Prenatal testosterone and autism

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Summary of Dissertation

Work at the cognitive level suggests that autism may be an extreme manifestation of some sexually dimorphic traits or an "extreme male brain." Although this theory was originally defined purely psychometrically, it has since been suggested that foetal testosterone (fT) may be a risk factor for autism and may be responsible for the biased sex ratio seen in these conditions. In this dissertation I used several strategies to explore the extreme male brain theory and the potential role of prenatal testosterone in autism

Chapter 1 reviews the role of fT in sexual differentiation, the background of the extreme male brain hypothesis, and the methodologies available for studying the relation of prenatal testosterone to postnatal behaviour in human populations. Chapters 2, 3 and 4 investigate whether fT, measured in second trimester amniotic fluid, is related to childhood cognition and behaviour. fT was negatively correlated to quality of social relationships and the tendency to ascribe intentions to ambiguous visual stimuli and directly correlated with restricted interests. fT did not contribute to individual differences in gender-typical game participation. Chapter 5 examines gender-typical game participation in children with autism. Results suggest a shift to male-typical play behaviour in girls with autism. Chapter 6 examines the pattern of puberty in women with autism spectrum conditions. Women with autism spectrum conditions showed a delay in age at menarche of approximately 8 months. In Chapter 7 I review the current status of the Cambridge Antenatal Predictors of Child Development Project and discuss future studies which can be carried out with this unique sample. Chapter 8 provides a summary of the empirical findings and a general discussion of limitations, outstanding questions, and future directions related to this work.

Although high testosterone on its own is probably not the cause of autism, once we understand the hormone's involvement, we will be able to unravel the other causes. With a fuller understanding of the causes of autism we can progress in diagnosing and treating the condition.

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This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

This dissertation does not exceed the word limit for the Biological Sciences Degree Committee.

CHAPTER 1: Introduction

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 foetal development, parturition/birth, and foetal 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 (e.g. testosterone (T), dihydrotestosterone (DHT)), oestrogens (e.g. oestradiol, oestrone, oestriol) and progestins (e.g. progesterone).

Figure 1.1

Structure of the major gonadal steroid hormones
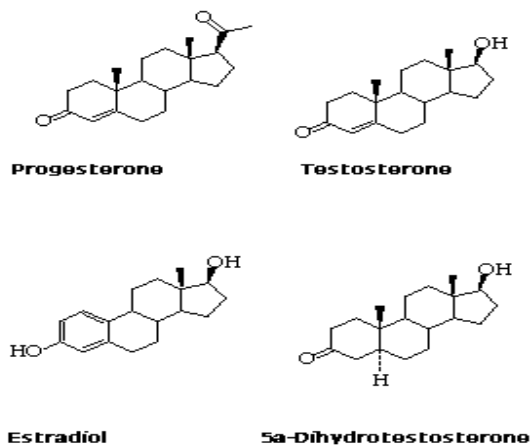
Hormone effects are usually classified as *organizational* (those that are permanent and happen early in development) or *activational* (those that occur later in development, are transient, and are superimposed on the early organizational effects). These later hormonal actions are often essential to allow the tissue or organ in question to perform its function. For example, the tissues of the genetic male are organized prenatally for male adult reproductive behaviour. However, the male will not display such behaviour, unless adequate sex hormones are present in adulthood. This project focuses primarily on organizational effects. However, the dichotomy between organizational and activational, although useful, is overly simplistic (Arnold & Breedlove, 1985). When studying the organizational effects of hormones on the developing foetus, it is important to remember that later activational effects may be essential to the function in question. For some hormones such as oestrogen the distinction is particularly problematic, as oestrogen appears to exert 'organizational' effects for a very long period of time (Fitch & Bimonte, 2002; Fitch & Denenberg, 1998).

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 may suffer extreme damage while the rest of the embryo remains essentially unaffected. Different behaviours can have different sensitive periods for development. For example, androgen exposure early or late in gestation has differential effects on male typical juvenile behaviours in the female rhesus macaque (Goy, Bercovitch, & McBrair, 1988).

It has been recognised since the 1940s that castration of males during neonatal or prenatal life prevents the development of masculine genitalia, while treatment of females with androgen masculinizes their genitalia (Jost, 1970). Phoenix, Goy, Gerall, & Young

(1959) reasoned that similar experiments would affect the development of the brain producing differences in sex-typical behaviour. They exposed female guinea pig foetuses to testosterone and found that, as adults, these females showed more male and less female copulatory behaviours. Similar experiments have been conducted in a wide range of mammals, comparing castrated males, normal males, normal females, and females treated with androgen on a range of sexually dimorphic features. Castrated males usually show feminized neural development, cognition, and behaviour; while females treated with androgen show masculinized neural development, cognition, and behaviour. Foetal testosterone (fT) has been shown to affect the anatomy of the brain, including the hypothalamus, limbic system, and neocortex, (Arnold & Gorski, 1984; Breedlove, 1994; MacLusky & Naftolin, 1981), sexually dimorphic behaviour such as aggression and activity level (Goy & McEwen, 1980) and sexually dimorphic cognitive skills like spatial navigation (C. L. Williams & Meck, 1991).

In human beings, sex differences are apparent both in brain structures and cognitive skills (Breedlove, 1994; Collaer & Hines, 1995; Halpern, 1992; Kimura, 1999; MacLusky & Naftolin, 1981). For example males tend to outperform females on some spatial and mathematical tasks, while females are superior at some language tasks (Kimura, 1999). However, the effects of prenatal hormones on these skills have been difficult to study because it is unethical to manipulate hormone levels in human foetuses. Certainly the differentiation of the male and female phenotypes in the developing human foetus follows a similar pattern to that in other mammals, although exact timings vary by species. Gonadal hormones appear to play the primary role in this process (Fuchs & Klopper, 1983; Kimura, 1999; MacLusky & Naftolin, 1981; Wilson, Foster, Kronenberg, & Larsen, 1998) although direct genetic influences on sexual differentiation of the brain are increasingly recognised (Arnold, 1996; De Vries et al., 2002; D'Esposito et al., 1995).

Under most circumstance 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 fertilising 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 can be described as heterogametic, and the female as homogametic. There are few differences between the genes of males and females, except for the Y chromosome in the male (Bardin & Catterall, 1981). The critical gene for starting the process of phenotypic differentiation of the sexes is the Sry gene, located on the Y chromosome. It is possible for the Sry gene to be translocated to another chromosome resulting in a child who may have a normal female karyotype, but develops as a phenotypic male.

Genetically male and female foetuses have undifferentiated gonads during early development– i.e. 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. This is thought to be the major function of the Y chromosome. The Leydig cells of the testis are capable of T synthesis by the end of week 8. Further development of the Leydig cells means that T secretion is high between week 10 and week 20. Foetal synthesis of T is probably controlled by HCG (human chorionic gonadotropin) and LH from the foetal pituitary. In addition males are exposed to T from the foetal adrenals.

In the female, differentiation of the ovaries begins around week 7 of gestation. The foetal ovary is generally considered inactive until late in development (Grumbach, Hughes, & Conte, 2003) but may produce a small amount of oestrogen (Smail, Reyes, Winter, & Faiman, 1981). The female foetus is also exposed to low levels of androgens. A small proportion may come from the foetal adrenals (a by-product of the production of corticosteroids) and some comes from the maternal adrenals, ovaries and fat (Geschwind & Galaburda, 1985; Martin, 1985).

The secretions of the gonads determine the *phenotypic sex.* If male sex hormones and the appropriate receptors are present, the male phenotype will develop and, if sufficient male sex hormones or functioning receptors are not present (i.e. in females), the female phenotype will develop (Donahoe, Cate, & MacLaughlin, 1987; George & Wilson, 1992; Jackendoff, 1987; Jost, 1961, 1972). The internal genitalia of both sexes are derived from the Wolffian (male) and Müllerian (female) Ducts, which co-exist in the undifferentiated developing embryo. In the male (between week 8 and week 14 of

gestation), the Wolffian Ducts, stimulated by T, develop into the male internal structures. The Müllerian Ducts regress, thus preventing the formation of female internal structures. Regression of the Müllerian Ducts is caused by anti-Müllerian hormone or Müllerian regression factor (secreted by the Sertoli cells of the testes). The formation of the male external genitalia is stimulated by DHT (dihydrotestosterone), which is formed from T by 5α-reductase. In the female, the low level of T causes the Wolffian Ducts to atrophy. The absence of anti-Müllerian hormone allows the Müllerian Ducts to become the female internal structures. Similarly, the low level of male sex hormones allows female external genitalia to develop, rather than male structures

The gonadal hormones are also thought to be involved in the sexual differentiation of the brain, determining the *neuronal sex*, which refers to male- or female- type gonadotropin secretion, sexual orientation, gender role behaviour, and *gender identity* (Dorner et al., 1987). In animal models, the general critical period for sexual differentiation of the brain usually occurs when sex-differences in serum testosterone are highest (L. L. Smith & Hines, 2000). 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 (Grumbach et al., 2003). The maximal sex difference in serum levels occur between week 12 and 18 (mean (sd) = 249 (93) ng/dL and mean (sd) = 29 (19) ng/dL for males and females respectively) (Abramovich, 1974). Testosterone levels in males are initially elevated in response to placental HCG (human chorionic gonadotropin) and remain high under the influence of LH (leutinizing hormone). However, HCG/LH receptors may not appear until weeks 10-12 of gestation raising the possibility that the earliest secretion of foetal testosterone (fT) is controlled by other, unknown factors. Levels of both gonadotropins, LH and FSH (follicle stimulating hormone), are controlled by the hypothalamic gonadotropin releasing hormone (GnRH) 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 oestrogen and progesterone produced by the placenta. Levels of gonadotropins and of fT fall (Abramovich & Rowe, 1973; Fechner, 2003;

Reyes, Boroditsky, Winter, & Faiman, 1974). 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 it is the only period for differentiation. Males are born with elevated testosterone levels (approximately 200 ng/dL), as a result of the sudden drop in inhibitory oestrogen levels produced by the placenta. Testosterone rapidly decreases in the first day of life and then begins to rise again after the first week (Fechner, 2003). Levels remain high for the first year of life, peaking around the 3rd to 4th months of life. Median levels are equivalent to those in the second stage of puberty (200-300 ng/dL) (Fechner, 2003; Forest, Sizonenko, Cathiard, & Bertrand, 1974); 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 (Mann, Gould, & Collins, 1989). As males have had a T surge at this time, and females have not, the same amount of subsequent T exposure will have very different effects on each sex (MacLusky & Naftolin, 1981).   Females have a postnatal surge in oestradiol production, which is thought to come from the ovaries (Bidlingmaier, Strom, Dorr, Eisenmenger, & Knorr, 1987). The post-natal surges in both sexes are stimulated by surges in gonadotropin levels (Bidlingmaier et al., 1987).

During childhood the gonads are quiescent. The GnRH pulse generator is inhibited by the very low levels of sex steroids present. This feedback system is ten times more sensitive to sex steroids than the adult feedback mechanism. Puberty occurs when the sensitivity of the GnRH 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 (Fechner, 2003).

In addition to these potential periods for steroid-induced sexual differentiation, sexual differentiation under direct genetic control could occur when no sex differences in testosterone are apparent (Arnold, 1996; Arnold, 2002; Arnold et al., 2004).

This project has focused 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 male sex hormones, female structures will develop. It has been assumed that no special hormonal environment is required for the formation of the female phenotype (Grumbach et al., 2003). However, this traditional model is now being replaced by a more complex one which recognises that small amounts of ovarian hormones may be required for active feminization of the female brain (Fitch & Bimonte, 2002; Fitch & Denenberg, 1998). Still, there are many stages, which must be successfully completed, in order 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 depends on exposure to ovarian hormones. In mammals, foetuses are exposed to high levels of female hormones from the mother, so it is adaptive for the default sex to be female. In species which 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 (Hadley, 2000). It is interesting to note that feminization of the brain in mammals by ovarian oestrogen is thought to occur at a later period than masculinization (in female rats this may extend from the late neonatal to the pubertal period and perhaps even into adulthood) (Fitch & Bimonte, 2002). This would mean ovarian oestrogen mediated feminization takes place after the individual is free from the maternal hormonal environment of the womb.

Human Sex Differences

In human beings, sex differences are apparent both in brain structures and cognitive skills (Breedlove, 1994; Collaer & Hines, 1995; Halpern, 1992; Kimura, 1999; MacLusky & Naftolin, 1981). The psychological study of sex differences has traditionally focused on spatial, mathematical, and verbal ability (Kimura, 1999). However, there is increasing interest in potential sex differences in social relationships.

Geary (1998, 2002) has argued that sexual selection can provide a unifying framework for incorporating hormonal, experiential, and evolutionary influences on human cognitive sex differences and has laid out a specific rational for why sex differences should be seen in social cognition.

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) (Darwin, 1871). It can be expressed as male-male competition, female-female competition, female choice, male choice or a combination of the above (Geary, 1998). The actual expression of sexual selection in a given species depends on the degree to which males and females 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 or OSR (the ratio of sexually active males to sexually active females in a given breeding population at a given point in time) (Halliday, 1994).

Humans, like other mammals, have internal gestation and obligatory postpartum female care (i.e. suckling). This means that the rate of reproduction in females is much lower than that of males (Clutton-Brock & Vincent, 1991). This leads to females putting relatively more effort into parenting. In contrast males can benefit by putting relatively more effort into mating. The difference in reproductive rates also biases the OSR so that there are usually more sexually receptive males than sexually receptive females 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 (Geary, 1998).

The importance of parenting effort to female reproductive success has significant implications for female social behaviour. Unstable social relationships have negative effects on the well being of children (Flinn & England, 1995; Flinn, Quinlan, Decker, Turner, & England, 1996) 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 (Hill & Hurtado, 1996), especially in societies which may be 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 (Geary, 1998; Geary & Bjorklund, 2000). This may explain why women appear to value reciprocal social relationships more than men and why their relationships with other females are more consistently communal than those among males-exhibiting greater empathy, nurturance, intimacy and emotional support. In contrast male relationships with each other are more often instrumental or agentic (an agentic role is characterized by concern for the self, in contrast to the communal role which is characterized by concern for others) (Winstead, Derlaga, & Rose, 1997). Concern for others may, in some cases, actually be disadvantageous for males as their reproductive success may have depended on dominating others, sometimes through physical aggression (Baron-Cohen, 2003; Geary, 1998).

The importance of reciprocal social relationships for female reproductive success may be further enlarged because human females have historically migrated to the social group of their mate while males remained in their birth group (males are the philopatric sex). This is also true for other great apes (Manson & Wrangham, 1991; Pasternak, Ember, & Ember, 1997; Seielstad, Minch, & Cavalli-Sforza, 1998). The probable result is that females, more than males, were forced to form social alliances with nonkin during evolutionary history.

However, women's social relationships are not always cooperative. Throughout primate species females compete with each other over access to high-quality food. Because human males often control resources which women need to raise their children successfully, women are expected to compete over these resource holding men. Human males show greater paternal investment than 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 (Hill & Hurtado, 1996) and in less harsh environments paternal investment seems to improve children's physiological, social, and psychological functioning (Emery, 1988). In contrast to men, where competition is often direct and physical, when females do compete with each other they use indirect methods such as gossip and social exclusion (Crick, Casas, & Mosher, 1997) in an attempt to damage the social networks crucial for female success and reduce their competitors' desirability as mates. In addition indirect aggression may be less apparent than physical aggression, thus maintaining the individual's appearance of empathy and kindness (Baron-Cohen, 2003).

Based on these evolutionary arguments, Geary (1998, 2002) suggests three socio-cognitive abilities that should show a female superiority: the ability to read nonverbal communication signals (i.e. body posture and facial expressions), language, and theory of mind. Baron-Cohen (2002, 2003; Lutchmaya, Baron-Cohen, & Knickmeyer, 2004) proposes that females, on average, are better at 'empathising.' He defines this 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 (1978, 1984) showed that females were on average better than males at interpreting body language, vocal tone, and facial expression. In a more recent study by Baron-Cohen, Jolliffe, Mortimore, & Robertson (1997), women were better at attributing subtle mental states to a person, when interpreting the eye region of the face. However, not all studies show this effect (Gitter, Black, & Mostofsky, 1972).

Part of this variation may depend on the specific emotions being examined. For example, a study by Rotter & Rotter (1988) showed that while females were better at identifying emotions overall, males were superior to females at recognising male anger.

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 (Gleason & Ely, 2002). There are well-replicated female advantages for verbal memory, spelling ability and verbal fluency in adulthood, although females do not have a larger vocabulary than males (Kimura, 1999). Developmentally, a number of studies have reported greater vocabularies and faster rates of language acquisition in girls (Fenson et al., 1994; Huttenlocher, Haight, Bryk, Seltzer, & Lyons, 1991; Hyde & Linn, 1988; Reynell & Huntley, 1985).

Theory of Mind is the ability to make inferences about the intentions, beliefs, and emotions of other people in order to predict and explain their behaviour. 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, including sex differences (Baron-Cohen et al., 1997). There are several studies, though, that suggest that theory of mind may develop earlier in females and that girls and women are, on average, better at making inferences about peoples mental states and adjusting their behaviour accordingly (Banerjee, 1997; Baron-Cohen et al., 1997; Baron-Cohen, O'Riordan, Stone, Jones, & Plaisted, 1999; Happe, 1995). These differences may arise, in part, from differences in social interest. Young girls show a preference for dyadic interactions (Benenson, 1993) and are more interested in facial than spatial/mechanical stimuli even at birth (Connellan, Baron-Cohen, Wheelwright, Ba'tki, & Ahluwalia, 2001).

Sex differences in social development can also be examined by looking at sex-biases in developmental conditions. Specific language delay, semantic-pragmatic disorder, and autism spectrum conditions are all more common in males (Bishop, 1990; Rutter, 1978b; Wing, 1981a). Autism in particular has been described as an extreme

manifestation of some sexually dimorphic traits or an "Extreme Male Brain" (Baron-Cohen, 2002).

The Extreme Male Brain Theory of Autism

Autism spectrum conditions (ASCs) are characterized by impairments in reciprocal social interaction, impairments in verbal and nonverbal communication, lack of imaginative play, and repetitive, stereotypical behaviours and interests. Autism, high-functioning autism, Asperger Syndrome, and pervasive developmental disorders, not otherwise specified, (PDD-NOS) are all considered ASCs. As many as 1 in every 200 people may have such a condition (Scott, Baron-Cohen, Bolton, & Brayne, 2002). Although the conditions are thought to have a strong neurobiological and genetic component, (Stodgell, Ingram, & Hyman, 2001) the factors causing ASCs are still unclear with many competing hypotheses discussed in the literature.

The starting point of the extreme male brain hypothesis is that factors during foetal 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 (Wellman, 1990) while folk psychology is our everyday understanding of people in terms of their mental states, sometimes referred to as "theory of mind" (Baron-Cohen, 1995). Baron-Cohen (2003) has reformulated this basic distinction, defining the male brain as being more developed at systemizing and the female brain being more developed at empathizing. Systemizing refers to the ability to understand systems in terms of logical 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 biological phylogenies, and structured social systems (dominance hierarchies for example). Empathizing includes what we normally define as empathy, appropriate emotional responding to another person, but also includes the cognitive

ability to model and predict people's emotions and behaviour using the idea of mental states (theory of mind) and the ability to read peoples' emotions in their faces, voices, and body language.

In this framework autism occurs when an individual's systemizing ability is intact or superior whilst their empathizing ability is impaired (Lawson, Baron-Cohen, & Wheelwright, in press). Evidence includes the fact that autistic individuals perform poorly on tests where females are usually superior to males such as "Reading the Mind in the Eyes" where subjects have to look at an individuals eyes and choose which of four emotions they are experiencing, but perform better than non-autistics on tests where males usually outperform females such as the "Embedded Figures Task" (Baron-Cohen & Hammer, 1997). Some individuals with Asperger syndrome (an autism spectrum disorder) have achieved extremely high levels of success in fields that require systemizing ability such as mathematics and engineering (Baron-Cohen, Wheelwright, Stone, & Rutherford, 1999). Baron-Cohen has also suggested that the restricted interests seen in autism are 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 (Baron-Cohen & Wheelwright, 1999).

If autism actually is an exaggeration of typical sex differences, then normally developing males should show more restricted interests, compared to females. Is this the case?

Intriguing evidence comes from a study of the Autism-Spectrum Quotient (AQ). This self-administered instrument measures autistic traits, and consists of 5 subscales (communication, social, imagination, local details, and attention switching). Males 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 males are more likely to have restricted interests than females (Baron-Cohen, Wheelwright, Skinner, Martin, & Clubley, 2001). Another source of evidence comes from tests of the drive to understand systems ('systemizing' see (Baron-Cohen, Richler,

Bisarya, Gurunathan, & Wheelwright, 2003; Baron-Cohen, Wheelwright, Griffin, Lawson, & Hill, 2002)), which, by definition, involves a very narrow focus of attention on each variable in the system. Males score higher on both the "Systemizing Quotient" (SQ) (Baron-Cohen et al., 2003) and on an experimental test of systemizing (Lawson et al., in press).

Lines of evidence implicating testosterone in the aetiology of autism include the following:

1) Autism is much more common in males than females-3:1 for classic autism, 9:1 for Asperger syndrome (Rutter, 1978a; Wing, 1981b).
2) High levels of fT are associated with less frequent eye contact in male children and lack of eye contact is common in children with autism (Lutchmaya, Baron-Cohen, & Raggatt, 2002a).
3) Normal males develop socially more slowly than do normal females and autistic people are even more delayed in social development (Baron-Cohen, O'Riordan et al., 1999)
4) Left-handedness is more common among male subjects and in individuals with autism (Fein, Humes, Kaplan, Lucci, & Waterhouse, 1988; Satz, Soper, Orsini, Henry, & Zvi, 1985; Soper et al., 1986).
5) In the normal population the male brain is heavier than the female and people with autism have even heavier brains than those in normal males (Piven, Bailey, Ranson, & Arndt, 1997)
6) A subset of adolescents with autism show elevated androgen levels and precocious puberty (Tordjman, Ferrari, Sulmont, Duyme, & Roubertoux, 1997).
7) The ratio of the second to the fourth digit (2D:4D ratio), a possible index of prenatal testosterone exposure, is lower in individuals with autism (Manning, Baron-Cohen, Wheelwright, & Sanders, 2001).

Social and environmental factors certainly play a significant role in the development of sex differences in social development, but biological contributions are also suggested by genetic and neurobiological studies. Social skills, social reciprocity, and empathy have a heritable component (Constantino & Todd, 2000; Scourfield, Martin, Lewis, & McGuffin, 1999; Zahn-Waxler, Robinson, & Emde, 1992). Several brain regions appear to subserve social cognition and theory of mind in particular. This neural network comprises the amygdala, superior temporal sulcus (STS), and the orbital and medial prefrontal cortex (Adolphs, 2001; Baron-Cohen et al., 2000). In addition, both specific language delay and autism are considered to have a neurobiological basis and a strong genetic component (Bishop, 2001; Stodgell et al., 2001).

Strategies for Studying fT in Humans

The effects of prenatal hormones on human sex-differences have been difficult to study because it is unethical to manipulate hormone levels in human foetuses. A role for fT in human development is suggested by studies of:

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 foetal testosterone has been measured in umbilical cord blood.
5) Maternal testosterone levels during pregnancy
6) Individuals whose foetal testosterone has been measured in fluid obtained at amniocentesis

Each of these lines of evidence is reviewed next.

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 characterised by an abnormal hormonal environment. This can be due to genetic or environmental factors.

Congenital Adrenal Hyperplasia (CAH) 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 males and females. The most common biochemical cause is 21-hydroxylase deficiency (making up 90-95% of cases). When there is a complete or almost complete absence of functioning enzyme, classical CAH occurs. This occurs in 1 in 15,000 live births and results in a significant overproduction of androgen beginning in the third month of foetal life (New, 2003).

Although it can affect both males and females, the most interesting psychological findings have come from studies of females, who are usually compared to unaffected female relatives. Most girls with the condition are recognised at birth or during early childhood at which point the hormonal abnormalities can be ameliorated through cortisone-replacement therapy (White & Speiser, 2002). This means that for individuals that received early neonatal diagnosis and treatment androgen is elevated only in the prenatal and early neonatal period. If testosterone effects human brain development, CAH girls should have masculinized cognition and behaviour. Studies of girls with CAH report that they have superior spatial reasoning (Hampson, Rovet, & Altmann, 1998); prefer masculine toys and activities (Berenbaum & Hines, 1992; Berenbaum & Snyder, 1995); draw pictures with masculine characteristics (Iijima, Arisaka, Minamotot, & Arai, 2001); are more likely to report the use of physical aggression in conflict situations (Berenbaum & Resnick, 1997); are less interested in marriage, motherhood and physical appearance (Dittman et al., 1990; Ehrhardt & Baker, 1974); score lower on measures

assessing empathy, intimacy and the need for close social relations (Helleday, Edman, & Ritzen, 1993; Resnick, 1982); are less likely to engage in heterosexual activity and more likely to fantasize about other women (Dittman, Kappes, & Kappes, 1992; Zucker et al., 1996) as predicted.

In contrast to girls with CAH, in most male foetuses with CAH, testosterone appears to be in the high normal range, and males with CAH are born with normal male external genitalia (Grumbach et al., 2003; Pang et al., 1980; Wudy, Dorr, Solleder, Djalali, & Homoki, 1999). Prenatal levels of the relatively weak androgen, androstenedione appear to be elevated and neonatally, there is some evidence that treatment to reduce corticosteroid exposure can also produce abnormally low testosterone levels in boys with CAH (Pang, Levine, Chow, Faiman, & New, 1979). Despite these abnormalities, the majority of studies of cognition and behaviour in males with CAH show no differences from control males (Collaer & Hines, 1995; Pang et al., 1980; Pang et al., 1979). Boys with CAH showed decreased rough and tumble play compared to control males in one study (Hines & Kaufman, 1994) and there are two reports of reduced mental rotations ability in males with CAH (Hampson et al., 1998; Hines, Fane et al., 2003). In contrast, two recent studies have found that the 2D:4D ratio is lower in CAH males, as well as females, suggesting that they are exposed to elevated fT levels (Okten 2002, Brown 2002), but see Buck, R. M. Williams, I. A. Hughes, & Acerini, (2003) for a study showing no affect of CAH on 2D:4D ratios in males or females. The negative result in that study may reflect that bone length was measured as opposed to soft tissue and only the left hand was included. Both Okten (2002) and Brown (2002) measured both hands and included soft tissue measures. Hypermasculinization of boys with CAH has also been reported for activity level, and preference for right handed writing (Mathews et al., 2004). There are 2 potential explanations for these paradoxical findings. Firstly, 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 in males with CAH). Androgenization may be spatially and temporally specific: a concept McFadden (2002) refers to as localized effects. For example, digit length might be affected during early pregnancy before negative feedback reduces testosterone levels. Secondly, the effect of testosterone on certain behaviours may be nonmonotonic. That is, increasing testosterone exposure may produce increasing masculinization up to a certain dose, but increase beyond this dose would cause a reversal toward the original state (demasculinization). There is support in the animal literature for nonmonotonic effects (Baum & Schretlen, 1975; M. M. Clark, Robertson, & Galef, 1996). In the human literature, it has been suggested that males are overrepresented in the extremes (both high and low) of abilities that show mean sex-differences favouring males, although this idea is controversial (Hyde & McKinley, 1997). Grimshaw, Sitarenios, & Finegan (1995) in their study of foetal testosterone and mental rotation found an inverted U-shape relationship, with the best scores in the low male range. The levels of circulating prenatal testosterone in males with CAH are clearly complex, making it substantially more difficult to test specific hypotheses about testosterone-behaviour relations in this group.

No study has directly examined whether autism is more common in girls with CAH, although there is a case report of a boy born with CAH and an XYY chromosomal karyotype who was described as autistic (Mallin & Walker, 1972). In reference to developmental conditions, Plante, Boliek, Binkiewicz, & Erly (1996) reported that individuals with CAH showed higher levels of language/learning difficulties than non-CAH family members. The authors report 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 CAH, separate sex analyses are crucial. Perlman (1973) found no evidence of learning disabilities in girls with CAH, but the sample was small and the measures used were less diagnostic than current ones. Nass & Baker (1991) found that females with CAH had a discrepancy between their verbal and performance IQ, which was considered indicative of a learning disability. However, this result may reflect the enhanced spatial ability often reported in CAH as opposed to a

learning disability (Berenbaum, 2001). It is interesting that children with classical autism often show a discrepancy between their verbal and performance IQ in the same direction. However, individuals with Asperger syndrome often have better verbal scores than performance scores (Gillberg & Coleman, 2000). Further study is clearly needed to determine whether there is a raised incidence of developmental disabilities in CAH. Such studies will require large sample sizes, since the population base 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.

Although many studies have supported the idea of masculinization in CAH, others have found no such effect. One study, for example, reported that females with CAH did not differ from controls on hand preference or dichotic listening asymmetry (Helleday, Siwers, Ritzen, & Hugdahl, 1994) and another reported no difference on tests of visuo-spatial ability as well as noting deficits in quantitative ability (Baker & Ehrhardt, 1974.) They do not appear to have atypical gender identity or playmate preference (Berenbaum & Snyder, 1995). In addition, females with CAH were found not to differ from their unaffected sisters with regard to assertiveness, dominance, acceptance in peer groups and energy expenditure (Dittman et al., 1990). These negative findings do not disprove the androgen theory of sexual differentiation in humans; rather it suggests that testosterone plays a role only in a sub-set of sex differences. Researchers must be careful in evaluating large categories such as "visuo-spatial" skills when interpreting various studies. A male superiority does not exist for all visuo-spatial skills. Most of these show no sex difference. Remembering an object in a busy environment shows a female superiority (James & Kimura, 1997). Even for those skills that do show a male superiority this varies in strength. Mental rotation shows a very strong male superiority, while finding an embedded figure shows only a moderate effect (Kimura, 1999). Timing of exposure must also be considered. Hines, Fane et al. (2003) found that girls with CAH performed better than unaffected girls on a targeting task, but not on a mental rotation task. In contrast, boys with CAH performed equally well to 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 rotations. 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 may be suppressed, so boys in their sample might have had lower testosterone levels than normal during the neonatal testosterone surge).

Studies of individuals with CAH provide some of the most compelling evidence that prenatal androgens have a lasting affect on human behaviour. 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 CAH are also exposed to high levels of adrenocorticotropic hormone (ACTH) and treatment may result in glucocorticoid excess (Berenbaum, 2001). In contrast to the androgen hypothesis, there is no clear theoretical reason why these changes would masculinize play behaviour. In addition, masculinization appears to be correlated with degree of androgen excess (Nordenstrom, Servin, Bohlin, Larsson, & Wedell, 2002; Servin, Nordenstrom, Larsson, & Bohlin, 2003). An additional potential confound is that parents may treat daughters with CAH differently because they are virilized at birth (Quadagno, Briscoe, & Quadagno, 1977). There is currently no experimental support for this position,(Berenbaum, 2001; Berenbaum & Hines, 1992; Dittman et al., 1990; Goy et al., 1988; Nordenstrom et al., 2002) but it has yet to receive a strong test (Berenbaum, 2001).

Androgen insensitivity (AI) occurs when there is a partial or complete deficit of androgen receptors. This insensitivity can be complete (CAIS) or partial (PAIS). Both disorders are X-linked recessive and hence occur more often in genetic males. Prevalence is estimated at between 1 in 20,000 and 1 in 60,000 live male births. At birth, genetic males with CAIS are phenotypically normal females, 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 oestrogen derived from testicular androgens. Diagnosis usually takes place when menarche fails to occur (Grumbach et al., 2003; Hines, Ahmed, & Hughes, 2003). Quality of life (self-esteem and psychological general well-being), gender-related psychological characteristics (gender identity, sexual

orientation, and gender role behaviour in childhood and adulthood), marital status, personality traits that show sex differences, and hand preferences do not differ between genetic males with CAIS and control women (Hines, 2002; Money, Schwartz, & Lewis, 1984). They also perform in a female-typical fashion on tests of visuo-spatial ability (Masica, Money, Ehrhardt, & Lewis, 1968). Findings argue against the need for two X chromosomes or ovaries to determine feminine-typical psychological development in humans and reinforce the important role of the androgen receptor in influencing masculine-typical psychological development. There are far fewer studies of psychological outcome and sex-typical behaviour in CAIS individuals than there are for CAH (possibly because such studies cannot separate prenatal hormone exposure from sex of rearing; or because the condition is rarer than CAH). Physical appearance in PAIS varies enormously from essentially that of a CAIS individual to uncomplicated hypospadias, infertility or gynaecomastia in an otherwise healthy-appearing male (Hines, 2002).

Idiopathic hypogonadotrophic hypogonadism (IHH) 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. Males with congenital IHH are usually diagnosed when they fail to undergo normal puberty. Males with congenital IHH 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 HCG in stimulating the human foetal testis. Possibly, maternal gonadotropins also stimulate the testes to produce hormones prenatally. Cappa et al. (1988) found no deficits on the block design subtest of the Weschler Adult Intelligence Scale (which shows a small sex difference, e.g. $d = 0.34$ (Colom, Garcia, Juan-Espinosa, & Abad, 2002)) in a group of men with IHH. In contrast, Hier & Crowley (1982) found that men with IHH performed significantly worse on the block design subtest, the embedded figures test, and the space relations subtests from the differential aptitude tests when compared to normal men and men with acquired hypogonadotrophic hypogonadism after puberty. This would be

consistent with androgens exerting a permanent organizing influence of the brain before or at puberty.

Individuals with Turner Syndrome and Klinefelter's 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 psychological development than the study of some other individuals. However, they may suggest direct genetic affects on sexual differentiation.

Individuals with Klinefelter's 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 know if prenatal levels are low, but they are not so low as to cause ambiguous genitalia at birth. Individuals have a lower than normal verbal I.Q, delayed emotional development, poor motor control (Grumbach et al., 2003), abnormal cerebral asymmetry (Warwick, Lawrie, Beveridge, & Johnstone, 2003), and an impairment in inhibitory skills (Temple & Sanfilippo, 2003). They are at greater risk of developing schizophrenia (Bassett, Chow, & Weksberg, 2000; DeLisi et al., 1994). They may have particular strengths in spatial planning, organisation and memory (Temple & Sanfilippo, 2003). 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 due to a number of somatic stigmata. Gender identity and attitudes are female; however because of gonadal dysgenesis individuals have extremely decreased oestrogen levels (Collaer, Geffner, Kaufman, Buckingham, & Hines, 2002; Grumbach et al., 2003). Individuals often have cognitive deficits in performance IQ, visuo-motor skills, visuo-spatial processing, visuo-spatial memory, and

mathematics with preserved or enhanced verbal skills (Bruandet, Molko, Cohen, & Dehaene, 2004; Grumbach et al., 2003; Temple & Carney, 1996). Collaer et al. (2002) specifically examined skills which 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. Impairments in social functioning are also common among individuals with Turner syndrome and they have significant impairments in face and emotion processing (Lawrence, Campbell et al., 2003; Lawrence, Kuntsi, Coleman, Campbell, & Skuse, 2003; McCauley, Feuillan, Kushner, & Ross, 2001; Ross, Stefanatos, Roeltgen, Kushner, & Cutler Jr., 1995; Ross et al., 2002; Siegel, Clopper, & Stabler, 1998).

Turner syndrome, like Klinefelter's, 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 be genetic factors and which are mediated by other causal factors (such as steroid hormones) which may correlate with genetics. Researchers can attempt to separate hormonal and genetic effects on theoretical grounds by extrapolating from animal research (Everhart, Shucard, Quatrin, & Shucard, in press), and by comparing individuals with full Turner syndrome to those who are mosaic for the disorder and testing for X-chromosome dosage effects (D. G. M. Murphy et al., 1997). Skuse et al. (1997) has 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. He has 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 socio-cognitive abilities and for the greater vulnerability of males to developmental disorders including autism (Skuse, 2000; Skuse et al., 1997; N. S. Thomas et al., 1999). Any study on Turner syndrome individuals should check for imprinting effects as well as X-chromosome dosage and hormonal effects.

Exposure to chemicals that mimic or block endogenous hormones

Diethylstilbestrol (DES) is a synthetic oestrogen. Beginning in 1946 DES was marketed for numerous gynaecological conditions, including prevention of threatened miscarriage in high-risk pregnancies. It became widely prescribed even during normal pregnancies, up until 1971 when a report associated DES with a rare form of vaginal cancer in the daughters of women who took the drug. DES was shown to cross the placenta and have a direct effect on the developing foetus (Newbold, 1998). Initial predictions were based on rodent models. In rodents, oestrogen 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 oestrogen. It is oestrogen and its receptor that actually effect gene transcription. Therefore exogenous oestrogen masculinizes rodents when administered at the correct time. Females are protected from maternal and placental oestrogen by binding proteins (Fitch & Bimonte, 2002; Newbold, 1998). Some studies of DES exposed females are consistent with this model (Hines & Shipley, 1984; Schacter, 1994). However, in another study no group differences were seen for DES exposed women on a range of abilities at which females excel on average or for abilities at which males excel on average (Hines & Sandberg, 1996).

J. H. Clark (1998) strongly argues against extrapolating from rodent models where oestrogen is concerned. A study of prenatal DES exposure in rhesus monkeys (Goy & Deputte, 1996) suggests that oestrogen has little or no effect on behavioural masculinization in primates (with the possible exception of rough and tumble play). It has also been suggested that oestrogen has demasculinizing/feminizing effects that occur at a later period than the testosterone initiated effects discussed above (Fitch & Bimonte, 2002). Studies of DES exposed human males offers some support for this position (Reinisch & Sanders, 1992) (laterality and spatial ability).   When administered to rats, DES (and 4-octylphenol, a putative environmental oestrogen) exposure results in a decreased expression of CYP17 mRNA (CYP17 codes for P450c17, an enzyme in the testosterone synthesis pathway). Suppression of CYP17 could have an adverse affect on foetal masculinization, suggesting an alternative explanation for the feminising effects

seen in the above study (Grumbach et al., 2003). Given that oestrogen may 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 DES. Additional problems are that DES may have different effects from endogenous oestrogen 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 oestrogen 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 (MPA) 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 (Diamond, llacuna, & Wong, 1973; Hull, 1981; Hull, Franz, Snyder, & Nishita, 1980; Shapiro, Goldman, Bongiovanni, & Marino, 1976). Androgenic progestins mimic the action of androgen and include Danazol and 19-nortestosterone derivatives such as norethindrone and ethisterone. Thus we would predict that progesterone based progestins would impair masculine-typical development and that androgen based progestins would promote it (Hines, 2002).

Reinisch, Ziemba-Davis, & Sanders (1991) reviewed 19 studies on the behavioural effects of prenatal exposure to hormones administered as treatment for at-risk pregnancies. Three findings on play (one a trend) and one on gender identity/role (also a trend) suggested that natural progesterone has a demasculinizing effect in males (Kester, Green, Finch, & Williams, 1980; Zussman, unpublished data). In contrast, Kester, Green, Finch, & Williams (1980) also found a trend for increased participation in sports, indicating masculinization. Aggression/assertion was found to be masculinized in both studies. Two studies examined natural progesterone in females and found exposed girls to be feminized on multiple aspects of gender identity/role, and on aggression (Lynch, Mychalkiw, & Hutt, 1978). 3 studies examined progesterone-based synthetic

progestins (Ehrhardt, Meyer-Bahlburg, Feldman, & Ince, 1984; Kester et al., 1980; Meyer-Balhburg, Grisanti, & Ehrhardt, 1977) in males. Of 43 comparisons made to matched controls, 41 were non-significant suggesting that these drugs did not affect sexual differentiation. One group of girls exposed to synthetic progesterone based progestins had been studied at that time. 26 of 33 findings were nonsignificant. Three findings on gender identity/role (2 trends), one finding relating to marriage and maternalism (also a trend), one finding on play behaviour, and one finding on athleticism suggest that these substances may be demasculinizing/feminising (Ehrhardt, Grisant, & Meyer-Bahlburg, 1977; Ehrhardt et al., 1984). One study of males exposed to androgen-based progestins found increased aggression (Reinisch, 1981). Females exposed to androgen-based progestins (drawn from 2 studies) had masculinized play behaviour (2 findings), peer relations (1 finding), aggression (1 finding), interest in marriage/maternalism (1 finding), and gender-identity/role (2 findings) (Money & Ehrhardt, 1972; Reinisch, 1981).

Overall the reports do not support an effect of progesterone or synthetic progestins on sex differentiated behaviour in males or females. There were numerous non-significant findings and many of the reported findings are, in fact, trends ($0.05 \leq p \leq 0.10$). Because sample sizes were very small and non-parametric tests with limited power were used, one study also used a statistical alpha criterion of 0.10 instead of the more conventional 0.05 (Ehrhardt et al., 1984). Since sample sizes were small in all studies (5 to 18) there is 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. Dosage, duration of treatment, and combination of hormones administered varied widely even when using patients from the same doctor. 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 Reinisch & Karow (1977), 71 of the mothers in the study received at least one medication, 59 at least two, 43 at least 3, 20 at least four, and 8 received five different medications. 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 oestrogen exposure, as in Reinisch (1977) and Reinisch & Karow (1977) which found that individuals with exposure primarily to progestins were more independent, sensitive, individualistic, and self-sufficient than those exposed primarily to oestrogens, who were more group oriented and group-dependent.

In addition to the difficulty arising from differing treatment variables, as with DES, an association between consumption of a drug during pregnancy and a behavioural effect on the foetus does not necessarily imply causation. It may be impossible to differentiate a possible effect of illness on the foetus from an effect of the drug used to treat the illness. Well-matched controls are crucial, with sibling controls being the gold-standard (Reinisch & Karow, 1977). One of the benefits of studies using individuals exposed to drugs that mimic or block endogenous hormones during pregnancy is that, in contrast to CAH, 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 (Grumbach et al., 2003)). Therefore, parents could not have treated their exposed children differently on the basis of their external genitalia and it is unlikely 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 remember taking any medication during pregnancy (Noller & Fish, 1974; Reinisch & Karow, 1977).

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 rational for this strategy comes from experiments in mice: female mice adjacent to male mice in utero are masculinized (Clemens, 1974) possibly by testosterone diffusing across the amniotic membrane (Fels & Bosch, 1971) or being carried through the maternal circulation (Meisel & Ward, 1981). Human twin pregnancies are very different than multiple offspring pregnancies in rats, but fT may transfer from the male to the female foetus through amniotic diffusion in humans (Resnick, Gottesman, & McGue, 1993). The foetal

skin is permeable to fluid and some dissolved solutes up to week 18 of gestation and amniotic fluid moves through the entire foetoplacental unit (Abramovitch & Page, 1972; Brace & Resnik, 1989; Findlay, 1984). Human females with male co-twins are masculinized with regards to sensation seeking (Resnick et al., 1993) and spatial ability on a mental rotation task (Cole-Harding, Morstad, & Wilson, 1988). Further, females with a male co-twin are masculinized with regard to spontaneous otoacoustic emissions (SOAEs), which are continuous, tonal sounds produced by most normal hearing cochleas. SOAEs are more common in females than males (McFadden, 1993, 2002). 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 may 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 to controls with an opposite sex sibling of similar age.

Some behaviours, such as play preferences, do not show an opposite-sex twin effect (Henderson & Berenbaum, 1997; Rodgers, Fagot, & Winebarger, 1998) even though play preferences are dramatically affected by CAH. A further study of handedness in opposite and same-sex twins found no association between sex of co-twin and handedness (Elkadi, Nicholls, & Clode, 1999). It is not clear why this should be the case, but one limitation of opposite-sex twin studies is that they necessarily 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 foetuses 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 (Hull, 1981; Hull et al., 1980; Shapiro et al., 1976) and may serve a protective role in human females, as discussed previously (Ehrhardt & Meyer-Bahlburg, 1979; Ehrhardt et al., 1984).

Timing of exposure could also play a role. Henderson & Berenbaum (1997) argue that exposure to androgen from a twin brother should not occur after the 18th week

of gestation. If the critical period for a particular sexually dimorphic behaviour occurs after week 18, one would not expect to see an opposite twin effect. E. M. Miller (1994) argues that testosterone could be passed from a male foetus to a female foetus throughout pregnancy via the maternal blood stream. He cites a study (Meulenberg & Hofman, 1990) which reported that maternal blood testosterone levels depend on the sex of the foetus being carried and that unbound testosterone crossed the placenta via a diffusion gradient. The placenta is rich in aromatase, the enzyme that converts T to oestrogen. This should protect the foetus 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 foetus and the mother virilize during pregnancy (Grumbach et al. 2003) However this protection system is not perfect. Women with medical conditions causing elevated androgen during pregnancy or those taking androgenic hormones may give birth to females with ambiguous (somewhat masculinized genitalia) suggesting that maternal hormones can cross the placenta into the foetal system (Barbieri, 1999; Ehrhardt & Money, 1967; Wilkins, 1960). It does not appear that hormones pass from foetus to mother. Bammann, Coulam, & Jiang (1980), Dawood & Saxena (1977), Demisch, Grant, & Black (1968), Glass & Klein (1981), and Hines, Golombok et al. (2002) found no differences in maternal T levels during pregnancy in women carrying male versus female foetuses (but see Klinga, Bek, & Runnebaum (1978)), and women carrying foetuses with CAH do not show elevated androgen levels (Grumbach et al., 2003; Hines, Golombok et al., 2002).

Umbilical Cord Blood

Jacklin, Maccoby, & Doering (1983) measured 5 steroid hormones including T in umbilical cord blood at birth. They assessed timidity in home and laboratory observations using a set of novel toys. Girls had higher mean timidity scores in 2 of the 3 samples tested. Timidity in girls was not predicted by any of the hormones measured. In boys, testosterone and progesterone increased boldness while oestrogen decreased boldness.   The relation 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 oestrogen. The authors state that their findings are consistent with the Reinisch & Karow (1977) study discussed previously. However, in that study women were exposed to synthetic progestins (some of which were androgen based) while in the Jacklin et al. (1983) study only natural progesterone levels were measured. Their results are certainly not consistent with natural progesterone protecting against the effects of testosterone.

Jacklin, Wilcox, & Maccoby (1988) examined cognitive abilities at 6 years of age. In females, T was inversely correlated with spatial ability, but there was no correlation in boys. The T result in females was the opposite direction to that predicted. At term there is considerable overlap between male and female T levels measured in umbilical cord blood and significant sex differences are reported inconsistently (Abramovich, 1974; Abramovich & Rowe, 1973; Dawood & Saxena, 1977; Forest & Cathiard, 1975; Forest et al., 1974; Hines, Golombok et al., 2002; Maccoby, Doering, Jacklin, & Kraemer, 1979; Pang et al., 1979), so this might not be the best time to look for hormone correlates for sexually dimorphic behaviours. In addition, the abilities measured in Jacklin et al. (1988) did not show any sex differences and therefore are less likely to relate to gonadal hormone levels. Jacklin et al. (1988) also suggested that the onset of labour and the stress of labour might affect the levels of hormones found in cord blood.

Maternal Testosterone During Pregnancy

Udry, Morris, & Kovenock (1995) examined a broad questionnaire measure of gendered behaviour in women in relation to maternal testosterone levels. Total testosterone and sex hormone binding globulin (SHBG) were measured in maternal blood samples. The study focused exclusively on women because, as discussed previously, testosterone may pass from the mother to the female foetus along a diffusion gradient, but it does not appear that testosterone can pass from the male foetus to the mother. Only free testosterone can pass through the placenta; so total testosterone and SHBG are proxies for actual testosterone exposure. Androgen exposure (as indicated by SHBG

levels) in the second (and no other) trimester of foetal life, combined with and in interaction with adult androgens, masculinized women's behaviour, explaining 16 to 18% of the within-sex variation in scores.

Hines, Golombok et al. (2002) related levels of T and SHBG in maternal blood samples to children's gender-role behaviour as assessed by the Pre-school Activities Inventory (PSAI). Gender-role behaviour 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 foetus and promote masculine-typical development. Males will usually have higher endogenous testosterone levels than their mothers, and it does not appear that testosterone produced by male foetuses 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, Golombok et al. (2002) also suggest as an alternative or additional explanation, that mothers with relatively high testosterone have daughters with relatively high testosterone because of a genetic predisposition passed from mother to daughter. In either case, studies using this strategy can only be applied to females.

Levels of post-pubertal testosterone are determined, in part, genetically, with heritability estimates around 40% to 60% (Harris, Vernon, & Boomsma, 1998; Sluyter et al., 2000). The second study only included males, but the first included both sexes and provided some evidence that the genetic connection is clearer in females. In contrast, Sakai, Baker, Jacklin, & Shulman (1992) found that variation in testosterone levels at birth was accounted for primarily by environmental variation, not genetic variation. However, sexes were not examined separately, so different relations in boys and girls could not be detected. No-one has evaluated whether testosterone levels at midgestation are heritable.

Hines, Golombok et al. (2002) also drew attention to the lack of a consistent relationship between prenatal hormone variability and gender-role behaviour in boys when other research strategies were used, including studies of CAH and of synthetic

progestins. They suggest 2 reasons why consistent relations are less common in boys than girls across studies. Firstly, they suggest that because foetal testosterone levels are much higher in males than females, that normal variability may be insignificant in relation to the high levels seen in the majority of males. Secondly, they suggest that hormone-related predispositions in boys may be reduced or eliminated by other forces. For example, boys are more strongly encouraged to behave in a sex-typical way, and more likely to be discouraged from behaving in a cross-gendered way (Fagot, 1978).

Although this strategy may only be applicable to females, 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 fT via amniocentesis

Both male and female foetuses produce some testosterone. In males the main source is the testes. Females are exposed to small amounts of fT from the foetal adrenal glands and from the maternal adrenals, ovaries and fat (Geschwind & Galaburda, 1985; Martin, 1985). Testosterone can be measured in amniotic fluid collected during midtrimester amniocentesis (Finegan, Bartleman, & Wong, 1989). Testosterone is thought to enter the amniotic fluid via diffusion through the foetus' skin in early pregnancy, and later from foetal urination (Klopper, 1970; Nagami, McDonough, Ellegood, & Mahesh, 1979; J. D. Robinson, Judd, Young, Jones, & Yen, 1977). Although the exact correlation between testosterone levels in the foetal serum and the amniotic fluid is unknown, the maximal sex difference in amniotic testosterone between males and females occurs between weeks 12 and 18, closely paralleling peak serum levels (Finegan et al., 1989). In animal models, the general critical period for sexually differentiation of the brain usually occurs when sex-differences in serum testosterone are highest (L. L. Smith & Hines, 2000). Therefore it is likely that this is an important period for sexual differentiation of the human brain as well. This is supported by the Udry et al. (1995) study reviewed previously which found that only prenatal androgen exposure in the 2nd trimester related to adult gendered behaviour and the Hines, Golombok et al.

(2002) study which measured testosterone in maternal blood during pregnancy at a mean gestational age of 16 weeks.

The first study to use this methodology was carried out by Finegan, Niccols, & Sitarenios (1992). 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 may be because the abilities studied did not show a sex difference in their, or other, samples (Tierney, Smith, Axworthy, & Ratcliffe, 1984). Later studies by the same group have produced results more consistent with predictions. At age eight, girls with higher levels of fT performed a mental rotation task faster than girls with lower levels (Grimshaw, Sitarenios et al., 1995). At age 10, girls with higher levels of fT showed a more masculine pattern of cerebral lateralization (Grimshaw, Bryden, & Finegan, 1995). In a previous study carried out by the Autism Research Centre at Cambridge University, fT was negatively related to vocabulary size (Lutchmaya, Baron-Cohen, & Raggatt, 2002b) and frequency of eye contact (Lutchmaya et al., 2002a) at 12 months of age when examining a combined sex group. In addition, within boys, fT was negatively related to frequency of eye-contact.

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 fT are related to gendered behaviour have used groups with large abnormalities in prenatal endocrine conditions due to genetic flaws or foetal exposure to synthetic progestins. This can be a weakness as well, as larger sample sizes may be needed to show an effect then in studies where 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. Addenbrooke's Hospital, which analyses amniocentesis samples from 6 hospitals in East Anglia, processes approximately 1000 samples a year (Lutchmaya, 2000). 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, since one can only include in a study those individuals who have decided/been advised to have an amniocentesis due to late maternal age or other factors that increase the risk of foetal abnormality.

Each of the above methodologies has strengths and weaknesses (see Table 1.1 for a synopsis), but taken together they indicate that foetal testosterone does play a role in the sexual differentiation of the human brain. A role for foetal oestrogen in sexual differentiation of the brain appears much less likely, but should not be ruled out. Each strategy could, potentially, be applied to studying whether fT is a risk factor for autism. In this report, I will focus primarily on studies using the amniocentesis design. I also report studies examining sex-typical behaviour in children with autism, and examine whether elevated androgen activity might also be detectible in postnatal life.

Table 1.1

Available research strategies for studying foetal sex hormones and later behaviour: Strengths and weaknesses

Research Strategy	Pros	Cons
Congenital Adrenal Hyperplasia (CAH)	High dosage of fT in females means effects can be observed with small sample sizes	Nature of fT exposure in males unclear, may be high, low, or normal
	Genotype and sex of rearing is female, but exposure to fT was high	Virilization of genitalia may affect parents' response to CAH girls
	Degree of T exposure correlates well with genotype	Also exposed to high levels of ACTH during prenatal period
	Treatment of female usually begins early in neonatal life: restricting fT exposure to prenatal period	With treatment girls may 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 (CAIS)	Good comparison to CAH, as genotype is male but exposure to T is absent	Hormonal sex and sex of rearing confounded Condition rare, so sample size is limited Does not measure normal variability in T
Idiopathic Hypogonadotrophic Hypogonadism (IHH)	Sex of rearing is male but T levels are low Could reveal effects of neonatal and pubertal testosterone as the condition is not usually diagnosed until puberty	Not clear if T levels are low prenatally; maternal hormones may stimulate the foetal testes Condition rare, so sample size limited
Turner Syndrome	Can examine direct genetic effects of having only 1 X chromosome Can examine effects of low oestrogen levels	Direct genetic effects and hormonal effects confounded Extensive clinical problems Condition rare, so sample size limited
Klinefelter's Syndrome	Can examine direct genetic effects of having 2 X chromosome and 1 Y chromosome Low testosterone levels	Direct genetic effects and hormonal effects confounded. Not known if prenatal T levels are low Extensive clinical problems Condition rare, so sample size limited

Exposure to Diethylstilbestrol (DES)	Can examine effects of high oestrogen exposure Genitalia normal	Unclear what role oestrogen has in human sexual differentiation Different researchers have predicted both masculinizing and feminising effects Dosage and timing of exposure vary widely and may 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	May act as anti-androgens, providing a good comparison to CAH studies Roughly comparable to animal experiments Genitalia normal in most cases	Some progestins are virilizing, while some act as anti-androgens. Patients often given a mix of progestins of different types (also oestrogens) Dose and timing of exposure varies 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 females Results may 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 T levels, significant sex differences reported inconsistently May miss the critical period for sexual differentiation Hormone levels may be affected by the stress of labour
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 T levels throughout pregnancy	May only be applicable to female samples

Amniotic Fluid	No genetic disorder, maternal disease, or drug exposure	Cannot select a truly representative sample
		Will probably not reveal effects on behaviours that are organized late in pregnancy
	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 males and females	

CHAPTER 2: Foetal Testosterone, Social Relationships, and Restricted Interests in Children

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, presumably due to changing diagnostic criteria and greater awareness, it is still a relatively rare disorder. Autism spectrum conditions (ASCs) may occur as often as 1 in every 200 people (Scott et al. 2002). Only a small proportion of pregnant women will be asked to undergo amniocentesis. As explained previously, Addenbrooke's Hospital in Cambridge processes approximately 1000 amniocentesis samples every year. Based on population samples, we would estimate that of 4000 children whose mothers underwent amniocentesis approximately 20 would go on to be diagnosed with an ASC, assuming that 100% of those pregnancies resulted in healthy births. So in order to carry out a sufficiently powerful study of amniotic testosterone and ASCs a minimum of 4000 samples would be needed if all subjects were contactable, and all agreed to participate. To add the difficulties, autism is seldom diagnosed before age 3, 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. Currently, the Autism Research Centre in Cambridge has access to 3000 amniocentesis samples dating from 1996 to 2000. Later on, we will describe the progress that has been made in recruiting these potential participants and describe the characteristics of the families contacted thus far. However, we can begin investigating possible links between foetal testosterone and autism spectrum conditions in a less direct way.

It is increasingly suggested that autism is part of a spectrum of disorders 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 non-clinical groups are also important in the clinical population. As discussed earlier, autism can be viewed as an extreme manifestation of male typical traits. Specific behaviours of interest include the ability to read nonverbal communication signals (i.e.

body posture and facial expressions), language, and theory of mind (Geary, 1998, 2002) and skills which Baron-Cohen (2002, 2003) classifies as 'empathising' and 'systemising.' In the present study we examined the relationship between fT measured in fluid obtained at amniocentesis and scores on the children's communication checklist (CCC) (Bishop, 1998) in typically developing four-year-old children.

This instrument measures speech, syntax, pragmatic language skills, quality of social relationships, and restricted interests. The speech scale measures the intelligibility of speech and could be considered a measure of articulation. The syntax scale assesses basic grammar. Hyde & Linn (1988) argue from the results of a meta-analytic study that the effect sizes for sex differences in such basic language skills are so slight as to be nonexistent. Therefore we predicted no relation between these scales and fT. However, it should be noted that some authors suggest that in very young children, girls are superior in both areas (Kimura, 1999) There are no reported sex-differences on these scales of the CCC.

Pragmatic language skills have been relatively little studied in this age group. Pragmatic skills are intrinsically social. The ability to adapt one's speech to different listeners would be facilitated by using a theory of mind. In a sample of 6 year old twins, girls scored higher on the pragmatic composite of the CCC when completed by teachers. When completed by parents, girls scored higher on one of the subscales of the pragmatic composite (rapport), but not on the entire composite (Bishop and Laws, personal communication). Therefore we predicted that higher fT levels would be associated with poorer scores on the pragmatic language scale, although we were aware that the pragmatic scale included multiple components and this might reduce associations with fT.

The CCC includes the quality of social relationships and restricted interest scales so as to determine whether a diagnosis of autism should be considered. Given that autism is characterized by social deficits coupled with restricted interests and occurs far more often in males, we predicted that testosterone would affect these scales as well. In the

sample of 6 year old twins, there was a significant sex difference in restricted interests when parents completed the scale, but the scale was unreliable. No sex differences were seen on either scale when teachers completed the questionnaire (Bishop and Laws, personal communication) However, the quality of social relationships scale is comparable to instruments such as the "Social Cognitive Skills Questionnaire" (Scourfield et al., 1999; Skuse et al., 1997), which shows a small but consistent sex difference. Sex differences in restricted interests are suggested by studies of the AQ and SQ, as described earlier. Finally, girls with CAH perform more like males on the AQ (Knickmeyer et al., Submitted). This further suggests a role for fT in these areas. We predicted that fT would be negatively correlated to quality of social relationships and positively correlated to restricted interests.

Methods

Participants

Participants were 58 children (35 male, 23 female), age 4.0 to 4.25 years. 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. We chose to study these children at age 4 because children's social development has been well studied at this age (Bartsch & Wellman, 1995; Flavell, Miller, & Miller, 1993; Maccoby, 1990).

The final sample of 58 subjects represents those who responded from a larger sample of 103 families taking part in the Cambridge Child Development Study, a long-term project on the effects of foetal testosterone on child development (an attrition rate of 45%). All families were originally contacted during their baby's first year of life. Each of the 103 families had taken part in one or more of 3 follow-up projects (at 12 months, 18 months, and 24 months). Exact data on the causes of the attrition are unavailable, but may represent families moving out of the area and the relative difficulty of participating

in projects once mothers have returned to work and children are regularly attending pre-school.

Initial recruitment for the longitudinal study was carried out according to a multi-stage procedure. First medical records of 500 patients were screened. These represented all women who had undergone amniocentesis at the Rosie Hospital (Cambridge), West Suffolk Hospital (Bury St. Edmunds) and the Ipswich Hospital between June 1996 and June 1997. Subjects were excluded if the amniocentesis revealed a chromosomal abnormality, the pregnancy ended in miscarriage or termination, the child suffered neonatal or infant death, the child suffered significant medical problems after birth (for example requiring long stays in the Special Care Baby Unit), twin pregnancy, or if the relevant information was absent from medical records. Families could also be excluded due to exceptional circumstances (such as maternal illness or child in foster care). Of the 500 patients screened approximately 400 were included and 100 were excluded based on these criteria. If subjects were not excluded following medical records checks, a letter was sent to their GP briefly explaining the study. GPs were asked to indicate whether there was any subsequent reason why contacting the family would be inappropriate. Approximately 400 GPs were contacted of whom 250 gave consent and 150 either withheld consent, did not reply, or informed us that the patient was no longer registered. Finally, 250 families were invited to take part in the study. 112 families consented while 138 did not. In 9 of the consenting families the amniocentesis sample could not be traced, resulting in a final sample of 103. Figure 2.1 illustrates the process of subject recruitment and subject numbers involved at each stage (including participation in follow-ups).

Figure 2.1

Chart to illustrate the process of subject recruitment and subject numbers involved at each stage of the Child Development Project.
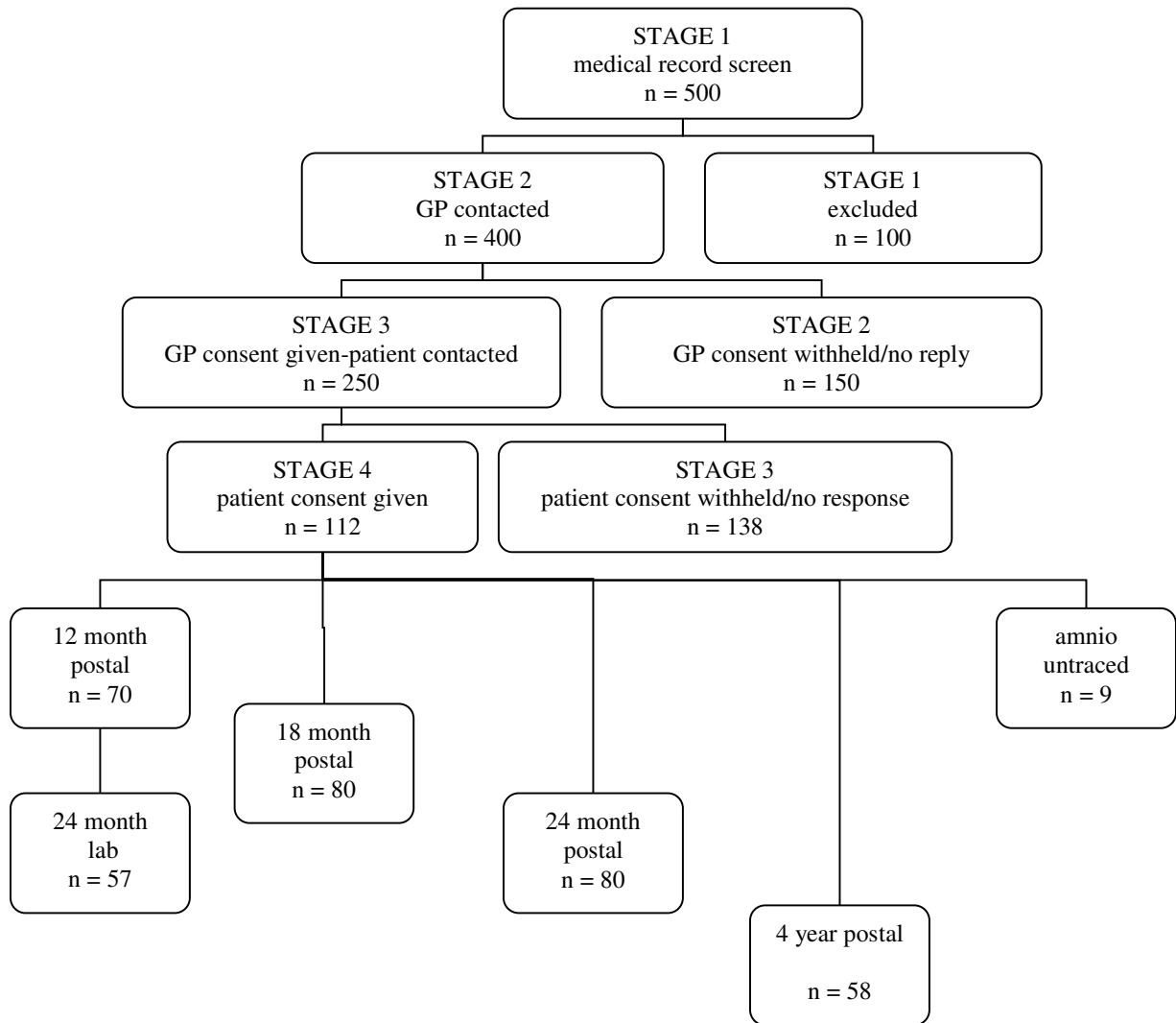
It must be kept in mind that this is an opportunity sample and not a random sample of the population. The majority of mothers were referred for amniocentesis based on late maternal age (25%) or high results on the triple test (indicating an increased risk for Down's syndrome) (60%). The remaining mothers underwent amniocentesis for several reasons including a family history of Down's Syndrome, maternal anxiety concerning the pregnancy, and soft markers for Down's or other chromosomal abnormalities seen on ultrasound scan. All amniotic samples tested negative for Down's and other chromosomal abnormalities. Children who have had amniocentesis show no evidence of decreased well being or impaired brain development (Finegan, Sitarenios, Bolan, & Sarabura, 1996).

Outcome Variable: The Children's Communication Checklist (CCC) (Bishop, 1998)

The Children's Communication Checklist (CCC) consists of 9 subscales. Scales A (speech) and B (syntax) measure traditional language skills. Scales C to G (inappropriate initiation, coherence, stereotyped conversation, use of context, and rapport) are combined to make a single pragmatic language score. Scale H measures the quality of social relationships. Scale I measures restricted interests. Table 2.1 shows the possible range of scores on each scale, scores indicating possible impairment and sample items. Note that the scoring is designed so that higher scores always correspond to 'better' performance. At present, reliability and validity for the CCC are restricted to 2 clinical studies. The first study looked at children aged 7 to 9, who have been identified as having language problems (Bishop, 1998). The second included children age 5 to 17 who had been diagnosed with either an autism spectrum condition, attention-deficit hyperactivity disorder, or specific language delay (Bishop & Baird, 2001). A small normally developing comparison group (31 children aged 6 to 16) was also included in the latter study. Alpha indices of internal consistency suggested that the items in each scale were homogenous. Parent-teacher inter-rater reliability ranged from 0.30 to 0.64 (Pearson's correlations), all of which were significant at the 0.01 level.

Table 2.1:

Score ranges and sample items from the Children's Communication Checklist (CCC)

Subscales	Range	Impairment	Sample item
(A) Speech	16-38	<27	1. people can understand virtually everything he/she says
(B) Syntax	24-32	<29	12. speech is mostly 2 or 3 word phrases such as "me got ball" or "give dolly"
Pragmatic composite	86-162	<132	
(C) Inappropriate initiation	18-30	<24	16. talks to any-one and everyone
(D) Coherence	20-36	<22	22. it is sometimes hard to make sense of what he/she is saying because it seems illogical or disconnected
(E) Stereotyped conversation	14-30	<24	33. often turns the conversation to a favourite theme, rather than following what the other person wants to talk about
(F) Use of context	16-32	<24	38. tends to repeat back what others have just said
(G) Rapport	18-34	<26	50. makes good use of gestures to get meaning across
(H) Quality of social relationships	14-34	<24	54. is popular with other children
(I) Restricted interests	20-34	<28	65. has one or more over-riding specific interests (e.g. computers, dinosaurs) and will prefer doing activities involving this to anything else.

Note: Higher scores correspond to "better" performance

The CCC was chosen for several reasons. Firstly we were interested in looking at the pragmatics of language. This is an area where sex differences have been reported (Maccoby, 1999), but the development of pragmatics in childhood has been relatively little studied. The CCC is one of the first instruments designed to explore pragmatics in childhood. Although its reliability and validity are so far restricted, the questions are applicable to anyone using complete sentences. Secondly, the CCC also covers "traditional" language skills, quality of social relationships, and restricted interests, all of which we were interested in. We could have used a different test for each area, but using a single instrument facilitates the comparison of results. Finally, the CCC was designed, in part, to distinguish children with semantic-pragmatic disorder from children with specific language delay or autism. As discussed earlier these conditions may represent extreme examples of typical sex differences. If scores on the checklist are related to fT it could have implications for understanding the sex ratio in these conditions.

Predictor variables

Foetal testosterone levels (fT) (nmol/L). The predictor of greatest interest in this study is foetal testosterone. Testosterone levels in amniotic fluid were measured by radioimmunoassay by the Department of Clinical Biochemistry, Addenbrooke's Hospital Cambridge, a method our group has reported previously (Knickmeyer, Baron-Cohen, Raggatt, & Taylor, 2005; Lutchmaya et al., 2002a, 2002b) (Appendix A). There were significant differences between male and female testosterone levels, mean (SD) = 1.04 nmol/L (0.40) and 0.40 (0.19) nmol/L for boys and girls respectively (see table 2.2), t (56) = 8.12, p = 0.00, d = 2.0. Note, d is an effect size index. It is calculated by dividing the difference in means for the two groups by the standard deviation. It provides a standardized measure of the magnitude of group differences that can be compared across samples of different size. A d of 0.2 is considered a small effect size. A d of 0.5 is considered a medium effect size. A d greater than 0.8 is considered a large effect size (Cohen, 1988). Equal variances were not assumed on any t-tests. The probability of a type I error was maintained at 0.05 for all t-tests. If the lowest fT levels in our sample

were near the detection limit of the assay (0.1 nmol/L), it would raise the possibility of a floor effect (particularly for girls). We further investigated the distribution of scores to determine whether this was the case. No girls had undetectable levels of fT. Only 2 girls (about 9% of the female sample) scored below 0.2 nmol/l, indicating that there was not a strong floor effect. However, the distribution of female scores was skewed to the left in comparison to the distribution of male scores. Transformation of scores was not necessary. There was also a degree of overlap between fT levels in boys and girls in this study, which raises the possibility that fT levels were declining in males. The mean fT levels in both males and females (1.02 and 0.39 nmol/l respectively) were slightly lower in our study than those in Finegan et al. (1989): 1.34 and 0.58 nmol/l respectively). The effect size for the sex-difference in fT was also lower in our study, $d = 1.87$ vs. $d = 2.7$. We also included the following control variables in our analysis.

Prenatal oestrogen levels (pmol/L). Oestradiol is the most biologically active oestrogen. In rodents it masculinizes the brain when it is synthesized in vivo via aromatization of testosterone and related precursors, so it is important to consider oestradiol when looking at the biological activity of fT. However, studies of individuals with complete androgen insensitivity syndrome and of girls exposed in utero to the synthetic oestrogen, diethylstilbestrol (DES), suggest that in humans, testosterone directly influences sexual differentiation without being converted to oestrogen (Hines, 2002; Hines, Ahmed et al., 2003). Amniotic oestradiol levels were also assayed by the Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge (Appendix A). There were no significant differences between oestrogen levels in males and females, mean (SD) = 932.55 (432.88) pmol/L and 1009 (424.63) pmol/L for boys and girls respectively (see table 2.2), $t (55) = -0.57$, $p = 0.57$, $d = 0.18$. Examination of the univariate distributions indicated that prenatal oestrogen level was positively skewed (skewness = 1.70) so a natural logarithmic transformation was carried out. This reduced the skewness considerably (skewness = 0.49). The transformed variable was used in all subsequent correlations and regressions. There were no significant differences between males and females when the transformed version of the variable was used, $t (55) = -0.64$, $p = 0.57$, $d = 0.12$.

Prenatal alpha-foetoprotein level (MU/L). Alpha-foetoprotein (AFP) is thought to be a general marker for severe foetal ill health and also provides a specific control for any unexpected abnormalities of amniotic fluid dilution (Wathen, Campbell, Kitau, & Chard, 1993). Amniotic AFP levels were also assayed by the Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge (Appendix A). There were no significant differences between AFP levels in males and females, mean (SD) = 10.42 (3.35) MU/L and 10.91 (2.82) MU/L for boys and girls respectively (see table 2.2), t (56) = -0.81, p = 0.41, d = 0.21.

Gestational age at amniocentesis (weeks). Levels of fT vary during gestation. Although amniocentesis occurs on average at week 16, it can occur as early as week 12 and as late as week 22. Therefore it was important to determine whether fT was related to gestational age in our sample. Gestational age at amniocentesis (as calculated from date of last menstrual period) was obtained from hospital records. There were no significant differences in gestational age between males and females, mean (SD) = 16.81 (1.55) weeks and 16.61 (1.11) weeks for boys and girls respectively (see table 2.2), t (47) = -0.68, p = 0.50, d = 0.19. Males showed no linear relationship between gestational age and fT, r (28) = 0.18, p = 0.37, and no quadratic relationship was apparent. However, for females a significant linear relationship was seen. The correlation between amniotic testosterone and gestational age in our sample was significant, r (22) = -0.54, p = 0.01. This is unexpected given that Reyes et al. (1974) report no change in foetal serum concentrations of testosterone for females during this period. One girl had a fT level more than 2 standard deviations above the mean and a gestational age more than 2 standard deviations below the mean. When this case was removed the correlation with gestational age was no longer significant, r (21) = -0.37 p = 0.10. This case was excluded from the regression analyses.

Sociodemographic variables. A range of sociodemographic variables were also included in this study because of their possible importance in determining the child's environment. Maternal age, maternal education level, and number of older siblings could

influence the amount and nature of interaction between children, their parents, and their peers. Young children's understanding of beliefs and feelings is influenced by their interactions with their mother and siblings (Dunn, Brown, Slomkowski, Tesla, & Youngblade, 1991). Maternal age was particularly important to include because women undergoing amniocentesis have a higher mean age than the general childbearing population. If a particular variable was related to maternal age within our group it would have implications for the wider applicability of our findings. Maternal education level was measured according to a 5 point scale: 1 = no formal qualifications, 2 = 'O' level / G.C.S.E. (A General Certificate of Secondary Education represents successful completion of exams after schooling to age 16) or equivalent, 3 = 'A' level, HND (A levels are generally in academic subjects while the Higher National Diploma is awarded for vocational subjects. Both usually represent schooling to age 18 + exams) or vocational qualification, 4 = university degree, 5 = postgraduate qualification.

<div align="center">Results</div>

Descriptive Statistics

The first set of analyses provided basic descriptive statistics. Examination of the univariate distributions indicated that several variables had distributions that deviated from the Gaussian distribution. Prenatal oestrogen level was positively skewed (skewness greater than 1) so a natural logarithmic transformation was carried out. The speech, syntax, and restricted interests scales of the CCC were negatively skewed (skewness less than -1). Skewness was confirmed with a Kolmogorov-Smirnov test. Scores were reflected and then a natural logarithmic transformation was carried out. We investigated the distribution of scores on these CCC subscales to determine whether this was due to a ceiling effect. For the majority of scales there was no significant ceiling effect. For the speech scale, only four percent of the children obtained the maximum score. For the restricted interests scale, only nine percent obtained the maximum score. The syntax scale did, however, show some ceiling effects: fifty-seven percent of children achieved the maximum score. Although it was not transformed, the quality of social

relationships scale did show some ceiling effects, with 30 percent of the children achieving the maximum score. The majority of the children did not achieve a maximum score. Transformation of the scale reduced skewness but increased kurtosis. Residual plots for the regression analyses were better when the untransformed variable was used.

Table 2.2 presents means, standard deviations, and ranges for each sex separately. Sex differences were tested for using a t-test and equal variances were not assumed. Because several scales of the CCC correlate with each other, both in our sample (see Table 2.3) and other reported samples, one would usually perform a correction for multiple comparisons. However, because of the sample size we did not have the power to perform these. Therefore we have reported the t-test results for the CCC scales in terms of confidence intervals instead of p values. This provides greater information on the statistical relationships observed. Males had more restricted interests, mean (SD) = 30.73 (2.30) and 32.09 (1.60) for boys and girls respectively (see table 2.2), CI = -0.21 to -0.015, d = 0.64. There was a trend for females to score better on quality of social relationships, mean (SD) = 32.38 (1.62) and 33.00 (1.00) for boys and girls respectively (see table 2.2), CI = -0.13 to 1.32, d = 0.47. The existence of sex differences on these scales indicates a possible role for fT. Therefore these scales were explored further. Speech, syntax, and pragmatics scores did not show sex differences, based on confidence intervals and effect sizes. Confidence intervals that do not include 0 are generally considered to be significant.

Examining the correlations between subscales, it is notable that within the group as a whole, quality of social relationships and restricted interests were correlated, r (52) = 0.40, p = 0.00. They were also correlated within the boys, r (31) = 0.44, p = .01. These scales are also significantly correlated in the mixed-sex sample reported by Bishop (Bishop, 1998), r(59) = 0.45 and r(51) = 0.65 for rater A and rater B, respectively. This raises the possibility that scores on both scales are related to a single factor.

Table 2.2

Means, standard deviations and ranges for outcome and predictor variables by sex

Variable	Girls N=23			Boys N=35			
	Mean	SD	Range	Mean	SD	Range	Cohen's d
Age	4.10	.10	4.0-4.25	4.12	.08	4.0-4.25	.22
fT** (nmol/l)	.40	.19	.17-.80	1.04	.40	.13-1.80	2.0
Gestational Age at Amnio	16.61	1.11	14-19	16.81	1.55	14-21	.19
AFP (MU/l)	10.91	2.82	6.17-19.70	10.42	3.35	3.10-17.90	.21
Oestrogen (pmol/l)	1009	424.63	496-1950	932.55	432.88	404-2630	.18
Maternal Age	34.36	4.70	23-40	35.47	4.61	25-43	.23
Paternal Age	36.80	6.91	25-53	36.96	5.79	29-52	.03
Maternal Education	3.13	.81	2-4	3.27	.83	2-4	.17
Number of Older Siblings	1.09	.92	0-3	1.10	1.01	0-3	.01
Speech	33.43	3.87	22-38	32.84	3.80	21-37	.15
Syntax	31.10	1.04	28-32	31.30	1.15	28-32	.18
Pragmatic Composite	145.28	7.95	130-158	145.97	6.41	131-157	.10
Quality of Social Relationships	33.00	1.00	31-34	32.38	1.62	29-34	.47
Restricted Interests*	32.09	1.60	29-35	30.73	2.30	25-34	.64

**Difference is significant at the 0.01 level (2-tailed).

* Difference is significant at the 0.05 level (2-tailed).

Note: d is an effect size index. It is calculated by dividing the difference in means for the two groups by the standard deviation. It provides a standardized measure of the magnitude of group differences that can be compared across samples of different size. A d of .2 to .4 is considered a small effect size. A d between .5 and .7 is considered a medium effect size. A d greater than .8 is considered a large effect size (Cohen 1988)

Table 2.3

Correlation matrix showing relationships between the Children's Communication
Checklist (CCC) scales (n=47-54)

	Speech	Syntax	Quality of Social Relationships	Restricted Interests	Pragmatic Composite
Speech					
Syntax	.64**				
Quality of Social Relationships	-.03	-.15			
Restricted Interests	-.00	-.19	.40**		
Pragmatic Composite	.23	-.31*	.25	.25	

Note: n varies due to missing data for some participants

Note: Correlations are Pearson correlations

* $p < 0.05$, ** $p < 0.01$

Relations between Outcome Variables and Foetal Testosterone Levels

Previous studies investigating fT measured in amniotic fluid have found quadratic relationships with some cognitive measures (Grimshaw, Sitarenios et al., 1995; Lutchmaya et al., 2002a). Examination of scatterplots suggested linear relationships as opposed to quadratic ones for our measures, so a hierarchical multiple regression analysis was used. In the first block, any predictor variable that correlated significantly with the outcome variable at $p < 0.2$ was forced into the model (as recommended by Altman (1991)). Suppressor variables were also included when possible; these were predictors that correlated highly ($p < 0.01$) with the other predictors in the model, but were not significantly correlated with the outcome variable (See Table 2.4 for correlations between all predictor variables). In the second block, the main effects of fT and child's sex were tested for inclusion with a stepwise analysis. In the third block, the interaction of sex and fT was tested for inclusion with a stepwise analysis. This would test whether boys and girls showed consistent differences in terms of the relations between fT and the CCC subscales. The entry criterion was $p < 0.05$; the removal criterion was $p > 0.1$.

For quality of social relationships the following predictors were entered: paternal age, gestational age (suppressor) and maternal age (suppressor). Inclusion of fT in the 2nd stage produced a significant *F* change, *F* change = 6.24, β = -0.46, $p = 0.02$. A main effect of fT was included in the model, while child's sex was excluded. This indicates that fT explains more of the variance than child's sex. This suggests that the sex differences seen in the scores are testosterone dependent. The interaction of fT and child's sex was also excluded from the model. This suggests that the relationship between fT and quality of social relationships is the same for boys and girls. Those with lower fT levels score higher on quality of social relationships. The only significant predictor in the final model was fT (see Table 2.5). Residual analysis showed acceptable plots and no outliers.

Table 2.4

Correlation matrix showing relationships between the independent variables for all subjects of both sexes (n=37-58)

	fT nmol/liter	gestational age at amnio	AFP (μmol/l)	oestrogen	maternal age	paternal age	maternal education	number of siblings
fT (nmol/l)								
gestational age at amnio	.09							
AFP (μmol/l)	.03	-.59**						
oestrogen	.15	-.07	.36**					
maternal age	.07	-.36*	.17	-.05				
paternal age	-.07	-.37*	.29	.04	.71**			
maternal education	-.08	-.26	.12	.04	.24	.29		
number of siblings	.01	-.12	.16	-.16	.37**	.20	.19	

Note: n varies due to missing data on some participants

Note: Correlations are Pearson correlations

* $p < 0.05$, ** $p < 0.01$

For restricted interests the following predictors were entered: gestational age, oestrogen, paternal age (suppressor) and AFP (suppressor). Inclusion of fT in the 2nd stage produced a significant F change, F change = 5.27, β = 0.41, p = 0.03). A main effect of fT was included in the model, while child's sex was excluded. This indicates that fT explains more of the variance than child's sex. This suggests that the sex differences seen in the scores are testosterone dependent. The interaction of fT and child's sex was also excluded from the model. This suggests that the relationship between fT and restricted interests is the same for boys and girls. Those with higher fT levels had more restricted interests. The only significant predictor in the final model was fT, although gestational age also approached significance (see Table 2.5). Residual analysis showed acceptable plots and no outliers.

Table 2.5

Final model of hierarchical regression analyses testing the contribution of sex and fT to scores on the CCC.

Dependent Variable	R^2	Predictors	B	SE B	Sig.
Quality of social relationships	.22	Constant	35.674	4.769	.000
		Gestational age at amnio	-.097	.191	.616
		Maternal age	.014	.093	.878
		Paternal age	-.021	.074	.784
		fT	-1.636	.615	.012*
Restricted interests[a]	.25	Constant	.806	.775	.305
		Gestational age at amnio	-.054	.027	.055
		AFP	-.020	.012	.118
		Oestrogen[b]	.265	.196	.185
		fT	.173	.069	.017*

[a]Scores were reflected and logged before analysis. [b]Scores were logged before analysis.

$p^* < .05$

Note: For quality of social relationships: power >0.60.   For restricted interests: power >0 .75.

The analyses did not indicate different relationships with fT and quality of social relationships or restricted interests for boys and girls. Also a main effect of sex was excluded as a predictor, but fT was included. This suggests that the sex differences seen in the scores are testosterone dependent. However, to further investigate whether the previous result might be due to a sex difference (not necessarily involving testosterone),

we analysed the relationship between these scores and fT within each sex. It should be kept in mind that this reduced the sample-size by half and therefore the power of the analysis.

For quality of social relationships no significant relationship with fT was observed for boys or girls, r (32) = 0.21, p = 0.24 and r (21) = -0.22, p = 0.34, respectively.

Within boys none of the background variables correlated significantly with restricted interests at $p < 0.2$. When fT was entered in the regression analysis, F change = 4.11, β = 0.35, p = 0.05 and the model explained 12.4% of the variance in restricted interests. Boys with higher fT levels had more restricted interests. Residual analysis showed acceptable plots and no outliers. Figure 2.1 shows a scatterplot of the relation between restricted interests and fT in boys. fT and restricted interest did not correlate in girls, r (21) = 0.29, p = 0.21, therefore a regression analysis was not performed for girls.

Figure 2.2

Scatterplot of the relation between fT and restricted interests in boys (n = 32)
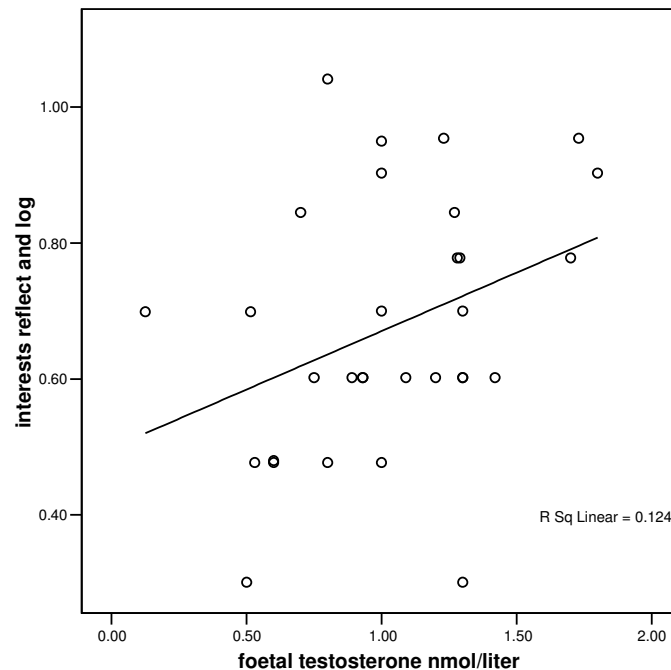
Discussion

This study tested for correlations between foetal testosterone (fT) and the 5 subscales of the Children's Communication Checklist (CCC) (Bishop, 1998). We predicted that no sex differences would be seen on the first two scales of the CCC, which measure non-social aspects of language skills. In contrast, we predicted that females would score better on pragmatic language abilities as measured by the pragmatic composite and on quality of social relationships and that this would be related to fT. We also predicted that boys would have more restricted interests and that this would be related to fT. In general our predictions were supported. No sex differences were seen on the first two scales of the CCC. There was a trend for girls to score better on quality of social relationships and this was related to fT levels in the group as a whole. There was a significant difference between boys and girls on the restricted interests scale. Boys

had more restricted interests and this was related to fT levels in the group as a whole. fT was also related to restricted interests when boys were examined separately. In addition, the study raises the intriguing possibility that quality of social relationships and restricted interests instead of being two independent dimensions as proposed (Baron-Cohen, 2002) may be controlled by a single factor.

Sex Differences and Pragmatic Language

There was a lack of any significant sex-difference on the pragmatic composite. It is possible that sex differences in pragmatic language are not present, at least in this age group and on this particular test. However, it is also possible that the analysis was underpowered. The sample size used is comparable to other studies using amniotic fluid, but might not be sufficient to detect differences on this scale. It should be noted that a significant sex-difference for the pragmatic subscale was observed in a sample of 6 year old twins when completed by their teachers (girls: mean (SD) 153.08 (6.9); boys: mean (SD) 146.91 (10.15); $t = 3.92$; $p < 0.0001$) (Bishop and Laws, personal communication). The difference in our sample may reflect a type II error in our study, but could also indicate an age effect. Sex-differences in pragmatic language may arise after age 4. Finally, the lack of relationship might reflect the fact that the pragmatic composite comprises a mix of cognitive skills.

Quality of Social Relationships and Restricted Interests

There was a trend for girls to score better on quality of social relationships and there was a main effect of fT on this scale when the group was examined as a whole (the analysis excluded a main effect of sex, or an interaction of sex and fT). This indicates that in both boys and girls, higher fT levels are associated with poorer quality of social relationships. Causal interpretations are, of course, unjustified from this correlational study, but the observed correlations are consistent with the hypothesis that fT diminishes social cognition or social interest in both boys and girls, resulting in poorer quality of social relationships. Another explanation would be that relations between fT and social

relationships are mediated by other variables, or that fT is serving as an index for an unknown third variable. It should be noted that in the sample of six year old twins reported earlier no sex differences were seen on this scale (Bishop and Laws, personal communication). However, significant sex-differences are seen on similar instruments such as the Social Cognitive Skills Questionnaire (Scourfield et al., 1999; Skuse et al., 1997) and the Social Responsiveness Scale (Constantino, Przybeck, Friesen, & Todd, 2000; Constantino & Todd, 2000). No significant relationships were seen when the sexes were examined separately, but this may be because the analysis was underpowered. A sample size of approximately 80 would be required to give the model a power of 0.6, assuming a similar effect size as was detected when both sexes were examined together. It would be necessary to run this experiment with a larger sample before drawing any strong conclusions.

Boys had significantly more restricted interests than girls. It should be noted that in the sample of six year old twins reported earlier no sex differences were seen on this scale (Bishop and Laws, personal communication). However, higher male scores on the imagination and attention scales of the AQ do suggest that males may have more restricted interests than females (Baron-Cohen et al., 2001). There was a main effect of fT on this scale when the group was examined as a whole (the analysis excluded a main effect of sex, or an interaction of sex and fT). This indicates that in both boys and girls, higher fT levels are associated with more restricted interests. This relationship was also seen when boys were examined separately. It is possible that the sample size was too small to observe a relation in girls (there were 35 boys in the sample, but only 23 girls). A sample size of approximately 200 would be required to give the model a power of .6, assuming a similar effect size as was detected in the boys. It is also possible that because the lower range of fT in this sample is close to the detection limit of the testosterone assay, that less variability is detectable in girls than boys. However, in the study by (Finegan et al., 1992) the lowest fT levels in girls were also close to the detection limit, but in that case, fT-behaviour relationships were seen in girls but not boys. We further investigated the distribution of scores to determine whether their might be a floor effect among the girls. No girls had undetectable levels of fT. Only 2 girls (about 9% of the

female sample) scored below 0.2 nmol/l, indicating that there was not a strong floor effect. However, the distribution of female scores was skewed to the left in comparison to the distribution of male scores. Transformation of scores was not necessary.

As with the observed relations between fT and social relationships, the relation between fT and restricted interests may indicate that fT increases restricted interests, but it is also possible that relations between fT and restricted interests are mediated by other variables or that fT is serving as an index for an unknown third variable.

The quality of social relationships and restricted interests scales were included in the design of the CCC in order to determine whether a diagnosis of autism should be considered (Bishop, 1998). As mentioned in the introduction, autism has been described as an extreme manifestation of some sexually dimorphic traits (Baron-Cohen, 2002). Our results are compatible with this framework. In our study, normal boys had a poorer quality of social relationships than normal girls. People with autism have even greater difficulty with social relationships. In our study, normal boys had more restricted interests than girls. People with autism have even more restricted interests. Our results suggest that high fT levels are associated with poorer quality of social relationships and more restricted interests, particularly in boys. Although we cannot extrapolate directly from this study to autism, the results strengthen our argument that it is worthwhile to explore whether fT is involved in the male vulnerability to autism.

Limitations

When evaluating our results, it is important to keep in mind that our sample of 4-year old, typically developing children is different to those populations for which published data on the reliability and validity of the CCC are available. Comparing the scores for our sample to the 31 normally developing controls reported in Bishop & Baird (2001), mean scores are very similar; the speech and syntax score are slightly lower; the pragmatic composite about 8 points lower. The speech and syntax scores were also slightly more variable in our sample. We realize that relying on maternal report has some

drawbacks, notably that different mothers may interpret items differently. An advantage of maternal report is that mothers have the opportunity to judge their children's skills in a variety of contexts over an extended period of time. A crucial part of pragmatic language is the ability to adjust speech for different contexts and speakers. Likewise social skills will vary widely depending on whether a child is relating to a parent, stranger, or a friend. To further test our hypotheses we performed a laboratory study of social cognition in this same group of children. The design and results of that study are described in the next chapter.

CHAPTER 3: Foetal Testosterone and Interpreting Behaviour in Terms of Mental and Affective States

Our study of the Children's Communication Checklist in a group of children whose fT levels were measured in amniotic fluid suggested that fT is associated with poorer quality of social relationships. To further test this apparent relationship we invited these children and their families for a laboratory test of social cognition.

Research on social cognition in autism spectrum disorders has primarily focused on Theory of Mind (ToM), the ability to attribute independent mental states to others and to oneself in order to explain and predict behaviour. ToM also constitutes an important component of the category of skills Baron-Cohen (2002, 2003) classifies as "empathising" in his extreme male brain theory of autism. The gold-standard laboratory test for ToM has been the false belief task, where a participant must be aware of a character's mistaken mental state (as opposed to the participant's own belief or reality) in order to predict that character's behaviour. False belief tasks have played an important role in characterizing the difficulties in social understanding typical of autism and in tracking the development of ToM in young children. However, false belief tasks also have a number of limitations.

Firstly, false belief tasks require additional capacities to ToM, including executive functions such as inhibition. Normal 3-year olds routinely fail false belief tasks despite evidence of social insight in real-life and other experimental tasks, and this may represent their inability to inhibit responses based on reality (Leslie & Thaiss, 1992). Russell, Saltmarsh, & Hill (1999) argued that children with autism may fail false-belief tasks for the same reason. Secondly, many high-functioning people with autism may pass standard false-belief tasks while still having serious real-life difficulties in recognising others' mental states (Frith, Happe, & Siddons, 1994). Interventions have been designed that attempt to teach ToM skills to individuals with autism. Although this training results in better performance on experimental ToM tasks it does not seem to increase social and communicative competency in real-life (Hadwin, Baron-Cohen, Howlin, & Hill, 1997;

Ozonoff & Miller, 1995). Most ToM tasks are presented as problems to be solved, with the problem explicitly identified. Cognitively able individuals with autism may be helped by this format, but in real life, social situations rarely present themselves as problems to be solved. Before deciding on the appropriate behaviour for a social situation, an individual must perceive the relevant social elements of that situation. Thirdly, most ToM tasks are dichotomous: either a response is ToM or non-ToM, correct or incorrect. This fails to capture the continuum of social ability (or difficulty) displayed by individuals with autism and typically developing individuals. The dichotomous nature of most ToM tasks also means they have a limited ability to detect subtle individual differences, including sex differences (Baron-Cohen et al., 1997) and differences related to variations in fT. Therefore, we chose to use a different type of task: one that explored children's capacity to spontaneously attribute social qualities to ambiguous visual stimuli.

This paradigm was first created by Hieder & Simmel (1944). They showed a film of two triangles and a circle moving within and around a rectangle and found that adults viewed the moving shapes as having goals and intentions, and that they described the moving shapes with personal and mental state terms. Springer, Meier, & Berry (1996) showed Heider and Simmel's film to 3, 4, and 5 year old children and found that character attributions were more differentiated in older than younger children. Mongomery & Montgomery (1999) showed that even 3-year olds can detect the intended goal of an animated shape, on the basis of a simple pattern of motion. Bowler & Thommen (2000) showed the original Heider and Simmel animation to children with autism and Asperger syndrome and controls. They found that the groups were equally able to distinguish intentional action from mechanical motion and used comparable amounts of propositional language for actions between animate agents (such language was infrequent in all groups). Abell, Happe, & Frith (2000) suggest that although the original Heider and Simmel animation does involve goal-directed action, it may not elicit complex mental state attribution and this may explain the lack of differences between groups in the Bowler and Thommen study. More recently, two independent groups have designed animation sequences specifically designed to elicit mental state attributions. Klin (2000) found that individuals with autism showed marked deficits on all seven

indices of social cognition on his Social Attribution Task (SAT), including the number of mental and affective state terms used. This is in keeping with the Baron-Cohen, Leslie, & Frith (1986) study, in which children with autism gave fewer mentalising descriptions than controls on a picture-sequencing task. Abell et al. (2000) found that although children with autism used mentalising description less often than normally developing 8-year olds when describing their computer-presented animations, they did so as often as children with general intellectual impairment. However, children with autism frequently referred to mental states that were inappropriate to the animation. No-one has examined whether there is a sex difference in the tendency to attribute social meaning to these films, but research on sex differences in social cognition suggests that a difference may be apparent.

Methods

Participants:

Participants were 38 typically developing children (24 male, 14 female), age 4.0 to 4.25 years, taking part in the Cambridge Child Development Study, a longitudinal study on the relations between foetal testosterone, assayed in amniotic fluid, and later social, communicative, and cognitive development. These children are a sub sample of the 58 children who took part in the study of the Children's Communication Checklist (CCC) discussed in the previous chapter. Many of the families in the sample live several hours from our testing centre and given the work schedules of the parents and the fact that all children are enrolled in various schools, travelling for testing is difficult and time-consuming. This meant that not all families who completed the CCC questionnaire by post were able to attend the Autism Research Centre for laboratory tests. See pages 44-47 for a review of how the Cambridge Child Development Study was originally ascertained and recruited.

Outcome Variable:

Computer-presented animations were provided by Fulvia Castelli, and were used in the studies by Abell et al. (2000) and Castelli, Happe, Frith, & Frith (2000). The animations showed one large red and one small blue triangle moving around a screen which contained a rectangular enclosure. One animation (Random) showed the triangles moving about purposelessly (bouncing off the sides) and not interacting with each other (this was chosen randomly from a possible set of 4). Two animations were designed to show ToM (these were also taken from a possible set of 4). One film showed the big triangle coaxing the little one out of the enclosure (see Figure 3.1). One showed the little triangle hiding behind a door and surprising the big triangle. These 2 films were chosen on the grounds that the social events they depicted would be familiar to young children. The 2 excluded films demonstrated seduction/bluffing and mocking; the social events depicted in these films would have been unfamiliar to most children the age of our participants. Sequences lasted 34 to 45 seconds each. Children watched the film once and were then asked what was happening. They were then asked to describe the film again as it was playing (to reduce the memory burden). For the random film only these initial descriptions were recorded. For the ToM films, after the first descriptions were given children were asked a series of questions designed to elicit more information and to encourage them to view the sequence in human terms. Figure 3.2 shows a sample transcript with the interviewer's questions and the child's response. The childrens' narratives were tape-recorded and then transcribed.

Figure 3.1

Stills from animation scripted as 'coaxing'

a) Mother tries to interest child in going outside   b) Child is reluctant to go out

c) Mother gently nudges child towards door   d) Child explores outside

e) Mother and child play happily together
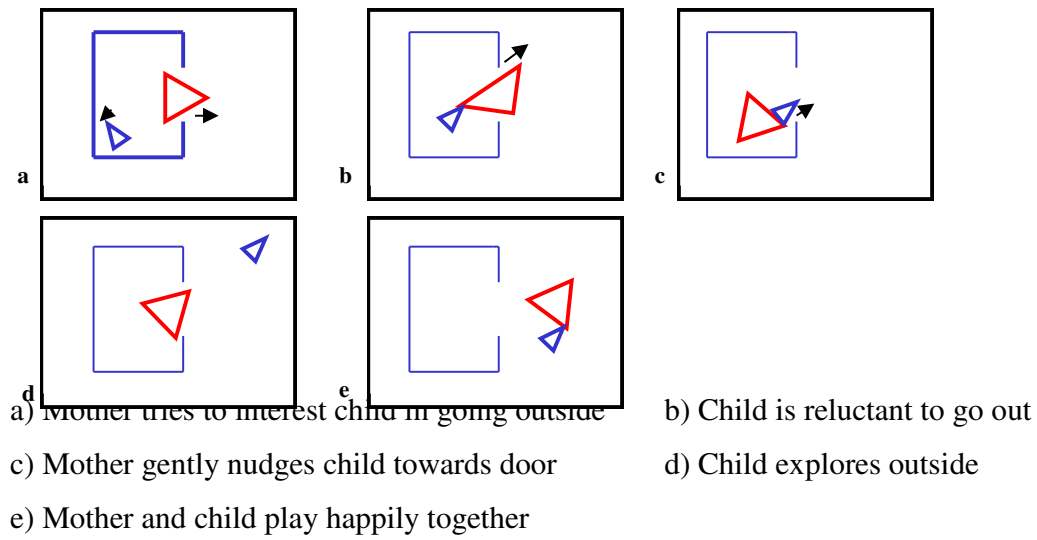
Figure 3.2

Sample transcript of a child's description of the Castelli animations

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Star Film:

1. **QUESTION:** Now tell me what you saw in the cartoon
   RESPONSE: triangles
2. **QUESTION:** Tell me what happened
   RESPONSE: they're dancing about

Coaxing Film:

1. **QUESTION:** Now tell me what you saw in the cartoon
   RESPONSE: twisting around

2. **QUESTION:** Tell me what happened
   RESPONSE: no response

3. **QUESTION:** Let's pretend the big triangle and the little triangle are people. If they were people, what were they doing?
   RESPONSE: walking

4. **QUESTION:** what do you think the people were feeling?
   RESPONSE: happy…cause they're going to see their friends

5. **QUESTION:** why is the big triangle pushing the little one?
   RESPONSE: to get him out

6. **QUESTION:** What are they doing now that they're outside the square?
   RESPONSE: no

7. **QUESTION:** what kind of person is the big triangle?
   RESPONSE: dad

8. **QUESTION:** What kind of person is the little triangle?
   RESPONSE: a little sister

Surprise Film:

1. **QUESTION:** Now tell me what you saw in the cartoon
   RESPONSE: no response

2. **QUESTION:** Tell me what happened
   RESPONSE: so the little one was knocking on the door and the big one came went down outside

3. **QUESTION:** Let's pretend the big triangle and the little triangle are people. If they were people, what were they doing?
   RESPONSE: playing so they have fun

4. **QUESTION:** what do you think the people were feeling?
   RESPONSE: pink (?)

5. **QUESTION:** Look the little triangle knocks on the square and then goes away-why do you think it does that?
   RESPONSE: so the big one doesn't know where he is

6. **QUESTION:** What are they doing now that they're inside the square?
   RESPONSE: they fight cause the big one and the tiny one is tricking the big one and the other one the big one got cross

7. **QUESTION:** what kind of person is the big triangle?
   RESPONSE: a dad

8. **QUESTION:** What kind of person is the little triangle?
   RESPONSE: a little sister

A rater, blind to the childrens' identities, counted the number of mental state terms and affective state terms used. Mental state terms included terms expressing one character's belief, desire, thought, imagination, intention, plan, motivation and behaviours that could not exist without shared cognition between characters (tricking, bullying, sneaking, hiding). Affective state terms included emotional terms (happy, sad, afraid) and behaviours that could not exist without a shared emotional state between the characters (cheering, hugging, kissing). Behaviours which were not uniquely human and implied (but did not necessitate) emotions were not included (fighting, playing). In order to control for the length of narrative, number of terms was divided by the total number of propositions used; a proposition is defined as a verb plus its complement. 22 of 38 transcripts were rated by a second rater. Inter-rater reliability for mental state terms was 0.88. Inter-rater reliability for affective state terms was 0.68. Inter-rater reliability for number of propositions was 0.98. We predicted that females would use more mental and affective state terms than males.

Each proposition was also defined as one of the types set out by Bowler and Thommen (2000). The resulting classes of events are undirected actions (Act), action between animates (ActA), action on the rectangle (ActR), relation with the rectangle (RelR), intentional (Int), and neutral (Neu). ActA and ActR can be considered a subset of what might be called 'intentional acts.' The category intentional refers to all intentional actions that do not fall into ActA or ActR categories. In practice this contained many propositions describing emotional states, beliefs, and desires. Bowler and Thommen (2000) found that individuals with autism made less reference to actions between animate objects (ActA) than controls. We therefore predicted that females would make more ActA propositions than males. We also predicted that females would make more Int propositions than males, if they are more likely to ascribe intentional actions to the stimuli in general. Table 3.1 provides examples of each proposition type. Number of each type of proposition was divided by the total number or propositions to control for length of narrative. Inter-rater reliability for each category is also presented in Table 3.1.

Inter-rater reliability was good to excellent, except in the case of relation to rectangle (RelR). This may reflect the difficulty in ascertaining whether the children were referring to the rectangular enclosure or the screen itself when describing the movements of the triangles.

Table 3.1

Examples of proposition types

PROPOSITION TYPE	EXAMPLE	RELIABILITY
undirected actions (Act)	He's messing about	0.79
action between animates (ActA)	The big one's trying to hit the little one	0.86
action on the rectangle (ActR)	He's closing it up	0.65
relation with the rectangle (RelR)	He went in the square	0.48
intentional (Int):	The triangle knew the way	0.74
neutral (Neu).	There's a small triangle	0.90

Predictor Variables

Predictor variables were the same is in the study of the Children's Communication Checklist, although because of the reduced sample size, we did not use variables where missing data would have reduced the sample size further: this included paternal age and maternal education.

Results

The first set of analyses provided basic descriptive statistics. Table 3.2 presents means, standard deviations, and ranges for outcome variables for each sex separately. Examination of the univariate distributions indicated that number of mental state terms, ActA, ActR, RelR, and Int were positively skewed. Kolmogorov-Smirnov tests indicated that only ActR was significantly skewed. ActR scores were logged. Where the original score was 0, the logged score was recorded as -2.00. Transformation reduced skewness, but increased kurtosis. Kolmogorov-Smirnov tests indicated that ActR was still significantly skewed. We investigated the distribution of scores to determine whether there were floor effects for any of the variables. ActR showed a strong floor effect with 50% of children not producing the category. The other categories showed small floor effects. For number of mental state terms 20% of children used no such terms at all. For ActA, 13.5% of children did not produce this category of proposition. For RelR, 22% of children did not produce this category. For Int, 17% of children did not produce this category. ActR was discarded from the subsequent analyses.

Sex differences were tested for using a t-test and equal variances were not assumed. Females used significantly more affective state terms than males, mean (SD) = 0.04 (0.05) and 0.09 (0.07) for boys and girls respectively, t (19) = -2.17, p = 0.04, d = 0.82. Males produced significantly more neutral propositions, mean (SD) = 0.45 (0.15) and 0.37 (0.10) for boys and girls respectively, t (33) = 2.01, p = 0.05, d = 0.63. There was a trend for females to produce more intentional propositions, mean (SD) = 0.06 (0.07) and 0.11 (0.09) for boys and girls respectively, t (21) = -1.90, p = 0.07, d = .62. Females also used more mental state terms than males, mean (SD) = 0.07 (0.07) and 0.11 (0.09) for boys and girls respectively, t (22) =-1.53, p = 0.14, d = 0.49. Although the t tests for this comparison does not meet traditional standards for significance, the effect size is medium (and quite comparable to the effect size seen in our earlier studies), suggesting that sex-differences would be apparent in a larger sample. Therefore these scales were explored further.

Table 3.2

Means, standard deviations and ranges for predictor variables by sex

Variable	Boys n = 25			Girls n = 14			
	Mean	SD	Range	Mean	SD	Range	Cohen's d
Number of Propositions	27.9	9.03	9-41	26	7.82	15-43	0.21
Mental State Terms	0.07	0.07	0.00-0.33	0.11	0.09	0.00-0.26	0.50
Affective State Terms	0.04	0.05	0.00-0.14	0.09	0.07	0.00-0.22	0.82*
Act	0.22	0.12	0.00-0.46	0.22	0.11	0.10-0.42	0.00
ActA	0.11	0.10	0.00-0.33	0.12	0.11	0.00-0.43	0.00
ActR	0.03	0.04	0.00-0.15	0.04	0.04	0.00-0.10	0.25
RelR	0.07	0.07	0.00-0.26	0.08	0.07	0.00-0.23	0.14
Int	0.06	0.07	0.00-0.33	0.11	0.09	0.00-0.26	0.62
Neu	0.45	0.15	0.24-0.89	0.37	0.10	0.19-0.53	0.63*

* Difference is significant at the 0.05 level (2-tailed).

Note: All variables except number of propositions represent total counts divided by the number of propositions.

Relations between Outcome Variables and Foetal Testosterone Levels

Hierarchical regression analysis was used to explore the contributions of the predictor variables to variation in the outcome variables. However, it should be kept in mind that with a sample size of 39, the analysis had limited power. In the first block, any predictor variable that correlated significantly with the outcome variable at $p < 0.2$ was forced into the model (as recommended by Altman (1991)). Suppressor variables were also included when possible; these were predictors that correlated highly ($p < 0.01$) with the other predictors in the model, but were not significantly correlated with the outcome variable (See Table 3.3 for correlations between all variables). In the second block, the main effects of fT and child's sex were tested for inclusion with a stepwise analysis. In the third block, the interaction of sex and fT was tested for inclusion with a stepwise analysis. This would test whether boys and girls showed consistent differences in terms of the relations between fT and the outcome variables. The entry criterion was $p < 0.05$; the removal criterion was $p > 0.1$.

Table 3.3

Correlation matrix showing relationships between the independent variables for all subjects of both sexes (n=35-53)

	Mental State Terms	Affective State Terms	Int	Neu	fT (nmol/L)	Child's Sex	Gestational Age	AFP (MU/L)	Oestrogen (pmol/L)	Maternal Age	Number of Siblings
Mental State Terms											
Affective State Terms	-0.07										
Int	0.70**	0.05									
Neu	-0.11	-0.24	-0.05								
fT (nmol/L)	-0.31	-0.14	-0.43**	0.23							
Child's Sex	-0.26	-0.24	-0.32	0.29	0.70**						
Gestational Age	0.09	-0.03	-0.13	0.29	0.10	-0.61**					
AFP (UM/L)	0.15	-0.02	0.32	-0.25	0.03	-0.03	-0.61**				
Oestrogen (pmol/L)	-0.09	0.05	0.21	0.11	0.15	-0.30	-0.03	0.36**			
Maternal Age	0.08	-0.01	0.07	-0.28	0.07	-0.35*	-0.35	0.17	-0.06		
Number of Siblings	0.17	0.07	0.01	-0.19	0.01	-0.13	-0.13	0.16	-0.16	0.37**	

Note: n varies due to missing data for some participants
Note: Correlations are Pearson correlations
* $p < 0.05$, ** $p < 0.01$

None of the background predictor variables correlated with either the number of mental state or number of affective state terms used at the $p < 0.2$ level (See Table 3.3 for correlations between all predictor variables). Therefore, in the first block, the main effects of fT and child's sex were tested for inclusion with a stepwise analysis. In the third block, the interaction of sex and fT was tested for inclusion with a stepwise analysis. For mental state terms neither sex or fT or the interaction of sex and fT met inclusion criteria for the model. For affective state terms, inclusion of sex in the first stage produced a significant F-change, F change = 6.09, β = -0.02, p = 0.02. This model explains 14% of the variance in frequency of affective state terms. Residual analysis showed acceptable plots and no outliers. Although our results did not suggest that fT played an important role in frequency of mental and affective state terms, because of the correlation between fT and sex, we cannot rule out a role for fT. To further investigate any potential relationship, we analysed the relationship between these scores and fT within each sex. It should be kept in mind that this reduced the sample-size by half and therefore the power of the analysis. Neither mental state terms nor affective state terms correlated significantly with fT within either sex, r (25) = 0.003, p = 0.99 and r (14) = 0.16, p = .58 for boys and girls respectively and r (25) = 0.03, p = 0.90 and r (14) = -0.18, p = 0.53.

For intentional propositions the following variables were entered: AFP, gestational age (suppressor) and oestrogen logged (suppressor). Inclusion of fT in the 2nd stage produced a significant F change, F change = 7.73, β = -0.45, p = 0.01. A main effect of fT was included in the model, while child's sex was excluded. This indicates that fT explains more of the variance than child's sex. This suggests that the sex differences seen in the scores are testosterone dependent. The interaction of fT and child's sex was also excluded from the model. This suggests that the relationship between fT and quality of social relationships is the same for boys and girls. Those with lower fT levels produced more intentional propositions. The only significant predictor in the final model was fT (see Table 3.4). Residual analysis showed acceptable plots and no outliers. To further investigate whether the previous result might be due to a sex

difference (not necessarily involving testosterone), we analysed the relationship between Int and fT within each sex. It should be kept in mind that this reduced the sample-size by half and therefore the power of the analysis. Within females there was little correlation between fT levels and Int, r (13) = -0.14, p = 0.64. Within males fT levels and Int did correlate and the relation approached traditional standards of significance, r (24) = -0.37, p = 0.07 (one tailed p = 0.04). Figure 3.3 shows a scatterplot of the relation between intentional propositions and fT in boys.

For neutral propositions the following variables were entered: gestational age, AFP, maternal age, and number of siblings (suppressor). Neither fT or sex or the interaction of sex and fT was included in the final model. None of the background variables included in the final model were significant.

Table 3.4

Final model of hierarchical regression analyses testing the contribution of sex and fT to use of intentional propositions

Dependent Variable	R^2	Predictors	B	SE B	Sig
Int	0.28	Constant	-0.211	0.344	0.54
		AFP (MU/L)	0.006	0.006	0.35
		Gestational Age	0.007	0.012	0.65
		Oestrogen (pmol/L)	0.07	0.083	0.42
		fT (nmol/L)	-0.08	0.030	0.01**

$*p \leq 0.01$

Figure 3.3

Scatterplot of the relation between intentional propositions and fT in boys (n = 24)
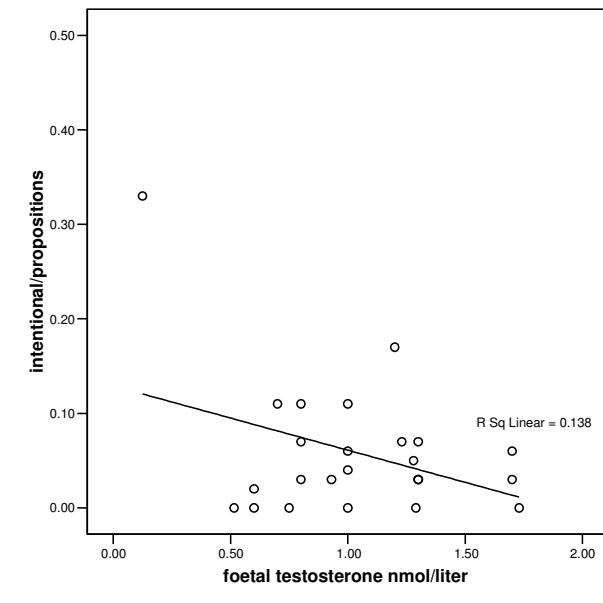
Discussion

In this study we examined whether fT was related to the tendency to interpret ambiguous visual stimuli in intentional and human terms. We showed typically developing children a series of films featuring shapes whose movements were designed to elicit theory of mind attributions and recorded the children's descriptions. We analysed their narratives for the frequency of mental and affective state terms and classified all the propositions in their narratives according to the criteria set out by Bowler & Thommen (2000). We predicted that females would use more mental and affective state terms than males. We also predicted that females would make more intentional propositions as classified by Bowler & Thommen (2000); this would include propositions describing actions between animates and all intentional propositions other than action between animates and action with the rectangle. We also predicted that variation in fT levels would account for the predicted sex differences. In general our

predictions have been supported. Our results are compatible with the extreme male brain theory of autism and provide some support for the hypothesis that fT is a risk factor for autism.

Mental and Affective State Terms

Females used mental state terms more frequently than males, but the relationship did not meet traditional standards of significance. The effect size for the relationship was medium ($d = 0.49$), according to Cohen's guidelines, and was similar to the effect sizes seen in our earlier studies. The majority of psychological studies demonstrate moderate effect sizes (i.e. $d = 0.5$) (Eagly, 1995). This suggests that the primary reason we did not observe a significant difference was insufficient sample size. Assuming that the mean difference seen in this sample accurately reflects the mean difference in the population, a sample size of approximately 80 would be required to give the model a power of 0.6. Neither child's sex nor fT was included in the final regression model. Once again, this may indicate that the analysis was underpowered. Ideally, regression analyses should include at least 50 individuals. Although we invited approximately 60 families to take part in the study, difficulties in attending the lab for testing meant that only 38 families participated. It should also be kept in mind that mental state terms were relatively infrequent, and that 20% of the children produced no such terms at all. In order to determine whether the films were effective at eliciting mental state attributions we compared the frequency of such terms in our sample to those found in other, similar, studies. Bowler & Thommen (2000) reported a near floor effect on mental state terms for all groups in their study using the original Hieder and Simmel film; suggesting that the film was not a good instrument for eliciting the use of mental state terms. For the category 'think/know' they reported mean numbers (sd) per group as 0.18 (0.71), 0.10 (0.32), 0.00, and 0.18 (0.60) for those with autism, chronological age matches, verbal mental age matches, and IQ matches respectively. For the category 'want' they reported mean numbers (sd) per group as 0.36 (0.84), 0.00, 0.36 (0.63), 0.09 (0.30) for those with autism, chronological age matches, verbal mental age matches, and IQ matches respectively. In contrast, in our study the mean mental state terms (includes both

think/know and want) for the entire group was 2.0 (1.7). Abell et al. (2000) reported mean number of mentalising descriptions as 1.73 (1.03) for typically-developing 8-year olds and 3.57 (0.65) for typical adults (note that the films we used are a subset of those used by Abell et al.). Klin (2000) reported 13.6 (10.7) for the cognitive index on their film. Comparison is difficult because each study used different methods and produced narratives of varying length. If we divide the reported mean for each study by the number of propositions (or in the case of Abell et al. (2000) the number of explanations), we find that at 0.08, the children in our study produced terms more frequently than the best scoring group in Bowler & Thommen (2000) (0.02), but far fewer than the typically developing groups in Abell et al. (2000) and Klin (2000); 0.32, 0.90, and 0.30 respectively. Abell et al. (2000) found that use of mentalising language increased from age 8 to adulthood. The children in our sample were age 4. It is possible that if the children were retested at a later age they would use a greater number of mental state terms. Sex-differences and relations to fT might be more apparent at that age.

Our results suggest that with a larger sample or at a later age, a sex-difference would be apparent. However, we cannot speculate on whether this difference would be related to fT. Sex differences may be produced by many factors, both biological and social. Within sex correlations of fT and mental state terms were not significant. It is true that the sample size was very small. However, significant results have been obtained in amniocentesis studies with similar sample sizes. Grimshaw, Sitarenios et al. (1995) found significant within-sex correlations for amniotic fT and mental rotation speed for both girls and boys, $r(12) = 0.67$ and $r(13) = -0.62$. r values were much smaller in the current study, suggesting that even in a larger sample no strong relationship between fT and mental state terms would be observed. However, sex differences in mental rotation are larger ($d = 0.9$) than those we measured for mental state terms. If the correlation of 0.16 in girls and 0.18 in boys seen in this study reflected an actual population correlation sample sizes of 110 and 150, for girls and boys respectively, would be needed for the model to have a power of 0.6. Even if within sex variations in fT do not contribute to differences in the frequency of mental state terms, this does not rule out a role for fT in

producing a sex-difference on this variable. fT may only exert its effects on mental state terms at very high doses.

For use of affective state terms a significant sex difference was seen, with girls using such terms more frequently than males. Sex was the only significant predictor in the final models of our regression analyses for both male and female items; fT was excluded from the analysis. Again, this may indicate that the analysis was underpowered. It is also possible that sex-differences in the use of affective state terms are the result of other variables, either biological or social. Dunn et al. (1991) reported that children who grew up in families in which they engaged in conversations about feelings and causality were better able to explain the feelings and actions of puppet characters when tested 7 months later. Their results are compatible with a role for such conversations in promoting conceptual development, although because the child is an active instigator and participant in such conversations, the relationship could also reflect a common underlying ability. Dunn, Bretherton, & Munn (1987) reported that mothers talk more about feelings to their daughters, although Fivush (1989) notes that this may depend on the type of emotion. None of the background variables in our study were significantly related to use of affective state terms, but the background variables are quite broad. When looking within sex, fT was not significantly correlated with scores on either scale and r values were low. Our results suggest that amniotic fT, measured at this period, does not account for individual variation in use of affective state terms, but because of the sample size issues we cannot rule out a role for fT in the differences seen between boys and girls.

In conclusion, our results for mental and affective state terms support the extreme male brain theory in that boys performed more like individuals with autism. Klin (2000) found that individuals with autism used fewer mental and affective state terms when describing films similar to the ones we used and Baron-Cohen (1989) found that children with autism used fewer mental state terms when performing a picture-sequencing task. It should be noted that Bowler & Thommen (2000) did not find a difference between individuals with autism and controls when describing the Heider & Simmel (1944) film, but this probably reflects the very low frequency of such terms in all groups. Abell et al.

(2000) found that children with autism did not provide fewer mental state explanations than controls with moderate learning disability, but they did find the children with autism were less accurate. We were unable to meaningfully test whether normally developing 4 year olds showed sex-differences in the accuracy of their descriptions, as overall accuracy was very low. Abell et al. (2000) classified accuracy for the ToM sequences according the following criteria.

Surprising:

2 Any mention of the little triangle tricking, surprising the big triangle; hiding; or playing hide and seek

1 Description which gives part of the story but misses the critical point (see above)

0 Description which only gives a minor part of action i.e. knocking on the door, or does not relate to any of the events in the sequence

Coaxing:

2 Description that conveys the child's reluctance to go out and the mother's attempts to get the child out, e.g. persuading

1 Partially correct description focusing on one aspect of the story or one character only, e.g. child does not want to go out; or, mother is pushing child to go out

0 Actions that do not relate to the events or relate to a minor aspect of the sequence only (e.g. dancing together) or unrelated description

Only 4 children in our sample achieved a score of 2 on the surprising sequence. No child achieved a score of 2 on the coaxing sequence.

Intentional Propositions

Bowler & Thommen (2000) found that children with autism were significantly less likely to use propositions describing actions between animates (ActA) than a variety of control groups. They interpreted this as reflecting a difficulty in perceiving the goal-directedness of an action when it is embedded in a complex, time-pressured context.

They suggest that for the child with autism, some-one engaging in an action is doing just that and the consequences and directedness of the action are less likely to be taken into consideration when describing an event. In keeping with the extreme male brain theory we predicted that boys would perform more like children with autism and produce less ActA propositions than girls. There was no significant sex-difference on this measure and the effect size was very small, indicating that even in a larger sample no differences would be apparent. This suggests that there are deficits in autism beyond those predicted by the extreme male brain theory. However, we did find a significant sex difference in the frequency of intentional propositions (Int), with girls using more than boys. These are intentional propositions not involving action with animates or action with the rectangle. In practice, these included many propositions describing mental and affective states, but also included a broader range of utterances. There was a main effect of fT on this variable when the group was examined as a whole (the analysis excluded a main effect of sex, or an interaction of sex and fT). This indicates that in both boys and girls, higher fT levels are associated with fewer intentional propositions. This relationship was also seen when boys were examined separately. It is possible that the sample size was too small to observe a relation in girls (there were 24 boys in the sample, but only 13 girls). A sample size of approximately 26 would be required to give the model a power of 0.6, assuming a similar effect size as was detected in the boys. It is also possible that because the lower range of fT in this sample is close to the detection limit of the testosterone assay, that less variability is detectable in girls than boys. See page 64 for a discussion of this potential problem.

The results of this laboratory study are consistent with the relationship between fT and quality of social relationships we observed using a maternal questionnaire measure in the previous chapter. Causal interpretations are, of course, unjustified from this correlational study, but the observed correlations are consistent with the hypothesis that fT diminishes social attributions in both boys and girls. Another explanation would be that relations between fT and social relationships are mediated by other variables, or that fT is serving as an index for an unknown third variable. The results strengthen our argument that fT may be involved in the male vulnerability to autism. It is also possible

that testosterone promotes a general "male vulnerability" (Kraemer, 2000). It is worth keeping in mind that testosterone is not the only factor that varies between males and females. Arnold (1996) suggests that there may be genetically triggered differences between males and females that do not involve hormone intermediates. Skuse (2000) for example, suggests that an imprinted gene on the X chromosome is responsible for the sex ratio in autism. It is also possible that there is an interaction between these postulated factors.

We also observed a significant sex difference for frequency of neutral propositions, with boys producing more of these than girls. Bowler & Thommen (2000) reported that children with autism did produce more neutral propositions, particularly in comparison with IQ matched controls, so this result is in keeping with the extreme male brain theory. They interpreted this as a pragmatic impairment. Although a few such statements are necessary to create a coherent story, children with autism peppered their accounts with these statements which describe non-action related aspects of the film. Neither sex or fT (or the interaction of sex and fT) was included in the final regression model. Again, this may indicate the analysis was underpowered.

Limitations

All amniocentesis studies are limited by the problems inherent in studying foetal endocrinology. We will fully discuss these limitations in the final chapter. Scarr & McCartney (1983) and others have also pointed out that there is no such thing as a purely biological or purely environmental contribution to behaviour. Addressing all potential factors and their interactions would require an immense sample size. Our focused, relatively small-scale studies are valuable in demonstrating that foetal testosterone potentially has long-term effects on social cognition, social relationships and restricted interests.

CHAPTER 4: Foetal Testosterone and Gender-typical Play

Thus far, we have examined whether foetal testosterone levels are related to skills that are of relevance to autism spectrum conditions. However, our sample of typically developing children whose mothers underwent amniocentesis is also a valuable resource for examining known sexually dimorphic behaviours and testing whether they are related to foetal testosterone. Such studies also provide insight into the strengths and weaknesses of the amniocentesis design. In this chapter, we report the first attempt to measure foetal testosterone (fT) and correlate it with gender-typed play in a normative sample of humans.

Play, in a variety of forms, exists in most mammals. Although many definitions have been proposed for play, the most frequently occurring theme is that play incorporates many physical components of adult behaviours, such as aggression, but without their immediate functional consequences. For example, during play fighting animals may chase each other, take up offensive and defensive postures, and mouth their opponent, but unlike serious fighting the activity is of a low intensity, the muscles are relaxed, and biting is inhibited (Aldis, 1975).

Primates have one of the longest developmental periods relative to their body size and lifespan (Aldis, 1975; Joffe, 1997). Comparative studies of primate species show that length of developmental period is related to the complexity of the species' social system and the size of the neocortex (Aldis, 1975; Dunbar, 1993; Joffe, 1997). This suggests that social factors contributed to the evolution of mind and brain and that a long developmental period is needed to practice and refine social skills (Geary, 2002). An extended developmental period is also related to enhanced tool use and a greater knowledge of local ecology in some species (Byrne, 1995; Geary, 1998). Play may be an important method by which individuals practice behaviours that are critical for survival and reproduction in adulthood. "Animals do not play because they are young, but they have their youth because they must play" (Groos, 1976).

Sex differences in play patterns are found in many species, including human beings. One of the most consistently found differences is in the nature and frequency of rough and tumble play. Rough and tumble play includes vigorous behaviours such as wrestling, grappling, kicking, and tumbling that would appear aggressive outside the playful context. Boys engage in rough and tumble play more often than girls in most cultures that have been examined (DiPietro, 1981; Humphreys & Smith, 1984; Pellegrini & Smith, 1998). This sex difference is also apparent in many other mammalian species, including mice and rats (Casto, Ward, & Bartke, 2003; Meaney & McEwen, 1986; Meaney & Stewart, 1981), savannah baboons (DeVore, 1963), hamadryas baboons (Kummer, 1968), and macaques (Harlow & Harlow, 1970; Symons, 1973). Rough and tumble play may have long-term benefits in preparing males for adult inter-male competition, but may also serve immediate purposes, such as the creation and maintenance of dominance hierarchies (Pellegrini & Smith, 1998).

Another sex difference, this one favouring girls, is in the frequency of play parenting (e.g. baby doll play). Play mothering by juvenile females is common among primates, including chacma baboons (Bolwig, 1959), pigtail macaques (Zehr, 1998), rhesus monkeys (Herman, Meaday, & Wallen, 2003; Lovejoy & Wallen, 1988), and vervet monkeys (Lancaster, 1976). Primate males may also display protective and affectionate responses towards infants, but this behaviour is more variable in pattern and extent (Lancaster, 1976).

Most of the research on sex-related differences in children's play has focused on cultural determinants of these differences, such as parental socialization (Eagly, 1987). Given the widespread occurrence of these differences in other species, it is appropriate to explore potential biological contributions as well. Evidence suggests that circulating hormones influence sex differences in adults, but this is unlikely to account for sex differences in childhood as the gonads are quiescent at this time and sex hormone levels are low. Therefore, it is appropriate to investigate prenatal influences, keeping in mind that such organizational effects may predispose individuals to behave in a certain way, but do not act independently of social and contextual influences.

The occurrence of rough and tumble play is related to prenatal or early postnatal exposure to male hormones in many mammalian species (Pellis, 2002; Young, Goy, & Phoenix, 1964). Testosterone implants into the amygdala during the neonatal period masculinize play in juvenile female rats (Meaney & McEwen, 1986). Conversely, exposure to antiandrogens such as vinclozolin and flutamide either during prenatal or early neonatal life, feminizes play behaviour in male rats (Casto et al., 2003; Hotchkiss, Ostby, Vandenbergh, & Gray, 2003). Female rhesus monkeys exposed to a long period of prenatal androgen or to a short period of androgen late in pregnancy show a dramatic increase in the amount of rough and tumble play. Exposing males to higher prenatal androgen levels had no effect (perhaps because their natural levels were already high). Suppressing testicular function after birth did not affect rough and tumble play in males, suggesting that this preference had its root in the prenatal period (Wallen, 1996).

Gender-typical play has been examined in individuals with classic congenital adrenal hyperplasia (CAH). Girls with CAH show increased play with boy's toys and decreased play with girl's toys when compared to matched controls or their unexposed female relatives (Berenbaum & Hines, 1992; Berenbaum & Snyder, 1995; Dittman et al., 1990; Nordenstrom et al., 2002; Servin et al., 2003; Zucker et al., 1996). In keeping with the belief that CAH boys are not exposed to elevated androgens prenatally, boys with CAH do not differ from their male relatives in play with boy's and girl's toys (Berenbaum & Hines, 1992; Berenbaum & Snyder, 1995). Girls with CAH also show less interest in infants and play less with dolls (Berenbaum & Snyder, 1995; Ehrhardt, Epstein, & Money, 1968; Leveroni & Berenbaum, 1998).

Studies of individuals with CAH provide some of the most compelling evidence that prenatal androgens have a lasting affect on human behaviour including gender-typical play. 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. As discussed previously, girls with CAH are also exposed to high levels of adrenocorticotropic hormone (ACTH) and treatment may result

in glucocorticoid excess (Berenbaum, 2001). In contrast to the androgen hypothesis, there is no clear theoretical reason why these changes would masculinize play behaviour. Masculinization appears to be correlated with degree of androgen excess (Nordenstrom et al., 2002; Servin et al., 2003). An additional potential confound is that parents may treat daughters with CAH differently because they are virilized at birth (Quadagno et al., 1977). However, there is no experimental support for this position (Berenbaum, 2001; Berenbaum & Hines, 1992; Dittman et al., 1990; Goy et al., 1988; Nordenstrom et al., 2002).

There is also evidence suggesting that fT levels affect play behaviour in boys. Boys exposed to the synthetic progestin medroxyprogesterone acetate (MPA) for at least 1 week during the second to eighth month of pregnancy showed some demasculinization and feminization of play on a scale derived from the CGPQ (Meyer-Bahlburg, Feldman, Cohen, & Ehrhardt, 1988). MPA decreases T levels and is known to reduce rough and tumble play in male rats (Birke & Sadler, 1983). Exposure to polychlorinated biphenyls (PCBs) also suppresses T levels (Hany et al., 1999; Kaya et al., 2002). In a study of Dutch school children, higher prenatal exposure to PCBs was related to less masculine play as assessed by the Pre-School Activity Inventory (PSAI) (Vreugdenhil, Slijper, Mulder, & Weisglas-Kuperus, 2002).

In this chapter, we report the first study to directly measure foetal testosterone and relate this to play behaviour in a normative sample of children. We measured gender-typed play via maternal report with a questionnaire adapted from the CGPQ. We predicted that boys and girls would show strong differences in their scores for male and female items. We also predicted, based on previous research in humans, that fT would be directly related to scores for male items and inversely related to scores for female items.

Methods

Participants

Participants were 53 children (31 male, 22 female), age 4.75 to 5.8 years, taking part in the Cambridge Child Development Study, a longitudinal study on the relations between foetal testosterone, assayed in amniotic fluid, and later social, communicative, and cognitive development. The final sample of 53 subjects represents those who responded from a larger sample of 103 families. See pages 44-47 for a review of how the Cambridge Child Development Study was originally ascertained and recruited. The majority of these children also took part in the studies reported in Chapters 2 and 3.

Outcome Variable

Mothers completed a modified version of the Child Game Participation Questionnaire (CGPQ). The original CGPQ (Bates & Bentler, 1973) shows highly significant gender-differences (Meyer-Bahlburg et al. 1985) and has been used in a previous study on gender-typed play and exposure to synthetic progestins (Meyer-Bahlburg et al, 1988) and on gender-typed play in CAH (Meyer-Bahlburg, Dolezal, S. W. Baker, Carlson, Obeid, & New, 2004). In order to make the questionnaire more manageable and increase response rate we created a pared down version (See Appendix B for full details on the development of the shortened version). The questionnaire included 10 masculine items, 10 feminine items, and 8 neutral items. For each game, mothers indicated their child's interest on a Likert scale where 1 was not at all interested and 5 was very interested. A total femininity score was calculated by adding together the score on each female item (1=0, 2=1, 3=2, 4=3, 5=4). A total masculinity score was calculated by adding together the score on each male item in the same way. The femininity and masculinity scores had a possible range of 0 to 40.

Predictor Variables

Predictor variables were the same as in the study of the Children's Communication Checklist, although paternal age was excluded because of missing data. Number of older siblings was removed and replaced with gender of older siblings, as several studies show that sex-typed behaviour may be influenced by the sex of a sibling, especially an older one (Abramovitch, Corter, Pepler, & Stanhope, 1986; Henderson & Berenbaum, 1997; Stoneman, Brody, & MacKinnon, 1986). For the variable "older brothers," children were coded 1 if they had elder brothers (regardless of number) and 0 if they had no elder brothers. For the variable "older sisters," children were coded 1 if they had elder sisters (regardless of number) and 0 if they had no elder sisters.

Results

Descriptive Statistics

Table 4.1 presents means, standard deviations, ranges, and gender effects sizes for outcome variables for each sex separately. Table 4.2 presents frequencies and percentages for the gender of older siblings. Males scored higher on the masculinity scale, mean (SD) = 28.45 (5.41) and 7.61 (6.04) for boys and girls respectively, t (41) = 12.7, p = 0.00, d = 3.6. Females scored higher on the femininity scale, mean (SD) = 10.7 (4.89) and 30.7 (7.14) for boys and girls respectively, t (34) = -11.1, p = 0.00, d = 3.2. The existence of very strong sex differences on these scales indicates a possible role for fT. Therefore these scales were explored further.

Table 4.1

Means, Standard Deviations and Ranges for Outcome Variables by Sex

| | Girls | | | Boys | | | |
| | $n = 22$ | | | $n = 31$ | | | |
Variable	Mean	SD	Range	Mean	SD	Range	d
Male Items[**]	7.61	6.04	0-20	28.45	5.41	18-40	3.63
Female Items[**]	30.7	7.14	16-40	10.7	4.89	1-19	3.27
Neutral Items[*]	32.7	3.96	22.5-40	29.4	4.50	20-37.5	0.77

[*] $p < 0.05$, [**] $p < 0.01$

Table 4.2

Number of children with older brothers and sisters

	Girls n = 22			Boys n = 31	
	Frequency	Percentage		Frequency	Percentage
Older Brother/s					
No	11	50		17	54.8
Yes	4	18.2		11	35.5
Missing	7	31.8		3	9.7
Older Sister/s					
No	10	45.5		16	51.6
Yes	5	22.7		12	38.7
Missing	7	31.8		3	9.7

Note: Chi-square was used to test whether girls and boys differed in the number of children having older brothers and in the number of children having older sisters, χ^2 (1, N = 43) = 0.69, p = 0.41 and χ^2 (1, N = 43) = 0.37, p = 0.54 respectively. Data on gender of older sibling was missing for approximately one third of the girls.

Hierarchical Regression Analyses

The first analyses explored the contribution of fT to scores on the masculinity and femininity scales. In block one, sex was entered. In block 2, fT was entered. In block 3, the interaction of fT and sex was entered. Table 4.3 summarizes the results of these analyses. For the masculinity scale, inclusion of sex in the 1rst stage produced a significant F change, *F* change = 161, β = -0.87, *p* = 0.00. This model explained 76% of the variance in masculinity scores. Inclusion of fT in the 2nd stage did not produce a significant F change, *F* change = 0.06, β = -0.02, *p* = 0.80. Examination of the beta weight sizes suggests that fT does not account for a significant proportion of variance not accounted for by sex. The inclusion of a sex by fT interaction in the 3rd stage also did not produce a significant F change, *F* change = 0.81, β = 0.24, *p* = 0.37. Examination of the beta weight sizes suggests that the interaction of fT and sex does not account for a significant proportion of variance not accounted for by sex. Residual analysis showed acceptable plots and no outliers. The only significant predictor in the final model was sex. However, to further investigate, we analyzed the relationship between the masculinity scores and fT within each sex. It should be kept in mind that this reduced the sample-size by half and therefore the power of the analyses. The masculinity scale did not correlate with fT levels in either boys or girls, *r* (31) = 0.00, *p* = 0.98; *r* (21) = -0.19, *p* = 0.40, respectively.

For the femininity scale, inclusion of sex in the 1rst stage produced a significant F change, *F* change = 134, β = 0.85, *p* = 0.00. This model explains 72% of the variance in femininity scores. Inclusion of fT in the 2nd stage did not produce a significant F-change, *F* change = 0.26, β = 0.05, *p* = 0.61. Examination of the beta weight sizes suggests that fT does not account for a significant proportion of variance not accounted for by sex. The inclusion of a sex by fT interaction in the 3rd stage also did not produce a significant F change, *F* change = 3.91, β = -0.57, *p* = 0.054. The only significant predictor in the final model was sex. However, in the 3rd stage, both fT and the sex by fT interaction approached significance, *p* = 0.058 and *p* = 0.054 respectively. Residual analysis showed acceptable plots and no outliers. We explored the potential interaction by analyzing the

relationship between femininity scores and fT within each sex. It should be kept in mind that this reduced the sample-size by half and therefore the power of the analysis. The femininity scale did not correlate with fT levels in boys, r (31) = -0.01, p = 0.94. In girls the femininity scale showed a relationship with fT in a direction opposite to that expected, but the relationship was not significant, r (21) = 0.36, p = 0.10.

Table 4.3

Summary of hierarchical regression analyses testing the contribution of sex and fT to
scores on male and female items

	Variable	B	$SE\ B$	β
Male Items				
	Step 1			
	Sex	-20.8	1.63	-0.87**
	Step 2			
	Sex	-21.2	2.23	-0.89**
	fT	-0.58	2.30	-0.02
	Step 3			
	Sex	-17.9	4.22	-0.75**
	fT	3.70	4.17	-0.15
	Interaction of Sex and fT	3.75	4.17	-0.24
Female Items				
	Step 1			
	Sex	19.6	1.70	0.85**
	Step 2			
	Sex	20.4	2.30	0.89**
	fT	1.22	2.38	0.05
	Step 3			
	Sex	13.3	4.23	0.58**
	fT	8.12	4.19	0.34
	Interaction of Sex and fT	-8.28	4.19	-0.57

Note. For Male Items: $R^2 = 0.76$ for Step 1 ($p < 0.01$); $\Delta R^2 = 0.00$ for Step 2 (*ns*); $\Delta R^2 = 0.00$ for Step 3 (*ns*). For Female Items: $R^2 = 0.72$ for Step 1 ($p < 0.01$); $\Delta R^2 = 0.00$ for Step 2 (*ns*); $\Delta R^2 = 0.02$ for Step 3 (*ns*).

$^* p < 0.05$, $^{**} p < 0.01$

Finally, we performed a hierarchical regression analysis that took into account the background variables we had measured. In block 1, any predictor variable that correlated significantly with the outcome variable at $p < 0.20$ was entered into the model (Altman, 1991). Suppressor variables were also included when possible; these were predictors that correlated highly, $p < 0.01$, with the other predictors in the model, but were not significantly correlated with the outcome variable (See Table 4.4). In block 2, sex and fT were tested for inclusion using a step-wise analysis (Entry criteria was $p < 0.05$; removal criteria was $p > 0.1$). In block 3, the interaction of sex and ft was tested for inclusion using a stepwise analysis (entry and removal criteria as above). Table 5 summarizes the results of these analyses. The only significant predictor in both final models was sex. Residual analysis showed acceptable plots and no outliers.

Table 4.4

Correlation matrix showing relationships between the independent and dependent variables for all participants of both sexes (n = 40-53)

Variable	Male Items	Female Items	Sex	fT	Estrogen[a]	AFP	Gest. Age	Mat Age	Mat Edu	Older Bro	Older Sis
Male Items											
Female Items	-0.75**										
Sex	-0.87**	0.85**									
fT	0.57**	-0.54**	-0.67**								
Oestrogen[a]	-0.01	0.05	-0.05	0.29							
AFP	0.10	-0.04	-0.01	0.11	0.35*						
Gest. Age	0.07	-0.13	-0.10	0.16	-0.10	-0.57**					
Mat. Age	0.27*	-0.20	-0.16	0.15	-0.14	0.18	-0.50**				
Mat. Edu	-0.04	-0.19	-0.02	-0.12	0.03	0.06	-0.33	0.34*			
Older Bro	0.19	-0.16	-0.11	0.13	-0.16	0.24	-0.05	0.22	0.01		
Older Sis	0.07	-0.12	-0.14	-0.04	-0.24	0.13	-0.30	0.33	0.29	0.13	

Note. *n* varies from 40-53 due to missing data from some participants
Note: Correlations are Pearson correlations
[a] Scores were logged prior to analysis
* $p < 0.05$, ** $p < 0.01$

Table 4.5

Final Model: hierarchical regression incorporating the contribution of background variables, maternal age and gestational age, to scores on male and female items

Variable	B	$SE\ B$	β
Male Items			
Gestational Age	0.38	0.75	0.05
Maternal Age	0.33	0.22	0.14
Sex	-20.4	1.84	-0.85[**]
Female Items			
Gestational Age	-0.78	0.76	-0.10
Maternal Age	-0.25	0.22	-0.11
Sex	18.3	1.87	0.83[**]

[*] $p < 0.05$, [**] $p < 0.01$

Discussion

This study confirmed very large sex differences for both the masculinity and femininity scales of the Children's Play questionnaire. For comparison, in Meyer-Bahlburg, Sandberg, Dolezal, & Yager's (1994) factor analytic study of the CGPQ in 6-10 year old children, the masculinity scale had a d of 1.90 and the femininity/preschool scale had a d of 1.67. The majority of psychological studies demonstrate moderate effect sizes (i.e. $d = 0.5$) (Eagly, 1995).

We also predicted that T levels measured in amniotic fluid would be related to scores for male and female items. However, sex was the only significant predictor in the final models of our regression analyses for both male and female items. Within sex fT was not significantly correlated with scores on either scale, and r values were low. Our results suggest that prenatal testosterone, measured at this period, does not account for individual variation in gender-typical play behaviour. There are several possible explanations for this, each of which reveals important factors to consider when investigating hormone-behaviour relations.

(1) T present at this time is related to gender-typical toy preferences, but amniotic testosterone is not a reliable proxy measure for prenatal testosterone exposure of the brain. Amniotic fluid studies of T make the assumption that amniotic levels are correlated with actual exposure levels, but there is no direct evidence to either support or contradict this assumption. It is important to note that in all existing studies, including the present one, hormones are assayed at a single timepoint. However, given that previous studies with this group have shown a relationship between amniotic T and sex-dimorphic variables such as frequency of eye contact (Lutchmaya et al., 2002a), vocabulary development (Lutchmaya et al., 2002b), and quality of social relationships (Knickmeyer et al., 2005), it seems likely that amniotic testosterone is an adequate proxy measure for actual exposure. These studies used similar analytical strategies to the present study and all showed significant sex differences, $d = 0.53$, $d = 0.66$, $d = 0.47$, and $d = 0.61$ respectively.

(2) T present at this period is related to gender-typical play, but the questionnaire we used did not accurately measure the children's behaviour. Relying on parental report has some drawbacks, including the possibility that different parents may interpret items differently. It is also possible that parents' reports reflected their expectations of their child's behaviour rather than the child's actual behaviour. Lytton & Romney (1991), in a meta-analytic study of differential socialization of boys and girls, found that parents in North American studies encouraged sex-typed activities, thus lending credence to the idea that parents would assume their child had sex-typical play. However, a study by

Meyer-Bahlburg, Erhardt, & Feldman (1985) showed that parental ratings were very similar to those produced when children themselves were given the CPGQ. It would be possible to test this hypothesis by using a laboratory observation of toy play in this group. Observational studies of girls with CAH indicate that parents do accurately represent their children's gender atypical behaviour (Nordenstrom et al., 2002).

(3) T present at this period does contribute to gender-typical toy preferences, but the effect is only detectable when the child is exposed to highly atypical levels of prenatal T. It is possible that fT contributes to the sex difference in toy preference, but is not detectable with the inter-individual variations that normally occur within sex. For example, an effect of fT on play behaviour is easily observed when a female foetus is exposed to the abnormally high levels of fT that occur in CAH or when a male foetus is subject to abnormally low fT. While the degree of masculinization and defeminization in girls with CAH is associated with severity of the disorder (Servin et al., 2003), we do not know how fT variations occurring in CAH girls compare to less extreme physiological variations that normally occur within either sex. Dosage effects could explain why studies of females with CAH show an effect on toy preference, but our amniotic fluid study did not. Grimshaw, Sitarenios et al. (1995), in their study of amniotic T and mental rotation, reported no relationship between fT and spatial play. Several studies of play in opposite-sex twins have also yielded paradoxical results. The rationale for opposite-sex twin studies comes from experiments in rats: female rats adjacent to male rats in utero are masculinized (Clemens, 1974), possibly by T diffusing across the amniotic membrane (Fels & Bosch, 1971) or being carried through the maternal circulation (Meisel & Ward, 1981). Human twin pregnancies are very different than multiple offspring pregnancies in rats, but T may transfer from the male to the female foetus through amniotic diffusion also in humans (Resnick et al., 1993). The foetal skin is permeable to fluid and some dissolved solutes up to week 18 of gestation, and amniotic fluid moves through the entire foetoplacental unit (Abramovitch & Page, 1972; Brace & Resnik, 1989; Findlay, 1984). Human females with male co-twins have been reported to be masculinized with regards to sensation seeking (Resnick et al., 1993) and spatial ability on a mental rotation task (Cole-Harding et al., 1988). However, studies of play preferences in opposite sex twins

show no effect (Henderson & Berenbaum, 1997; Rodgers et al., 1998). Perhaps the level of fT passed from male twins to female co-twins is not sufficient enough to produce changes in play.

(4) The strength of the relationship between fT and outcome may vary for different behaviours. Differences in the effect size of fT have important implications for sample size. If the effect of normal variation of fT on play is very small, then only very large studies will reveal it. Most amniocentesis studies are based on relatively low sample sizes (55 in this study, 60 in Grimshaw, Sitarenios et al. (1995)), but have consistently found significant relationships. Lutchmaya et al. (2002b) examined vocabulary size at 18 months in 87 children (40 girls and 47 boys). fT was a significant predictor with a β of 0.6; sex was also a significant predictor with a β of 1.3 (using a backward stepwise regression analysis). In contrast, in the current study beta weight sizes for fT were much smaller. Grimshaw, Sitarenios et al. (1995) found significant within-sex correlations for fT and mental rotation speed for both girls and boys despite small sample sizes, r (12) = 0.67 and r (13) = -0.62. Our within sex correlation analyses had sufficient power (greater than 0.80) to detect a similar effect size to that seen in Grimshaw, Sitarenios et al. (1995) but would have had reduced power in detecting smaller effect sizes. Our multiple regression analysis had sufficient power (at least 0.80) to detect medium and large effect sizes (as defined by Cohen (1988)). A sample size of 250-500 would be needed to detect small effect sizes. Therefore, we cannot rule out a small effect of fT. However, the extremely low β and r values seen in the current study, suggest that even in a larger sample no stronger relationship between fT and play would be observed.

(5) fT contributes to gender-typical toy preferences, but does so at a different time period than that examined in this study. Various sex dimorphic behaviours have different organizational periods, i.e. the restricted temporal period of development during which brain tissues that mediate a given behaviour can be modified. It is possible that the sensitive period for gender-typical play occurs later in pregnancy than the time our amniotic fluid samples were taken. This would fit in with the observation that female

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rhesus monkeys show a dramatic increase in rough play when exposed to a long period of prenatal androgen (≥35 days) or to a short period of androgen late in pregnancy (days 115-139), but not to a short period of androgen early in pregnancy (days 40-64) (Goy, 1978, 1981; Goy et al., 1988). Children with CAH would be exposed to high levels of T throughout pregnancy, and thus would be exposed regardless of when the critical prenatal period for gender-typical play occurs in humans. Although weeks 8 to 24 have been considered the most important in human sexual differentiation (Abramovich & Rowe, 1973; Finegan et al., 1989; Hines, Golombok et al., 2002; Udry, 2000), sex differences in T levels have been reported at birth (Forest & Cathiard, 1975; Forest et al., 1974). There is also a surge in T in males during months 1-5 of the neonatal period (Chemes, 2001; Forest et al., 1974). The behavioural effects of this surge are currently unknown. It is possible that gender-typical toy preference is related to neonatal T levels as opposed to prenatal levels. In Berenbaum & Snyder's (1995) study of play in children with CAH, the median age at diagnosis for girls was 7 days, well before the neonatal surge. However, age at diagnosis ranged from 0 days to 5.3 years. A more recent study (Nordenstrom et al., 2002) separated girls with extremely late diagnoses (3-6 years) from girls diagnosed during the neonatal period and examined these separately, although they did not specify when they considered the neonatal period to end. This study supported both prenatal and postnatal effects of androgens. Berenbaum, Duck, & Bryk (2000) also examined prenatal versus postnatal androgen excess and found that sex-atypical play was associated with inferred prenatal exposure, but not with early inferred postnatal exposure, but note that androgen levels were not directly measured. By examining our results in the context of experimental studies in rhesus monkeys (Goy, 1978, 1981; Goy et al., 1988) and studies of CAH which compare prenatal and postnatal exposure (Nordenstrom et al. 2002; (Berenbaum et al., 2000), we can narrow down the critical period for gender-typed play to later pregnancy.

(6) fT does not contribute to the development of gender-typical toy preferences. The changes in play behaviour observed in CAH may be the result of other characteristics of the condition or because parents treat CAH daughters virilized at birth differently (Quadagno et al., 1977). There are clearly many potential factors, both biological and

social that could produce sex differences in play. As the Lytton & Romney (1991) study shows, parents encourage their children to use sex appropriate toys. Children recognize "appropriate" toys and roles at an early age and emulate the behaviour of same-sex models in preference to opposite-sex ones (Greif, 1976). As discussed earlier, there is currently no empirical support for any one factor, or the way they potentially interact (Berenbaum, 2001; Berenbaum & Hines, 1992; Dittman et al., 1990; Goy et al., 1988; Nordenstrom et al., 2002). Reviews of other variables which may explain gender-typed play can be found in (F. P. Hughes, 1991; Powlishta, Sen, Serbin, Poulin-Dubois, & Eichstedt, 2001). These include peer influences, societal/media influences, and other biological factors.

In conclusion, although we found little evidence that normal variation in prenatal testosterone levels is related to gender-typical play, we would be reluctant to dismiss prenatal hormone influences altogether, particularly considering the well-replicated findings in CAH and the results reported by Hines, Golombok et al. (2002). Instead, our study draws attention to the complexity of hormonal influences on behaviour and highlights the need to consider dose and timing of exposure.

CHAPTER 5: Gender-typical Play in Children with Autism

The extreme male brain theory of autism (EMB) states that autism is an exaggeration of typical sex differences. However, it is unlikely that all sexually dimorphic behaviours are of relevance to autism. For example, given the evidence for a strong biological and genetic component to autism (Stodgell et al., 2001), one would not assume that sex differences caused purely by the social environment would be exaggerated in autism. Equally, it is unlikely that all of the symptoms of autism are present to a lesser degree in anyone with a male brain type. Some symptoms of autism, such as hypersensitivity to sound, are probable independent of brain type. The exact subset of sexually dimorphic skills relevant to autism has yet to be defined.

As originally formulated the EMB stated that the "male" brain had stronger folk physics than folk psychology, while the "female" brain had stronger folk psychology than folk physics (Baron-Cohen & Hammer, 1997). Subsequently, the distinction was reformulated in terms of empathising and systemising ability (Baron-Cohen, 2002). Both these abilities are rather broadly defined and it is not always clear how some well-replicated sex differences fit into this conception. In addition, the EMB theory does not rule out the possibility that sex differences not related to empathising and systemising may also be important in autism. Discovering the subset of sexually dimorphic behaviours that are exaggerated in autism could provide a window into the causes of the condition. The group of sexually dimorphic behaviours relevant to autism should share a common cause: either location in the brain, timing of development, common genetic origins, or a developmental sensitivity to a specific chemical. Although the original EMB defined the "male" and "female" brain psychometrically, it has since been suggested that increased levels of prenatal androgens may produce excessive masculinization of the brain and thereby increase the risk for autism spectrum conditions.

As discussed in the previous chapter, gender typical play represents one of the best replicated sex differences studied so far. The effect sizes are extremely large compared to the majority of effect sizes observed in the study of sex differences and in

psychology in general. It is evident on questionnaires, whether they are completed by parents or children themselves, and in laboratory and naturalistic conditions. Studies of individuals exposed to abnormally high or low levels of androgen as a result of genetic disorders (notably CAH) and exposure to synthetic progestins have indicated a role for prenatal androgens in the development of sex-typical play. In this chapter, we report a study examining gender-typical play in children with autism spectrum conditions. Our data extends previously studied relationships between sexually dimorphic human behaviour and autism to include sex-typical play and tests the hypothesis that prenatal masculinization of the brain is a risk factor for autism spectrum conditions.

Methods

Participants

66 children (20 female, 46 male) diagnosed with an autism spectrum condition (ASC) participated in the study. All families were members of the Cambridge Autism Research Centre Volunteer database. Families join the database through the Centre's web-page (http://www.autismresearchcentre.com/volunteers/default.asp). Individuals have been directed to the website by the National Autistic Society (UK), specialist clinics carrying out diagnostic assessments, and adverts in newsletters/web-pages for people with autism. To qualify as a volunteer a diagnosis must have been made by a suitably qualified professional, such as a psychiatrist or psychologist using established criteria for autism spectrum disorders (APA, 1994). For the current study we contacted all families in the database who had a girl, between the ages of 5 and 14, who was diagnosed with an autism spectrum condition (ASC). The families were sent invitation letters along with consent forms and a copy of the play questionnaire. The families of 76 girls with an ASC were contacted and 20 families returned the questionnaire (a response rate of 26%). We then contacted families of 102 boys with an ASC who were similar to the contacted girls in age and diagnosis. 46 families with a diagnosed boy returned the questionnaire (a response rate of 46%). We based the sample size on the number of girls available because we expected to see a stronger EMB affect in girls then boys. This was because

typical males have a high preference for male games and a low preference for female games, resulting in limited power for demonstrating an EMB effect in males with autism. Of the females, specific diagnoses were available for 19: 7 were diagnosed with Asperger syndrome (AS), 8 with autism, 1 with high-functioning autism (HFA), 1 with atypical autism, and 1 with pervasive developmental disorder not otherwise specified (PPD-NOS). Of the males, specific diagnoses were available for 41: 12 were diagnosed with AS, 27 with autism, 1 with HFA, and 1 with PDD-NOS. Rates of specific diagnoses did not differ between responders and non-responders.

55 typically developing children (24 female, 31male) acted as controls; all typically developing children were drawn from our longitudinal study of foetal testosterone and cognitive development (these are the same children discussed in Chapter 4). Participants ranged in age from 4.58 to 14.17 decimal years mean (SD) = 7.92 (3.00) for the entire group and 9.92 (2.06), 5.16 (0.28), 10.42 (2.12), 5.08 (0.28) for girls with an ASC, girls without an ASC, boys with an ASC, and boys without an ASC, in order). Children with autism were significantly older than children without autism, t (67) = -19.57, p = 0.00, d = 3.45. When completing the children's play questionnaire, all parents of children with autism were asked to describe their child's choices at age 5.

Outcome Variable

Mothers completed a modified version of the Child Game Participation Questionnaire (CGPQ) (Bates & Bentler, 1973). This is the same questionnaire as used in the study described in Chapter 4 (See Appendix B for full details on the development of this questionnaire). The questionnaire included 10 masculine items, 10 feminine items, and 8 neutral items. For each game, mothers indicated their child's interest on a Likert scale where 1 was not at all interested and 5 was very interested. A total femininity score was calculated by adding together the score on each female item (1=0, 2=1, 3=2, 4=3, 5=4). A total masculinity score was calculated by adding together the score on each male item in the same way. The femininity and masculinity scores had a possible range of 0 to 40.

Analyses of Variance (ANOVA) was used to examine the effects of sex (male, female) and diagnosis (ASC, typical control) on masculinity and femininity scores. In addition, planned comparisons were used to evaluate specific hypotheses. Based on the Extreme Male Brain Theory we predicted that females with an ASC would score higher than typical females on the masculinity scale and lower than typical females on the femininity scale. There was a possibility that this would result in a complete change of preference with masculinity scores being higher than femininity scores We predicted that males with ASCs would show the same pattern of preferences as typical males (with higher masculinity scores than femininity scores). Because typical males already show high scores on the masculinity scale and low scores on the femininity scale, there was limited power for demonstrating an EMB effect in males with autism. We predicted that males with autism would either show no difference to normal males, or would score even higher on the masculinity scale and lower on the femininity scale. Equal variances were not assumed on any t-tests. The probability of a type I error was maintained at 0.05 for all t-tests.

Results

Mean scores from each group are displayed in Table 5.1. ANOVA on the femininity scale demonstrated a main effect of sex, $F(1,119) = 167.5$, $p = 0.00$ (females scoring higher than males); a main effect of diagnosis, $F(1,119) = 93.1$, $p = 0.00$ (typical controls scoring higher than people with ASCs); and an interaction of sex and diagnosis, $F(1,119) = 12.4$, $p = 0.00$. Planned comparisons demonstrated that females with ASCs had significantly lower scores than typical females on the femininity scale, $t(32) = 5.72$, $p = 0.00$. Males with ASCs also had significantly lower scores than typical males on this scale, $t(49) = 7.03$, $p = 0.00$. Both these results are compatible with the extreme male brain hypothesis.

ANOVA on the masculinity scale demonstrated a main effect of sex, F (1,119) = 97.3, p = 0.00 (males scoring higher than females); a main effect of diagnosis, F (1,119) = 52.9, p = 0.00 (typical controls scoring higher than people with ASCs); and an interaction of sex and diagnosis, F (1,119) = 41.5, p = 0.00. Planned comparisons demonstrated that females with ASCs did not score differently than typical females on the masculinity scale, t (38) = 0.54, p =0.60. Males with ASCs scored significantly lower than typical males on this scale, t (73) = 11.7, p = 0.00. These results do not provide support for the extreme male brain hypothesis

Table 5.1

Mean, standard deviation and range for male and female items by sex and diagnosis

			Mean	SD	Range	N
Male Items						
	Controls					
		Boys	28.3	5.68	18-40	31
		Girls	7.61	6.04	0-20	23
	Diagnosed					
		Boys	10.9	7.31	0-34	46
		Girls	6.55	6.82	0-26	20
Female Items						
	Controls					
		Boys	10.2	5.21	1-19	31
		Girls	30.5	7.22	16-40	24
	Diagnosed					
		Boys	2.65	3.62	0-13	20
		Girls	14.3	10.8	0-36	46

The extremely low scores for individuals with ASCs on both scales suggested that there was an overall lower level or variety of play in children with autism and this might conceal any changes relating to the extreme male brain. Children with autism are known to have restricted interests and stereotyped behaviours (Frith, 1989; Happe, 1996; Hutt & Hutt, 1968; Piven, Palmer, Jacobi, Childress, & Arndt, 1997). We hypothesized that this might lead to low scores on the CGPQ. To test this possibility we compared scores on the neutral items of the questionnaire. A total neutral score was calculated by adding together the score on each neutral item (1=0, 2=1, 3=2, 4=3, 5=4). The neutral score has a possible range of 0 to 32. Children with ASCs did have lower scores on the neutral

items, t (113) = 8.10, p = 0.00. However the mean difference in scores was only 6.83. In contrast, the mean difference between females with and without ASCs on the female items was 16.2 and the mean difference between males with and without ASCs on the male items was 17.4. This suggests that the low masculinity and femininity scores cannot be accounted for by an overall lower participation in multiple games. One notable difference between the neutral items on the questionnaire and the male and female items is in the number of games requiring pretence. None of the neutral games required pretence, while approximately half of the female and male games did require pretence. Children with autism participate in less pretend play than typical children (Frith, 1989; Happe, 1996; Jarrold, Boucher, & Smith, 1993). A repeated measures analysis was run with gender of item (male, female) and pretence of item (pretence, nonpretence) as within subjects factors and child's sex (male, female) and diagnosis (ASC, typical control) as between subjects factors. The following items were classed as requiring pretence (1,2,4,5,6,11,12,17,19,26,28); neutral items were not included in the analysis; all other items were classed as non-pretence (See Appendix B for full details on the adapted CGPQ). See Table 5.2 for means.

The repeated measures analysis showed a main effect of pretence, F (1,116) = 44.2, p = 0.00, with children, on average, participating in more games that did not involve pretence. There was a significant 2-way interaction between pretence of item and diagnosis, F (1,116) = 18.1, p = 0.00. Paired t-tests showed that individuals with ASCs had a strong preference for items that did not involve pretence, t (65) = -9.71, p = 0.00. Typical controls did not show a significant preference for items that did not involve pretence, although the direction of their preference was the same as the group with ASCs, t (52) = -1.50, p = 0.14. There was a trend for male items to be more popular than female items, F (1,116) = 3.09, p = 0.08. The interaction of sex of item and diagnosis approached significance, F (1,116) = 3.30, p = 0.07. Paired t-tests showed that individuals with ASCs had a strong preference for male items, t (65) = 2.47, p = 0.02. This probably reflects the greater number of males than females in this group. There was a significant interaction between gender of item and sex of child. Paired t-tests showed that males strongly preferred male items and female strongly preferred female items, t

(76) = 12.6, *p* = 0.00 and *t* (41) = -7.95, *p* = 0.00 respectively. There was a significant 3-way interaction between gender of item, diagnosis, and sex, *F* (1,116) = 58.5, *p* = 0.00. Examination of the means showed that individuals with ASCs had less strong sex typical play preferences. However, play preferences in children with autism were in the direction expected for their biological sex and were statistically significant, *t* (19) = -3.09, *p* = 0.01; *t* (21) = -11.3, *p* = 0.00; *t* (45) = 8.51, *t* = 0.00; *t* (30) = 13.08, *p* = 0.00 for girls with an ASC, typical girls, boys with an ASC, and typical boys in order. There was a significant interaction between pretence of item and gender of item, *F* (1,116) = 31.2, *p* = 0.00. Paired t-tests showed that non pretence games were more popular than pretence games for male items, *t* (118) = -8.12, *p* = 0.00. For female items, non-pretence games and pretence games were equally popular, *t* (118) = -1.25, *p* = 0.21. There was a 3-way interaction of pretence of item, gender of item, and diagnosis, *F* (1,116) = 11.8, *p* = 0.00. Diagnosed cases showed a greater preference for male items, but only when the items did not involve pretence, *t* (65) = 5.20, *p* = 0.00.

Finally, there was a significant 4-way interaction between pretence of item, gender of item, diagnosis, and sex of child, *F* (1,116) = 6.01, *p* =0.02. Table 5.2 shows the mean, standard deviation, and range for male pretence items, male nonpretence items, female pretence items, and female nonpretence items. Diagnosed males show no difference between male and female pretence items; both are extremely low. In contrast, females with ASCs show a significant preference for female pretence items, in comparison to male pretence items, *t* (19) = -4.96, *p* = 0.00, in keeping with their biological sex. Their score is lower than typical females, which could be interpreted as a defeminization, as predicted by the EMB. However, the more conservative interpretation is that problems with pretence have caused the lower score. The finding that male scores seem to be more severely affected by the pretence of item may indicate that pretence is some-how protected in females with ASCs. For items not involving pretence, diagnosed

Table 5.2

Mean, standard deviation, and range for male pretence items, male nonpretence items, female pretence items, and female nonpretence items

			Mean	SD	Range	N
Male Pretence						
	Controls					
		Boys	13.8	4.95	4-20	31
		Girls	2.82	3.07	0-9	22
	Diagnosed					
		Boys	1.89	3.83	0-14	46
		Girls	1.20	2.22	0-9	20
Male Non-pretence						
	Controls					
		Boys	14.4	3.02	10-20	31
		Girls	4.77	3.61	0-12	22
	Diagnosed					
		Boys	8.93	4.54	0-18	46
		Girls	5.30	4.87	0-17	20
Female Pretence						
	Controls					
		Boys	5.03	3.02	0-11	31
		Girls	15.6	4.67	4-20	22
	Diagnosed					
		Boys	1.07	1.85	0-8	46
		Girls	6.96	5.42	0-17	20
Female Non-pretence						
	Controls					
		Boys	5.77	3.17	1-15	31
		Girls	14.9	3.82	5-20	22
	Diagnosed					
		Boys	1.77	2.64	0-9	46
		Girls	7.44	6.26	0-20	20

males show the expected pattern: preferring male items to female items, t (45) = 11.8, p = 0.00. They had slightly reduced scores on male nonpretence items compared to control males. Females diagnosed with ASCs do not show a preference for female items on nonpretence items, t (19) = -1.30, p = 0.21. This suggests that girls with ASCs are masculinized compared to typical girls in games where pretence is not involved.

Discussion

The Extreme Male Brain Theory of autism proposes that autism is an exaggeration of typical sex differences in empathising and systemising ability, but does not specifically address whether other sex-typical traits are relevant to the condition. The current study examined whether sex-typical play showed masculinization and/or defeminization in children with autism. Although the original EMB defined the "male" and "female" brain psychometrically, it has since been suggested that increased levels of prenatal androgens may produce excessive masculinization of the brain and thereby increase the risk for autism spectrum conditions. Studies of individuals exposed to abnormally high or low levels of androgen as a result of genetic disorders (notably CAH) and exposure to synthetic progestins have indicated a role for prenatal androgens in the development of sex typical play. Thus, the current study also tests the hypothesis that prenatal masculinization of the brain increases the risk of autism spectrum disorders.

Examination of the total score on male items and on female items showed that children with autism had significantly lower scores than typical controls. This suggested that an overall lower level or variety of play in children with autism could be concealing any changes relevant to the EMB. Approximately half of the sex-typical games required pretence and pretend play is known to be absent or limited in children with autism spectrum conditions (Frith, 1989; Happe, 1996; Jarrold et al., 1993). We therefore performed an analysis that took account of both gender of item and whether the game required pretence or not. Examination of items not requiring pretence provided the most direct test of the EMB theory and the androgen hypothesis.

For games that did not require pretence, girls with autism did not show a preference for female items as would be predicted by their gender. These results suggest a shift to male-typical play behaviour in girls with autism. Girls exposed prenatally to increased endogenous adrenal androgens (Berenbaum & Hines, 1992; Berenbaum & Snyder, 1995; Hines & Kaufman, 1994) or to exogenous androgenic progestins (Ehrhardt et al., 1968) also exhibit increased male-typical play. The similarity of our findings to those reported for girls with known exposure to male-typical androgen levels during prenatal life is consistent with the hypothesis that greater exposure to or sensitivity to prenatal androgens alters the brain is such a way as to contribute to the development of autism spectrum disorders. Alexander & Peterson (2004) have reported an increased preference for male-typical play styles, as well as increased gender dysphoria and a more typically "masculine" pattern of performance on two sex-typed spatial tasks, in a group of women with tic-symptoms. Tic symptoms, especially in the context of Tourette Syndrome, may also be related to excessive masculinization of the brain under the influence of androgens (Peterson et al., 1992). It is interesting to note that autism and Tourette Syndrome are often comorbid (Gillberg & Billstedt, 2000). In the previous chapters, we reported that variations in prenatal testosterone levels during midtrimester were related to poorer quality of social relationships and increased restricted interests (both areas being relevant to autism), but did not appear to affect individual differences in sex-typical play in a group of typically developing children. We suggested that play behaviour was affected by testosterone levels later in pregnancy. If this is the case, the current study suggests that girls with autism are exposed to high levels of testosterone during the midtrimester and later in pregnancy.

Boys with autism showed a male-typical pattern of play behaviour for items that did not require pretence, as we predicted. Because typical males already show high scores on the masculinity scale and low scores on the femininity scale, there was limited power for demonstrating hypermasculinization in males with autism. In fact, boys with autism did have a slightly lower score on male items than typical boys. This probably reflects an overall lower level or variety of play in children with autism, possibly as a

result of restricted interests. This interpretation is supported by the finding that children with autism had slightly lower scores on neutral items when compared to typical controls. Alternatively, this could be regarded as "hypomasculine" and therefore inconsistent with elevated exposure to prenatal androgens. This interpretation assumes that androgen levels are positively and linearly associated with play behaviour in males, which is not necessarily the case. As discussed in the introduction, the effect of testosterone on certain behaviours may be nonmonotonic. That is, increasing testosterone exposure may produce increasing masculinization up to a certain dose, but increase beyond this dose would cause a reversal toward the original state (demasculinization). Hines & Kaufman (1994) reported that boys with CAH showed decreased rough and tumble play compared to control males in one study, possibly demonstrating a nonmonotonic effect. In contrast, Alexander & Peterson (2004) found that men with tic symptoms reported elevated male-typical play preferences relative to other groups of men. In fact, the severity of their tic symptoms correlated significantly with their preference for male-typical play.

Examination of items requiring pretence also revealed an interesting pattern. Diagnosed boys showed no difference between male and female pretence items. Both scores were extremely low, as would be expected for a group that engages in relatively little pretence. In contrast, girls with ASCs showed a significant preference for female pretence items in keeping with their biological sex. Their score is lower than typical females, which could be interpreted as a defeminization, as predicted by the EMB, but the more conservative interpretation is that problems with pretence have caused the lower score.

Why were males more severely affected than females by the pretence of item? One possibility is that the boys in our ASC group had more severe forms of the condition than the girls in our ASC group. No quantitative measure of severity or of IQ was available. However, we did have specific diagnoses for many of the children in the sample. There are 4 recognised subgroups on the autistic spectrum: classic (or Kanner's) autism, high-functioning autism (HFA), Asperger Syndrome (AS), and pervasive developmental disorders not otherwise specified (PDD-NOS). The differential diagnostic

criteria for these 4 subgroups are not yet well specified, but the clinical rule of thumb is that classic autism invariably involves language delay and general developmental delay whilst AS and HFA do not; that classic autism and HFA invariably involve conspicuous communication difficulties whilst in AS this may be very subtle (in the domain of pragmatics) if apparent at all (Baron-Cohen, 1988) and that PDD-NOS may involve some but not all of the core diagnostic features. These subgroups vary in severity of symptoms with classic autism being far more severe than AS, HFA and PDD-NOS, if severity is measured in terms of conspicuousness.

Approximately half of the girls in our sample were diagnosed with autism, while the other half were diagnosed with conditions usually described as less severe, i.e. AS, HFA, and PDD-NOS. Within the boys, relatively more children were diagnosed with autism than with AS, HFA, and PDD-NOS. However, the difference was not significant when tested with a Fisher's exact test, $p = 0.16$.

An alternative explanation of the finding that boys with ASCs were more severely affected by the pretence of item than girls with ASCs is that pretence is some-how protected in females with ASCs. This protection could result from the social environment. Parent-daughter dyads, particularly mother-daughter dyads, are more likely to engage in pretence play than parent-son dyads (Lindsey & Mize, 2001; Lindsey, Mize, & Pettit, 1997; Tamis-LeMonda & Bronstein, 1991). The directionality of this relationship is not clear. Daughters might initiate pretend play more often than sons, or parents may initiate pretend play more often with daughters than with sons. If parents of children with ASCs do attempt to engage daughters in pretend play more often than they do sons with ASCs, this could provide a richer environment in which girls with ASCs could learn to engage in pretend play. Protection could also result from biological factors. Creswell & Skuse (1999), Skuse (2000), Skuse et al. (1997) and N. S. Thomas et al. (1999) report 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 and that autism is elevated in Turner girls with a paternally derived X chromosome. Skuse has argued that there is an imprinted locus on the short arm of the

paternally derived X-chromosome which is responsible for the female superiority on socio-cognitive abilities and protects them from developmental disorders such as autism. Although pretend play has not been examined specifically in Turner Syndrome, Leslie (1987, 1988) has argued that pretence is a prerequisite for the acquisition of Theory of Mind and impairments in ToM may be responsible for a number of core features in autism (Baron-Cohen, 1995, 1998, 2001). Several studies have reported that girls are more likely to engage in pretence than boys (Howes, Unger, & Matheson, 1991; Lindsey & Mize, 2001; Lindsey et al., 1997). Typically developing boys and girls did not show a sex difference in pretend play in the current study. However, the questionnaire we used asks how much a child would be interested in a particularly activity, not the frequency with which they engage in that activity.

Limitations

When evaluating our results it is important to keep in mind that the control children were taking part in a longitudinal study of amniotic testosterone and development. All their mothers had decided/been advised to have an amniocentesis due to late maternal age or other factors that increase the risk of foetal abnormality. Children who have had amniocentesis show no evidence of decreased well being or impaired brain development (Finegan et al., 1996) and any child whose medical records indicated ill health at birth, for example requiring long stays in the SCBU (Special Care Baby Unit), was excluded from the study. Maternal age may influence a person's own attitudes toward gender roles; and this could affect the degree to which mothers encourage and expect children to show gender-typical play. Some studies have shown that older persons tend to be more traditional in their gender-role perceptions (Lackey, 1989), but other studies have not found this relationship (Kulik, 2002). We explored whether maternal age in our sample was related to scores on male items and scores on female items within each sex. Maternal age was not related to gender-typical play in boys, $r(31) = 0.26$, $p = 0.16$ and $r(31) = -0.18$, $p = 0.33$ (for male and female items respectively), or girls, $r(22) = 0.32$, $p = 0.14$ and $r(22) = -0.06$, $p = 0.80$ (for male and female items respectively).

Our diagnosed cases were also significantly older than the control group. Although all mothers were asked to report their child's behaviour at age 5, mothers of the children with ASCs had to recall their child's earlier behaviour, while parents of controls reported present behaviour. This raises the possibility that parents of children with ASCs were less accurate. Several commonly used instruments that measure identification with gender group, including preference for sex typical play, require adults to report on their behaviour as children; this requires a similar or longer period of recall than in the present study. One example of this is the Recalled Childhood Gender Identity Scale used by Zucker et al. (1996) in a study of girls with CAH, and by Alexander & Peterson (2004) in their study of individuals with tic-symptoms.

Finally, it must be kept in mind when evaluating our results that this study relied on opportunistic samples. Ideally the girls and boys with autism would be perfectly matched for diagnosis and clinical characteristics, including symptom severity, IQ, comorbid diagnoses and prevalence of tics and associated motor difficulties. For the majority of volunteers in the Cambridge Autism Research Centre Volunteer database this information is not readily available. 2 of the boys in our sample were known to have a comorbid diagnosis of ADHD and one boy had comorbid diagnoses of bipolar disorder, sleep disorder, and ADHD. Among the girls, 1 individual had a diagnosis of ADHD, 1 had been diagnosed with both ADHD and developmental articulation disorder and 1 had been diagnosed with ADD and non-verbal learning difficulties. We did attempt to include similar numbers of children with autism and less severe diagnoses such as Asperger syndrome in each gender group. In practice approximately half of the girls who participated in the study were diagnosed with autism, while the other half were diagnosed with conditions usually described as less severe, while within the boys, relatively more children were diagnosed with autism than with other conditions. The difference was not significant when tested with a Fisher's exact test, $p = 0.16$. The sample size was not sufficient to test if the pattern of results was different in different diagnostic groups.

In conclusion, the current study has explored the association between gender-typed play behaviour and autism spectrum disorders. Our analysis of sex-typical play that does not involve pretence supports the extreme male brain theory of autism and the androgen hypothesis. Our results suggest that it would be worthwhile to document actual prenatal androgen levels in children who go on to develop autism spectrum conditions. In addition, our examination of sex-typical play that does involve pretence raises the intriguing possibility that pretence is protected in girls who develop ASCs.

CHAPTER 6: Age of Menarche in Females with Autism Spectrum Conditions

If fT does play a role in the development of autism, the question arises as to what factors might cause elevated prenatal androgen levels. Levels of T are determined, in part, genetically, with heritability estimates ranging from 40% to 60% (Harris et al., 1998; Sluyter et al., 2000). If the cause of elevated androgen activity is intrinsic to the foetus this deficit might also be detectible in postnatal life. Children with autism do not show elevated testosterone levels (Tordjman et al., 1995), but this may reflect the fact that in childhood the gonads are quiescent and sex hormone levels are extremely low. Hormone levels rise again at puberty suggesting that intrinsic differences in hormone levels or sensitivity may be more observable at this time. It is also possible that the pattern of puberty itself is different in individuals with ASCs as a result of these differences. There are anecdotal reports of precocious puberty in individuals with ASCs (Mouridsen & Larsen, 1989; Tordjman & Ferrari, 1992) and Tordjman et al. (1997) suggested that a subset of diagnosed individuals experience hyperandrogeny during puberty, which is associated with aggressive behaviours. No-one has studied whether the form of the testosterone surge at puberty differs in autistic individuals compared to controls.

The progression of puberty in individuals with ASCs has been relatively little studied, despite several indications that this is an important period of change for some individuals. Although it appears that the majority of children with ASCs do not experience more dramatic difficulties during puberty than other children, some studies report that between one-tenth and one-third of children experience deterioration and aggravation of symptoms soon after entering puberty and this may be particularly the case for girls (Gillberg, 1984; Gillberg & Schaumann, 1982). Some studies suggest that a quarter to one-third who have not suffered from epilepsy previously develop it during this period, and that in those who did have childhood epilepsy the frequency of attacks increases (Gillberg, 1984). A study by Ballaban-Gil, Rapin, Tuchman, & Shinnar (1996) compared changes in symptom severity in a group of 54 adolescents. 44% showed more

problem behaviours in adolescence compared to when they were children. 24% showed more self-injurious behaviour and 21% showed worsened stereotypies. Seltzer et al. (2003) report that adults with ASCs were less symptomatic than adolescents with respect to restricted, repetitive behaviours and interests, possibly suggesting a developmental rise in these behaviours at puberty.

Symptom changes during puberty could be mediated by circulating hormone levels. During adolescence there is a 30-fold increase in boys and a 100-fold increase in girls in the release of luteinizing hormone, resulting in a rapid and dramatic increase in the levels of circulating androgens and estrogens from the gonads (Veldhuis, 1996). In addition to being present in the hypothalamus, androgen and oestrogen receptors are concentrated in the medial amygdala, bed nucleus of stria terminalis and lateral septum (Riley, 1943). The amygdala has been implicated in the aetiology of autism and, in particular, in relation to social behaviour and emotion regulation (Baron-Cohen et al., 2000; Brambilla et al., 2003; Machado & Bachevalier, 2003; Sweeten, Posey, Shekhar, & McDougle, 2002) (but see Amaral, Bauman, & Mills Schumann, 2003), suggesting that increased hormone levels could lead to changes in social symptoms in individuals with ASCs during puberty. The hippocampus is also a target for steroid hormones (Beyenburg et al., 2000) and has been implicated in autism, particularly with regard to specific memory problems (Bachevalier & Loveland, 2002; Ben-Shalom, 2003; Brambilla et al., 2003). Sears et al. (1999) report that caudate volume is associated with ritualistic-repetitive behaviour in autism. Putatative oestrogen receptor alpha mRNA has been reported in the caudate (Pau, Pau, & Spies, 1998). Other areas which may be abnormal in autism and may also carry steroid hormone receptors are the corpus callosum (Egaas, Courchesne, & Saitoh, 1995; Fitch, Berrebi, Cowell, Schrott, & Denenberg, 1990; Moffatt, Hampson, Wickett, Vernon, & Lee, 1997, Brambilla et al., 2003), frontal cortex (Brambilla et al., 2003; Finley & Kritzer, 1999; Grady & Keightley, 2002; Pau et al., 1998; Shallice, 2001; Wang et al., 2004; Zilbovicius et al., 1995), and cerebellum (Abdelgadir, Roselli, Choate, & Resko, 1999; Brambilla et al., 2003; Pau et al., 1998). A link between circulating hormone levels and psychiatric symptoms has been shown for schizophrenia, where low oestrogen levels exacerbate symptoms of the disorder (Reicher-

Rossler et al., 1994).   Administration of oestradiol as an adjunct to traditional antipsychotics may help alleviate symptoms (Kulkarni et al., 2001; Lindamer, Buse, Lohr, & Jeste, 2001).

Sexual maturation in individuals with ASCs needs to be studied for several reasons. Firstly it is not known if sexual maturation proceeds differently in individuals with ASCs than in the general population. Secondly, if sexual maturation does differ in individuals with ASCs this may reveal something about the causes of these conditions. Thirdly, if pubertal events and symptoms are related this may suggest new treatment possibilities.   Finally, parents of children with ASCs often raise concerns about progression through puberty. Ideally, a study of pubertal development in ASCs would be longitudinal and track both sexual maturation and changes in symptom severity. However, longitudinal studies are difficult, time-consuming and expensive. Prior to beginning such an investigation researchers must have sufficient justification. In this chapter we report the first study that specifically examines the development of secondary sexual characteristics in individuals with ASCs using a cross-sectional design. We focused on age at onset of menarche since this is a very salient event, and can be recalled accurately many years later. Must et al. (2002) report that even after 30 years, recall of age at menarche is generally good both in precision ($r = 0.79$) and accuracy (mean error (recalled-original) =-0.08 yrs).

Methods

Participants

Group 1 comprised 38 women diagnosed with an ASC. Their mean age was 31.5 yrs (sd = 9.87). The majority of these women were members of the Cambridge Autism Research Centre Volunteer database. Individuals join the database through the Centre's web-page (http://www.autismresearchcentre.com/volunteers/default.asp).   Individuals have been directed to the website by the National Autistic Society (UK), specialist clinics carrying out diagnostic assessments, and adverts in newsletters/web-pages for people

with autism. To qualify as a volunteer a diagnosis must have been made by a suitably qualified professional, such as a psychiatrist or psychologist using established criteria for autism spectrum disorders (APA, 1994). For the current study we contacted all women in the database age 20 or above. The women were contacted either via email or post. 65 women were contacted and 35 agreed to participate (a response rate of 54%). 3 additional women were recruited via the National Autistic Society (UK). Specific diagnoses were available for 35 of the women. 10 were diagnosed with autism, 2 with high functioning autism, and 23 with Asperger syndrome. Several subjects also had comorbid diagnoses. 2 individuals had severe learning disabilities; one individual was diagnosed with Tourette's syndrome, one individual was diagnosed with ADD, one individual was diagnosed with dyslexia, one individual was diagnosed with both dyslexia and dyspraxia, one had an unofficial diagnosis of dyslexia and dyspraxia, one individual was diagnosed with epilepsy, one individual was diagnosed with depression and one individual was diagnosed with anxiety.

Group 2 comprised 38 healthy control women who had been age-matched to the women with ASCs. Their mean age was 32.2 yrs (sd = 9.14). This was not significantly different from the group with ASCs, t (74) = -0.10, p = 0.92, d = 0.07. The final 38 were selected from 100 women who responded from a larger sample of 126. We chose to match the participants by age because the accuracy of recall is reduced as the time interval between menarche and recall increases (Koo & Rohan, 1997). In addition, there is some debate regarding secular changes in age at menarche. It is well documented that age at menarche has fallen sharply since the 19th century. For example, in Norwegian maternity hospitals menarcheal age was almost 16 years in 1840, but had fallen to 13.5 years by 1940, a falling trend of 3 months/decade (Liestol, 1982). Similarly, Okasha, McCarron, McEwen, & Smith (2001) report that mean age at menarche decreased from 13.2 years in the earliest born to 12.5 years in the latest born in Scotland during the first half of the 20th century. Several studies suggest that age at menarche has stabilized in the second half of the 20th century (L. Coleman & Coleman, 2002). However, other studies report a continuing decline (Anderson, Dallal, & Must, 2003; Chumlea et al., 2003; Demerath et al., 2004; Freedman et al., 2002; Ostersehldt & Dankerhopfe, 1991;

Wattingney, Srinivasan, Chen, Greenlund, & Berenson, 1999). Some studies even report a recent reverse trend (Hulanicka & Waliszko, 1991). The reported changes in these recent studies are generally small, for example a 2.5 month decline during the time period 1963-1970 and 1988-1994 (Anderson et al., 2003), but could have concealed any changes in menarche related to diagnosis with an ASC if age was not controlled.

All women were asked the following question: "How old were you when you had your first period?" They were asked to indicate age in years and months. They were also asked "What date was it (month/year)?" If the subjects answered both questions, recalled age was used in the subsequent analysis as this is remembered more accurately (Brooke-Gunn, Warren, Rosso, & Gargiulo, 1987). For the analysis all ages were recorded in decimals (so 13.5 years = 13 years and 6 months). Participants were excluded if they reported having a hormonal condition that could influence the variable being tested, such as thyroid gland abnormalities, diabetes, and anorexia. 3 women were removed from the ASC group for exclusionary conditions. One women with AS and ADD had raised hormone levels resulting in an absence of periods. One women with AS and anxiety had serious eating disorders. One women with AS had thyroid gland abnormalities. Because timing of the onset of menarche differs among ethnic groups (Grumbach & Styne, 1998), only subjects with a Caucasian background were included in this study.

Results

We began by exploring whether any individuals in our sample had extremely delayed puberty or extremely early (precocious) puberty. Figure 6.1 presents box-plots for age at menarche. 3 of the women in the ASC group had their first period at a very late age (20.3, 20.1, and 20). This represents approximately 8% of the women with ASCs, a significant minority. 1 control woman had a delayed age at menarche (17.7). These women were excluded from subsequent analyses.

Table 6.1 compares age at onset of menarche between women with and without ASCs. A t-test shows that the ASC women, on average, began their periods at a later age (13.3 vs. 12.6 yrs), t (67) = 1.95, p = 0.055, d = 0.45. Equal variances were assumed. The probability of a type I error was maintained at 0.05.

Figure 6.1

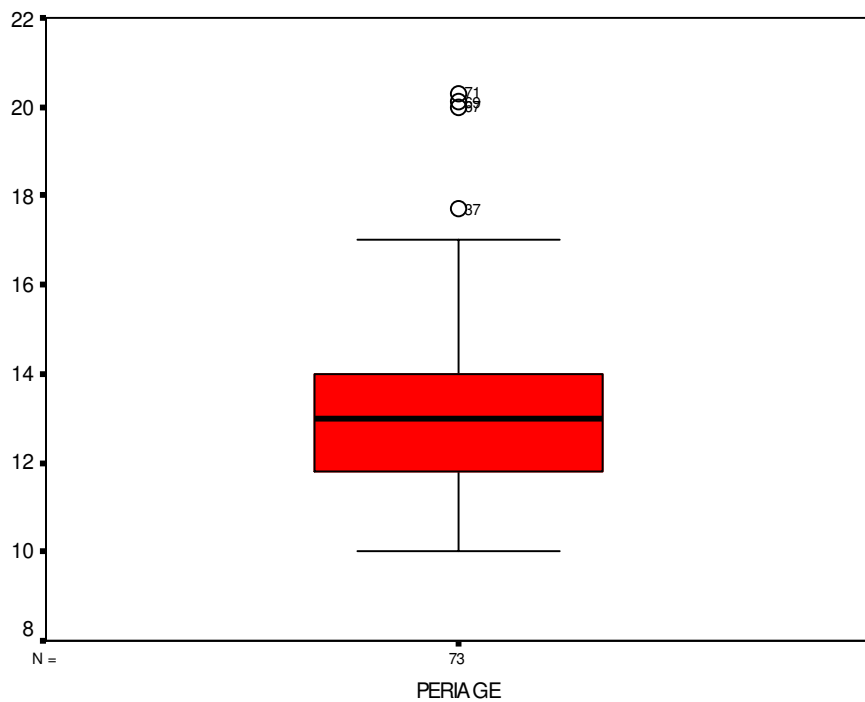<u>Box-plot indicating outliers for age at onset of menarche</u>

Table 6.1

Mean, standard deviation, and range for age at onset of menarche in women with and without autism spectrum conditions

	n	Mean	SD	Range
Women with ASCs	32	13.3	1.71	10-17
Control Women	37	12.6	1.35	10-16.8

Discussion

This is the first study that specifically examines the development of secondary sexual characteristics in individuals with ASCs. Women with ASCs had a later age at onset of menarche than age-matched controls, suggesting the possibility of a general shift in age at menarche in women with ASCs of about 9 months. This is potentially compatible with the hypothesis that prenatal exposure of the brain to high levels of testosterone increases the risk of autism spectrum disorders. Female rhesus monkeys exposed to high levels of androgens early in gestation experience delayed menarche (Abbot, Dumesic, Eisner, Kemnitz, & Goy, 1997). Female rhesus monkeys exposed to high levels of androgens late in gestation also showed delayed menarche in one study (Zehr, 2003), but showed normal menarche in another study (Goy, Uno, & Sholl, 1989). There is an association of the short alleles of the GGC repeat polymorphism of the androgen receptor gene with early age of menarche (Comings, Muhleman, Johnson, & MacMurray, 2002) suggesting that sensitivity to testosterone may be related to age at menarche in humans. Exon 1 of the androgen receptor gene contains 2 polymorphic repeats, GGC which encodes the polyglycine tracts and CAG which encodes the polyglutamine tracts. The number of CAG repeats has been shown to modulate AR expression (greater numbers of CAG repeats produces lower AR function), but the

functional significance of the GGC repeat is unclear (Kazemi-Esfarjani, Trifiro, & Pinsky, 1995).

Whether there is a systemic shift in age of menarche in women with CAH, a group with known exposure to elevated prenatal androgens is not entirely clear. New & Levine (1984) in their monograph on CAH state that the expected age of menarche in treated classical CAH is delayed as would be expected from the studies in rhesus monkeys. They cite reports from a number of clinics to support this proposition (Ghali, David, & David, 1977; Jones & Verkauf, 1971; Klingensmith, Garcia, Jones, Migeon, & Blizzard, 1977; Pang, Kenny, Foley, & Drash, 1977; Richards, Styne, Conte, Kaplan, & Grumbach, 1977). White & Speiser (2002) also stated that the average age at menarche in girls with CAH is late compared with healthy peers. In contrast, I. A. Hughes (2004) argues that in most reported series age at menarche for girls with CAH is no different from that of the normal population. There are several weaknesses in the literature reporting age of menarche in CAH. Most of the studies have relied on small sample sizes; some do not include any control groups for comparison; and often age at menarche was not the outcome measure of primary interest. For example, Premawardhana, Hughes, Read, & Scanlon (1997) report a mean age of 13 years and 4 months for a group of 16 girls with CAH. No control group was included. I. A. Hughes (2004) cites this study as supporting an average age at menarche. Certainly, the study does not indicate a dramatic delay, but the mean age is slightly higher than age at menarche for the general U.K. population as reported by J. Kaiser & Gruzelier (1999) (mean age 12.8 years) and Whincup, Gilg, Odoki, Taylor, & Cook (2001) (median age 12.9 years). Age at menarche in CAH is also affected by treatment regimes. Inadequate glucocorticoid replacement therapy for a prolonged period in childhood can lead to early onset of puberty (I. A. Hughes, 2004). Well-substituted patients may actually be hypoandrogenic and this could also affect pubertal development (Helleday, Siwers, Ritzen, & Carlstrom, 1993).

Even if one accepts that high prenatal testosterone levels could produce a delay in age at menarche, it would be inappropriate to conclude that this was the cause of the

delay we observed in women with ASCs in the current study. The timing of menarche may be set *in utero* or infancy, but post-natal factors are clearly important modulators (dos Santos Silva et al., 2002). Age at menarche may be affected by multiple factors: metabolic, endocrine, and emotional. Metabolic factors include nutritional status, physical training and weight (Hopwood et al., 1990; Job, Chaussin, & Toublanc, 1990; M. P. Warren, 1990). The possibility of a "critical level" of body composition related to puberty onset has been widely debated in the literature (M. P. Warren, 1990). Endocrine disturbances including hypothyroidism, hyperthyroidism, hypercortisolism, hyperprolactinemia, and isolated growth hormone deficiency as well as hyperandrogenism may cause puberty delay (Job et al., 1990). It is possible that the women with ASCs may have taken medications during the pre-pubescent or pubescent years that disrupted their metabolic or endocrine status. Stress is also thought to affect menarche, but due to the highly subjective nature of this phenomenon, it is difficult to study (M. P. Warren, 1990). Given that our study included no clinical controls, it is possible our results reflect stress associated with having a clinical condition. To determine the likelihood of this being the case we can review other studies of age at onset of menarche in clinical conditions.

Dalton & Dalton (1978) carried out a study of blind, deaf, and physically handicapped girls in England and Wales, and found that the majority of their diagnostic categories had an early onset of menarche. Evans & McKinlay (1988) report that a group of severely mentally handicapped girls in Manchester showed an average delay of 13 months. However, Down's syndrome formed a recognizable subgroup with a mean menarcheal age 11 months earlier than normal. Takano, Takaki, Kawano, & Nonaka (1999) also report an early age of menarche in Down's syndrome girls in Japan. Age of menarche shows no deviation in fragile X syndrome (Burgess, Partington, Turner, & Robinson, 1996). White girls with cerebral palsy have a later age at menarche (Worley et al., 2002). This suggests that there is no absolute association between having a clinical condition in childhood and age at onset of menarche. The factors producing this variation across conditions merit further study.

Extreme Scores

3 women with ASCs had a very late onset of menarche (around age 20) while only 1 of the control women was indicated as an outlier for age at menarche (age 17.7). All of these cases had a diagnosis of classical autism. In addition one case had a comorbid diagnosis of severe learning difficulties. The current sample size was not large enough to test whether the rate of extremely delayed menarche is elevated in populations with ASCs, but this is a possibility that deserves further research. The background details on these 3 cases suggests that extreme delays in puberty may be more common in classical autism than in high functioning autism or Asperger syndrome, but it would be inappropriate to draw strong conclusions from the current study, given the sample size.

Limitations

There are several limitations to our study. Firstly, we relied on women recalling their age at menarche (rather than performing a longitudinal study of individual pubertal development). The average time between age at onset of menarche and when we asked these women about their first period was 18.5 years (sd = 9.8). Although this is a significant period of time, age of menarche is a highly salient and memorable event during pubertal development for typical populations. Must et al. (2002) followed up 448 women who had taken part in the Newton Girls Study (NGS), a prospective study of growth and physical development that was run from 1965 to 1975. Girls were followed through their first mentrual period and for 2 years post-menarche. They recontacted the women in 1998. As in the current study participants were asked to recall their age at menarche in years and months. Mean recall ranged from 23 to 33 years (with a mean of 29 years). Despite the long recall period, original and recalled menarcheal age were highly correlated ($r = 0.79$). Multiple other studies support the reliability of recalled age of menarche across the lifespan (Bean, Leeper, Wallace, Sherman, & Jagger, 1979; Casey, Dwyer, Coleman, Krall, Gardner & Valadian, 1991; Koprowski, Coates, & Bernstein, 2001). The accuracy of our control sample's self-report can be evaluated by comparing their age of onset with that reported in other studies. Our controls reported a

mean age at menarche of 12.6. This is comparable to the age at menarche reported by Whincup et al. (2001) for a sample of 1166 girls from across Britain (median 12.9). More comparable to our sample in birth year are the studies by Cooper, Kuh, Egger, Wadsworth, & Barker (1996) and Zacharias, Wurtman, & Schatzoff (1970) which report a median age of 13 yrs and a mean age of 12.65 yrs respectively. The validity of self-report of menarche in women with ASCs has not been studied. Menarche may be well remembered in controls because it signals entry into the childbearing years, a social event. Therefore it may not have the same salience in women with autism and may be recalled less accurately.

In addition, we relied on self-report of exclusionary clinical conditions as it was not possible to perform physical and endocrine examinations on all subjects. It is possible that some of the autism group had an exclusionary condition but were unaware of the fact. This might be particularly relevant to the 3 extreme scores who would have merited a clinical diagnosis of delayed puberty.

The response rate for women with ASCs was moderate (58%). This is comparable to other postal studies run by our lab in the Cambridge area, but lower than the response rate for the control women (79%). Responders and non-responders did not differ in the rates of specific diagnosis: 26% classic autism, 5.2% HFA, and 60% AS for responders compared to 23.9% classic autism, 4.3% HFA, 70.1% AS, and 1.4% PDD-NOS for all ASC women contacted.

When interpreting our findings it is also important to note that menarche occurs relatively late in the pubertal process, and may not be an indicator for the age of pubertal onset. For example, Worley et al. (2002) report that white children with cerebral palsy begin puberty earlier than the general population, but age at menarche and the completion of puberty are delayed. Also, because of the nature of our sample, our analysis was restricted to one racial group which limits the generalizability of our findings.

In conclusion, we found a 9 month delay in age of menarche in females with ASCs which may fit in with the 'extreme male brain' theory of autism (Baron-Cohen & Hammer, 1997; Baron-Cohen, 2003). It will be necessary to perform a comprehensive study of pubertal development which takes account of nutritional status, medication, and other variables relevant to pubertal development in women with ASCs before any strong conclusions can be drawn, but our results suggest that such a study would be worthwhile. In addition, any longitudinal study of individual pubertal development should include boys with ASCs. We have focused on women in this study, because of the salience of age at menarche; no comparably salient event exists in men. Determining the progression of puberty in individuals with ASCs could potentially help determine the causes of the disorder and would be an important source of information for parents of children with ASCs.

CHAPTER 7: Foetal Testosterone and Risk for Autism Spectrum Disorders: Current Status of the Cambridge Antenatal Predictors of Child Development Project

Although we cannot extrapolate directly from the studies reported in the previous chapters to autism, our results suggest that it is worthwhile to explore whether fT is involved in the male vulnerability to autism. The best test of this theory would be to compare fT levels in children who go on to develop autism with normally developing children and appropriate clinical comparisons. As discussed previously, because autism is a relatively rare disorder, a large sample size is required for any study attempting to address this question directly. Work is currently underway to build a sufficiently large database of children whose mothers underwent amniocentesis in the Cambridgeshire region to perform a study comparing amniotic testosterone levels between different developmental conditions. In this chapter we review the recruitment procedure for the Antenatal Predictors of Child Development study, describe the statistical analyses planned for the completed sample, report the background characteristics of families which have already been recruited into the study, and compare foetal testosterone levels in the present sample to scores on the Social Responsivity Scale (SRS) (Constantino et al., 2000), a quantitative measure of autistic traits.

Subject Recruitment

The Autism Research Centre in Cambridge potentially has access to 2500 to 3000 existing amniocentesis samples from women who have undergone amniocentesis in Cambridgeshire between 1996 and 2000; these represent samples from Addenbrooke's Hospital, Peterborough Hospital, Hinchingbrooke Hospital, West Suffolk Hospital, and the Queen Elizabeth Hospital in King's Lynn. Addenbrooke's hospital, where all chromosome analysis in the area is performed, routinely stores these samples for several years.

Contacting the women from whom these samples were collected presents a number of important ethical considerations, which must be taken into account by any researcher working in this area. The following considerations were of prime importance.

1) Women did not give consent at the time of the amniocentesis to be contacted about later research. The data protection act encourages that subjects are informed at the time of collection of the ways in which their information may be processed. In this case, samples were collected for diagnostic purposes. The MRC guidelines on human biological samples in research acknowledge that samples may be of considerable value for research that was not and could not have been envisaged when they were collected. In this case they encourage contacting the donors for new consent.

2) We did not want to contact inappropriately a woman whose pregnancy did not end successfully due to miscarriage, termination or stillbirth. Our recruitment design includes 3 separate safety checks to ensure that we only contact appropriate women. First we check the cytogenetics records at Addenbrooke's hospital and eliminate from our database any woman who tested positive for Down's syndrome or another clinically important chromosomal abnormality. Secondly, we check the patient's obstetric records and ensure that the pregnancy of interest did not end in a miscarriage, termination, or stillbirth. We also eliminate from our database any woman who has had a miscarriage, termination, or stillbirth since the pregnancy of interest as they may be sensitive about any pregnancy related topics. We also eliminate any woman whose records show she has had a miscarriage or termination following an amniocentesis, as they may be sensitive about any research related to amniocentesis. Lastly, we contact the GP and ask them to indicate whether the pregnancy of interest ended successfully and whether there are any subsequent reasons why contact would be inappropriate (See Appendix C for the GP contact letter and consent form). This also prevents our contacting a woman who is recently bereaved or likely to be distressed for some other reasons.

3) Amniocentesis is a stressful procedure and contacting women regarding it may bring up difficult memories. We acknowledge in the first paragraph of our patient letter that we are contacting them regarding their amniocentesis and that this may stir up difficult memories. We apologise if the letter reminds them of something they would prefer to keep in the past. We have not yet received a complaint that being contacted regarding the amniocentesis was stressful. We also include in our patient questionnaire a section on amniocentesis, which gives the women an outlet to express any concerns and complaints that they may have felt about the initial procedure (See Appendix C for the patient contact letter, consent form, and initial questionnaire).

4) The women might be concerned that their information was passed outside of the hospital. Our research team includes the hospital. All patient letters are sent in the name of a health professional associated with the original amniocentesis and analysis. This has been the consultant responsible for carrying out amniocenteses in Addenbrooke's Hospital. Fluid from these, together with samples taken from other hospitals in the region, is sent to the lab in Addenbrooke's for analysis. We have never had a patient from one of the surrounding hospitals express concern at their samples having been sent to Addenbrooke's. Our patient letter explains that this is how all amniocentesis samples are analysed. The letter also reassures individuals that their information was not passed outside the hospital.

5) Women might be concerned that we were investigating the effects of amniocentesis on their child. We assure patients in our letter that we are not investigating the effect of amniocentesis on their child, and there is no reason to be concerned that there are any ill effects. We simply want to make use of the information collected at that time which can teach us about how children develop.

6) Women might be concerned that we approached them because we have some idea that their child is at increased risk for the developmental conditions we ask about. We stress in our patient letter that the developmental conditions we ask about are fortunately relatively rare, so for the majority of people receiving the letter this will not be relevant. We restate this in our questionnaire, when we ask whether their child has been referred for any of these conditions.

Study Design

Once contacted, the women are asked for their consent to analyse their amniotic fluid for foetal testosterone. Given the prevalence of autism in the population we can expect up to 10-20 of this group to have a child who meets criteria for an autism spectrum condition. This represents our primary group of interest. Since autism is often characterized by language delay and learning disabilities, it is necessary to have control groups representing these conditions. Otherwise we could not determine whether a high level of testosterone in the sample of children with autism indicated a specific correlation between autism and testosterone or a broader relationship between testosterone and language development or learning disabilities. Autism has also been characterized as a disorder of executive functions (Griffith & Pennington, 1998; C. Hughes, Russell, & Robbins, 1994a, 1994b; Ozonoff, 1995; Pennington et al., 1997; Russell, 1997). Attention deficit hyperactivity disorder (ADHD) is also characterized by executive dysfunction (Shallice et al., 2002). Therefore the group of 20 children with autism will be matched with 20 children referred for language delay, 20 children referred for learning disabilities, and 20 referred for ADHD, all drawn from the broader sample of 2500. This will result in a sample of 80 individuals. If more than 20 children have been referred for one of these conditions, the twenty will be selected randomly. We will also have a control group of 80 typically developing children who will be matched to the autism group by sex, age, and gestational age at amniocentesis. If we compared mean testosterone between 5 groups (the 4 clinical groups described above plus a normal group) with 20 individuals in each group the comparison would have satisfactory power (.80) to identify a difference in means of 0.34 nmol/L (Sokel and Rolf 1981). For comparison the difference between mean amniotic testosterone levels for males and females is 0.65 nmol/L.

In addition to comparing testosterone levels between clinical groups, we will also relate scores on a series of questionnaires to amniotic testosterone levels (See Table 7.1). These are used in screening for each disorder, but also show normal variation in the

population. With a sample size of 160, the analysis would have satisfactory power (.80) to reveal a medium effect size, even if up to eight predictor variables were included

Table 7.1

Screening questionnaires for the conditions of interest

	Autism Spectrum Disorders	**Language Delay**	**Attention Deficit-Hyperactivity**
Test	The Social Responsiveness Questionnaire (SRS)	The MacArthur Communication Development Inventory: Words and Sentences	The Preschool Behaviour Checklist and The Connors Rating Scale
References	(Constantino et al., 2000; Constantino & Todd, 2000, 2003)	(Fenson et al., 1994; Hamilton, Plunkett, & Shafer, 2000; Thal, O'Hanlon, Clemmons, & Fralin, 1999)	(McGuire & Richman, 1986) (Conners, Sitarenios, Parker, & Epstein, 1998; Goyette, Connors, & Ulrich, 1978)

Current Sample

Recruitment has been successfully completed in 3 Hospital areas. In the King's Lynn and West Suffolk area, 658 samples were potentially available. Medical records checks eliminated 18% of the sample because of material found in the records and 7% because the records were unavailable. 499 GPs were contacted and asked to indicate whether they gave consent for their patients to be contacted. 45% of GPs responded and granted consent; 12% of GPs responded to indicate the patient had left their practice; 7% of GPs responded indicating their patient should not be contacted; 36% of GPs did not reply. 227 women were contacted and invited to join the project. 92 women returned consent forms, agreeing to join the project. The response rate for all women contacted is thus 41% and the response rate for all women whose samples were available is 14%. Addenbrooke's hospital chose to complete the medical records checks and contact of GPs themselves, so full numbers are not available on the initial stages of recruitment. 332 GPs responded granting consent to contact their patients. 169 women returned the consent form, agreeing to join the project (a response rate for all women contacted of 51%). 4 children were excluded from the study as they were twins. 1 child was excluded from the study because their amniotic sample was contaminated with maternal blood. The total size of the current sample is thus 256. Figure 7.1 illustrates the process of subject recruitment and subject numbers involved at each stage.

Figure 7.1

Chart to illustrate the process of subject recruitment and subject numbers involved at each stage of the Cambridge Antenatal Predictors of Child Development Project
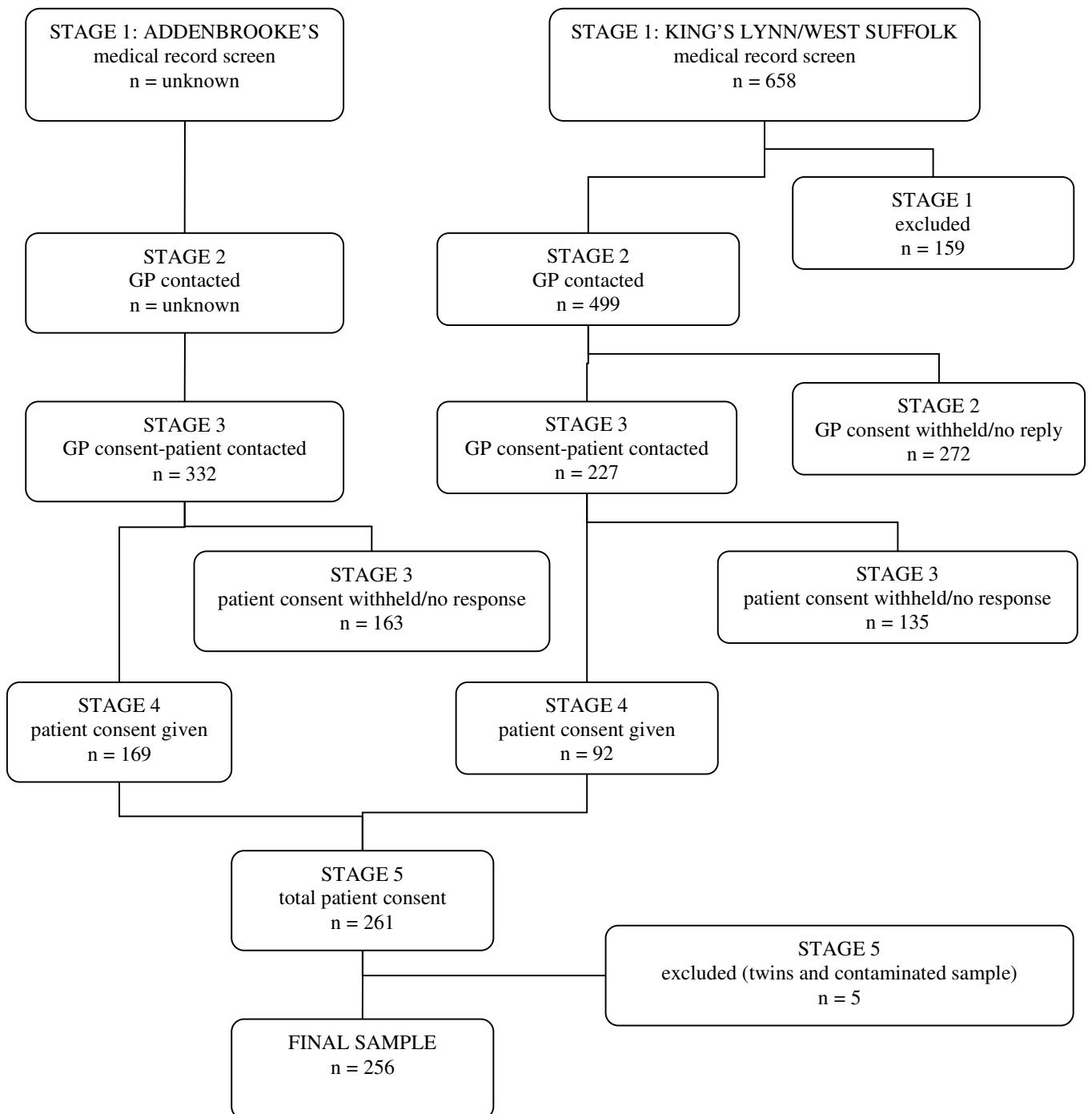
Predictor Variables

Foetal testosterone levels (fT) (nmol/L). The predictor of greatest interest in this study is foetal testosterone. Testosterone levels in amniotic fluid were measured by radioimmunoassay by the Department of Clinical Biochemistry, Addenbrooke's Hospital Cambridge (Appendix A). There were significant differences between male and female testosterone levels, mean (SD) = 0.83 (0.39) nmol/L and 0.30 (0.18) nmol/L for boys and girls respectively (see table 7.3), t (197) = 14.3, p = 0.00, d = 1.75. Equal variances were not assumed on any t-tests. The probability of a type I error was maintained at 0.05 for all t-tests. Examination of the univariate distribution suggested that fT levels were positively skewed in the combined sample and within each sex. Kolmogorov-Smirnov tests confirmed that fT levels were significantly skewed, p = 0.001, p = 0.003, and p = 0.03 in the combined sample, females, and males respectively. 3 females (2.5% of the total females) had undetectable levels of fT; their fT levels were recorded as 0.

Because multiple earlier studies failed to find an affect of either oestrogen or alphafoetoprotein on outcomes relevant to autism, these variables will not be measured for the current study.

Gestational age at amniocentesis (weeks). Levels of fT vary during gestation. Although amniocentesis occurs on average at week 16, it can occur as early as week 12 and as late as week 22. Therefore it was important to determine whether fT was related to gestational age in our sample. Gestational age at amniocentesis was obtained from hospital records. Gestational age can be estimated from the date of last menstrual period and from sonographic measures including biparietal diameter, head circumference, and femur length. When multiple measures were available, sonographic measures were used as these are more accurate. When different sonographic measures disagreed with each other, the average gestational age was calculated. Females had a significantly later gestational age than males, mean (SD) = 16.3 (1.33) weeks and 16.8 (1.77) weeks for boys and girls respectively (see table 7.3), t (189) = 2.38, p = 0.02, d = 0.31, although the effect size was fairly small. The mean difference was 0.5 weeks. Gestational age was

not correlated with fT levels, r (122) = 0.05, p = 0.61 and r (104) = 0.04, p =0.68 in males and females respectively.

Sociodemographic variables. A range of sociodemographic variables were also collected. Obviously, all these variables are not necessarily important for our initial outcome variables; i.e. diagnostic category and scores on the screening instruments. However, they give an excellent indication of the general characteristics of the sample and may be relevant to future studies. These included:

A. *Variables describing the parents at time of delivery*
>   1: Maternal Age at subject's birth, measured in decimal years
>   2: Paternal Age at subject's birth, measured in decimal years
>   3: Maternal Education
>   4: Paternal Education
>   5: Mother's current occupation
>   6: Father's current occupation
>   5: Mother's handedness
>   6: Father's handedness

Education level was measured according to a 5 point scale: 1 = no formal qualifications, 2 = 'O' level / G.C.S.E. (16 years schooling + exams) or equivalent, 3 = 'A' level, HND (18 years schooling + exams) or vocational qualification, 4 = university degree, 5 = postgraduate qualification. Current Jobs were classified according to the major group structure of the Standard Occupational Classification (SOC). SOC has been designed as a classification applicable to all paid jobs currently done by economically active persons in Great Britain. The major group structure is a set of broad occupational categories which are designed to be useful in bringing together unit groups which are similar in terms of qualifications, training, skills and experience. The division between major groups are distinguished on criteria similar to the International Standard Classification of Occupations (ISCO). Table 7.2 shows the nine major groups of SOC, defined in terms of the general nature of the qualifications, training and experience

associated with competent performance of tasks in the occupations classified within each major group. Homemakers were coded as 10. Those who reported that they were unemployed and did not indicate that they acted as stay-at-home parents were coded as 0. Individuals had the option of describing themselves as right-handed, left-handed, or ambidextrous.

Table 7.3 presents means, standard deviations, and ranges for all quantitative variables for each sex separately. There were no significant differences between males and females for maternal age, mean (SD) = 40.2 (5.13) years and 39.8 (4.87) years for boys and girls respectively, t (249) = 0.69, p = 0.49, d = 0.08; paternal age, mean (SD) = 41.2 (6.22) years and 42.1 (6.22) years for boys and girls respectively, t (235) = 1.20, p = 0.23, d = 0.14; maternal education, mean (SD) = 3.24 (1.18) and 3.15 (0.98) for boys and girls respectively, t (251) = 0.66, p = 0.51, d = 0.08; or paternal education, mean (SD) = 3.16 (1.19) and 3.08 (1.07) for boys and girls respectively, t (244) = 0.52, p = 0.60, d = 0.07. Table 7.4 shows the number of mothers and fathers in each occupational category. Chi-square was used to test whether girls and boys differed in the number of parents in each occupation. Results were not significant, χ^2 (10, N = 254) = 11.7, p = 0.31 and χ^2 (10, N = 254) = 9.98, p = 0.44 for mothers and fathers respectively. Table 7.5 shows the number of mothers and father's in each handedness category. Chi-square was used to test whether girls and boys differed in the number of parents in each handedness group. Results were significant for mother's handedness, χ^2 (3, N = 254) = 8.37, p = 0.04. Mothers of girls reported being ambidextrous more often than mothers of boys. Results were not significant for father's handedness, χ^2 (3, N = 254) = 2.40, p = 0.49.

Table 7.2

The major and minor group structure of the Standard Occupational Classification (R. Thomas & Elias, 2000)

Major Group	General nature of qualifications, training and experience for occupations in the Major Group	Constituent Minor Groups
1 Managers and administrators	A significant amount of knowledge and experience of the production processes administrative procedures or service requirements associated with the efficient functioning of organisations and businesses.	General managers and administrators in national and local government, large companies and organisation Production managers in manufacturing, construciton, mining and energy industries Specialist managers Financial institution and office managers, civil service executive officers Managers m transport and storing Protective services officers Managers in farming, horticulture, forestry and fishing Managers and proprietors in service industries Managers and administrators nos
2. Professional occupations	A degree or equivalent qualification, with some occupations requiring post graduate qualifications and/or a formal period of experience-related training.	Natural scientists Engineers and technologists Health professionals Teaching professionals Legal professionals Business and financial professionals Architects and surveyors Librarians and related professionals Professional occupations nos
3. Associate professional and technical occupations	An associated high-level vocational qualification, often involving a substantial period of full-time training or further study. Some additional task-related training is usually provided through a formal period of induction.	Scientific technicians Draughtspersons, quantity surveyors and other surveyors Computer analysts /programmers Ship and aircraft officers, air traffic planners and controllers Health associate professionals Legal associate professionals Business and financial associate professionals Social welfare associate professionals Literary, artistic and sports professional Associate professional occupations nos
4. Clerical and secretarial occupations	A good standard of general education. Certain occupations will require further additional vocational training to a well defined standard (eg. typing or shorthand)	Administrative /clerical officers and assistants in civil service and local government Numerical clerks and cashiers Filing and record clerks Clerks (not otherwise specified) Stores and despatch clerks, storekeepers Secretaries, personal assistants, typists, word processors operators Receptionists, telephonists and related occupations Clerical and secretarial occupations nos
5. Craft and related occupations	A substantial period of training, often provided by means of a work-based training programme	Construction trades Metal machining, fitting and instrument, making trades Electrical/electronic trades Metal farming, welding and related trades Textiles, garments and related trades Printing and related trades Woodworking trades Food preparation trades Other craft and related occupations nos
6. Personal and protective service occupations	A good standard of general education. Certain occupations will require further additional vocational training, often provided by means of a work-based training programme.	NCOs and other ranks, armed forces Security and protective service occupations Catering occupations Travel attendants and related occupations Health and related occupations Childcare and related occupations Hairdressers, beauticians and related occupation Domestic staff and related occupations Personal and protective service nos

Table 7.2 cont.

<u>The major and minor group structure of the Standard Occupational Classification (R. Thomas & Elias, 2000)</u>

7. Sales occupations	A general education and a programme of work-based training related to sales procedure. Some occupations require technical knowledge but are included in this major group because the primary task involvess selling.	Buyers brokers and related agents Sales representatives Sales assistants and check-out operators Mobile, market and door-to-door salespersons
8. Plant and machine operatives	The knowledge and experience necessary to operate vehicles and other mobile and stationary machinery, to operate and monitor industrial plant and equipment, to assemble products from component parts according to strict rules and procedures and subject assembled parts to routine tests. Most occupations in this major group will specify a minimum standard of competence that must be attained for satisfactory performance of the associated tasks and will usually have an associated period of formal experience-related training	Food drink and tobacco process operatives Textiles and tannery process operatives Chemicals, paper, plastics and related process operatives Metal making and treating process opeeratives Metal working process operatives Assemblers/lineworkers Other routine process operatives Road transport operatives Other transport and machinery operatives Machne and plant operatives nos
9. Other occupations	The knowledge and experience necessary to perform mostly simple and routine tasks often involving the use of hand.-held tools and, in some cases, requiring a degree of physical effort. Most tasks in these occupations have limited scope for personal initiative and judgement, and may not require formal educational qualifications, but will usually have an associated short period of formal experience-related training All non.managerial farming occupations are also included in the major group, primarily because of the difficulty of distinguishing between those occupations which require only a limited knowledge of agricultural techniques from those which require specific training and experience in these areas.	Other occupations m agriculture, forestry and fishing Other occupations in mining and manufacturing Other occupations in construction Other occupations in transport Other occpations in communication Other occupations in sales and service Other occupations nos

B. *Variables describing the course and outcome of pregnancy*

   1: Pregnancy and Birth Complications

   2: Birth Method

   3: Use of Epidural during labour

If a mother reported no complications, she was coded as 0 for the Pregnancy and Birth Complications variable. If a mother reported any complications, she was coded as 1. Mother's had a choice of describing their birth method as normal, caesarean, ventouse/forceps, or other. Use of epidural was coded 0 for no and 1 for yes. Table 7.6 summarizes obstetric data for each sex. Chi-square was used to test whether girls and boys differed in any of the obstetric variables. Results were not significant, χ^2 (2, $N =$

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254) = 2.66, p = 0.26 for birth complications; χ^2 (3, N = 254) = 1.90, p = 0.59 for birth method; and χ^2 (2, N = 254) = 1.68, p = 0.43 for use of epidural respectively.

C. *Variables describing the subject*

    1: Child's sex

    2: Child's age at recruitment (calculated from date of birth)

    3: Number of older siblings

The current sample includes 118 girls and 136 boys. Data on gender is missing for two children. Table 7.3 presents means, standard deviations, and ranges for all quantitative variables for each sex separately. There were no significant differences between males and females for age at recruitment, mean (SD) = 4.64 (1.38) years and 4.73 (1.65) years for boys and girls respectively, t (229) = 0.48, p = 0.63, d = 0.06, or number of siblings, mean (SD) = 1.36 (0.95) and 1.30 (0.96) for boys and girls respectively, t (246) = 0.55, p = 0.58, d = 0.06. Examination of the univariate distribution suggested that the variable, number of siblings, was positively skewed in the combined sample and within each sex. Kolmogorov-Smirnov tests confirmed that this variable was significantly skewed, p = 0.000, p = 0.000, and p = 0.000 in the combined sample, females, and males respectively.

Table 7.3

Means, standard deviations and ranges for quantitative predictor variables by sex

Variable	Girls n = 104-118			Boys n = 122-136			
	Mean	SD	Range	Mean	SD	Range	d
fT (nmol/L)	0.30	0.18	0.00-1.00	0.83	0.39	0.05-2.05	1.75
Gestational Age (weeks)	16.8	1.77	12-22	16.3	1.33	13-20	0.31
Maternal Age (decimal years)	39.8	4.87	22.8-48.3	40.2	5.13	26.3-51.74	0.08
Paternal Age (decimal years)	42.1	6.22	27.2-62.0	41.2	6.22	28.3-64.4	0.14
Maternal Education	3.15	0.98	1-5	3.24	1.18	1-5	0.08
Paternal Education	3.08	1.07	1-5	3.16	1.19	1-5	0.07
Child's Age (decimal years)	4.73	1.65	1.98-7.90	4.64	1.38	1.92-7.76	0.06
Number of Siblings	1.30	0.96	0-5	1.36	0.95	0-5	0.06

Note: n varies due to missing data on some participants

Table 7.4

Number of mothers and fathers in each occupational category

		Occupational Category												
		0	1	2	3	4	5	6	7	8	9	10	Missing	Total
Mother														
	Girls	0	8	20	13	11	1	19	2	1	30	10	3	118
	Boys	0	12	31	16	15	2	9	7	0	35	7	2	136
	Both	0	20	51	29	26	3	28	9	1	65	17	5	254
Father														
	Girls	0	16	29	8	6	23	9	4	11	3	0	9	118
	Boys	1	25	33	15	2	28	10	5	10	4	0	3	136
	Both	1	41	62	23	8	51	19	9	21	7	0	12	254

Table 7.5

Number of mothers and fathers in each handedness category

		Handedness Category				
		Right	Left	Ambidextrous	Missing	Total
Mother						
	Girls	101	11	6	0	118
	Boys	118	17	0	1	136
	Both	219	28	6	1	254
Father						
	Girls	98	15	1	4	118
	Boys	118	13	3	2	136
	Both	216	28	4	6	254

Table 7.6

Obstetric variables

	Birth Complications			
	Yes	No	Missing	Total
Girls	51	67	0	118
Boys	50	84	2	136
Total	101	151	2	254

	Birth Method				
	Normal	Caesarean	Ventouse/Forceps	Missing	Total
Girls	71	38	9	0	118
Boys	80	42	12	2	136
Total	151	80	21	2	254

	Epidural			
	Yes	No	Missing	Total
Girls	51	65	2	118
Boys	54	76	6	136
Total	105	141	8	254

Outcome Variable: Clinical Referral

Currently 20 children in the sample have been referred for language delay; 4 have been referred for ADHD, 6 have been referred for learning difficulties, and 6 have been referred for autism spectrum conditions. Some children in this group have been referred for multiple diagnoses. 1 female and 2 males have been referred for language delay, learning difficulties, and autism spectrum conditions. 1 female and 1 male have been referred for both language delay and learning difficulties. 1 male has been referred for both language delay and ADHD. A preponderance of males is expected for language delay, ADHD, and autism spectrum conditions (Rutter, Caspi, & Moffitt, 2003). Table 7.7 displays the number of males and females in each diagnostic group. As expected, a greater number of males were found in each of these categories. At present the number of clinical cases is too small to test whether males are significantly over-represented in any one clinical group. However, if we examine all children who have received a diagnosis of language delay, ADHD, and autism spectrum disorders, a significant excess of males is observed, $p = 0.01$ using a Fisher's Exact Test.

Table 7.7

Proportion of males and females referred for specific childhood developmental conditions

| Condition	Males referred	Females referred	Total referred
Language Delay	0.10	0.05	0.08
ADHD	0.03	0.00	0.02
Learning Difficulties	0.03	0.02	0.02
Autism Spectrum Conditions	0.04	0.01	0.02

Outcome Variable: Social Responsivity Scale

The number of clinical cases participating at present is too small to test whether fT levels are higher in any clinical group. However, 174 families have completed a copy of the Social Responsivity Scale (SRS) (Constantino et al., 2000; Constantino & Todd, 2000), an instrument for measuring where a given individual lies on the autism spectrum.

The SRS is a 65-item questionnaire whose psychometric properties have been tested in a study including 445 children age 4-14 years (Constantino et al., 2000). 158 of these children were child psychiatric patients and 158 were an epidemiological sample of school children. Total scores on the SRS were continuously distributed in all groups of subjects. Children with autism spectrum conditions scored significantly higher than did clinical and nonclinical controls. Clinical controls (including children diagnosed with conduct disorder, psychosis, mood disorder, and ADHD) did not differ significantly from nonclinical controls. Constantino et al. (2003) showed that SRS scores correlated with algorithm scores on the Autism Diagnostic Interview-Revised (ADI-R) on the order of 0.7 in a group of 61 child psychiatric patients (including both children with ASCs and children with other diagnosis). Inter-rater reliability was on the order of 0.8 and SRS scores were not related to I.Q. Figure 7.2 illustrates the mean and standard deviation for each group included in Constantino et al. (2000). Higher scores represent a greater degree of deficits in social behaviour. Latent class analysis and factor analysis failed to demonstrate separate categories for core autistic symptoms and more general impairments, consistent with the idea of a "broader autism phenotype." The mean SRS score for 4-to 7-year old boys (53.5 ± 36) was significantly higher than that for 4-to 7-year old girls (35.2 ± 27), $p < 0.0001$, $d = 0.58$, but there were no gender differences in 8-to 14-year old schoolchildren. In a recent study of 788 twin pairs aged 7 to 15 years (randomly selected from the Missouri Twin Study), the mean SRS score for boys (35.3 ± 22.0) was significantly higher than the mean score for girls (27.5 ± 18.4), $t (1578) = 7.63$, $p < 0.001$, $d = 0.38$ (Constantino & Todd, 2003).

Figure 7.2

American population means and standard deviations of SRS scores (Constantino et al., 2000)
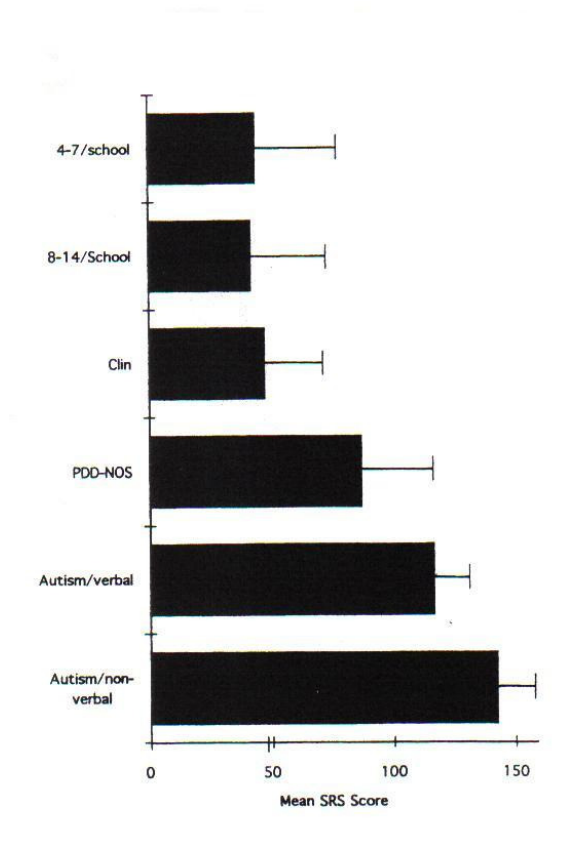
Descriptive Statistics

We began by exploring whether any children in our sample showed extremely high or extremely low SRS scores. Examination of boxplots indicated that 5 children were classified as extreme cases (all with extremely high scores) (see Figure 7.3). All 5 children were male. 4 had received a diagnosis of an autism spectrum disorder. 1 had received a diagnosis of global learning difficulties and language delay. In addition, 7 children were classified as outliers (again with high scores) (see Figure 7.3). All 7 children were male. 1 had been diagnosed with an autism spectrum disorder. 1 had been referred for both learning difficulties and language delay. 2 had been referred for

language delay alone. 1 had been diagnosed with ADHD. 2 of the outliers had not been referred for any diagnosis. Because, we were interested in the relationship of fT to social difficulties as well as to normal inter-individual variation in social responsivity and autistic traits, we included all cases in our initial analyses. We then reran the analyses, first removing only the extreme cases, then removing both the extreme cases and the outliers.

Figure 7.3

Box-plot indicating extreme cases and outliers for scores on the SRS (Constantino et al., 2000)
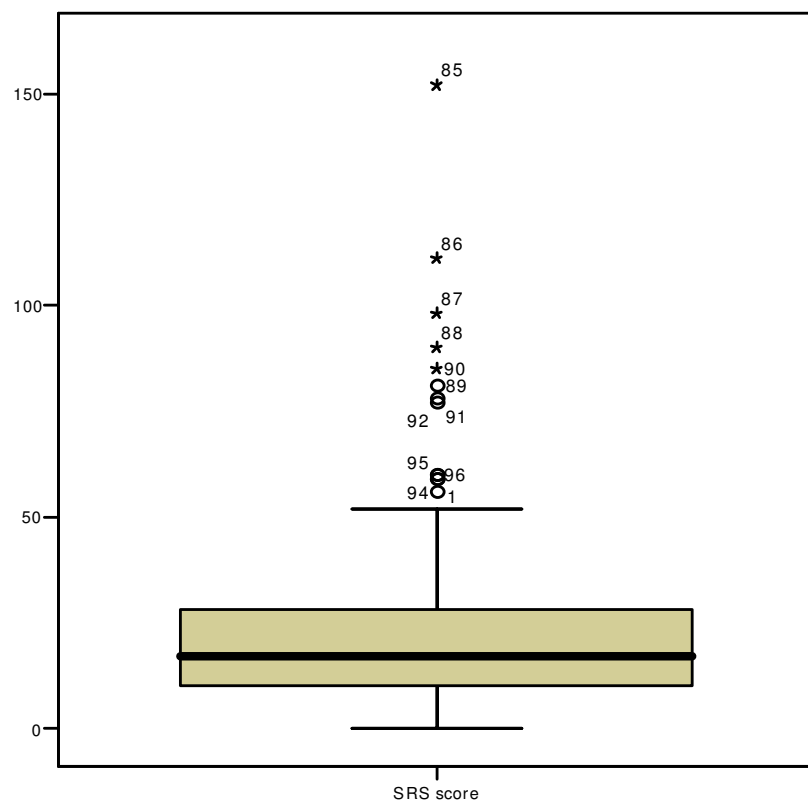
Note: Extreme cases have values more than 3 box lengths from the upper or lower edge of the box. Outliers have values between 1.5 and 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range.

Examination of univariate distributions suggested that both fT and SRS scores were positively skewed. Kolmogorov-Smirnov tests confirmed that both variables were significantly skewed, $p = 0.01$ and $p = 0.00$ for fT and SRS score respectively. A natural logarithmic transformation was carried out on fT. The transformed variable was not significantly skewed, $p = 0.21$. SRS scores were transformed by taking the square root of each score. The transformed variable was not significantly skewed, $p = 0.22$. t-tests indicated that there was a significant sex difference in SRS scores with males scoring higher than females, mean (SD) = 4.78 (2.20) and 3.93 (1.59) for boys and girls respectively, $t(162) = 2.93$, $p = 0.004$, $d = 0.44$ for the transformed variable. For the untransformed variable, mean (SD) = 27.7 (26.6) and 18.0 (12.5) for boys and girls respectively, $t = (172) = 3.04$, $p = 0.00$, $d = 0.47$.

Hierarchical Regression Analyses

Hierarchical regression analyses were performed as recommended by Altman (1991). In addition to fT and sex, we examined maternal age, paternal age, maternal education, paternal education, and number of siblings, as these socio-demographic variables could affect the frequency and quality of children's social experience. We also included child's age at the time the SRS was administered, as SRS scores are negatively related to age (Constantino et al., 2000). Table 7.8 reports means, standard deviations and ranges for each variable. Examination of the univariate distributions indicated that the variable, number of siblings, was positively skewed. Kolmogorov-Smirnov tests confirmed that this variable was significantly skewed. Number of siblings was transformed by taking the square root. This reduced skewness considerably, although Kolmogorov-Smirnov tests showed that the variable was still significantly skewed.

Table 7.8

Means, standard deviations, and ranges for predictor variables in the subset of children administered the Social Responsivity Scale (SRS) (Constantino et al., 2000).

	Girls			Boys			
Variable	Mean	SD	Range	Mean	SD	Range	d
fT (nmol/L)	0.28	0.19	0-1.00	0.81	0.38	0.05-2.05	1.76**
Maternal age (decimal years)	39.7	4.74	23.6-48.3	40.8	4.91	26.3-51.7	0.22
Paternal age (decimal years)	42.0	6.54	27.2-62.0	42.0	5.98	29.9-64.4	0.01
Maternal education	3.04	0.84	2-5	3.18	1.16	1-5	0.14
Paternal education	3.02	1.02	1-5	3.07	1.20	1-5	0.04
Number of Siblings	1.38	0.96	0-5	1.35	1.06	0-5	0.03
Age at SRS (decimal years)	5.86	1.11	3.43-7.90	5.83	0.89	3.84-7.76	0.03

** $p < 0.01$, * $p < 0.05$

The first analyses explored the contribution of fT to scores on the SRS. In block one, sex was entered. In block 2, fT was entered. In block 3, the interaction of fT and sex was entered. Table 7.9 summarizes the results of this analysis. Inclusion of sex in the 1rst stage produced a significant F change, F change = 4.10, β = 0.16, p = 0.05. This model explained 2.4% of the variance in SRS scores. Inclusion of fT in the 2nd stage did not produce a significant F change, F change = 3.65, β = -0.21, p = 0.06. The inclusion of a sex by fT interaction in the 3rd stage also did not produce a significant F change, F change = 0.04, β = -0.02, p = 0.84. The only significant predictor in the final model was

sex. Residual analysis showed acceptable plots and no outliers. To investigate further we examined the correlation between fT and SRS score within each sex. It should be kept in mind that this reduced the sample-size by half and therefore the power of the analyses. SRS scores did not correlate with fT levels in either girls or boys, r (84) = -0.15, p = 0.18; r (90) = -0.19, p = 0.08, respectively. There was a trend in boys for lower fT levels to be associated with higher SRS scores, a direction opposite to that predicted. This may partially reflect the inclusion of a case, diagnosed with global learning difficulties, who had an extremely high SRS score and a very low fT level (0.05, beneath the published detection limit of the assay).   When this case is removed the trend disappears, r (89) = -0.09, p = 0.42.

When the above hierarchical regression analysis is run excluding extreme cases or excluding both extreme cases and outliers, neither sex or fT is a significant predictor of SRS scores.

Finally, we performed a hierarchical regression analysis that took into account relevant background variables. In block 1, any predictor variable that correlated significantly with the outcome variable at $p < 0.20$ was entered into the model (Altman, 1991). Suppressor variables were also included when possible; these were predictors that correlated highly, $p < 0.01$, with the other predictors in the model, but were not significantly correlated with the outcome variable (See Table 7.10). In block 2, sex and fT were tested for inclusion using a step-wise analysis (Entry criteria was $p < 0.05$; removal criteria was $p > 0.1$). In block 3, the interaction of sex and ft was tested for inclusion using a stepwise analysis (entry and removal criteria as above).   Table 7.11 summarizes the results of these analyses. Both sex and paternal education are significant predictors of SRS scores; fT was excluded from the final model. Residual analysis indicated that 5 cases were multivariate outliers; these were the 5 cases with the highest SRS scores.   When this hierarchical analysis is run excluding extreme cases the pattern of results is the same. Both sex and paternal education are significant predictors of SRS scores and fT is excluded from the final model. When the analysis is run excluding both

extreme cases and outliers, sex is no longer a significant predictor. A t-test run on the sample, excluding extreme cases and outliers, confirms that no sex difference is SRS scores is apparent, mean (SD) = 19.5 (12.5) and 17.4 (11.8) for boys and girls respectively, t (158) = -1.05, p = 0.29, d = 0.17.

Table 7.9

Summary of hierarchical regression analyses testing the contribution of sex and fT to scores on the SRS (Constantino et al., 2000)

	Predictor	B	$SE\ B$	β
Step 1				
	Sex	0.53	0.26	0.16*
Step 2				
	Sex	1.02	0.36	0.30**
	fT[a]	-0.44	0.23	-0.21
Step 3				
	Sex	1.02	0.37	0.30**
	fT[a]	-0.44	0.23	-0.21
	Interaction of Sex and fT[a]	-0.04	0.19	-0.02

Note. $R^2 = 0.02$ for Step 1 ($p < 0.05$); $\Delta R^2 = 0.02$ for Step 2 (*ns*); $\Delta R^2 = 0.00$ for Step 3 (*ns*).

[a] ln (fT)

** $p < 0.01$, * $p < 0.05$

Table 7.10

Correlations between predictor and outcome variables in the subset of children administered the SRS (Constantino et al., 2000).

	SRS Score[a]	fT (nmol/L)[b]	Child's sex	Maternal Age	Paternal age	Maternal Education	Paternal Education	Siblings[c]	Child's Age
SRS Score[a]									
fT (nmol/L)[b]	0.04								
Child's sex	0.22**	0.71**							
Maternal Age	-0.02	-0.01	0.11						
Paternal age	-0.09	-0.02	-0.00	0.52**					
Maternal Education	-0.19*	0.08	0.07	0.06	0.05				
Paternal Education	-0.23*	0.06	0.02	0.15*	0.13	0.61**			
Siblings[c]	-0.04	0.01	-0.05	0.05	-0.04	-0.03	-0.02		
Child's Age	0.02	-0.13	-0.02	0.24**	0.16*	-0.06	-0.05	0.13	

Note: correlations are Pearson correlations
[a] square root of SRS score, [b] ln (fT), [c] square root of Number of Siblings
** $p < 0.01$, * $p < 0.05$

Table 7.11

Final model: Hierarchical regression incorporating background variables, maternal education and paternal education, to scores on the SRS (Constantino et al, 2000)

Predictor	B	$SE\ B$	β
Maternal Education	-0.16	0.16	-0.10
Paternal Education	-0.28	0.14	-0.18*
Sex	0.59	0.26	0.17*

$* p < 0.05$

Discussion

fT and the Social Responsivity Scale (Constantino et al., 2000)

The prenatal androgen hypothesis of autism would predict that children exposed to higher levels of fT should show a greater number of autistic traits than children exposed to lower levels of fT. This prediction has been supported by previous studies which suggested that children with higher levels of fT, measured in amniotic fluid, had poorer quality of social relationships and more restricted interests (as measured by the Children's Communication Checklist (Bishop, 1990)) and were less likely to interpret stimuli in intentional terms. However, sex and paternal education were the only significant predictors in the final model of our regression analyses for scores on the Social Responsivity Scale (SRS) (Constantino et al., 2000), a questionnaire designed to measure autistic traits in children and young adolescents. Within sex fT was not significantly correlated with SRS scores and *r* values were low. Our results suggest that prenatal testosterone, measured at this period, does not account for individual variation in SRS scores. The discrepancy between results in this study and the earlier studies requires explanation.

One possibility is that the questionnaire we used did not accurately measure the children's behaviour. Our primary reasons for selecting the SRS were that it was normally distributed in all groups of subjects in which it has been tested, including a large epidemiological sample of school children; that it showed a significant sex difference; particularly in children ages 4-7, the same age range found in the current study; and that it was higher in children with autism spectrum conditions, but not other childhood disorders. Our results suggest that none of these attributes may apply in the British population (all previous studies of the SRS had used American samples). The current sample is most comparable to the epidemiological sample of school children, age 4 to 7, studied in Constantino et al. (2000). Constantino et al. (2000) did not screen their epidemiological sample for children diagnosed with any clinical condition and one would expect at least a portion of students in mainstream schooling to be diagnosed with a childhood disorder. They reported that SRS scores were normally distributed in their sample, while SRS scores in the current sample were significantly positively skewed. Both samples showed a significant sex difference, however the effect size was lower in the current study, $d = 0.47$ vs. 0.58. Overall scores were also much lower in the current study. Constantino et al. (2000) report a mean SRS score of 53.5 (sd = 36) for 4-to 7-year old boys and a mean SRS score of 35.2 (sd = 27) for 4-to 7-year old girls. In the current study the mean SRS score for boys was 27.7 (sd = 26.6) and the mean SRS score for girls was 18.0 (sd = 12.5). The occurrence of 5 cases of autism in a group of 174 children, seen in the current study, is far higher than one would expect based on published prevalence rates. Because our invitation letters specifically discuss autism and ask for information on clinical referrals, parents of children with autism are probably more likely to respond, leading to an over-representation of clinical cases compared to Constantino et al.'s (2000) sample of school children. However, even if we remove the cases with autism, SRS scores remain positively skewed. This also reduced the sex difference in scores. If all outliers are removed, the sample is no longer skewed, but the sex difference in scores disappears entirely, mean (SD) = 19.5 (12.5) and 17.4 (11.8) for boys and girls respectively, $t(158) = -1.05$, $p = 0.29$, $d = 0.17$. Also of interest is the high representation of children with clinical conditions other than autism among high scorers on the SRS. Constantino et al. (2000) found no significant differences in SRS scores

between children diagnosed with clinical conditions other than autism (they included ADHD but not children with language delay or learning difficulties) and the epidemiological sample of school children. In the current sample children diagnosed with a clinical condition (either language delay, learning difficulties, or ADHD) had significantly higher SRS scores than the typically developing children, mean (SD) = 18.4 (12.8) and 41.1 (24.2) for typically developing children and clinical cases respectively, t (16) = -3.7, p = 0.00, d = 1.18.

In conclusion, although we found little evidence that normal variation in prenatal testosterone levels is related to scores on the SRS, we would be reluctant to dismiss prenatal testosterone influences on social development and autistic traits, given the different pattern of responding in our sample compared to the American samples in which the SRS was developed. The positive skewness in scores suggests that parents in our sample evaluated the SRS in an "all or nothing" way and were reluctant to ascribe any traits to typically developing children. Our results also suggest that children with language delay and learning difficulties are likely to score higher than normal children on the SRS even though they are not expected to have a higher level of autistic traits. None-the-less, we should acknowledge the possibility that our earlier studies showing a relationship between fT and quality of social relationships, restricted interests and use of intentional language could be chance results. A further possibility is that fT is more strongly related to specific autistic traits, such as a reduced tendency to understand events in intentional terms, than it is to the broader autism phenotype.

Future Uses of the Cambridge Antenatal Predictors of Child Development Population

Currently 256 families are participating in the Cambridge Antenatal Predictors of Child Development Project. Based on current response rates, in the next several years a further 300 families are expected to join the project via retrospective consent. In addition, Addenbrooke's hospital has arranged that every woman undergoing amniocentesis in the future will be asked for prospective consent to store her sample. Each woman will also be given the opportunity to express an interest in being invited to

participate in relevant research projects. Not only will this eventually enable a sufficiently powerful comparison of fT levels in children who go on to develop autism, language delay, ADHD, and learning difficulties with normally developing children, it will also provide a unique resource for studying the contribution of prenatal testosterone to later human behaviour. Several potential projects are outlined below.

1) Testosterone across the lifespan

It is currently unknown whether testosterone levels at different developmental stages are independent of one another. It would be possible to measure testosterone levels (either in serum or saliva) in this unique population during infancy, childhood, adolescence, and adulthood, thereby answering this important question

2) Sexual Dimorphism in the Brain

There is a great deal of evidence for sexual dimorphism in the human central nervous system. Specific brain areas of interest include the amygdala, the corpus callosum, and the hypothalamus (Collaer & Hines, 1995; MacLusky & Naftolin, 1981; Witelson, 1991). Animal studies suggest that prenatal testosterone may play an important role in creating this dimorphism, although a role for direct genetic factors cannot be ruled out. Brain imaging studies of the children in this sample could provide important evidence as to whether prenatal testosterone is, in fact, the critical factor.

3) Testosterone-Environment Interactions

Scarr & McCartney (1983) and others have also pointed out that there is no such thing as a purely biological or purely environmental contribution to behaviour. While this is generally accepted, in practice most psychologists focus primarily on either biological or social factors. This partially reflects the difficulty in finding a sample large enough to evaluate multiple factors and their interactions. We have frequently referred to sociodemographic factors that might be of importance in the development of different

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behaviours, but with the exception of paternal education and SRS scores, we have not found significant relationships. This may reflect the broad nature of the sociodemographic variables we used. The creation of the Cambridge Antenatal Predictors of Child Development Project will allow more focused studies of testosterone-environment interactions. For example, it might be possible to investigate whether prenatal testosterone levels modulate a child's response to peer reinforcement of gender-stereotypes.

Chapter 8: General Discussion

Summary of Results

Work at the cognitive level suggests that autism may be an extreme manifestation of some sexually dimorphic traits or an "extreme male brain" (Baron-Cohen, 2002). Although this theory was originally defined purely psychometrically, it has since been suggested that prenatal testosterone may be a risk factor for autism and may be responsible for the biased sex ratio seen in these conditions (Lutchmaya, Baron-Cohen, & Knickmeyer, 2004). In this dissertation I used several strategies to explore the extreme male brain theory and the potential role of prenatal testosterone in autism. A summary of each study is provided below.

Chapter 2: Foetal Testosterone, Social Relationships, and Restricted Interests in Children

58 typically developing children (35 male and 23 female), whose foetal testosterone (fT) level was analysed in amniotic fluid, were followed up at age 4. Their mothers completed the Children's Communication Checklist (CCC) (Bishop, 1998), a questionnaire assessing language, quality of social relationships and restricted interests. fT was negatively correlated to quality of social relationships and directly correlated with restricted interests, taking sex differences into account. fT was also positively correlated with restricted interests when boys were examined separately. These findings implicate fT in both social development and attentional focus. Because social development and attentional focus are severely affected in autism spectrum conditions, these results may also have implications for understanding the sex ratio in ASCs.

Chapter 3: Foetal Testosterone and Interpreting Behaviour in Terms of Mental and Affective States

The study of the CCC in a group of children whose fT levels were measured in amniotic fluid suggested that fT is associated with poorer social development. To further test this apparent relationship we invited these children and their families for a laboratory test of social cognition. 38 children (24 male, 14 female), age 4.0 to 4.25 years, were shown cartoons with 2 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. This is consistent with the extreme male brain theory in that boys performed more like individuals with autism. Females also used more intentional propositions than boys. 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 boys were examined separately. These results are consistent with the relationship between fT and quality of social relationships we observed using the CCC. Boys and girls did not differ in the use of propositions describing actions between animates, although children with autism are less likely to use such descriptions. This indicates that there are deficits in autism beyond those predicted by the extreme male brain theory.

Chapter 4: Foetal Testosterone and Gender-typed Play

Chapter 4 reports the first attempt to correlate gender-typed play in a normative sample of humans with measurements of amniotic testosterone. Participants were 53 children (31 male, 22 female), age 4.75 to 5.8 years, taking part in our longitudinal study of fT and child development. Mothers completed a modified version of the Child Game Participation Questionnaire (CGPQ) (Bates & Bentler, 1973). A strong sex-difference was observed on the modified CGPQ. Hierarchical regression analyses on the entire group and within sex correlations suggested that variations in fT did not contribute to individual differences in game participation, as reported by the mother. The results draw attention to the complexity of hormonal influences on behaviour and highlight the need to

consider dose and timing of exposure when designing and evaluating studies of prenatal hormones.

Chapter 5: Gender-typical Play in Children with Autism

The Extreme Male Brain Theory of autism proposes that autism is an exaggeration of typical sex differences in empathising and systemising ability, but does not specifically address whether other sex-typical traits are relevant to the condition. Mothers of 66 children (20 female, 46 male) diagnosed with an autism spectrum disorder completed a modified version of the CGPQ asking them to report their child's play preferences at age 5. 55 typically developing children (24 female, 31male), drawn from our study of fT and development, acted as controls. For games that did not require pretence, girls with autism did not show a preference for female items as would be predicted by their gender. These results suggest a shift to male-typical play behaviour in girls with autism, which is similar to that reported for girls with known exposure to high androgen levels during prenatal life. This is consistent with the hypothesis that greater exposure to or sensitivity to prenatal androgens alters the brain is such a way as to contribute to the development of autism spectrum disorders.

Chapter 6: Age of Menarche in Females with Autism Spectrum Conditions

To determine whether the age at menarche is different in females with autism spectrum conditions (ASCs) compared to non-clinical controls, 38 women with ASCs were asked when they had their first period. 38 healthy women who had been age-matched to the women with ASCs served as controls. Women with ASCs had a significantly later age at onset of menarche than age-matched controls, suggesting the possibility of a general shift in age at menarche in women with ASCs of about 8 months. In addition, 3 women with ASCs had a very late onset of menarche (around age 20) (these women were excluded from the group comparison). This is potentially compatible with the hypothesis that prenatal exposure of the brain to high levels of testosterone increases the risk of autism spectrum disorders, but could also reflect differences in

nutritional status, medication, and other variables relevant to pubertal development in women with ASCs.

Chapter 7: Foetal Testosterone and Risk for Autism Spectrum Disorders: Current Status of the Cambridge Antenatal Predictors of Child Development Project

Chapter 7 reviews the recruitment procedure for the Antenatal Predictors of Child Development study, describes the statistical analyses planned for the completed sample, and reports the background characteristics of the 256 families which have already been recruited into the study. In addition, the relationship between foetal testosterone levels and scores on the Social Responsivity Scale (SRS) (Constantino et al., 2000), a quantitative measure of autistic traits, is examined in a group of 174 children (84 female, 90 male). Hierarchical regression analyses on the entire group and within sex correlations suggested that variations in fT did not contribute to individual differences in SRS scores. However, we would be reluctant to dismiss prenatal testosterone influences on social development and autistic traits, given the different pattern of responding in our sample compared to the American samples in which the SRS was developed.

Limitations of the Amniocentesis Design

Amniocentesis studies are limited by the problems inherent in studying foetal endocrinology. Firstly we have assumed that amniotic testosterone levels accurately represent serum levels of testosterone and brain exposure. Regarding the relation between amniotic and serum levels, although the maximal sex difference in amniotic T occurs between weeks 12 and 18, closely paralleling peak serum levels (Finegan et al., 1989), no studies have tested empirically how strongly amniotic T levels correlate with serum T levels. In the serum, binding proteins and degradation enzymes affect the availability of the hormone. Only unbound T is biologically active. The assay used in this study measured total T in the amniotic fluid. However, because T is thought to enter the amniotic fluid via foetal urination and bound T is protected from excretion in the urine, the amniotic levels should primarily reflect unbound T. Regarding brain exposure,

due to its small size, circulating T can easily cross the blood brain barrier. It is lipophilic and can therefore pass through cell membranes and enter the cytoplasm of brain cells. Backstrom, Cartensen, & Sodergard (1976) found that in adult women and men there was a clear correlation between plasma T levels and levels of T in the cerebrospinal fluid (CSF) ($r = 0.831$). The fraction of the plasma level of T measured in the CSF was 2.5%. This drop in levels presumably reflects the fact that bound testosterone does not cross the blood brain barrier. There was no significant difference in the calculated level of free testosterone in the plasma and CSF levels. Again the correlation was high ($r = 0.92$). These results suggest that serum levels do represent brain exposure, however no study has measured the correlation between foetal testosterone levels in serum and CSF. Finally, the presence and sensitivity of appropriate receptors also determines whether and how potent T's effects may be. Currently, there is no method for determining receptor numbers and receptor sensitivity in vivo.

Determining gestational age at amniocentesis is not exact. In our early studies we calculated gestational age using the date of last menstrual period. More accurate estimates would be obtained using sonographic measures (such as femur length), but these were available for fewer children. In the sample discussed in chapter 7 we have used sonographic measures whenever they were available.

A final limitation of research using the amniocentesis method is that a truly random sample cannot be collected because one can only include individuals who have decided/been advised to have an amniocentesis due to late maternal age or other factors that increase the risk of foetal abnormality. Previous studies investigating the relationship of prenatal T to cognitive development in humans have relied upon individuals with abnormal hormonal environments during pregnancy or those exposed to drugs that mimic or block natural hormones. Compared to these groups, our sample is more representative of the general population. In addition, since all of our children's mothers had undergone amniocentesis, whatever may be unusual about that population will be shared by all the participants. It is unlikely that amniotic testosterone levels are different in mothers who undergo amniocentesis compared to those that do not, because

within this group no relationship was seen between fT and maternal age, alpha-fetoprotein level, paternal age, or parental education level (Lutchmaya, 2000).

Confidence in our results would be increased if we could show similar relationships between prenatal testosterone exposure and traits relevant to autism using different research strategies. In the analysis of sex-typical play in children with autism spectrum conditions, reported in Chapter 5, we found that for play that did not require pretence, girls with autism did not show a preference for female items. Their pattern of performance is similar to that of girls with CAH, who have known prenatal exposure to high testosterone levels. In the study reported in Chapter 6, we found an 8 month delay in age at menarche, which is also potentially compatible with the hypothesis that prenatal exposure of the brain to high levels of testosterone increases the risk of autism spectrum disorders.

In addition, a recent study (Knickmeyer et al., submitted) reports the first examination of autistic traits in females with CAH; this provides an additional test of the foetal testosterone theory of autism. 60 individuals with CAH (34 female, 26 male) and 49 unaffected relatives (24 female, 25 male) completed the Autism Spectrum Quotient (AQ), a brief, self-administered instrument for measuring the degree to which an adult with normal intelligence has traits associated with the autistic spectrum. It comprises 50 questions, made up of 10 questions assessing 5 different areas: social skills, attention switching, attention to detail, communication and imagination. Higher scores always indicate a greater number of autistic traits (i.e., poor social skills, poor imagination, poor communication, exceptional attention to detail, and poor attention-switching/strong focus of attention). People with HFA or AS score significantly higher than typical controls on total score and all subscales. Typical males score higher than typical females on total score and all subscales except local details. Females with CAH scored higher than their unaffected female relatives on the total AQ score and on the subscales measuring social skills and imagination. Males with CAH did not differ from their unaffected relatives on these subscales, but both males and females with CAH scored lower on the subscale measuring attention to details. These results suggest that prenatal testosterone may be

involved in the male vulnerability to autism, but that not all autistic traits are necessarily more male-typical, or necessarily increased by prenatal exposure to androgen.

Finally, there is also an important theoretical limitation to the EMB theory. It is a top-down theory which takes as its starting point cognitive differences between the sexes. Although there is an extremely large body of evidence supporting an important role for fT in brain development and in the expression of sexually-dimorphic behaviour, we do not yet know how fT actually works in the brain to produce sex differences in behaviour. Until developmental neuroscience can fill this gap, the EMB theory and the androgen-exposure hypothesis as an explanation of autism spectrum conditions must be speculative.

Further Questions

### What contributes to individual variation in fT levels?

Factors producing inter-individual variation in prenatal testosterone levels are not fully known. Variation could result from environmental factors and/or an individual's genetic makeup. Some variation may be random. The relative importance of different factors may vary depending on the sex of the individual. As indicated earlier, individual variation in the fT level of boys is primarily determined by testicular testosterone production. In girls, individual variation in fT levels is primarily determined by adrenal testosterone production, which is a by-product of the production of corticosteroids. This occurs in males as well, but the levels are very small compared to that produced by the testes. Female levels may also be affected by maternal testosterone levels. Women with medical conditions causing elevated androgen during pregnancy or those taking androgenic hormones may give birth to females with ambiguous (somewhat masculinized genitalia), suggesting that maternal hormones cross the placenta into the foetal system (Barbieri, 1999; Ehrhardt & Money, 1967; Wilkins, 1960). In contrast, hormones do not appear to pass from foetus to mother.

Regarding <u>genetic factors</u> levels of pubertal and post-pubertal testosterone in males are determined, in part, genetically, with heritability estimates around 40% to 60% (Harris et al., 1998; Sluyter et al., 2000). In women around 40% of the variance in testosterone is heritable, both in adolescence and adulthood (Harris et al., 1998). In contrast to their results in adolescent twins (which indicated that testosterone is highly heritable), Harris et al. (1998) found no correlation between testosterone levels in middle-aged fathers and their sons, suggesting that distinct genetic mechanisms influence testosterone levels over time. A moderate correlation was seen between mothers and their daughters, further supporting the hypothesis that genetic factors affecting testosterone levels may differ between the sexes. Sakai et al. (1992) found that genetic influences did not account for with-in sex variation if fT levels at birth. This is potentially compatible with the view that specific genetic contributions to fT levels only occur at specific time-points. However, the Sakai study did not examine the sexes separately, and may therefore have missed genetic influences which affected only one sex. Inter-individual variation in testosterone levels could be caused by genetic differences in the enzymes that are involved in the production of testosterone (see Figure 8.1) or the transcriptional factors regulating those genes. Overproduction of any of the enzymes leading to testosterone in the pathway could result in higher than normal levels. However, it should be kept in mind that 17 ketoreductase (also known as 17β-hydroxysteroid dehydrogenase) is a bi-functional enzyme. 11 isoforms have been isolated thus far. Some isoforms (including types 1 and 5) preferentially convert DHEA into androstenediol, while other isoforms (including types 2 and 4) carry out the reverse reaction. Likewise the formation of T from androstenedione is catalyzed by type 3 and 5, and the reverse reaction by type 2 (Chung & Hu, 2002). Underproduction of aromatase could also produce high fT levels by impairing conversion of testosterone to oestrogen. Dihydrotestosterone (DHT) is produced from testosterone and may be a stronger activator of the androgen receptor than testosterone itself. Therefore overproduction of 5α-reductase could also produce hypermasculinization. However, relevant genes are not restricted to these. For example, transforming growth factor-α (TGFα), produced by immature Leydig cells in response to leutinizing hormone (LH) may have significant autocrine effects on the proliferation of Leydig cells (the cells which produce

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testosterone) (Millena, Reddy, Bowling, & Khan, 2004). Greater proliferation of cells could lead to higher testosterone levels.   TGFα may also lead to increased androgen production in individual cells via its effect on enzymes in the testosterone production pathway, including P450ssc (Millena et al., 2004).   Steroidogenic acute regulatory protein (StAR) is a key regulator of cholesterol transport to the inner mitochondrial membrane (where P450scc is localized). When StAR levels are high there is an increase in cholesterol transport and steroid secretion. In addition, the effects of exposure to high fT levels could be mimicked by increased sensitivity to normal fT levels (for example because of an overproduction of androgen receptors). Therefore androgen receptors and related transcriptional factors could also be of important.

Figure 8.1

Testosterone synthesis pathway
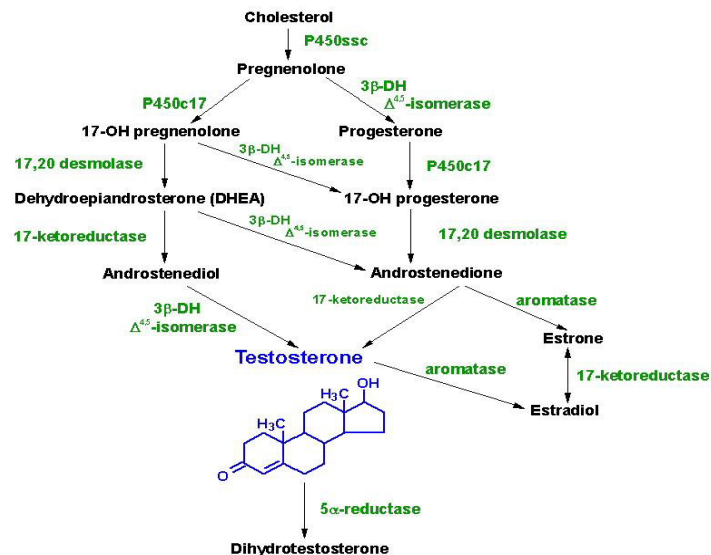
    Stress also has a potential affect on testosterone levels. Because the adrenal gland is involved in the production of stress hormones (the corticosteroids) and female fT levels are primarily adrenal in origin, it would seem that stress should have a greater influence on inter-individual variation in females than it does in males. However, in rat models the females appears to be less sensitive to maternal stress exposure than males. Long term neuroendocrine effects of prenatal stress on male rats include feminization of sexual behaviour, increase in aggressive behaviour, morphological changes in sexually dimorphic brain regions, suppression of the pituitary response to leutinizing hormone releasing hormone (LHRH), decrease of hypothalamic catecholamine response to acute stress, decrease of adrenocortical response to acute stress, noradrenergic hypersensitivity of the hypothalamic-pituitary-adrenal (HPA) axis, and a decrease in glucocorticoid receptor density in the hippocampus. Effects in females include decrease of fertility and

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fecundity, increase in aggressive behaviour, delay of sexual maturation, changes in the oestrous cycle, and a moderate increase of adrenocortical response to acute stress (Reznikov, Nosenko, & Tarasenko, 1999). Several of these reported effects on males are similar to those seen when testicular hormones are removed during development. Prenatal stress apparently results in a trough in Δ5-3β-hydroxysteroid dehydrogenase (3β-HSD) activity which disrupts a surge of testosterone that normally occurs in the developing male rat (Ward et al., 2003; Ward, Ward, Hayden, Weisz, & Orth, 1990). Affects in females, though less widely reported, resemble the affects of exposure to abnormally high testosterone and stress has been reported to produce "extraordinarily high levels of testosterone" in pregnant rats (Beckhardt & Ward, 1983). Behavioural and endocrine masculinization in females and feminization in males has also been reported for the guinea pig, a precocial animal whose developmental timing may correspond more closely to humans than that of the rat (S. Kaiser, Kruijver, Straub, Sachser, & Swaab, 2003; S. Kaiser, Kruijver, Swaab, & Sachser, 2003). Behavioural masculinization in the female guinea pig corresponds with elevated adrenal activity (S. Kaiser & Sachser, 1998).

Stress during pregnancy has also been suggested to affect human sexual differentiation. Dorner, Schenk, Schmiedel, & Ahrens (1983) reported that homosexual and bisexual men in East Germany reported more moderate to severely stressful events during their mother's pregnancy. Several subsequent studies have failed to find the same affect (Bailey, Willerman, & Parks, 1991; Schmidt & Clement, 1990) (but see Ellis, Ames, Peckham, & Burke, 1988 and Ellis & Cole-Harding, 2001). Interpretation of these studies is difficult as most used relatively small samples (with the exception of Ellis & Cole-Harding, 2001) and all relied on retrospective report of prenatal stress. Not only did this often involve recall over several decades, but mothers of homosexual children may have been biased towards reporting increased stress. Hines, Johnston et al. (2002) were the first to assess the relationship between maternal reports of prenatal stress and human gender development prospectively. In a sample of 14,138 children, they found no relationship between stress reported during pregnancy and gender-role behaviour in male children at 42 months. In female children maternal reports of stress during pregnancy

were correlated with masculine typical behaviour at 42 months and this relationship remained significant when other factors that related to stress were controlled. The authors are careful to point out that the correlations between stress and gender role behaviour were small and that other factors made larger contributions to girls' gender-role behaviour. None-the-less, their results suggest that prenatal stress in girls may lead to increases in adrenal fT and affect later behaviour.

Maternal smoking is positively associated with the mother's circulating testosterone levels during pregnancy, as well as with the testosterone levels of her female offspring (Kandel & Udry, 1999). Remembering that maternal testosterone is believed to cross the placenta and affect sexual differentiation of female offspring, it is reasonable to expect a positive correlation between maternal smoking and behavioural masculinization in girls. Ellis & Cole-Harding (2001) found that prenatal smoking was associated with an elevated occurrence of lesbianism. One possibility is that nicotine actively increases foetal plasma testosterone levels. L. M. Smith, Cloak, Poland, Torday, & Ross (2003) have reported that maternal nicotine exposure resulted in a chronic increase in plasma testosterone in female rats (but not in males) and acute increases in plasma testosterone in both female and male ovine foetuses. In contrast, Lichtensteiger & Schlumpf (1985) and Segarra & Strand (1989) have reported that prenatal nicotine suppresses the testosterone peak in male rat foetuses and feminizes their later behaviour. Alternatively, nicotine levels may not directly cause alterations in testosterone, but may be a behavioural marker for naturally elevated testosterone levels. James (2001) has suggested that smoking is a risky behaviour that is more likely to be engaged in by those with high levels of a human character trait known as "sensation-seeking". Sensation-seeking is higher in males than females and is associated with high levels of gonadal hormones, especially testosterone. Genes for atypically high female testosterone levels may be passed from mother to daughter as discussed previously.

There is currently a great deal of concern over environmental endocrine disruptors that could adversely affect developing human foetuses. The focus has primarily been on environmental xenoestrogens (including multiple herbicides, pesticides, polychlorinated

biphenyls (PCBs), plasticizers, and polystyrenes) that mimic estrogens and environmental antiandrogens (including polyaromatic hydrocarbons, linuron, vinclozolin, and (pp'-dichlorodiphenyl) dichloroethylene (pp'-DDE)). These are not always antiandrogens in the classic sense (compounds which bind and deactivate the androgen receptor), but are a diverse group of chemicals which disrupt the synthesis, transport, activity, and metabolism of androgen (Sharpe, 2001; Sultan et al., 2001). All these compounds would be expected to result in feminization/demasculinization and, as such, are unlikely to increase the risk of autism spectrum conditions if these are, in fact, related to high testosterone levels. A search of the literature on pubmed and ISI Web of Science essentially revealed no reports of environmental endocrine disruptors with androgenic activity. Research on endocrine disruptors was identified as one of the six high-priority topics in the U.S. Environmental Protection Agency ORD Strategic Plan (USEPA, 1996) leading to a greater interest in developing tools to test chemicals for androgenic, anti-androgenic, oestrogenic, and anti-oestrogenic properties (Kojima, Katsura, Takeuchi, Niiyama, & Kobayashi, 2004; Korner et al., 2004). This may reveal compounds which have androgenic activity. However, Kojima et al. (2004) tested 200 pesticides and found that none of them had androgen receptor mediated androgenic activity. In contrast 66 of them were antiandrogens.

Birth Spacing. Hormone levels during pregnancy are also influenced by how recently the mother has had a previous child. First-borns of both sexes have higher oestrogen and progesterone levels, and male first-borns have higher T than later borns, when these are measured in umbilical cord blood. This is not due to maternal age, length of labour or birth weight. Close spacing of childbirths (i.e. less than 4 years) results in hormone levels being lower than normal, and the effects of spacing is greater for boys than girls. After 4 years, levels return to first-born levels or above (Maccoby et al., 1979).

Where in the brain does fT exert its effects on Autism Spectrum Conditions?

Due to its small size, circulating T 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 (AR) is a classic steroid receptor found in the cell cytoplasm. Once bound to T (or DHT which is synthesized within the target cell from T), it passes into the nucleus of the cell where it binds to DNA and thereby affects transcription. T can also be aromatized to oestrogen within the target cell where it can bind to the oestrogen receptor and influence transcription in a similar fashion. T may 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 (De Vries and Simerly, 2002).

In the foetal primate brain, significant AR binding is observed in the cerebral cortex, cerebellum, mediobasal hypothalamus, amygdala, corpus callosum, and cingulate cortex of both sexes. Detectable levels of 5α-reductase and aromatase are also found in these regions (Handa, Connolly, & Resko, 1988; Handa, Roselli, & Resko, 1988; Sholl, Goy, & Kim, 1989). ARs 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 (Handa, Connolly et al., 1988). Sex differences are seen in the distribution of AR in the developing cortex. AR binding is higher in the right frontal lobe and the left temporal lobe of males compared to the contralateral side. Females do not show the same asymmetry (Sholl & Kim, 1990). These findings support the hypothesis that androgens can act to differentiate the brain in a site-specific fashion. Information on the distribution of AR in the human foetal brain is extremely limited. However, the distribution in monkeys is similar in many respects to that described in the male rat, suggesting that AR distribution may be conserved across species. The human foetal hypothalamus does uptake radioactively labelled T during the midtrimester (Abramovich & Rowe, 1973), but oestrogen, androgen and progestin receptors were not found in a study of midtrimester foetal brains (Abramovich, Davidson, Longstaff, & Pearson, 1987). However, this is only a single study and the results have not been replicated. In addition, it is also

possible that T 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 (Collaer & Hines, 1995; MacLusky & Naftolin, 1981; Witelson, 1991); however it must be kept in mind that genetic factors other than those mediated by hormonal factors may also be involved. The hypothalamus is the brain area most widely recognised as sexually dimorphic and is important in mediating the sexual behaviour of males and females. A subregion of the preoptic area of the hypothalamus is larger in male than female rats and enlarges under the influence of testosterone. This region has been labelled the sexually dimorphic nucleus of the preoptic area or SDN-POA. The human analogue of the SDN-POA is thought to be located in the interstitial nuclei of the anterior hypothalamus (INAH), but there is not an absolute consensus on which of the 4 INAH nuclei is actually analogous to the SDN-POA (Kimura, 1999). Several studies have reported that parts of the INAH are smaller in women than men (Allen, Hines, Shryne, & Gorski, 1989; Swaab & Hofman, 1995). The bed nucleus of the stria terminalis (BST), another hypothalamic structure involved in sexual behaviour, is also larger in women than men (Kimura, 1999).

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 favouring women. This difference may be restricted to the posterior region (the splenium) and several reports suggest the difference is only apparent when a correction is made for the larger size of men's brains (Dreisen & Raz, 1995; Kimura, 1999). 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 women (Allen & Gorski, 1991). Overall, this suggests that women have greater inter-hemispheric connectivity than men, but the functional significance of this may vary for different cognitive skills (Kimura, 1999).

The amygdala may also be sexually dimorphic. Caviness, Kennedy, Richelme, Rademacher, & Filipek (1996) found that the amygdala was smaller in females than males aged 7 to 11 (based on a sample of 15 girls and 15 boys) using magnetic resonance imaging. Goldstein et al. (2001) also found that the amygdala was smaller in females than males in a community sample of 48 normal adults using magnetic resonance imaging, but note that G. M. Murphy, Jr. (1986) found no sex difference when examining 17 post-mortem brains from gestation through age 94.

Sexual dimorphism is also present in the cortex. The planum parietale (part the parietal lobe at the posterior end of the Sylvian fissure) shows an overall rightward-larger asymmetry which is greater in right-handed men than right-handed women. The pattern is reversed in left-handers (Jancke, Schlaug, Huang, & Steinmetz, 1994). The degree of left-ward 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 women, but studies in large groups suggest the difference is fairly trivial (Kimura, 1999). Goldstein et al. (2001) found that women had larger cortical volumes relative to cerebrum size, particularly in the frontal and medial paralimbic cortices than men, while men had larger volumes of frontomedial cortex relative to cerebrum size.

Areas of the brain that show gross anatomical differences between the sexes are certainly of interest, but it should be kept in mind that differences in cognitive functioning between the sexes may arise in the absence of gross visible structural variations. Differences can occur at the cellular level (e.g. 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 physiological differences, even if they are not visible to the naked eye.

If fT does play a role in the development of autism spectrum disorders we would expect a significant degree of overlap between brain areas which show sex differences and/or are known targets of androgen and brain areas which are implicated in autism.

Brambilla et al. (2003) recently reviewed all original MRI research papers involving autistic patients, published from 1966 to May 2003. They found that increased total brain, parieto-temporal lobe and cerebellar hemisphere volumes were the most frequently reported abnormalities, while recent findings suggested that the amygdala, hippocampus and corpus callosum may 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 8.1 compares brain areas consistently implicated in autism with brain areas which express the androgen receptor during development and with sexually dimorphic brain areas. Regions which meet all three criteria are highlighted and as predicted there is a substantial degree of overlap. However, this list can not be considered complete. As noted previously, there are doubtless physiological differences between the sexes that are not observable at a gross anatomic level. Equally, there may be physiological differences in autism that are not observable at a gross anatomic level. Also, it is increasingly recognised that steroid hormones may not exert all their effects through classical receptors. The existence of a novel membrane-bound AR 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 (SHBG) receptor. The SHBG receptor activates cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) in response to binding by an androgen-SHBG complex: SHBG must first bind to the receptor and then to the steroid. SHBG molecules already bound to steroid are not able to react with the receptor (Heinlein & Chang, 2002). Testosterone may also act as a functional antagonist at the 5-HT receptor (Rupprecht, 2003). Nongenomic effects could also potentially be induced directly by androgens in the absence of a receptor. DHT has been found to alter membrane fluidity in some cell types lacking the classical androgen receptor, but only at extremely high levels which may not be relevant *in vivo* (Heinlein & Chang, 2002).

Table 8.1

Comparison of brain regions implicated in autism with those showing gross anatomical sex differences and those expressing androgen receptors

Autism	Androgen Receptors	Sexually Dimorphic (Gross anatomical level)
Parietal-temporal lobe	Temporal lobe	Parietal and Temporal lobe
Cerebellum	Cerebellum	
Amygdala	Amygdala	Amygdala
Hippocampus		
Corpus callosum	Corpus callosum	Corpus callosum
Frontal cortex	Frontal cortex	Frontal cortex
	Hypothalamus	Hypothalamus
	Cingulate Cortex	

Note: with major reference to Brambilla et al. (2003), Handa, Connolly et al. (1988), Handa, Roselli et al. (1988), Sholl et al. (1989), Kimura (1999), and Goldstein et al. (2001).

When does fT exert its effect on Autism Spectrum Conditions?

It is not currently known at what point in development the neurological changes relevant to autism spectrum conditions take place. If we accept that foetal testosterone does play a role in the aetiology of autism this could direct attention at certain periods of development. The onset of testosterone biosynthesis occurs at about 9 weeks (Grumbach et al., 2003) and the maximal sex difference in serum levels occurs between weeks 12 and 18. In the studies reported in this dissertation, we found that fT levels measured during the midtrimester were associated with poorer quality of social relationships, restricted interests, and a reduced tendency to view stimuli in mentalistic terms. Previous studies have demonstrated that fT levels at the midtrimester are related to reduced

frequency of eye contact and slower vocabulary development (Lutchmaya et al., 2002a, 2002b). These qualities are typically found in an extreme form in individuals with autism. Therefore, it appears that the midtrimester may be an important period of development in autism. This is compatible with the relatively normal physical appearance of children with autism, as damage to the nervous system in the first three months of foetal life usually results in major physical stigmata. It is also compatible with the observation that the ratio of the lengths of the 2nd to the 4th digit (2D:4D) is lower in children with autism than in the general population (Manning et al., 2001). 2D:4D is thought to be negatively associated with prenatal testosterone levels, a hypothesis that is supported by studies of children with CAH and normal children whose fT levels have been measured in amniocentesis (Brown, Hines, Fane, & Breedlove, 2002; Lutchmaya, Baron-Cohen, Raggatt, Knickmeyer, & Manning, 2004; Manning, Scutt, Wilson, & Lewis-Jones, 1998; Okten, Diren, Karaagaoglu, & Anlar, 2001). The relative lengths of the digits is set before birth and most probably by week 14 of pregnancy (Garn, Burdi, Babler, & Stinson, 1975). High fT exposure in children with autism may continue throughout pregnancy, as we found some evidence for masculinized play preferences in girls with autism and play behaviour was not influenced by midtrimester fT measures, but might be affected by fT later in pregnancy.

How does this time-scale compare with that suggested by other research in autism? M. T. Miller et al. (in press) have recently reviewed the findings of 5 studies involving individuals showing characteristics of autism spectrum disorders associated with malformations and dysfunctions known to result from early embryogenic defects. These included studies of children with exposure to thalidomide and misoprostol and those with Möbius sequence, CHARGE association, and Goldenhar syndrome of unknown aetiology. These studies suggest an origin for autism in the first trimester. Gillberg & Coleman (2000) suggest that immaturity or abnormal development of cranial nerve nuclei during the first trimester may be particularly relevant to mutism. Malformations of cortical development that occur during the second trimester can also be found in autistic subgroups, possibly including foetal alcohol and other toxic syndromes (Gillberg & Coleman, 2000). To what extent the timing of abnormal development

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associated with specific syndromes is relevant to idiopathic autism is unclear. Rodier (2000) and Rodier, Bryson, & Welch (1997) report that minor ear malformations are more common in children with autism than in typically developing children, as are dysfunctions of eye movement and lack of facial expression. Rodier argues that these anomalies represent dysfunction of the cranial nerves compatible with damage as early as the fourth week of gestation. Rodier, Ingram, Tisdale, & Croog (1997) report a single autopsy case of a female with autism who showed a striking reduction in the number of motor neurons in the facial nucleus compared to a control. They suggested the autistic case completely lacked the 5th rhombomere; a deficit potentially caused by a defect in *Hoxa-1,* a gene involved in early brain development. The case also completely lacked the superior olive, which is generally present in the brains of individuals with autism (Bauman, 2002), suggesting this case may be atypical. In addition Rodier, Ingram et al. (1997) provide no background information on the single control. M. Coleman (1994) presents a variety of evidence suggesting that insult during the 2nd trimester is responsible for the development of children with autism who do not bear any physical stigma, including evidence from one of the only prospective studies of prenatal factors in autism. This was the Collaborative Study of the National Institutes of Health in the United States. The study found that second-trimester bleeding was significantly higher in children who went on to develop autism than in typical and mentally retarded controls. Coleman also argues that neuronal migrational deficits seen in some patients with autism are most compatible with insults occurring in the second trimester. Bauman (2002; Bauman, Filipek, & Kemper, 1997) has argued that abnormal neurodevelopment must occur prior to weeks 28-30 of gestation based on the pattern of cerebellar abnormalities often found in autism. Post-mortem studies of autistic brains show a dramatic decrease in the number of Purkinje cells throughout the cerebellar hemispheres, while showing a normal number of olivary neurons (though the size of these neurons may be unusual). Cerebellar lesions which destroy Purkinje cells lead to a retrograde loss of olivary neurons after week 30 of gestation, therefore the presence of olivary neurons combined with the lack of Purkinje cells indicates an earlier advent of abnormal development. Kern (2003), however, argues that Purkinje cells may be selectively vulnerable and that neuronal cell death or brain

damage after birth, perhaps related to significant illness or clinical events, is responsible for some cases of autism.

Is the neonatal surge in testosterone relevant to Autism Spectrum Conditions?

In Chapter 6, we discuss the possibility that if the cause of elevated prenatal androgen activity is intrinsic to the foetus this deficit might also be detectible in postnatal life. In that chapter we specifically reviewed evidence that puberty is an important time of symptom change for some individuals with ASCs and that the pattern of puberty itself might be different in these cases. However, we have not yet discussed whether the neonatal testosterone surge may be relevant to autism spectrum conditions. This partially reflects the fact that the consequences of the neonatal rise in testosterone levels on brain development are unclear. G. R. Brown & Dixson (1999) treated 7 male infant rhesus macaques with avorelin, a GnRH (gonadotropin releasing hormone) agonist which reversibly suppresses testicular androgen function, and treated 10 female infant rhesus macaques with testosterone from postnatal day 5 until 6 months of age. Behavioral development was recorded during the same period. Sexually dimorphic patterns of play and mounting were not affected by treatment. Similar results were found in a study of male infant macaques whose testosterone levels were suppressed using a GnRH antagonist and whose play and mounting behaviour was recorded at 1 year of age (Wallen, Maestripieri, & Mann, 1995). In contrast, manipulation of the postnatal T surge does have dramatic affects on phallic and clitoral development (G. R. Brown, Nevison, Fraser, & Dixson, 1999). Thus far only one primate study has found a link between the postnatal T surge and later sexually dimorphic behaviour; female marmosets exposed to testosterone in the neonatal period have partially masculinized genitalia and exhibit more rough and tumble play and male sexual behaviour than controls (Abbott & Hearn, 1978). However, marmosets have a very short gestation period and the majority of pregnancies are dizygotic twins which share a common placental circulation. The unique nature of their early development necessitates caution in generalizing these results to other species.

The neonatal T surge may also be important for phallic development in humans as indicated by several case studies of progressively impaired genital development (i.e. lack of penile growth and involution of the scrotum) in boys lacking the early physiological rise in T (Main, I. M. Schmidt, & Skakkebaek, 2000). There has been no study of normal physiological variation in neonatal T and later behaviour in humans. Berenbaum et al. (2000) examined prenatal versus postnatal androgen excess in girls with CAH and found that sex-atypical play was associated with inferred prenatal exposure, but not with early inferred postnatal exposure, but note that androgen levels were not directly measured. Some authors have speculated that the occasional reports of hypomasculinization in boys with CAH may reflect suppression of the neonatal T surge following cortisone-replacement therapy (Hines, Fane et al, 2003). Although, there is currently little evidence for a link between neonatal T and later behaviour, this is an area that deserves further research in both humans and other primates. Given the extremely high circulating T levels in the neonatal period it will be surprising if they have no effect on the brain.

<center>Alternative Explanations of the Sex Ratio in Autism</center>

Despite the dramatic sex ratios reported for autism spectrum conditions, research on potential mechanisms underlying the sex ratio has been extremely limited. Thompson, Caruso, & Ellerbeck (2003) reviewed publications abstracted in PsychLit between 2000 and 2002 under the keyword 'autism'. Of 563 articles listed only 24% provided information about the number of males and females and more than half of these were small sample, single subject designs that were not powerful enough to analyze sex differences. Only 2% of the articles which reported numbers of male and female subjects analyzed dependent variables separately for males and females. The majority of studies discussing sex differences focused on incidence and prevalence and not on the potential causes of biased sex ratios or differences in phenotypic expression of autism and treatment outcome that would be informative regarding potential causes. The studies reported in this dissertation suggest that prenatal hormones may be involved in the biased sex ratio, but several alternative models exist which could also explain the excess of males with autism.

Tallal, Ross, & Curtiss (1989) examined the pattern of family history from a set of 62 4-year-old children with specific developmental language disorders (developmental dysphasia), a disorder with a sex ratio of approximately 2.5:1, in order to investigate possible sex-linked transmission. To their surprise they found that families where the mother was affected with language impairment had a disproportionate background frequency of male births. These mothers were 3 times more likely to have male children. In the general population roughly equal numbers of boys and girls are born. This suggests an increased rate of spontaneous abortion early in pregnancy (increased wastage) restricted to female offspring of affected mothers. One possible explanation for this is based on an adaptation of the Trivers-Willard hypothesis (TWH) (Trivers & Willard, 1973). The TWH states that for all species for which male fitness variance exceeds female fitness variance, parents in better conditions should produce more male than female offspring while those in poorer conditions should produce more female than male offspring. While the TWH specifically highlights the material and economic conditions of parents, it has been argued that whenever a particular environment results in males experiencing greater reproductive success than females (or vice versa), the possibility exists for the evolution of parental mechanisms which vary the sex ratio accordingly (Kanazawa & Vandermassen, submitted; Ridley, 1993). Social and communication problems may be more detrimental to women (in evolutionary terms) because women may rely more heavily on social-communication skills than men do. In fact, McLennan, Lord, & Schopler (1993) found that despite the observation that girls with high-functioning autism (HFA) were less handicapped in regard to social and communicative behaviour in comparison to their male counterparts during childhood, older females with HFA were described as having more severe social deficits than males, particularly in regard to peer relationships. The authors speculate that men with autism were able to take part in social activities with other men, such as spectator sports, that were less verbal and intensely interactive. The peer activities of young women rely more heavily on communication and social interests which put the women with autism at a

severe disadvantage. No study has examined whether women with ASCs (or the broader phenotype) are more likely to produce sons than daughters. However, an analysis of the 1994 U.S. General Social Survey suggests that individuals (both men and women) in careers which require high systemising skills (e.g. engineers) produce more sons than daughters, while those in careers which require high empathising skills (e.g. nursing) produce more daughters than sons (Kanazawa & Vandermassen, submitted). Some authors have suggested that maternal testosterone levels may be involved in varying the offspring sex ratio (James, 1987a, 1987b, 1990, 1992). Grant (1996) has suggested that high testosterone levels may select for ova specifically designed to receive a Y-chromosome bearing spermatozoan or nothing, but no adequate physiological mechanism is proposed.

Immunoreactive Theory of Selective Male Affliction

The possibility that maternal immunoreactivity to male and female foetuses differs has been a source of speculation for many years (Gualtieri & Hicks, 1985). The idea is that male-specific antigens from male foetuses may enter the maternal circulation and activate the mother's immune system. The mother then produces antibodies to the male antigen which cross the placental barrier and enter the foetal brain where they alter brain development. The probability (or strength) of maternal immunization increases with each male foetus. This means that risk to a male offspring increases with the number of older brothers, a phenomenon known as a fraternal birth order effect. Although Gaultieri & Hicks (1985) were primarily concerned with whether maternal immune response could explain the male preponderance in developmental psychopathology, the theory has been most thoroughly investigated with regards to male homosexuality. Male homosexuals show a strong fraternal birth order effect. It has been estimated that each additional brother increases the odds of homosexuality by 33% and that 15% to 30% percent of homosexual men owe their sexual orientation to fraternal birth order (Blanchard, 2001, 2004; James, 2004; Quinsey, 2003). Blanchard (2004) suggests that the hypothesized male antigen is an H-Y antigen. H-Y antigens refer to a wide range of molecules that are found on the surface of male cells, but not female cells,

that stimulate immune reactions in females, and whose structural or regulatory genes reside on the Y-chromosome. Animal research indicates that the immune system of pregnant females does recognize and react to foetal H-Y antigens, male foetuses are more antigenic to human mothers than female foetuses, and H-Y antigens are strongly represented on the surface of brain cells. Blanchard suggests two potential candidate proteins: protocadherin 11 Y-linked and neuroligin 4 Y-linked, but also notes alternative mechanisms of action such as the trans-placental transfer of pro-inflammatory cytokines (a different product of the immune system) and an altered hormonal milieu caused by immune system mediated enlargement of the placenta. It should be noted that the maternal immune hypothesis is not universally accepted as an explanation of the fraternal birth order affect in homosexual males (James, 2004; Rahman & Wilson, 2003). James (2004) argues that the affect is best explained by postnatal learning. T. J. Williams et al. (2000) found that men with a greater number of older brothers had lower 2D:4D ratios and therefore suggested that male foetuses preceded by a greater number of male pregnancies are exposed to higher levels of testosterone. This result was not replicated by S. J. Robinson & Manning (2000) and is in contrast to Maccoby et al. (1979) who found lower testosterone levels in the umbilical cord blood of later born males (at least when birth spacing was within 4 years).

Is there any evidence for a fraternal birth order affect in developmental disorders? Fraternal birth order is positively correlated with fluctuating asymmetry and fluctuating asymmetry is thought to be marker for disturbed prenatal development (Lalumiere, Harris, & Rice, 1999). Male newborns with older brothers also weigh less than male newborns with older sisters (Cote, Blanchard, & Lalumiere, 2003). Gualtieri & Hicks (1985) reported that pregnancy complications were more common in autistic boys with older brothers than in autistic boys with older sisters. Lord (1992) examined 16 multiplex families (those having 2 offspring diagnosed with ASCs). A general, though not perfect, pattern of decreasing non-verbal IQ with birth order was found. The study did not directly test whether older male siblings were a risk factor for autism. Neither did it show that severity of autism was associated in any simple way with birth order. However, it did suggest that unidentified factors, potentially including maternal

immunoreactivity, influence cognitive level within autism spectrum conditions. Surprisingly, the study found similar birth order effects in families where one of the affected children was female. Maternal immunoreactivity would not predict any relation with or in females. Spiker et al. (2001) replicated Lord's study in a much larger sample of 144 autism multiplex families. Again, firstborns with autism had higher non-verbal IQ scores than second born children with autism. The decreasing IQ score was related to autistic birth order rather than actual birth-order, another finding that is difficult to understand in the context of maternal immunoreactivity. There was no interaction between birth order and gender and there was no birth order affect for social skills or nonverbal language as measured by the ADI-R (Autism Diagnostic Interview-Revised). Rituals scores were higher for firstborn siblings.

R. P. Warren et al. (1990) investigated the possible role of maternal immunoreactivity in autism in a very different way. They investigated whether mothers showed an aberrant immune response to their child's lymphocytes. They found that 6 out of 11 mothers (54%) with an autistic child showed a significantly elevated complement dependent cytotoxic reaction to the lymphocytes of their child. In contrast 2 out of 20 (10%) of control mothers with a healthy child showed the same reaction. In 3 of the 6 families with an immunoreactive mother, maternal antibodies reacted to the lymphocytes of a healthy older sibling as well as to those of the autistic child and, in fact, in all cases where maternal reactivity was observed, the child had a healthy older sibling. This is consistent with the hypothesis that mother's of an autistic child were sensitised to foetal antigens during pregnancy with the older child. Unfortunately, family composition and sex of the children were not reported in this study.

Genetic Explanations of the Sex Ratio

The biased sex ratio in autism could result from a variety of genetic reasons including 1) autism is caused by the transmission of an autosomal gene with reduced penetrance in females 2) autism is a polygenic disease and females have a higher threshold for developing the disorder 3) autism is caused by the transmission of an X-

linked gene 4) autism is a genetically heterogenous disorder where both autosomal and X-linked transmission takes place. The majority of research has focused on polygenic thresholds and X-linkage.

The expectation from a polygenic threshold model is that the sex with the lower incidence of a particular disorder (in the case of autism, females) should require a higher 'dose' of genetic risk (as indexed by high familial loading) to develop the condition. One would therefore expect an excess of affected relatives in the extended families of female probands, but the majority of studies have failed to find this affect (Pickles et al., 2000; Rutter, 2000; Szatmari et al., 2000).

The principle characteristics of X-linked recessive inheritance are as follows 1) males are predominantly affected 2) all the phenotypically unaffected but heterozygous daughters of affected males are carriers 3) among the sons of heterozygous women there will be a 1:1 ratio of affected to unaffected subjects 4) transmission occurs from affected grandfathers through healthy mothers to affected grandsons 5) all sons of affected men are unaffected (no male-to-male transmission) (Vogel & Motulsky, 1996). Hallmayer, Spiker et al. (1996) examined 11 extended pedigrees from 77 multiplex families and found male-to male transmission in 6, indicating that autism cannot be an exclusively X-linked disorder. Data from the other 5 families was consistent with either an autosomal or X-linked mode of transmission. However, a number of studies have failed to find a susceptibility gene on the X chromosome (Consortium, 1998; Hallmayer, Hebert et al., 1996; Hallmayer et al., 1994; Risch et al., 1999; Schutz et al., 2002) (but see Philippe et al., 1999). The Y chromosome has also been examined in relation to autism, although it is clear that no gene on the Y chromosome can be necessary for autism to develop or the condition would never be seen in girls (unless they had translocations of relevant Y genes). None-the-less, XYY males are at an increased risk of developing autism (Geerts, Steyaert, & Fryns, 2003; Nicolson, Bhalerao, & Sloman, 1998). Jamain et al. (2002) examined Y-haplotypes in males with autism and controls, but found no evidence for a specific Y chromosome affect in autism.

Rutter et al. (2003) have suggested that traditional molecular genetic studies which look for susceptibility genes on the sex chromosomes ignore numerous interesting complexities of the sex chromosomes that could play a role in sex differences in psychopathology. For example, although females have 2 X chromosomes, only one of these is generally active. X chromosome inactivation (the process by which one X is suppressed while the other remains active) acts to negate the 'dosage' difference in X chromosome genes between males and females. However, 10-15% of X chromosome genes may continue to be expressed from the supposedly inactive X. X chromosome gene dosage affects may therefore play a role in sex ratios. Inactivation does not involve the same X chromosome in all cells, meaning that females are all epigenetic mosaics. Although inactivation is largely random it may be influenced by the environment. Mosaicism effects could create greater variability in females or act as a protective factor. Genomic imprinting is also of interest. Imprinting refers to a process by which genetic effects are influenced according the whether the genes are transmitted through the father or the mother (Keverne, 1997). Ordinarily this would not result in sex differences in the rate of a condition, but it will do so if the imprinting affects the X chromosome. Skuse (2000; Skuse et al., 1997) has suggested that an imprinted X-locus may explain sex differences in social and communicative skills and the male vulnerability to social and communication impairment.

Imprinting in Social-Communication Skills and Autism

Skuse et al. (1997) found that in individuals with Turner's syndrome (which is characterized by the XO genotype), the rate of social difficulties varied according to whether the single X chromosome was inherited from the father or the mother. Social problems were greater when the X chromosome was maternal in origin. Normal females always inherit an X-chromosome from both parents, but normal males always have only a maternal X chromosome. Skuse argued that genomic imprinting was the key mechanism for the social deficit seen in some girls with Turner's, and it was hypothesized that a gene expressed on the paternal X chromosome acted as a protective factor against the social problems seen in Turner's and, by extrapolation, a protective factor against other

conditions, such as autism, that involve social-communication problems. Creswell & Skuse (1999) subsequently presented 5 cases of autism from an unselected sample of 150 subjects with Turner's syndrome. All the cases had an intact maternal X-chromosome and a structurally abnormal or absent paternal X-chromosome. It should be noted that all of the cases Skuse & Creswell report had moderate to severe learning difficulties and low verbal IQ scores, despite the fact that intelligence is usually normal in Turner's. This raises the possibility that the autism observed was a consequence of learning difficulties. Skuse & Creswell note that 6 other cases in their sample also had equally low verbal abilities, but did not show autistic features. 2 of these had a paternally derived chromosome and 4 had a maternally derived chromosome. Skuse & Creswell's finding has not been replicated, although there has been an additional case report of autism in association with Turner's syndrome (Donnelly et al., 2000). This case did have a maternally derived X-chromosome. However, 77% of Turner's have a maternally derived X-chromosome, while only 23% have a paternally derived chromosome (Grumbach et al., 2003). This means that purely by chance one would expect to find autism in association with maternally derived X-chromosome Turner's more often than autism in association with paternally-derived X-chromosome Turner's. It is clear that the putatative X-linked imprinted gene is not sufficient or necessary for the development of autism. If it were sufficient, all males would have autism as would all Turner's females with a maternally derived X. It is not necessary for the development of autism or autism would not be seen in females. Rather Creswell & Skuse propose that the genetic liability to autism depends primarily on the autosomes. The functions of the X-linked genetic locus are not directly linked to features of the autistic phenotype, but it acts upon other loci elsewhere in the genome that confer susceptibility to the autism phenotype. Therefore, the imprinted-X liability threshold model does not explain why a particular male develops autism; but it can explain why males, in general, are more likely to develop autism. Likewise, it can explain the sex difference in social and communicative skills between males and females, but does not explain inter-individual variation within either sex. In contrast, the prenatal androgen model does attempt to explain individual risk for autism spectrum conditions and within-sex variation in traits relevant to autism.

Skuse (2000) also argues that exposure to prenatal androgens is unlikely to account for the biased sex ration in autism. Firstly, he argues that the prenatal androgen hypothesis cannot account for the superior performance of mother's of autistic probands on visuo-spatial tasks that usually show a male advantage. He argues that this indicates androgen exposure is neither sufficient nor necessary for the enhancement of visuo-spatial abilities in autistic individuals and their relatives. Skuse's argument appears to assume that females are not exposed to any androgen during prenatal life, when, in fact, they are exposed to androgens from the adrenal gland and the maternal circulation. Some women will be exposed to greater levels of androgens than others. Because testosterone exposure may be heritable, it is quite possible that the mothers of autistic probands have also been exposed to higher than normal testosterone levels. This hypothesis is supported by Manning et al. (2001) who found that 2D:4D ratios were lower in the mothers and fathers of children with autism as well as in the children themselves.

Secondly Skuse (2000) argues that there is no evidence that females exposed to exceptionally high levels of androgens are more likely to develop autism or autistic-like behaviours. As discussed earlier, no study has specifically examined whether a diagnosis of autism is more likely in females exposed to abnormally high prenatal testosterone levels, for example as a result of CAH. Given that the genetic liability to autism is presumably rather rare and that CAH only occurs in 1 in 15,000 births, it would be unusual for such females to be at genetic risk. A recent study (Knickmeyer et al., submitted) suggests that many autistic traits, as measured by the Autism Questionnaire, are higher in women with CAH.

Finally, Skuse (2000) argues that the androgen-exposure hypothesis should predict a greater number of affected male relatives in the families of females with autism than those of males with autism. As discussed earlier several research studies have failed to find such a pattern. Skuse's argument appears to be that females with autism must have a greater genetic liability to autism than males, because their testosterone exposure (even if higher than a normal female) is not as high as that of a male (conversely, the male with autism, being exposed to higher testosterone levels, requires less genetic

liability). The male relatives of both the male and female with autism will have similar testosterone exposure, but because the genetic liability was greater in the family of the female, she should have more male relatives diagnosed with the condition than the male with autism. This argument contains multiple assumptions. Firstly, it again treats testosterone exposure as a basically dichotomous variable and doesn't consider the variations in testosterone levels within both sexes. Secondly, because testosterone levels are probably controlled by different mechanisms in males and females, one cannot assume that the testosterone levels will be similar in the relatives of male and female autistics. Consider the possibility that testosterone levels are more heritable between male relatives than between male and female relatives. The male relatives of the male with autism will not only share genetic liability which is unrelated to levels of testosterone, they will also share a genetic propensity for higher testosterone exposure, increasing their risk of autism. Male relatives of females with autism, on the other hand, wouldn't share a genetic propensity for higher testosterone exposure, so the male relatives of the females with autism would have lower testosterone levels than the relatives of the males with autism.

Both the imprinted-X liability threshold model and the androgen-exposure model offer interesting potential explanations for the biased sex ratio in autism. Given the current lack of support for either a polygenic threshold model or traditional X-linkage, both theories deserve further research attention. Although the preceding discussion and Skuse (2000) present these two models as conflicting explanations for the sex ratio, it should also be noted that this is not necessarily the case. Because the putative imprinted gene has not yet been identified, we cannot rule out the possibility that the gene is regulated by testosterone or that the gene product affects the production or sensitivity of an individual to testosterone. It is also possible that the putative X-linked imprinted locus and prenatal testosterone exposure are 2 independent risk factors for autism. The 2 theories could be explored further in individuals with intersex conditions, in particular Congenital Adrenal Hyperplasia (CAH) and Androgen Insensitivty (AI). Recall from the introduction that CAH cases have a normal female genetic complement (XX), but high exposure to fT. AI cases have a normal male genetic complement (XY) but lack a

functioning androgen receptor. According to the imprinted-X liability threshold model, individuals with CAH should perform like normal women (and X^p Turner's) on tests of socio-communicative ability such as the one used in Skuse (1997) while AI women should perform like normal males (and X^m Turner's). In contrast, the androgen-exposure model would predict CAH women to perform like normal males and AI women to perform like normal females. If both groups performed like normal males it would suggest that both theories have validity (although this could also indicate a non-AR mediated mechanism for testosterone).

Conclusion

Although it is widely accepted that autism is a biological disorder, there is no consensus on what biological factors are involved. The higher incidence of autism in males may provide important clues to the aetiology of the condition. The studies reported in this dissertation suggest that prenatal testosterone may be involved in the male vulnerability to autism, but further research is required. Several important questions remain including 1) Do variations in fT relate to variations in cognitive functioning and symptom severity within individuals with autism spectrum conditions? The studies reported in the first two chapters show that variations in fT 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) Is fT specifically involved in the development of autism or does it promote a general 'male vulnerability' (Kraemer, 2000)? Rutter et al. (2003) note 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 behaviour. In contrast, female-biased sex ratios largely occur in emotional disorders with a peak age of onset in adolescence. Although they acknowledge that there may be diagnosis-specific factors relevant to the sex-differences in individual disorders, they suggest that type of causal influence will be similar within these two groups of disorders that differ in both sex ratio and age of onset. 3) Is fT relevant to most cases of idiopathic autism or to a specific subset of cases? Gillberg & Coleman (2000) argue that autism is a syndrome or series of

syndromes caused by many different, separate individual diseases, all of which affect the same final common pathway in the brain that causes individuals to present with autistic symptoms. Exposure to elevated levels of fT could be an important component of one or several of these individual diseases that lead to the behaviour profile called autism. Although high testosterone on its own is probably not the cause of autism, once we understand the hormone's involvement, we will be able to unravel the other causes, and possibly discover why the condition is increasing in prevalence. With a fuller understanding of the causes of autism we will be able to think more fruitfully about diagnosing and treating the condition.

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Appendix A: Hormone Assays

Testosterone

Amniotic fluid was extracted with diethyl ether. Recovery experiments have demonstrated 95 percent recovery of testosterone using this method. The ether was evaporated to dryness at room temperature and the extracted material redissolved in assay buffer. The testosterone was assayed by the DPC 'Count-a-Coat' method (Diagnostic Products Corp, Los Angeles, CA 90045-5597), which uses an antibody to testosterone coated onto propylene tubes and a 125-I labelled testosterone analogue. The detection limit of the assay is approximately 0.1 nmol/L. Intra-assay coefficients of variation (i.e. 1 standard deviation expressed as a percentage of the mean value) were between 10 and 15%. This method measures total extractable testosterone.

Oestrogen

Amniotic fluid was extracted with diethyl ether. Recovery experiments have demonstrated 95 percent recovery of oestradiol using this method. The oestradiol was measured by fluorescence-labelled immunoassay. The Wallac-Delfia method was used (Wallac OY, Turku, Finland). This assay uses a polyclonal rabbit antibody to oestradiol in a competitive format in which sample oestradiol competes with europium-labelled oestradiol analogue for the antibody binding sites. A second antibody directed against rabbit IgG is coated to the microtitre plate and is used to capture the first antibody and its bound oestradiol analogue. After washing, the europium is measured by time-resolved

fluorescence. Calibration is with pure 17beta-oestradiol. The detection limit is 25 pmol/L. The cross reactivity with steroids other than 17beta oestradiol is very low. It should be noted that 16 hydroxy and 16 oxo-steroids, steroids that are formed in the foeto-placental unit, cross react to less than 0.9% by weight. Intra-assay coefficients of variation were 5.2% at 180 pmol/L and 3.9% at 875 pmol/L.

Alpha-foetoprotein (AFP)

AFP was measured by fluorescence-labelled immunoassay. The Wallac-Delfia method was used (Wallac OY, Turku, Finland). This assay is based on the direct sandwich technique in which two monoclonal antibodies (derived from mice) are directed against two separate antigenic determinants on the AFP molecule. The analytical sensitivity of the assay is typically better than 0.1 U/ml. Recovery experiments have demonstrated 101 percent recovery of AFP using this method. Serum albumin concentrations in the normal physiological range do not interfere with AFP determination. Intra-assay coefficients of variation were 1.0% at 10199 U/mL and 1.1 at 12438 U/mL.

Appendix B: Pilot Study of the Modified Child Game Participation Questionnaire

Methods

Participants

Participants were 28 girls and 35 boys from local schools in Cambridgeshire. All children were 4 to 5 years old and attended reception/nursery classes. This age was chosen to match that of the children in our group's longitudinal study of prenatal testosterone and child development. Children were given the questionnaire and a letter explaining the study in sealed envelopes at school and told to take them home to their parents. If parents agreed to participate they completed the questionnaire and returned it by freepost envelope. The 63 participating children represent those whose parents responded from a larger sample of 80.

The Children's Play Questionnaire

The questionnaire was adapted from the Child Game Participation Questionnaire (CGPQ) (Bates & Bentler, 1973). This instrument was originally developed to discriminate between boys with gender identity disorder and gender-typical boys. It also shows highly significant gender differences (Meyer-Bahlburg et al., 1985). The original item pool included 120 children's games. In order to make the questionnaire more manageable and increase the response rate, it was decided to shorten the questionnaire. In addition, given the young age of the sample, some items, such as softball, were not

appropriate. The final questionnaire included 10 items that were expected to be preferred by boys and 10 that were expected to be preferred by girls. 9 of the male items appeared in the bipolar Gender scale as masculine items in Meyer-Bahlburg et al.'s (1994) factor-analytic study of a modified CGPQ. The difference between boys and girls on this factor showed a large effect size, $d = 3.90$. We added one item ("playing with blocks or lego/duplo") that seemed gender dimorphic in our region. 9 of the female items appeared in the bipolar Gender scale as feminine items in Meyer-Bahlburg et al.'s (1994) study. We added one item ("playing with hair") that seemed gender dimorphic in our region. We split the item "plays with dolls" into 2 items: "playing with Barbie-type dolls" and "playing with baby dolls" in order to have equal numbers of male and female items. The questionnaire mailed to parents also included 10 items thought to be gender neutral. This was done to prevent biased answering, which might have occurred if parents realized the test focused specifically on gender-typical play.

For each game, parents indicated their child's interest on a Likert scale where 1 was not at all interested and 5 was very interested.

Results

Two scores were calculated for each individual. A total femininity score was calculated by adding together the score on each female item (1=0, 2=1, 3=2, 4=3, 5=4). A total masculinity score was calculated by adding together the score on each male item in the same way. The femininity and masculinity scores had a possible range of 0 to 40. Table B.1 shows descriptive data for each scale by gender. Both scales showed

significant differences and large effect sizes. Effect sizes are similar to that reported for the composite scale on the modified CGPQ used by (Meyer-Bahlburg et al., 1994) with 6-10 year old children.

Table B.1

Scores of 4-5 year old children on female and male items on the pilot version of the Children's Play Questionnaire

Scale	Girls n = 28			Boys n = 35			
	Mean	SD	Range	Mean	SD	Range	d
Female Items[**]	31.37	5.69	15-39	11.12	5.68	0-21	3.57
Male Items[**]	11.89	4.85	4-23	28.22	6.91	7-39	2.74

[**] $p < 0.01$

t-tests were run on every item in order to determine whether they showed the expected sex-differences (t-tests were also run on neutral items to ensure that they did not show a sex-difference). Equal variances were not assumed. The probability of a type I error was maintained at 0.05 for all analyses. Because this involved 30 comparisons, it was necessary to use a Bonferroni correction when evaluating the results. Sex-differences were considered significant if they had a t value greater than 3.32. Table B.2 shows an item analysis for the pilot questionnaire. 3 of the male items did not show significant sex-differences although they showed trends in the expected direction (Building play houses, forts, huts or dens; Playing with blocks or Lego/Duplo; Climbing

trees/rope ladders). All of the female items showed significant sex-differences in the correct direction. We decided to eliminate the three male items from the next version of the test. In order to keep the number of male and female equivalent we split the items "pretending to be a soldier or a super-hero," "Playing Cowboys and Indians or similar/play-fighting" and "Playing with toy vehicles (e.g. cars, trucks, planes, trains)" into 2 separate items each. 2 of the neutral items ("using colouring books" and "doing arts and crafts/painting" showed significant sex-differences in a female direction. These were eliminated from the next version of the questionnaire.

Table B.2

Mean scores of 4-5 year old children by sex for all items on the pilot version of the

Children's Play Questionnair*e*

Item	Sex	Mean	*t*-value
Playing with Barbie-type dolls*	Female	4.00	8.56
	Male	1.50	
Role-playing domestic activities (e.g. cooking, cleaning, bathing)*	Female	4.50	6.49
	Male	3.00	
Playing dress up (fashion/jewellery)*	Female	4.71	8.99
	Male	2.40	
Role-playing family relationships (e.g. parenting/marriage)*	Female	4.30	6.49
	Male	2.46	
Skipping rope or skipping*	Female	3.25	5.81
	Male	1.69	
Playing school (pretending to be a teacher)*	Female	4.25	5.90
	Male	2.34	
Dancing*	Female	4.64	6.56
	Male	2.77	
Playing with hair (e.g. brushing someone else's hair)*	Female	3.54	7.61
	Male	1.51	
Playing tea-parties*	Female	3.89	6.31
	Male	2.20	
Playing with baby dolls*	Female	4.14	12.29
	Male	1.49	

Pretending to be a soldier or super-hero*	Female	1.29	-10.58
	Male	3.91	
Playing with toy guns or other weapons*	Female	1.46	-8.28
	Male	3.88	
Playing with toy vehicles (e.g. cars, trucks, planes, trains)*	Female	2.54	-7.47
	Male	4.42	
Pretending to be an astronaut (space-man) or explorer*	Female	1.36	-8.41
	Male	3.46	
Playing with toy tools*	Female	2.18	-4.87
	Male	3.66	
Playing with electric trains*	Female	2.11	-4.84
	Male	3.71	
Playing Cowboys and Indian or similar/play-fighting*	Female	1.29	-8.59
	Male	3.69	
Building play houses, forts, huts, or dens	Female	3.46	-1.51
	Male	3.89	
Playing with blocks or Lego/Duplo	Female	3.50	-2.27
	Male	4.03	
Climbing trees/rope ladders	Female	3.04	-3.21
	Male	4.00	
Looking at picture books	Female	4.61	.975
	Male	4.41	
Using colouring books*	Female	4.59	3.74
	Male	3.69	
Playing with stuffed animals	Female	3.82	2.94
	Male	2.91	
Riding on tricycles/bicycles	Female	4.57	-1.29
	Male	4.77	

Swimming	Female	4.46	1.66
	Male	4.06	
Playing on swings	Female	4.54	1.42
	Male	4.23	
Playing on see-saws	Female	3.43	-1.04
	Male	3.71	
Doing arts and crafts/painting*	Female	4.75	3.49
	Male	4.09	
Watching cartoons	Female	3.79	-1.73
	Male	4.26	
Playing board-games (e.g. Ludo, Snakes and Ladders)	Female	3.61	.443
	Male	3.49	

Note. Raw scores were used for each item (lowest score =1, highest=5)

Note. First 10 items were expected to show a female preference, the next 10 items were expected to show a male preference, the last 10 items were expected to show no preference

[*]$p < 0.05$, using a Bonferroni correction

References

Bates, J. E., & Bentler, P. M. (1973). Play activities of normal and effeminate boys. *Developmental Psychology, 9*, 20-27.

Meyer-Bahlburg, H. F. L., Erhardt, A. A., & Feldman, J. F. (1985). Questionnaires for the assessment of atypical gender role behavior: A methodological study. *Journal of the American Academy of Child Psychiatry, 24*, 695-701.

Meyer-Bahlburg, H. F. L., Sandberg, D. E., Dolezal, C. L., & Yager, T. J. (1994). Gender-related assessment of childhood play. *Journal of Abnormal Child Psychology, 22*, 643-660.

Appendix C: Recruitment Documents

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Appendix C.1: GP Contact Letter

Information Sheet for GPs: Version 7 12/04/04

Title of Project: Antenatal Predictors of Child Development

Date: 19/07/04

Dear Dr. GP NAME,

We are researchers at Cambridge University carrying out a study on child development. We are writing to you for a little help with our project. We realise this will take a few minutes of your time, but we hope you will agree that this is a worthwhile project and be willing to help us. You have been selected because we are interested in your patient, PATIENT NAME, and want to invite her to participate.

The study will test whether foetal testosterone levels (which we measure in 2nd trimester amniotic fluid) predict the later social and communicative development of children, including the risk for developmental concerns including language delay, attention deficit hyperactivity, and autism spectrum conditions.

Your patient was selected because she underwent routine amniocentesis on DATE of AMNIO. Addenbrookes Hospital, which carries out tests on amniotic fluid for many hospitals in the Cambridgeshire area, routinely stores the samples for some time after the baby is born.

You may be concerned that this fluid was stored for clinical purposes, not for research purposes, and that this woman has not therefore given her consent for the fluid to be used in this way. We have gone through the Eastern MREC and your local ethics committee in RELEVANT DISTRICT, and obtained approval to seek her **retrospective consent.** It is of course true that this study would have been better designed to use prospective consent, but the DoH and MRC guidelines state that where fluid has been stored, potential researchers should attempt to obtain retrospective consent from the patient where possible. Naturally, if your patient does not consent, her fluid will be destroyed.

We are pleased to say that this study has been running since 1997 and several hundred women have been included. We have been pleasantly surprised by their positive interest in this study of the predictors of child development.

If we contact her she would be asked for permission to analyse this fluid for foetal testosterone. In addition she would be asked to complete a checklist indicating whether the child of that pregnancy has been referred for language delay, attention deficit hyperactivity, or autism spectrum conditions (including Asperger syndrome and pervasive developmental disorders) and to complete a questionnaire assessing her child's social and communicative development.

These developmental conditions listed above are fortunately relatively rare, so for the majority of people receiving this letter THESE CONDITIONS will not be relevant. HOWEVER, EVEN FOR CHILDREN WHO ARE DEVELOPING WITHIN THE NORMAL RANGE, OUR PLAN IS TO analyse the fluid for testosterone and compare levels in relation to the scores on the questionnaire. If you'd like to know more about the study, you can contact me at the phone number below.

In order to prevent any woman being contacted inappropriately we need you to make sure that your patient has **NOT** had any of the following since her amniocentesis on **DATE of AMNIO**

1. **A MISCARRIAGE**
2. **A TERMINATION**
3. **A STILL BIRTH**

Given the distress that would be caused if any woman was contacted inappropriately following one of these 3 sad outcomes, we would be extremely grateful if you would be willing to **check the patient's notes to confirm**

a) that she has not had one of these 3 events since her amniocentesis on DATE of AMNIO and
b) that the results of the amniocentesis on **DATE of AMNIO** were **NEGATIVE**. Errors have occurred in the past, hence the need for absolute accuracy.

In compliance with the Data Protection Act, we are not requesting that you reveal any of

this patient's private history. Rather all we ask that you SIMPLY indicate on the

enclosed form whether or not we may go ahead and approach this patient. You can return

the form in the freepost envelope provided.

We are extremely grateful for your assistance with this part of the project, since it is an essential step in the selection of potential volunteers for this study.

We realise this may incur you in some extra work, in an already very busy workload. If this is a straight forward task for you, and you can undertake it easily, you may feel you don't need any reimbursement for your time. If however this check of your patient's notes causes you significant work, we would be more than happy to reimburse you for this. Please let us know.

With many thanks for helping our research. In return for your help, we would be delighted to send you a copy of our results in due course.

With best wishes,

Yours sincerely,

Rebecca Knickmeyer
Cambridge University Department of Experimental Psychology, Doctoral Scientist
Phone: 01223-746030

Appendix C.2: GP Consent Form

FORM FOR GPS: Version 8 12/04/04

GP: GP NAME and GP ADDRESS

Below are details of the family we would like to contact:
Mother's name: PATIENT NAME   Date of amnio: DATE of AMNIO
Address: PATIENT ADDRESS   Current Year: 2004

Please consult this lady's medical notes and check that she has not had a miscarriage,

termination, or stillbirth since her amniocentesis on DATE of AMNIO. Please make sure

the amnio on DATE of AMNIO was negative and that there are no subsequent reasons

why contacting her regarding the child of that pregnancy would be inappropriate.

		Please check box
1.	**I confirm that I have read and understand the information sheet concerning the study Antenatal Predictors of Child Development (version 7 12/04/04)**	☐
2.	**I confirm that I have consulted this patient's medical notes**	☐
3.	**I know of no reason why this family should not be contacted regarding the study Antenatal Predictors of Child Development**	☐
4.	**There are reasons why this family should not be contacted**	☐
5.	**I would be interested in hearing the results of this study**	☐
6.	**I would appreciate reimbursement for this check**	☐

Signed_____ Date_____

Practice stamp:

PLEASE COMPLETE THIS FORM AND RETURN IT IN THE FREEPOST ENVELOPE
PROVIDED. THANK YOU FOR HELPING US WITH OUR RESEARCH.

Appendix C.3: Patient Contact Letter

Research Subject Information Sheet/Invitation Letter: Version 1 16/11/00

Dear Ms. PATIENT NAME,

We are writing to you about something which some of you may find stirs up difficult memories, namely, the amniocentesis that you had on DATE of AMNIO. We apologise if this letter reminds you of something that you would prefer was left firmly in the past.

I am the consultant responsible for carrying out amniocenteses in Addenbrooke's Hospital. Fluid from these, together with samples taken from hospitals in the region, is sent to our lab in Addenbrooke's for analysis. Rest assured that this information remains confidential to the hospital.

As we are a research based hospital, we need to follow up patients and their children, and I am hoping that you will agree to take part in a very interesting and unique study.

The aims of this research project are to find out if factors during pregnancy have any link with how your child is developing now. In particular, we would like to test if factors in your amniotic fluid obtained from the amniocentesis that you had while you were pregnant relate to your child's current social and communicative development. This study also hopes to address whether these factors are related to the risk of developmental concerns such as language delay, attention deficit hyperactivity, learning difficulties, and autism spectrum conditions.

We fully appreciate that undergoing amniocentesis can be a highly stressful process and we do not want to cause further anxiety. We are not investigating the effect of amniocentesis on your child, and there is no reason to be concerned that there are any ill effects. We simply want to make use of the information collected at that time which can teach us about how children develop. Regarding the developmental conditions listed above, these are fortunately relatively rare, so for the majority of people receiving this letter this will not be relevant.

What is involved?

If you agree to take part, which we very much hope you will, we would like to collect the following data:

1. Your consent for us to reanalyse the amniotic fluid that was stored following your amniocentesis, to measure foetal testosterone levels. Testosterone is produced by both normally developing male and female babies.
2. Some information on whether your child has been referred for any developmental concerns. A checklist of these is included with this letter.
3. A questionnaire on social and communicative development in your child. This is also included with this letter.
4. Permission to send you some additional questionnaires assessing language development and attention span at a later date, if you are still willing to be involved in the study.

We may also invite some mothers and their child to the Child Development Project at Douglas House, Trumpington Road, Cambridge so we can observe the child's behaviour first hand. This will only apply to a small number of families. If you are invited and decide to come we will, of course, reimburse you for the cost of your travel.

If you are unsure that you want to take part in any face-to-face assessment, but are willing to fill out the postal questionnaires for us, we would still be very grateful for your involvement with just this postal part of the project.

If you are happy to take part, a consent form is included with this letter, as well as the short checklist and the social-communicative questionnaire. You can complete these and return them to the research co-ordinator in the freepost envelope provided. You can withdraw from the study at any time, with no consequence to you and without giving any reason. You may obtain further information about the study directly from the research co-ordinator, Rebecca Knickmeyer, on 01223-746030. All details will of course remain strictly confidential.

This is a one off opportunity for us to study your child from before birth to the present day. We hope that you will find it of interest and would be most grateful if you would agree to take part.

With thanks

Yours sincerely,

Mr. G Hackett
Consultant Obstetrician & Gynaecologist

Appendix C.4: Patient Consent Form

Research Subject Consent Form: Version 1 16/11/00

Hospital/Institution Headed Paper

Title of Project: Antenatal Predictors of Child Development

Name of Researcher: Miss. Rebecca Knickmeyer, Doctoral Scientist: Department of
Experimental Psychology, Cambridge University
Phone: 01223-746030

                                                                    Delete as Appropriate

1. I confirm that I have read and understand the information in the   YES / NO
invitation letter for the above study and have had the opportunity
to ask questions.

2. I understand that my participation is voluntary and that I am free   YES / NO
to withdraw at any time, without giving any reason, without my
medical care or legal rights being affected.

3. I understand that all information will remain confidential   YES / NO

4. I agree to take part in the above study   YES / NO

Your name (BLOCK letters): …………………………………………………

Your signature: ……………………………………….   Date: ……………………

Appendix C.5: Patient Questionnaire

ANTENATAL PREDICTORS OF CHILD DEVELOPMENT

Questionnaire, v2, 28/01/01

MOTHER- Mother's Name
Date of Birth:_____   Occupation:_____   At what age did you leave school? _____   Did you complete any further education? YES / NO   If YES, please give brief details _____   Are you 1. Right-handed   2. Left-handed   3. Ambidextrous

FATHER
Date of Birth:_____   Occupation:_____   At what age did you leave school? _____   Did you complete any further education? YES / NO   If YES, please give brief details_____   Are you 1. Right-handed   2. Left-handed   3. Ambidextrous

CHILDREN

PARTICIPATING CHILD: Child's name_____

Child's date of birth_____

If you (mother) have any other children, please give details

<u>Sex</u>   <u>Date of Birth</u>

PREGNANCY & BIRTH

Was there anything unusual about your pregnancy when you were pregnant with the participating child (e.g. high blood pressure, bleeding during pregnancy, diabetes, toxaemia, prematurity) Please give details:

_____
_____
_____
_____

By what method was he/she born? Please indicate

5. normal
6. caesarean
7. ventouse/forceps
8. other, please specify _____

Did you have an epidural? YES / NO

YOUR CHILD

Has you child been referred for any of the following developmental concerns?

1 language delay   YES / NO

   Please give details
_____

2. attention deficit hyperactivity   YES / NO

3. learning difficulties   YES / NO

    Please give details
_____

4. autism spectrum conditions (including Asperger   YES / NO
    syndrome and pervasive developmental disorders)

    Please give details

_____

Regarding the developmental conditions listed above, these are fortunately relatively rare, so for the majority of people receiving this letter this will not be relevant.

AMNIOCENTESIS

Do you have any comments regarding the routine amniocentesis that you underwent when you were pregnant with your child?:

Did you feel the doctor adequately prepared you for the amniocentesis?

Did you feel that the <u>reasons</u> why you were given an amniocentesis were adequately explained?

Do you know why you were invited to have an amniocentesis?   YES / NO
If YES, please give the reason. (e.g. late maternal age)

Is there anything that you think should be changed about any aspect of the procedure?