Dosage-sensitive X-linked locus influences the development of amygdala and orbitofrontal cortex, and fear recognition in humans

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Summary
The amygdala, which plays a critical role in emotional learning and social cognition, is structurally and functionally sexually dimorphic in humans. We used magnetic neuroimaging and molecular genetic analyses with healthy subjects and patients possessing X-chromosome anomalies to find dosage-sensitive genes that might influence amygdala development. If such X-linked genes lacked a homologue on the Y-chromosome they would be expressed in one copy in normal 46,XY males and two copies in normal 46,XX females. We showed by means of magnetic neuroimaging that 46,XY males possess significantly increased amygdala volumes relative to normal 46,XX females. However, females with Turner syndrome (45,X) have even larger amygdalae than 46,XY males. This finding implies that haploinsufficiency for one or more X-linked genes influences amygdala development irrespective of a direct or indirect (endocrinological) mechanism involving the Y-chromosome. 45,X females also have increased grey matter volume in the orbitofrontal cortex bilaterally, close to a region implicated in emotional learning. They are as poor as patients with bilateral amygdalec-tomies in the recognition of fear from facial expressions. We attempted to localize the gene(s) responsible for these deficits in X-monosomy by means of a deletion mapping strategy. We studied female patients possessing structural X-anomalies of the short arm. A genetic locus (no greater than 4.96 Mb in size) at Xp11.3 appears to play a key role in amygdala and orbitofrontal structural and (by implication) functional development. Females with partial X-chromosome deletions, in whom this critical locus is deleted, have normal intelligence. Their fear recognition is as poor as that of 45,X females and their amygdalae are correspondingly enlarged. This 4.96 Mb region contains, among others, the genes for monoamine oxidase A (MAOA) and B (MAOB), which are involved in the oxidative deamination of several neurotransmitters, including dopamine and serotonin. Abnormal activity of these neurotransmitters has been implicated in the aetiology of several neurodevelopmental disorders in which social cognitive deficits are prominent. These associated deficits include a specific lack of fear recognition from facial expressions. We show that the thrombocytic activity of MAOB is proportionate to the number of X-chromosomes, and hypothesize that haploinsufficiency of this enzyme in 45,X females predisposes to their deficits in social cognition.

Keywords: amygdala; Turner syndrome; X-chromosome; fear; emotion

Abbreviations: SHOX = short-stature homeobox-containing gene; IQ = intelligence quotient; MAO = monoamine oxidase; OFC = orbitofrontal cortex; PAR = pseudoautosomal region

Introduction
The evolution of the sex chromosomes has given rise to unique mechanisms of regulation, so as to equalize gene expression between the sexes (Disteche, 1999). In normal females one of the two X-chromosomes is inactivated at
random in order to ensure equal expression of X-linked genes in male and female mammals (Lyon, 1961). Genes that escape X-inactivation are found at the tips of the X and Y chromosome arms in the so-called pseudoautosomal regions (PARs), where the equivalent nucleotide sequence is identical in both sex chromosomes, thus allowing meiotic recombination to take place. Surprisingly, many genes are now known to escape inactivation elsewhere on the X-chromosome. These are non-randomly distributed, lie mostly on the short arm, and do not necessarily have expressed Y-homologues (Carrel et al., 1999). Persistence of a dosage imbalance in such genes between males (46,XY) and females (46,XX) may be important for sex-specific functions (Disteche, 1999). We considered that it should be possible to identify the effects of dosage-sensitive loci on brain development by studying Turner syndrome females who have a single X-chromosome (45,X or X-monosomy) and who are therefore haploinsufficient for non-inactivated X-linked genes relative to normal females (Zinn and Ross, 1998).

Turner syndrome is a chromosomal disorder, with a prevalence of 1 per 2500 live female births, in which typically all or a substantial part of one X-chromosome is missing because of non-disjunction or chromosome loss during early cleavage of the zygote. In 70% of cases of monosomic (45,X) Turner syndrome the single X-chromosome is maternally inherited (Jacobs et al., 1997), the remainder being paternally inherited. In monosity X this single chromosome is never inactivated, although in normal 46,XX females one of the two X-chromosomes is inactivated at random during the blastocyst stage of development (Boumil and Lee, 2001). Dosage-sensitive genes that escape X-inactivation may contribute to some features of Turner syndrome if haploinsufficient in X-monosomy. For example, SHOX (Blaschke and Rappold, 2001) is now known to contribute to the short stature of the syndrome, and is normally expressed from the PAR1 of both the X- and the Y-chromosome.

**Amygdala anomalies and autism**

A number of recent studies have implicated the amygdala in the neurobiology of autism (e.g. Baron-Cohen et al., 2000; Howard et al., 2000; Grelotti et al., 2002; Salmond et al., 2003). They show a lack of amygdala activation in functional MRI studies of exposure to facial expressions that would normally evoke a response, and poor cognitive performance on tasks that involve neural processing by this structure. Specifically, amygdala-related cognitive deficits include poor facial recognition memory (Davies et al., 1994), failure to recognize specific facial emotions, especially fear (Howard et al., 2000) and lack of interest in and failure to follow eye gaze normally (Baron-Cohen et al., 1997). Post-mortem studies of autistic individuals have found increased cell density and abnormally small cells in the amygdala (Bauman and Kemper, 1985; Bailey et al., 1998; Kemper and Bauman, 2002). There have also been structural imaging studies that have identified abnormalities of amygdala volume (Aylward et al., 1999; Howard et al., 2000; Pierce et al., 2001). A recent structural imaging study in autism concluded that, although no neural area was abnormal in all cases of autism studied by a novel and sensitive brain imaging technique (voxel-based morphometry), abnormalities were present, particularly in the amygdala and in the orbitofrontal cortex (OFC), among those with an intelligence quotient (IQ) in the normal range (Salmond et al., 2003).

The caudal OFC receives direct projection from the amygdala in primates (Stefanacci and Amaral, 2002) and also sends projections back to the amygdala (Cavada et al., 2000). The anatomical connectivity of the OFC gives it the potential, like the amygdala, to integrate sensory information from diverse sources, to modulate sensory and other cognitive processing via feedback connections, and to influence motor and autonomic output responses. Thus, our (unpublished) findings of abnormal autonomic responses during fear conditioning of Turner syndrome subjects, and the evidence that autonomic reactivity is also abnormal in high-functioning autistic individuals (Hirstein et al., 2001), pointed to these two anatomical regions (the amygdala and the OFC) as being
potentially abnormal in terms of structure and/or function in Turner syndrome.

Deficits in social cognition in particular are highly variable in apparently non-mosaic X-monosomic individuals (45,X) when measured by conventional rating scales or interviews. Functional compensation has been described in autistic individuals of high intelligence (Teunisse and de Gelder, 2001), and recent imaging studies indicate that there is considerable variability in neural organization among such autistic probands (Grelotti et al., 2002). Because of the variability in the cognitive and behavioural phenotype of 45,X females, we hypothesized that structural brain changes might be more consistently abnormal than behavioural deficits of social cognitive skills, relative to normal 46,XX females. For example, by parental report >30% of Turner syndrome children made poor eye contact in social conversation, a proportion at least 6 times greater than the proportion displayed more overtly autistic behaviours.

**Deletion-mapping genes on the X-chromosome**

Phenotypic features of the Turner syndrome, relating to neural dysfunction, are due in large part to haploinsufficiency for X-linked gene products that are normally expressed in two copies from genes that escape X-inactivation in normal 46,XX females (Ross et al., 2002). Males are also potentially haploinsufficient for these gene products, because few X-linked genes that escape inactivation outside the PARs at the tips of the X-chromosome have expressed Y-homologues (Disteche, 1999). Accordingly, we reasoned that it should be possible, through a study of individuals with X-chromosomal anomalies, to map dosage-sensitive genes that influence sexually dimorphic cognitive abilities. This hypothesis has received considerable recent support from other species in terms of both theory (Zechner et al., 2001) and empirical evidence (Xu et al., 2002). Because of the predisposition for 45,X females to show autistic features of behaviour, we hypothesized that dosage-sensitive genes that contribute to this aspect of their phenotype may also explain male vulnerability to the development of disorders of social cognition (Baron-Cohen, 2002), in view of the fact that males, like Turner syndrome females, possess only a single X-chromosome.

Accordingly, we aimed to identify dosage-sensitive X-linked genetic loci that influence the structure and function of neural systems affecting social-cognitive development, through a deletion-mapping strategy based on a study of brain structural and functional phenotypes in females with variably sized deletions of the X-chromosome. The technique of deletion-mapping has been used before to map genes on the X-chromosome that contribute to the Turner syndrome (Rao et al., 1997). Small numbers of subjects are invariably used, because naturally occurring deletions of part of the X-chromosome are rare. Such deletions are usually terminal and affect the short arm of the X (known as Xp). Rarely, they are interstitial, meaning there is loss of material intermediate between the centromere of the chromosome and the telomere at the tip of the arm.

Very small, distal deletions in the PAR on Xp (the region is described as pseudoautosomal because it combines with the Y-chromosome’s equivalent region during meiosis) are associated with a mild phenotype which affects mainly stature (James et al., 1998). The PAR extends across ~2.5 Mb of DNA from the telomere of the short arm. Genes in this region have homology to the equivalent region of the Y-chromosome. They are expressed from both X-chromosomes normally and are dosage-sensitive. So, if one copy is mutated or missing because of a microdeletion, there will be an abnormal phenotype which usually affects stature preferentially. The only gene that certainly contributes to the Turner phenotype was mapped this way (SHOX); it was mapped to the PAR of the short arm of the X-chromosome by a study of 16 patients (Rao et al., 1997). The location of the gene was identified by a detailed molecular genetic investigation of just three crucial cases. SHOX is needed in two copies for normal development in stature. Individuals in whom it was deleted were excessively short relative to their parents’ heights.

**SHOX** was deletion-mapped in the following way. First, the order of unique genetic markers (essentially way-points) in the PAR of the X-chromosome was determined. It was then necessary to find a sample of individuals with variably sized deletions within this critical region, some of whom were short and others of whom had normal stature. By a comparison of the chromosomal breakpoints (as implied by the presence or absence of markers) among individuals who had a deletion but an apparently normal phenotype, and those with the short stature phenotype, the approximate location of the gene could be determined.

This task is not straightforward, for it is rarely possible to state with precision exactly where a break has occurred. Markers may be spaced several kilobases of DNA from one another, and even their correct order on the chromosome may be a matter of debate. Confirmation of a suspected candidate gene usually requires the ascertainment of a mutation in that gene in other individuals with the phenotype, but who have intact chromosomes. This confirmation was achieved in the case of **SHOX**, and suggested that a mutation in this gene could account for many cases of idiopathic short stature in the general population (Blaschke and Rappold, 2001).

Much larger deletions, which extend from the telomere of the Xp to the region for microphthalmia with linear skin defects (MLS), which is ~14 Mb from the telomere, are compatible with the abnormal X remaining active. This is important because patients with Xp deletions distal to Xp22.3 will be nullisomic for genes that are subject to X-inactivation and lie distal to the **MLS** gene. In other words, patients with shorter terminal deletions of the X-chromosome that end proximal to the **MLS** gene are likely to have a more severe phenotype than those with even greater deletions. Because of this complexity, deletion mapping within the region between the PAR and the **MLS** genes is exceptionally difficult.
Table 1 Details of chromosomal breakpoints, X-inactivation status and phenotypic characteristics of 13 females with partial deletions of Xp (46, XXp-)

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Age (years)</th>
<th>Cyto- genetic break-point</th>
<th>Deletion</th>
<th>Parental origin of deleted X-chromosome</th>
<th>Cells in which the abnormal X is active (HUMAR/ BrdU) (%)</th>
<th>Ascertainment</th>
<th>Most proximal deleted marker (minimum size)</th>
<th>Most distal heterozygous marker (maximum size)</th>
<th>Verbal IQ</th>
<th>Performance IQ</th>
<th>Ovarian failure</th>
<th>Short stature</th>
</tr>
</thead>
<tbody>
<tr>
<td>45,X[3]/46,X,del(X) (p22.32) [27]</td>
<td>9.0</td>
<td>p22.32</td>
<td>De novo</td>
<td>Paternal</td>
<td>64/not done</td>
<td>Short stature</td>
<td>DXYS230X</td>
<td>107</td>
<td>81</td>
<td>Too young to assess</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,t(X;Y) (p22.31;q11.21)</td>
<td>24.25</td>
<td>p22.31</td>
<td>De novo</td>
<td>Paternal</td>
<td>69/58</td>
<td>Short stature</td>
<td>DXS8105</td>
<td>DXS996</td>
<td>103</td>
<td>99</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,t(X;Y) (p22.31;q12)</td>
<td>11.1</td>
<td>p22.33</td>
<td>Familial</td>
<td>Maternal</td>
<td>34/33</td>
<td>Tumor syndrome</td>
<td>DXS7470</td>
<td>DXS7103</td>
<td>76</td>
<td>78</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,t(X;Y) (p22.33;q12)</td>
<td>48.9</td>
<td>p22.33</td>
<td>De novo</td>
<td>Paternal</td>
<td>05/12</td>
<td>Mother of case 95-02697</td>
<td>DXS7470</td>
<td>DXS7103</td>
<td>101</td>
<td>93</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,del (X) (p22)</td>
<td>16.4</td>
<td>P22</td>
<td>De novo</td>
<td>Paternal</td>
<td>0/0</td>
<td>Short stature</td>
<td>DXS8378</td>
<td>DXS207</td>
<td>130</td>
<td>112</td>
<td>Partial</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,del(X) (p22.1)</td>
<td>29.10</td>
<td>p22.12</td>
<td>De novo</td>
<td>Paternal</td>
<td>0/0</td>
<td>Short stature</td>
<td>DXS43</td>
<td>DXS8036</td>
<td>101</td>
<td>101</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,del(X) (p21.2) [17]</td>
<td>25.4</td>
<td>p21.2</td>
<td>De novo</td>
<td>Paternal</td>
<td>0/0</td>
<td>Tumor Syndrome</td>
<td>DXS985</td>
<td>DXS1036</td>
<td>105</td>
<td>85</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,del(X) (p11.33)</td>
<td>38.6</td>
<td>p11.33</td>
<td>Familial</td>
<td>Maternal</td>
<td>Not done</td>
<td>Daughter of affected female</td>
<td>DXS1368</td>
<td>DXS993</td>
<td>103</td>
<td>100</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,del(X) (p11.2)</td>
<td>37.5</td>
<td>p11.2</td>
<td>De novo</td>
<td>Paternal</td>
<td>0/0</td>
<td>Problems with IVF, Short stature, some stigmata of Turner syndrome</td>
<td>DXS8026</td>
<td>DXS8083</td>
<td>109</td>
<td>109</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>46,X,del(X) (p11)</td>
<td>14.6</td>
<td>P11</td>
<td>De novo</td>
<td>Paternal</td>
<td>0/0</td>
<td>Turner Syndrome</td>
<td>DXS1367</td>
<td>DXS1208</td>
<td>108</td>
<td>92</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,del(X) (p11.2)</td>
<td>16.9</td>
<td>p11.2</td>
<td>De novo</td>
<td>Paternal</td>
<td>Not done</td>
<td>Turner Syndrome</td>
<td>DXS8024</td>
<td>DXS8062/ DXS423E</td>
<td>91</td>
<td>80</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>45,X[45]/46,X,del(X) (p11) [55]</td>
<td>46.5</td>
<td>p11.2</td>
<td>De novo</td>
<td>Paternal</td>
<td>0/0</td>
<td>Turner Syndrome</td>
<td>DXS8023</td>
<td>DXS8032</td>
<td>95</td>
<td>83</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>46,X,der(X)(qter&gt;p11.2::q26&gt;qter)</td>
<td>14</td>
<td>p11.2</td>
<td>De novo</td>
<td>Paternal</td>
<td>Not done</td>
<td>Turner Syndrome, 423E (FISH probe)</td>
<td>DXS1199</td>
<td>93</td>
<td>46</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>
Our interest was primarily in a region close to the centromere on Xp (Xp11.3), in which there is a cluster of genes that escape X-inactivation and which are therefore presumably needed in two copies for the normal development of females (Disteche, 1999). We hypothesized that at least some aspects of the Turner cognitive or behavioural phenotype could be associated with haploinsufficiency of genes in this region. Fortunately, terminal deletions of the X that extend as far as Xp11.3, beyond the MLS gene, are invariably associated with preferential inactivation of the abnormal X-chromosome (James et al., 1998). Thus, interpretation of phenotypic data from a comparison of females with variably sized deletions in this region should be relatively straightforward, on the basis of the hypothesis that phenotypes found in larger deletions will be associated with haploinsufficiency of genes that are normally required in two copies.

**Imprinting of X-linked genes**

We had previously reported evidence (Skuse et al., 1997) that one or more X-linked loci that affect cognitive function are imprinted, in the sense that only the allele that is paternally inherited is expressed. The proposed mechanism entailed the systematic silencing of the imprinted locus on the X-chromosome in the maternal germ-line and the removal of imprints in the paternal germ-line. Accordingly, the expressed locus would invariably be passed from father to daughter (and would be expressed in her). The silent locus would be inherited by sons from their mothers, on their single X-chromosome, and also by daughters. The consequence of this proposed mechanism was a system for determining the expression of sexually dimorphic traits that could operate independently of sex steroids. We had found evidence for its existence by contrasting the cognitive and behavioural profiles of X-monosomic females, whose single X was derived either maternally (45,Xm) or paternally (45,Xp). In general, 45,X females with a single paternal X-chromosome had better social adjustment than those whose single X was maternal in origin. Autistic features were significantly more common among those with a maternally derived X-chromosome, and autism was only found in 45,Xm females (Skuse et al., 1999; Skuse, 2000). Accordingly, we predicted that we would find differences in structures that are important for social cognition between 45,Xm and 45,Xp Turner females.

**Hypotheses**

Prior to the neuroimaging study presented here we proposed the following hypotheses. (i) Abnormalities in the structure of the amygdala and OFC would be present in women with Turner syndrome (45,X) compared with normal females (46,XX) in adulthood. (ii) In females with partial deletions of one X-chromosome (46,XXp–), loss of a genetic locus in the Xp11.3 region would be associated with many typical neurocognitive deficits of Turner syndrome. In 46,XXp– females whose critical region was deleted there would be structural brain anomalies similar to those of 45,X Turner syndrome, when compared with 46,XX adult females. (iii) There would be structural differences in brain regions important for social cognition between women with X-monosomic Turner syndrome whose single X-chromosome was maternally inherited (45,Xm) and those with a paternally inherited single X-chromosome (45,Xp).

**Material and methods**

**Subjects**

This study, which was approved by the Ethics Committee of Great Ormond Street Hospital for Children and the Institute of Child Health, involved 64 females with X-chromosome anomalies (51 with Turner syndrome 45,X, aged 15–44 years, and 13 with partial X-chromosome deletions (46,XXp–), aged 9–52 years.). All were selected on the basis that they had no significant learning difficulties. Forty per cent had participated in some form of higher education and 20% had attended university. The subjects’ consent was obtained according to the Declaration of Helsinki. Clinical subjects were selected from a national survey of Turner syndrome and from the records of the Wessex Regional Genetics Laboratory. The mean (SD) age of the 45,X females was 25.1 (7.1) years and that of the 46,XXp– females was 26.4 (13.2) years. The mean (SD) verbal IQ of the 45,X and 46,XXp– females was similar [96.9 (13.3) versus 101.6 (12.3)], as was their performance IQ [90.3 (16.0) versus 91.0 (17.6)]. All subjects were healthy, with no significant neurological disease. All 45,X women had been receiving sex steroid replacement therapy since early adolescence. The comparison group of 45 normal 46,XX women (recruited from staff and students at Great Ormond Street Hospital for Children and the Institute of Child Health) was matched to the 45,X group in terms of mean age [24.1 (SD 4.6) years] and verbal IQ (Wechsler, 1986) [100.9 (11.7)]. Their mean performance IQ of 108.1 (14.4) was significantly greater than that of 45,X females, (P < 0.001), as expected (Temple and Carney, 1995). Our comparison group of 25 normal 46,XY males was recruited from the same volunteer sample as normal females.

**Genetic methods**

Karyotypes were determined by analysis of G-banded metaphase chromosomes harvested from peripheral blood. DNA for molecular studies was extracted by salt precipitation. Parental origin of the normal X-chromosome was determined using the PCR to amplify DNA polymorphisms from each proband and their parents.

Our sample of 13 46,XXp– females had variably sized breakpoints, all but one terminal, which ranged in size from 5 to 55 Mb from the telomere. For the purpose of analysis they were numbered 1–13 in increasing size of deletion (Table 1).
Deletion breakpoints (James et al., 1998) were mapped by PCR using a panel of short tandem-repeat polymorphisms spanning the X-chromosome short arm. In Table 1 the laboratory numbers of the subjects correspond to the numbers used by James and colleagues, and fuller information is given about the phenotypic characteristics of many partially deleted subjects there (James et al., 1998). Locus order was taken from Ensembl (http://www.ensembl.org/). Standard PCR conditions were used throughout and all primer sequences are available through the Genome Database (http://gdbwww.gdb.org/). The X-inactivation status of a deleted X-chromosome was determined at the androgen receptor locus (HUMAR). In deletions 5–13, the abnormal X was unilaterally inactivated. In order to detect possible mosaicism, a minimum of 50 cells per individual (usually 100 cells) were analysed cytogenetically. All 45,X females were apparently non-mosaic in peripheral blood, although this does not exclude the presence of an additional cell line in the brain. Among the deletion carriers, a 45,X cell line was also present in three cases: Case 1 (10% of cells), Case 7 (66% of cells) and Case 12 (45% of cells). Thus, these three cases have an X-chromosome complement intermediate between monosomic 45,X females and normal females.

**Structural neuroimaging**

In order to characterize the brain structural phenotypes we performed high-resolution MRI structural imaging (Deichmann et al., 2000) and voxel-based morphometry (Ashburner and Friston, 2000) on 21 45,X subjects and all subjects with partial deletions. This fully automated whole-brain imaging technique enables detection of subtle changes in grey matter on a voxel-wise basis between groups of patients and normal subjects. Classical MRI morphometric techniques are based upon region-of-interest-type metrics, which are inherently subjective and operator-dependent, perhaps explaining inconsistencies in previous descriptions of the structural phenotype of the brain in Turner syndrome (Murphy et al., 1993; Reiss et al., 1995).

High-resolution volumetric MRI was performed on a Siemens 2 T Magnetom scanner using an optimized MPRAGE (magnetization-prepared, rapid gradient echo) sequence, which affords enhanced grey/white matter contrast and segmentation (Deichmann et al., 2000). The acquisition parameters included TR-repetition time 11; TE-echo time 4; TI-inversion time 1000; flip angle 12°; matrix 256 × 224; field of view 256 × 224 mm; 176 sagittal slices and 1 mm isotropic voxels. An optimized method of voxel-based morphometry (Good et al., 2001b) was used, which involves a number of fully automated preprocessing steps: extraction of brain; spatial normalization into stereotaxic space; segmentation into grey and white matter and CSF compartments; correction for volume changes induced by spatial normalization; and smoothing with a 10 mm full width at half maximum isotropic Gaussian kernel. After smoothing, each voxel represents the local average amount of grey (or white) matter in the surrounding region, the size of which is defined by the size of the smoothing kernel. We used a customized grey matter template derived from all the Turner syndrome patients and control subjects in order to avoid any bias during the spatial normalization step. The smoothed data were analysed using Matlab 5.3 (MathWorks, Natick, MA, USA) and statistic parametric mapping (SPM99) employing the framework of the general linear model.

Regionally specific structural differences were assessed statistically using a two-tailed test, testing for increased or decreased grey matter. Corrections for the search volume and implicit multiple comparisons were made to the P values using Gaussian random field theory, which accommodates spatial correlations inherent in the data. Significance levels were set at P < 0.05, corrected for whole brain volume. Because the amygdala had been identified a priori as a possible candidate for structural change, amygdala changes were corrected for small volume using a sphere of diameter 40 mm as a generous estimate of total amygdala volume. The design matrix modelled the control group, the 45,X group (divided according to the maternal or paternal origin of the single X-chromosome) and individual partial deletions arranged in order of the number of megabases missing from the short arm of the X-chromosome. The mean global grey matter intensity was modelled as a covariate in order to facilitate the detection of regionally specific structural changes, having discounted global differences. Age was included as a covariate. In a separate analysis we compared normal males, normal females and 45,X females with mean global grey matter and age as covariates.

**Neuropsychological tests**

Standard tests of verbal and non-verbal IQ were employed, using the Wechsler scales (Wechsler, 1986). Because we had hypothesized that the social adjustment problems associated with Turner syndrome (McCauley et al., 1987; Ross et al., 2000) could be linked to amygdala dysfunction, subjects were tested using the Ekman Pictures of Facial Affect (Ekman and Friesen, 1976). Poor performance in the recognition of basic emotions has been specifically linked to amygdala dysfunction (Adolphs et al., 2002). Sixty pictures of male and female faces were shown to subjects, who had to decide which emotion was being conveyed by the facial expression: happiness, sadness, fear, surprise, anger or disgust. Fifty-one 45,X individuals were compared with 45 normal 46,XX females matched for age and verbal IQ, and 25 normal 46,XY males.

**Results**

**Neuropsychological tests**

Scores for the six emotions on the Ekman pictures (Ekman and Friesen, 1976) were entered into a MANOVA (multivariate analysis of variance), covarying for performance IQ,
with Bonferroni correction. The 45,X females were substantially impaired in recognizing fear ($P < 0.0001$, effect size = 1.3) and anger ($P < 0.006$, effect size = 1.0) relative to 46,XX females (Fig. 2), but they were unimpaired in the recognition of other facial emotions. None was aware of their perceptual deficit. They could provide plausible situations in which fear or anger might be experienced, but many could not recount times when they themselves had experienced these emotions. No significant differences were found in performance on these tasks between 45,X females whose single X-chromosome was maternal and those in whom it was paternal in origin. Face emotion perception was similar in normal females and an age- and IQ-matched sample of 16 normal males. Males lacked the fear recognition deficit of 45,X females, despite their single X-chromosome.

**Structural neuroimaging**

We selected 21 apparently non-mosaic Turner syndrome females with a 45,X karyotype (45,X females), 17 age- and verbal IQ-matched control females [25 (SD) 9.0 years] and all 25 age-matched normal males [27.1 (8.1) years] for neuroimaging investigations. Eleven of the 45,X females had a single X-chromosome of maternal origin (45,Xm) [mean (SD) age 24.3 (7.5) years] and in 10 cases it was of paternal origin (45,Xp) [22.3 (6.4) years].

In keeping with our hypotheses, we found that X-monosomic females possessed localized regions of altered brain structure compared with 46,XX controls. These abnormalities included significantly increased grey matter volumes of the amygdalae and OFC bilaterally (Fig. 1). Previous studies have shown gender differences in the amygdala structure of normal subjects; males (46,XY) have increased grey matter compared with females (over and above the global grey matter differences between the sexes) (Murphy et al., 1993; Good et al., 2001a). However, no sex differences have been reported in the OFC grey matter in this region. We compared matched samples of normal males with 46,XX and 45,X females and found that, although males did have larger amygdalae than normal females ($P < 0.001$ uncorrected), the...
amygdalae of 45,X females were larger still \( (P < 0.05 \text{ corrected}) \) (Fig. 1). Therefore a dose–response relationship seems plausible between the expression of one or more X-linked genes that escape inactivation (and which lack Y-homologues) and the structural development of the amygdala. Contrary to our hypothesis, we did not find any significant differences in amygdala or OFC structure according to the parental origin of the single X-chromosome.

There was no gender difference in OFC grey matter volume or density. Thus, the increased grey matter volume of the OFC in Turner syndrome individuals was seen relative to both males and females from our comparison groups.

**Deletion-mapping the critical region on X-chromosome, using cognitive and neuroimaging phenotypes**

Using our 46,XXp– subjects (Table 1), we attempted to map the putative dosage-sensitive locus with the structural brain phenotype as our outcome variable. Subjects with breakpoints including or proximal to the locus DXS8083 (44.07 Mb from Xpter), were phenotypically similar to 45,X females in terms of brain structure, with significantly increased grey matter in the amygdala and OFC (Fig. 2). In contrast, these brain structures were indistinguishable from normal in 46,XXp– females with breakpoints distal to the locus DXS1368 (39.11 Mb from Xpter). Subjects with partial deletions had fear recognition skills that closely mirrored their brain phenotypes, in the sense that those with no increased volume of OFC or amygdala also possessed normal fear recognition, whereas in general those with abnormal OFC and amygdala
volumes had a deficit in their ascertainment of fear, irrespective of the parental origin of their deletion (Fig. 3). No other aspect of cognitive or somatic phenotype differentiated females with deletions that extended beyond the critical region from the remainder (Table 1).

No correlation existed between menstrual history, or other evidence of ovarian failure, and amygdala structure or fear recognition skills in partial deletion subjects. The 46,XXp± female with a deletion to DXS8083 (Subject 9) had conceived spontaneously, and two subjects with larger deletions (Subjects 10 and 11) had normal menstrual histories. Accordingly, oestrogen deficiency is unlikely to be directly responsible for abnormalities of brain structure and function among the 46,XXp± sample.

**Molecular genetic studies**

These findings suggest that a dosage-sensitive locus influencing both amygdala and OFC development in 45,X females lies in the interval between 39.11 and 44.07 Mb from the telomere of the short arm (Fig. 4). This critical region is defined by loci DXS1368 (distally) and DXS8083 (proximally) and must contain one or more genes which escape X-inactivation, and are thus needed in two copies in 46,XX females for typical development to occur. Genes escaping X-inactivation tend to exist in clusters and, significantly, at least one cluster maps within the DXS1368–DXS8083 interval (Carrel et al., 1999; Distche, 1999; Sudbrak, 2001). Within this 4.96 Mb interval there are 21 confirmed genes (Table 2, Fig. 4). There are also 26 genes predicted with varying degrees of confidence of which 16 have expressed sequence tags. There is a block of homology between this region of the X-chromosome, corresponding to cytogenetic bands Xp11.3 and Xp11.3, and a non-recombining part of the Y chromosome, Yp11.2. Of the X-linked genes in our critical region that are known to escape X-inactivation, only USP9X, DDX3 and UTX have functional Y-homologues and all three are expressed in (mouse) brain (Xu et al., 2002) Completion of the human genome sequencing project and more extensive gene annotation may identify additional X/Y gene pairs.

**Neurochemical studies**

Among the genes in this critical region are those encoding monoamine oxidase A (MAOA) and monoamine oxidase B (MAOB). These are nuclear-encoded mitochondrial isoenzymes that catalyse the oxidative deamination of a number of biogenic amines, including the neurotransmitters serotonin (5-HT), norepinephrine and dopamine, and the neuromodulator phenylethylamine. Both enzymes have been implicated in predisposition to a range of psychiatric disorders (Shih and Thompson, 1999) and both genes are expressed in the human amygdala. MAOB is also expressed in platelets, in which activity levels are believed to reflect genotypic variation. Methylation analysis of the MAOB 5’ CpG island has found that all CpG dinucleotides tested in DNA extracted from lymphocytes are unmethylated on both the active (Xa) and inactive (Xi) X-chromosomes (Chen et al., 1992). This suggests the gene is expressed from both X-chromosomes in normal females and is not subject to X-inactivation. There is no functional Y-homologue of MAOB (males in whom it is deleted have no measurable MAOB activity). Accordingly, we hypothesized that males and 45,X females could be haploinsufficient for the gene products, relative to normal
### Table 2  Genes identified in critical region at Xp11.3-Xp11.4 between markers DXS1368 and DXS8083; Entrez Homo Sapiens Genome View (Build33)

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females, if upregulation of their single copy did not occur, and that 45,X females would show male-typical levels of activity.

We therefore conducted a further study specifically to test the hypothesis that the sexual dimorphism in platelet activity of MAOB could be due to haploinsufficiency of MAOB expression in males. This has not been investigated previously. We confirmed prior evidence that the platelet activity of MAOB is sexually dimorphic (e.g. Harro et al., 2001), levels in males being ~30% lower than levels in normal females. We also showed, in a preliminary investigation (of non-smokers), that mean (SD) levels were almost identical in males and in Turner syndrome (45,X) [9.5 (1.1) and (9.7) β-phenylethylamine deaminated per 10^10 platelets per min nmol, respectively]. In contrast, mean (SD) normal female levels of MAOB activity were significantly higher [13.7 (1.4), P = 0.05].

Discussion

Structural brain anomalies in Turner syndrome

The increase in the amygdala volumes of our 45,X subjects is consistent with previous studies that have found that amygdala volume correlates inversely with the number of X-chromosomes, irrespective of sex. Two studies (Goldstein et al., 2001; Good et al., 2001a) have reported larger volumes among 46,XY males than 46,XX females. 47,XXY males have amygdalae of similar volume to 46,XX females, whereas the amygdalae of 47,XXX females are significantly smaller than those of either group (Patwardhan et al., 2002). Individual nuclei within the amygdala subserve different functions, but we were unable, with the scanning resolution available to us, to determine whether these substructures were differentially affected.

The amygdala is a critical component of the social cognition circuitry in primates. Modular cognitive processes are devoted to making social judgements, and in particular our perception of facial expressions plays a critical role in social intelligence (Adolphs, 1999; Winston et al., 2002). An influential neurobiological model of social cognition (Brothers, 1997) postulates that socially relevant information, processed by sensory and association cortices, is imbued with emotional significance by the amygdala and OFC. This model has received widespread support from research in both primates and humans (Amaral et al., 2002). Lesion and functional imaging studies in humans have demonstrated its role in identifying emotions in others. Humans and primates with complete bilateral amygdala damage have difficulty

<table>
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<td>70205</td>
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<td>NDP</td>
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<td>C</td>
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C = confirmed gene model; E= expressed sequence tag only; PE = predicted gene plus expressed sequence tag; P = predicted gene only; I = interim locus ID; *blank field implies activation status unknown
recognizing fear (and anger) in facial expressions and they demonstrate subtle abnormalities of normal social responsiveness (Adolphs et al., 2002).

Healthy males, despite having a single X-chromosome and larger amygdalae than normal females, do not have deficits in their ability to recognize fear in another’s facial expression (Campbell et al., 2002). Consequently, the fear-recognition problems we describe in association with 45,X Turner syndrome must be due to a genetic or sex-steroid associated mechanism that is peculiar to that syndrome. We found increased grey matter volume bilaterally in the OFC of Turner syndrome individuals, relative to both normal females, and males. Unlike the amygdala, this region is not sexually dimorphic in structure. We propose that poor fear recognition among our TS subjects must therefore be causally linked to this OFC anomaly. It is well established that there are face-selective neurons in the primate OFC, and ventral frontal lobe damage in humans impairs the identification of facial expressions. The OFC receives inputs of the same general type as the amygdala, and is certainly connected intimately with it ((Rolls, 2002). Specifically, we hypothesize that there is a deficit in functional connectivity between the amygdala and the OFC as a consequence of X-monosomy in females. The areas of maximal OFC abnormality bilaterally (Fig. 1) are close to a region, at BA 11, which was co-activated with amygdala in an aversive conditioning paradigm, using facial expressions as the conditioned stimuli (Morris et al., 1997). The observation indicates these structures may act together as a functional unit. Males, who are of course also X-monosomic, could be protected from the OFC abnormality (despite their single X chromosome) because the critical gene(s) is expressed also from the Y-chromosome (hence there is dosage equivalence between males and females). Alternatively, there may be upregulation of the single male copy of an exclusively X-linked gene, by male sex steroids.

**Neurochemical anomalies**

Within the critical region mapped in our investigation of females with partial deletions of the short arm of the X-chromosome, the MAO genes are clearly contenders for a potential influence upon amygdala development. Recently, Borowsky and colleagues showed that three of four members of a family of G protein-coupled receptors that are activated by trace amines, such as β-phenylethylamine, are expressed exclusively in the human amygdala (Borowsky et al., 2001). Trace amines are exquisitely sensitive to the deaminergic actions of MAO genes (in the case of phenylethylamine it is especially MAOB; Grimsby et al., 1997). Accordingly, the relatively low levels of MAOB activity consequent upon haploinsufficiency in males and 45,X females may lead to male-typical patterns of amygdala responsiveness, for example in the context of emotional learning (Canli et al., 2002).

Genes that are subject to X-inactivation do not have functional Y-homologues, and because expression is from a single X-chromosome in males and females the dosage is likely to be equivalent in both sexes. Furthermore, because in Turner syndrome (45,X) the single X-chromosome is always active it is unlikely that such genes are contributing in any significant way to the Turner phenotype. In contrast to MAOB, methylation analysis of the MAOA CpG island has suggested the gene might be subject to inactivation (Hendriks et al., 1992). Although expression of MAOA has not been detected from Xi in cDNA from cloned human cell lines, the approach used (Hendriks et al., 1992)—reverse transcription of intronic polymorphisms using random hexamers—has limited sensitivity. Heterogeneous expression from Xi has been demonstrated for some X-linked genes, indicating variability of inactivation between tissues and even between individual females (Carrel et al., 1999). Testing of somatic cell hybrids suggests that some 5–15% of X-linked genes behave this way (Carrel et al., 1999). Accordingly, MAOA could escape X-inactivation in some tissues. If so, activity would be relatively lower in 45,X than 46,XX females, and potentially sexually dimorphic, with lower activity in males. Such a mechanism could exacerbate sex differences in vulnerability to certain disorders, affecting predominantly males, which result from MAOA functional polymorphisms (e.g. Caspi et al., 2002).

### Potential role of sex steroid deficiencies

Haploinsufficiency for genes at a dosage-sensitive X-linked locus is not the only possible explanation for the structural and functional characteristics of brains of 45,X females. One contributory factor that cannot be discounted by our data is interaction between sex steroid abnormalities and neurodevelopment. In 45,X Turner syndrome there is ovarian dysgenesis and failure of endogenous oestrogen production. Oestrogen receptors are widely expressed in the forebrain, with highest concentrations of the α-subtype in the amygdala, and certain hypothalamic nuclei, in both primates and humans (Osterlund and Hurd, 2001). However, as we have shown (Table 1, Figures 2 and 3) there is no strict relationship between ovarian function and either the structural anomalies reported in the OFC and amygdala or the results of our psychological tests.

It is also possible that environmental influences—perhaps a disadvantage relating to short stature—could have systematically affected brain development of the 45,X females relative to 46,XX females. This explanation cannot account for the within-group differences in brain phenotype of our 46,XXp–subjects, who were all of short stature.

### Implications of findings for male susceptibility to autism

Our findings could have relevance to the aetiology of male susceptibility to disorders that are associated with social cognitive impairment, such as autism. This condition is at
least four times as common in males as females, and among subjects whose IQ is in the normal range (full scale >70) the ratio is substantially greater. No explanation has yet been found for this discrepancy in prevalence, but one possibility is that the phenotypic penetrance of genetic susceptibility due to autosomal loci is decreased in females, because they possess two X-chromosomes (Folstein and Rosen-Sheidley, 2001). Serotonin (5-hydroxytryptamine, 5-HT) blood levels have often been reported as elevated in platelets in about one-third of autistic subjects and their first-degree relatives (Betancur et al., 2002). More than 99% of blood 5-HT is in the platelet fraction; normal levels of platelet 5-HT are higher in males than females. No explanation for the excessively high level in autism has been found, but a lack of MAOB activity, which is associated with relatively impaired aminergic degradation, is one possibility.

Individuals with high-functioning autism have a relatively specific failure to recognize—in the sense of being able consciously to label accurately—specifically fearful facial expressions (Howard et al., 2000). They also have impaired facial recognition memory (Klin et al., 1999) and deviant egocentric gaze monitoring (Calder et al., 2002). Such individuals suffer from impaired theory of mind skills (Frith, 2001) and are unable to make rational judgements about the trustworthiness of strangers (Adolphs et al., 2001; Tenyi et al., 2002). Each of these aspects of social cognitive impairment has been reported in individuals with acquired or developmental lesions of the amygdala, although such individuals are not classically autistic. There are functional and structural anomalies of the amygdala in some, if not most, cases of autism (Sweeten et al., 2002; Salmond et al., 2003).

As discussed, we are currently investigating the extent of similar cognitive and functional neural deficits in adult 45,X females of normal intelligence (e.g. Elgar et al., 2002; Lawrence et al., 2003a, b).

MAOB and male susceptibility to neurodevelopmental disorders

In order to take this work to its logical conclusion, we need to establish first whether haploinsufficiency of MAOB is indeed the aetiological factor in the abnormal amygdala development in 45,X individuals. Males in whom MAOB is ablated, in the rare microdeletion variant of Norrie syndrome (Suarez-Merino et al., 2001), are profoundly retarded in mental development. No systematic study has been done of female carriers of this microdeletion (who, like males, would have one functioning copy of the Norrie gene on their intact X-chromosome). We hypothesized that they may have a Turner-like phenotype in aspects of brain structure and function, because the microdeletion lies within the critical region we defined at Xp11.3. We did conduct a small study of this nature, in one family with a heritable deletion which encompassed not only the Norrie disease gene locus but also both MAO genes on one X-chromosome (deletion did not extend beyond the markers DXS1027 and DXS1239 and involved a little less than 3 Mb of DNA). The results were intriguing. One of two sisters possessing a deletion of MAOA and B, as well as the Norrie disease gene locus on one X-chromosome, had a long history of severe psychiatric disorders of emotional regulation. These were associated with excessively low MAOB thrombocytic activity. The other sister had a disinhibited personality characterized by abnormally aggressive outbursts. Larger numbers would be required to confirm these findings and to permit adequate interpretation of the associated neuroimaging data. It is possible that some apparently idiopathic cases of profound male mental retardation could be due to non-functional mutations of the MAOB gene, a hypothesis for which there is tangential evidence (Holinski-Feder et al., 1999). Confirmation of the X-inactivation status of MAOB is required (as well as the equivalent status of other genes in the critical region that are plausible candidates).

Conclusions

In summary, our data imply that one or more non-inactivated X-linked genes (within a 4.96 Mb interval at Xp11.3) are involved in the development of a neural system influencing social cognitive processing, in which the amygdala plays a critical role. Our findings suggest that the critical gene is expressed in two copies in normal 46,XX females, and that full dosage compensation does not occur in 46,XY males (with consequent structural amygdala changes), or in 45,X monosomy (in which more extensive structural and cognitive anomalies ensue). Sexual dimorphism in the expression patterns of one or more genes at this locus is therefore predicted. MAOB is proposed as a candidate gene, with an important contributory role in the development of the amygdala.

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