

Possible association between the androgen receptor gene and autism spectrum disorder

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Short title: Association between androgen receptor gene and autism

Summary

Autism is a highly heritable disorder but the specific genes involved remain largely unknown. The higher prevalence of autism in men than in women, in conjunction with a number of other observations, has led to the suggestion that prenatal brain exposure to androgens may be of importance for the development of this condition. Prompted by this hypothesis, we investigated the potential influence of variation in the androgen receptor (AR) gene on the susceptibility for autism. To this end, 267 subjects with autism spectrum disorder and 617 controls were genotyped for three polymorphisms in exon 1 of the AR gene: the CAG repeat, the GGN repeat and the rs6152 SNP. In addition, parents and affected siblings were genotyped for 118 and 32 of the cases, respectively. Case-control comparisons revealed higher prevalence of short CAG alleles as well as of the A allele of the rs6152 SNP in female cases than in controls, but revealed no significant differences with respect to the GGN repeat. Analysis of the 118 families using transmission disequilibrium test, on the other hand, suggested an association with the GGN polymorphism, the rare 20-repeat allele being undertransmitted to male cases and the 23-repeat allele being overtransmitted to female cases. Sequencing of the AR gene in 46 patients revealed no mutations or rare variants. The results lend some support for an influence of the studied polymorphisms on the susceptibility for autism, but argue against the possibility that mutations in the AR gene are common in subjects with this condition.

Key words: autism; androgens; testosterone; polymorphism; CAG repeat; GGN repeat; rs6152

Introduction

Autism is a disorder with early onset that is characterized by impairments of social relatedness and communication, repetitive behaviors, abnormal movements, and sensory dysfunction (Bailey et al., 1996), and that is often but not always associated with mental retardation (MR). Twin studies have demonstrated a much higher concordance for autism in monozygotic (>70%) than in dizygotic twins (0-10%), suggesting genetic factors to play a major role in the etiology (Bailey et al., 1995; Steffenburg et al., 1989). Certain monogenic causes of autism are well known, such as fragile X syndrome, Rett syndrome and tuberous sclerosis, and recently certain rare *de novo* mutations and copy number variations associated with enhanced risk for autism were identified (Durand et al., 2007; Jamain et al., 2003; Sebat et al., 2007). It is likely that common variants in susceptibility genes, exerting merely a modest influence on autism risk, are also of importance, although no such variants have as yet been proven beyond doubt to be associated with this condition.

Several lines of evidence suggest a role for androgens in the etiology of autism, presumably exerted during prenatal brain development, (Baron-Cohen et al., 2005): (1) The prevalence of autism in men is three to four times higher than in women (Ellefsen et al., 2007; Gillberg, 1984; Latif and Williams, 2007). (2) In a normal population, some of the characteristic features of autism – such as low empathizing and enhanced systemizing – are more common in males; in this respect, subjects with autism may hence be described as having an “extreme

male brain” (Baron-Cohen et al., 2005; Gillberg et al., 1984; Wing, 1981). (3) Both direct (assessment of androgen levels) and indirect (assessment of the ratio between the second and fourth digit as a marker for prenatal androgen exposure) (Manning et al., 1998) evidence suggests elevated fetal testosterone levels to be associated with autism and autism-related traits (Knickmeyer et al., 2005; Manning et al., 2001; Williams et al., 2003). (4) Girls with congenital adrenal hyperplasia, a trait associated with exposure to high prenatal androgens, display autism-like traits to a higher extent than their unaffected sisters (Knickmeyer et al., 2006).

Although the relationship between prenatal androgen exposure and androgen production at adult age is unclear, studies providing preliminary support for the notion that autistic traits are associated with enhanced serum levels of testosterone (Geier and Geier, 2007; Tordjman et al., 1997) may also be taken as support for the notion that autism is indeed associated with enhanced androgenization. In the same vein, one study reports testosterone-related disorders to be more common in women with autism than in controls (Ingudomnukul et al., 2007).

The androgen receptor (AR) is a ligand-activated transcription factor that belongs to the steroid and thyroid hormone receptor superfamily (Zhou et al., 1994) and is encoded by a gene located on the X chromosome (Xq11-12). Exon 1 of this gene, which encodes the amino-terminal domain of the AR involved in transcriptional activation of downstream genes (Faber et al., 1989), comprises two repeat polymorphisms: a CAG repeat sequence encoding a poly-glutamine stretch and a GGN repeat sequence encoding a poly-glycine stretch. Between these, a synonymous SNP rs6152 (G1733A) – also known as *StuI* – is situated. Whereas the length of the CAG repeat is inversely correlated with the activity of the AR as a transcription factor (Beilin et al., 2000; Chamberlain et al., 1994), findings on the functional consequences of the GGN repeat polymorphism are inconsistent (Brockschmidt et al., 2007; Ding et al., 2005). The functional effect of the SNP rs6152 has not yet been studied.

In the present study, these three polymorphisms in the *AR* were studied for possible association with autism using both case-control and family-based association tests. In addition, mutation screening of coding and regulatory regions of the *AR* was performed in a subset of patients.

Methods

Study population

In total, 267 subjects meeting DSM-IV criteria for autism spectrum disorder were genotyped. The individuals came from two different cohorts: (a) 204 were recruited from the Paris Autism Research International Sib-pair (PARIS) study (Philippe et al., 1999), and (b) 63 came from a separate Swedish cohort. All individuals in cohort (a) had been given a full clinical research protocol assessment including collateral diagnostic interviews, personal assessment, psychological testing and laboratory examinations to exclude associated genetic disorders, see (Philippe et al., 1999). The individuals in cohort (b) had been examined by an experienced neuropsychiatrist in an in-patient setting and diagnosed according to strict DSM-IV-criteria on the basis of this examination and psychological testing. In addition, DNA from 118 parents and 32 affected sibs of the PARIS subjects was obtained and analyzed.

In the PARIS study, the vast majority was of either French (50%) or Swedish (25%) origin, with 92% being Caucasian. Of the 118 subjects from the PARIS study included in the family-based analysis, 35 were female. Seventy-eight % of the male and 74% of the female subjects of this subgroup had mental retardation (MR). Mean age was 12 ± 7 years.

Of the remaining 86 subjects from the PARIS study, 20 were women. Fifty-four % of the male and 59% of the female subjects of this subgroup had MR. Ages were in the interval 18-60 years. Cases not meeting criteria for MR were categorized as “high-functioning”.

Of the 63 subjects from the separate Swedish sample, all were of Swedish origin and all had MR. The men ($n=39$) were between 16 and 60 years old (mean: 43) and the women were between 17 and 58 years old (mean: 34).

The control sample ($n=617$) used for the case-control association studies consisted of 100 French (41 women) and 517 Swedish subjects (270 women). The French controls were recruited among blood donors at the Pitié-Salpêtrière hospital in Paris. They were all of European descent and inclusion was made after interviews with the Diagnostic Interview for Genetic Studies and the Family Interview for Genetic Studies to confirm the absence of both personal and family history of major psychiatric disorders. Mean age was 43 ± 13 years. The Swedish

population was drafted from the general population. The women were born on uneven days in 1956 (Rosmond and Bjorntorp, 1998) and were 42 years old at evaluation. The men were a subsample of all men born during the first 6 months in 1944 and living in Gothenburg, and were 51 years old at evaluation. More detailed information has been previously reported (Rosmond et al., 1998).

These samples were used to conduct three analyses. First, a case-control study was undertaken, comprising all cases and controls. For subjects belonging to sib-pairs ($n=32$), one sib was randomly chosen by means of the generation of a random vector. Second, a case-control analysis where all individuals were included and relatedness was controlled for, was conducted. And third, a family-based association study was performed.

Using the results from the genotyping of the CAG and GGN repeats in the family-based association study, one of the siblings from affected sib-pairs sharing one or two alleles of the *AR* were selected for mutation screening ($n=13$). Thirty-three other patients were selected randomly to generate a final sample of 46 subjects (3 women) for this part of the study, two of whom had normal IQ.

The study was conducted in accordance with the declaration of Helsinki and approved by the local Research Ethics Boards. Written informed consent was obtained from parents or legal guardians, and, if possible (i.e. if the patient did not have severe MR) from patients, as well as from controls.

Genotyping and mutation screening

In the PARIS-study, DNA was extracted from blood leukocytes ($\approx 50\%$) or lymphoblastoid cell lines ($\approx 50\%$). In the separate Swedish population, DNA was extracted from blood samples from all subjects. Genotyping of repeat polymorphisms was performed by means of electrophoreses using an ABI 3730 genetic analyzer (Applied Biosystems, Foster City, CA). The number of repeats corresponding to fragment length was verified using sequencing. Genotyping of the SNP was performed by pyrosequencing technology (Pyrosequencing AB, Uppsala, Sweden). Except for fragments (20-30bp) surrounding the two microsatellites in exon one, all eight exons, intron-exon boundaries and the 600 bp upstream of the transcription start site containing the promoter (Tilley et al., 1990) were successfully sequenced using the BigDye Terminator Cycle Sequencing Kit (V3.1, Applied Biosystems). Samples were then subjected to electrophoresis, using the ABI 3730 genetic analyzer. For primer sequences and PCR conditions used in genotyping and mutation screening, see Supplementary Table 1.

Classification of genotypes

For the repeats, alleles were classified into one of four possible length categories: rare short (CAG: 7-19 repeat units, GGN: 12-22), common short (CAG: 20-21, GGN: 23), common long (CAG: 22-23, GGN: 24) and rare long (CAG: 24-33, GGN: 25-28). Although not possible due to the uneven distribution of alleles, the selection of cutoffs had the purpose of creating equal-sized groups. The two short and the two long categories were merged to create one short (S) and one long (L) length category, respectively. These cutoffs corresponded to the median repeat lengths in controls. The categorizations were used both for the case-control analyses and the family-based association tests.

Statistical analyses

In the case-control analyses, the groups were compared with respect to allelic length using Independent-samples t-tests, and with respect to distributions of length categories and of genotypes derived from dichotomized alleles using likelihood ratio based chi-square tests. One proband was chosen randomly from each affected sib-pair family; when sexes were analyzed separately, the proband of the relevant sex was always chosen from mixed-sex sib-pairs. Generalized estimating equations (GEE), where both individuals from sib-pairs could be included and dependencies controlled for, were performed with case-control status as the dependent binomial variable, a logit link function, and the polymorphisms as independent variables. Analyses of possible two-locus effects were conducted by means of GEE. These analyses were performed using SPSS, version 15.0.

Multiallelic Transmission Disequilibrium Tests (mTDT) were performed using the mTDT program in the gap package in R, producing the Stuart chi-square statistics and corresponding p -values using 10.000 simulations. TDTs were also performed for the grouping of the alleles into short and long repeat lengths. In addition, each length category was tested against all other alleles grouped together.

Because the *AR* is located on the X chromosome, the haplotype of every XY individual, and also for most mothers of probands, could be unambiguously determined. To shed light on the extent of linkage disequilibrium (LD) between the three polymorphisms, haplotype frequencies were assessed for the subgroup of individuals for

which haplotypes did not have to be estimated. LD measures are hence based on observed and not estimated haplotypes in the subgroup consisting of male controls and parents of probands.

The multiallelic modification of Lewinton's D' , a weighted average of the D' values, the weights being the products of the corresponding allele frequencies, is given as LD measure. The extent of LD was determined by means of chi-square tests; the sum of squared D' -values divided by the product of the corresponding allele frequencies and multiplied by the number of haplotypes in the sample is Chi-square distributed with $(n-1) \cdot (m-1)$ degrees of freedom, where n and m are the number of alleles for the two loci. Alleles with frequencies less than 1% were excluded.

Results

Allele and haplotype frequencies

The distributions of the repeat alleles are displayed in Figure 1. The allele frequency was 0.14 for the A allele of rs6152. Haplotypes could be unambiguously determined in men and in most parents of probands. Haplotype frequencies are displayed in Table 1. The D' between the CAG and GGN repeats was 0.25 ($p > 0.5$), between the CAG repeat and rs6152 it was 0.44 ($p < 0.001$) and between the rs6152 and the GGN repeat it was 0.72 ($p < 0.001$). The rare A allele of the rs6152 most often occurred on the same haplotype as the common 24-repeat allele of the GGN (74 times against 15 times on other GGN alleles). Furthermore, the A allele always occurred with CAG alleles of lengths 18–22 repeats, preferentially with 18- or 21-repeat alleles, both of which belong to the short category (see Table 1 for haplotype frequencies).

Case-control association study

The mean CAG allele length was shorter in patients than in controls, and the S allele frequency was significantly higher in cases than in controls. After splitting for gender, this difference was found to be significant only in females, among whom, in addition, cases had a higher frequency of the SS genotype (Table 2). There was also a trend for higher frequency of the two shorter allele categories in females when the allelic length was divided into four categories (Table 2). No significant differences were found for the GGN repeat. The rs6152 displayed higher frequency of the rare A-containing genotypes in female cases compared to female controls (Table 2).

The GEE, utilizing all individuals including both members of affected sib-pairs, produced similar results of higher frequency of the S allele ($p = 0.02$, OR = 1.6 for the S compared to the L allele) and of the SS genotype when treating genotype as a covariate ($p = 0.03$, OR = 1.6) in females with autism. There was also an association between the rs6152 A allele and autism in females using GEE ($p = 0.03$, OR = 1.7 for presence of the A allele compared to the GG genotype). No trend was observed for the GGN repeat.

Separate analysis of high-functioning subjects was not deemed meaningful due to the low number of cases ($n = 46$ for men and $n = 13$ for women). Removing the high-functioning cases did not reduce the level of significance, neither for the CAG repeat, nor for the rs6152 SNP (data not shown).

No two-locus effects of the polymorphisms were found when analyzing combinations of genotypes (data not shown). When the CAG repeat and the rs6152 SNP were entered in the same GEE model, only the CAG repeat had a significant effect on autism risk in women.

Family-based association study

When considering the four allele length categories, 82, 31 and 66 families were informative for multiallelic TDT— since they comprised heterozygous mothers— for the CAG repeat, the rs6152 and the GGN repeat of the *AR*, respectively.

Neither the TDTs for the CAG repeat, nor the TDTs for the rs6152 polymorphism, revealed excess transmission of any allele to probands from heterozygous parents (Table 3). The GGN repeat showed undertransmission of the rare short allele category to probands from mothers carrying one such allele and one of the longer alleles, an effect that was found to be prevalent only when considering transmissions to males (Table 3), and to be entirely due to undertransmission of the 20-repeat allele (transmitted 7 times and non-transmitted once only). The 20-repeat GGN allele always occurred on a haplotype with the rs6152 G allele. A significant overtransmission of the common short 23-repeat allele of the GGN polymorphism to female probands was found (Table 3).

Because of the indication of an association between the CAG repeat and autism in women, the short CAG allele categories were further investigated in the family sample, revealing that the 20-repeat allele was the only allele that was significantly overtransmitted to female probands ($p=0.02$, transmitted 8 times and non-transmitted only once).

Mutation screening

Sequencing of the *AR* did not reveal any previously unknown polymorphisms or rare variants.

Discussion

Although the results from the case-control study and the family-based study were not unequivocal, they do lend support for the notion that variants in the gene encoding the *AR* may influence the susceptibility for autism in women. In contrast, no support was obtained for the hypothesis that rare mutations in this gene are common in subjects with autism.

Two previous studies have assessed microsatellites in the *AR* to address the issue of altered X-inactivation patterns in autism (Gong et al., 2008; Talebizadeh et al., 2005), but they did not investigate the possible association between repeat length and the studied disorder. This is, thus, to our knowledge, the first study presenting results from association studies of the *AR* in autism. This is somewhat surprising since this gene is an obvious candidate gene for autism, due to its position on the X chromosome, the skewed male-to-female prevalence of autism and the suggested relationship between autism and the prenatal influence of androgens on brain development (Baron-Cohen et al., 2005; Geier and Geier, 2007; Gillberg and Schaumann, 1982; Knickmeyer et al., 2005; Leboyer et al., 1988; Manning et al., 2001; Tordjman et al., 1997; Williams et al., 2003; Wing, 1981).

The length of the CAG repeat is inversely correlated with the activity of the *AR* (Beilin et al., 2000; Chamberlain et al., 1994), and short repeat lengths have previously been shown to increase prenatal androgen sensitivity as measured by the ratio between the second and fourth digit (Manning et al., 2003). It has also been shown that, in women, short CAG repeats are associated with higher testosterone levels (Ibanez et al., 2003; Westberg et al., 2001) as well as with higher risk of conditions related to hyperandrogenicity (Shah et al., 2008). The outcome of our case-control association study, indicating a higher prevalence of short CAG repeat alleles in female patients, is hence consistent with the “extreme male brain” theory of autism suggesting enhanced prenatal influence of androgens on brain development to be a factor of importance for the development of this condition (Baron-Cohen et al., 2005). When based on a categorization of CAG repeats according to length, the TDT test did not reveal a significant influence of the CAG repeat on risk for autism, but a post hoc analysis, motivated by the outcome of the case-control study, suggested the short 20-repeat to be overtransmitted to women with autism.

It should be noted that neither the case-control study nor the family-based analysis provided any support for an influence of the CAG repeat in the *AR* on the risk for autism in males. Obviously, it is not unlikely that men and women differ with respect to the importance of inter-individual differences in prenatal androgen exposure, and that women may be more sensitive to subtle differences in this regard.

The common G allele of the rs6152 has previously been associated with androgenetic alopecia in men (Ellis et al., 2007; Ellis et al., 2001; Hillmer et al., 2005; Prodi et al., 2008); this association has however sometimes been proposed to be due to LD with other polymorphisms. When interpreting our finding of an association between the A allele and autism in women, it should be taken into consideration that the A allele occurred on haplotypes with certain CAG repeat alleles only, the majority of which belong to the short category; this observation is in line with previous reports (Saare et al., 2007). Notably, when the two polymorphisms were analyzed simultaneously, the CAG repeat, but not the rs6152, showed an influence on autism risk.

The high LD seen between the SNP and both the CAG and the GGN repeats is consistent with other reports of high LD over the *AR* (Hillmer et al., 2005; Rexrode et al., 2008); moreover, also in line with previous studies, the two repeats were not in LD (Kittles et al., 2001). In spite of the exclusion of alleles with frequencies smaller than one percent, the power of detecting LD may have been too low because of the large number of alleles.

While the case-control study provided no support for an influence of the GGN repeat polymorphism, the family-based analyses revealed an overtransmission of the 23-repeat allele to female patients and an undertransmission of the 20-repeat allele to males with autism. The possible functional consequences of the GGN repeat polymorphism remain controversial; three studies indicate a positive correlation between GGN

repeat number and AR activity or protein amount (Brockschmidt et al., 2007; Gao et al., 1996; Werner et al., 2006), one indicates an inverse correlation with AR protein amount (Ding et al., 2005), and one suggests higher activity for the most common 23-repeat allele (Lundin et al., 2007). Studies on androgen-related phenotypes in men, including prostate cancer, infertility and baldness, report different direction of association for the GGN repeat; most but not all studies however indicate that a short repeat length is associated with enhanced androgenicity (Chang et al., 2002; Edwards et al., 1999; Ferlin et al., 2004; Hillmer et al., 2005; Hsing et al., 2000; Lundin et al., 2006). Notably, one study indicating a direction for the relation between the GGN repeat and androgen exposure in women show shorter GGN repeats to be associated with higher androgen activity as illustrated by a smaller risk for endometrial cancer (Sasaki et al., 2005), a finding that is in line with our observation of overtransmission of the common short 23-repeat allele to female cases, considering the androgen theory of autism.

Even when not combined with autism, MR is much more common in men than in women. Since the majority of the subjects included in this study displayed autism combined with MR, the associations observed might obviously reflect an association between the *AR* and MR in general rather than between the *AR* and autism *per se*. Indeed, although *AR* mutations that *cause* MR have not yet been found (Gottlieb et al., 2004), genetic variation in the *AR* has been associated with MR (Kooy et al., 1999) and cognitive ability (Yaffe et al., 2003).

Although relatively few patients were screened for mutations, we can exclude a high frequency of rare *AR* variants in autism. This finding is in line with a previous small negative study (Yan et al., 2004).

In conclusion, our findings support the notion that genetic variation within the *AR* may influence the susceptibility of autism, hence lending support to the “extreme male brain” theory of autism. However, the results also indicate that this influence may be gender-dependent. Furthermore, our results suggest that rare mutations in the *AR* are not common in autism.

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