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Polymorphisms in the maternal sex steroid pathway are associated with behavior problems in male offspring

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Abstract

Objective—Slight perturbations in maternal sex steroid production and metabolism may interfere with normal fetal neurodevelopment. The balance of maternal estrogens and androgens may have direct fetal effects, may influence the fetal hypothalamic-pituitary-gonadal axis or may alter local hormonal activity within the fetal brain. We investigated maternal functional polymorphisms of *CYP17, CYP19* and *CYP1B1*, which control three major enzymatic steps in sex steroid biosynthesis and metabolism, in relation to childhood behaviors.

Methods—The Mount Sinai Children's Environmental Health Study enrolled a multiethnic urban pregnancy cohort from 1998–2002 (n = 404). DNA was obtained from maternal blood (n=149) and from neonatal cord blood (n=53). At each visit, mothers completed the Behavior Assessment System for Children (BASC), a parent-reported questionnaire used to evaluate children for behavior problems. We focused on problem behaviors more commonly associated with ADHD (hyperactivity, attention problems, externalizing behaviors, conduct disorder, poor adaptability) to see if maternal genetic variants in sex steroid production and metabolism influence sexually-dimorphic behaviors in offspring.

Results—The more active gene variants were significantly associated with Attention Problems and poorer Adaptive Skills in male compared to female offspring. The *CYP19* variant allele was also significantly associated with worse scores for boys on the Hyperactivity, Externalizing Problems Composite and Adaptive Skills Composite scales ($p < 0.05$).

Conclusion—We observed maladaptive behaviors in the male offspring of mothers who carried functional polymorphisms in the sex steroid pathway. The strongest associations were in domains commonly affected in Attention Deficit-Hyperactivity Disorder.

Keywords

single nucleotide polymorphism; estradiol; androgen; BASC; attention deficit-hyperactivity disorder; 17-alpha-hydroxylase; aromatase; cytochrome P-450; CYP1B1

Introduction

Sex steroids are major modulators of mammalian brain function, regulating neurotransmitters and influencing neuronal differentiation, growth, and synapse formation (Arnold and Gorski, 1984; Rubinow and Schmidt, 1996). Exposure to varying levels of sex steroids early in development can lead to permanent changes in behavior (Morris et al.,

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2004). Sex hormone synthesis is mediated by the cytochrome P450 (CYP) family of enzymes. CYP17 catalyzes both steroid 17{alpha}-hydroxylation and the 17, 20-lyase reaction necessary for the conversion of progesterone and pregnenolone into the androstendione and dehydroepiandrosterone and, further downstream, into testosterone and estradiol. CYP19 is the rate-limiting enzyme in the conversion of testosterone to estradiol. CYP1B1 is responsible for the 4-hydroxylation of estradiol, resulting in the catechol estrogen, 4-hydroxyestradiol (Parvizi and Ellendorff, 1983). CYP1B1 also catalyzes hydroxylation of testosterone, which may effectively decrease androgenic activity (Tang et al., 2000). Single nucleotide polymorphisms (SNPs) in the genes coding for these three enzymes have been described. A substitution of C for T at −34bp in the 5′ promoter region of the CYP17 gene increases gene expression, resulting in higher levels of estradiol activity in pre-menopausal women (Carey et al., 1994; Feigelson et al., 1998; Small et al., 2005). The Leu432Val variant of CYP1B1 results in a threefold higher 4-hydroxylase activity than the Leu432 allele (Hanna et al., 2000; Paracchini et al., 2007). The C/T polymorphism in the 3′UTR of CYP19 is associated with higher levels of mRNA expression (Kravitz et al., 2006) resulting in elevated levels of estradiol and decreased levels of testosterone (Fisher et al., 1998; Olson et al., 2007). A recent study suggests that the C/T polymorphisms in CYP19 may affect personality traits (Matsumoto et al., 2009) and sexual behavior (Jones et al., 2006) in human males.

In this study we focused on the functional polymorphisms for *CYP17, CYP1B1* and *CYP19* and behavior problems. In addition to maternal sources of sex steroid exposure, inherited variants of these alleles may predict patterns of sex steroid biosynthesis in the offspring; therefore, we analyzed a subset of the children with behavior outcomes for which the child's genotype was also available.

Disruption of endocrine pathways at critical stages in development may differentially affect non-reproductive behaviors in male and female offspring (Weiss 2002). Sexually-dimorphic responses to sex steroids could potentially occur in human brain regions such as the hippocampus, prefrontal cortex, striatum and amygdala, regions that are associated with attention, working memory and emotional regulation (Gillies and McArthur 2010). Attention-deficit/hyperactivity disorder (ADHD) is a sexually-dimorphic clinical disorder that appears to be influenced by hormone-sensitive neural pathways (Martel et al., 2009). We therefore examined sex interactions for behavior outcomes that characterize ADHD: attention, hyperactivity, adaptability (including leadership and social skills), aggression, conduct problems, and externalizing behaviors (Reynolds and Kamphaus, 1998). We hypothesized that maternal polymorphisms in the sex steroid pathway may influence these sexually-dimorphic behaviors in offspring.

Methods

Study population

The Mount Sinai Children's Environmental Health Study is a multiethnic pregnancy cohort which enrolled 479 pregnant women between May 1998 and July 2001. All mothers were primiparous and gave birth to singleton offspring. Seventy-five of the women were subsequently excluded for reasons detailed elsewhere resulting in a cohort of 404 mother-infant pairs for whom birth data were available (Berkowitz et al., 2003; Berkowitz et al., 2004). Maternal peripheral blood and child cord blood were collected in the third trimester and at birth, respectively. Study participants were asked to return for an assessment when the children were between the ages of 4 and 9 years old. During these follow-up visits, mothers completed the Behavior Assessment System for Children (BASC-PRS).

Genotype Analysis

Whole blood was collected using heparin-treated vacutainers. DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Applied Science) according to manufacturer's instructions. DNA was stored at −20°C. Genotyping for *CYP17, CYP19* and *CYP1B1* was performed at the Mount Sinai School of Medicine DNAcore Sequencing Facility using SNPlex method under standard condition as described by Applied BiosystemsTM (Foster City, California). It utilizes pre-optimized universal assay reagents kits and a set of SNP-ligation probes to perform genotyping up to 48-plex (48 SNPs genotyped in a single reaction). The analysis system collects and manages raw data and provides automated allele calling and quality metrics. All three genotypes followed the Hardy-Weinberg equilibrium (Table 1).

Measures of Childhood Behavior

The BASC is a norm-referenced instrument commonly used in both research and clinical environments to evaluate problematic behaviors in children and adolescents aged 2.5–18 years (Reynolds, Kamphaus 1998). The BASC–PRS includes nine clinical scales to assess a child's adaptive and problem behaviors in home and community settings. Parents respond to 130 items on a 4-point scale that ranges from never to almost always. Externalizing Problems is a composite scale derived from the Hyperactivity (including both hyperactivity and impulsivity items), Aggression, and Conduct Problems scale items. An additional scale encompasses Attention Problems. The Adaptive Skills Composite combines information from the Adaptability, Social Skills and Leadership scale items. For the clinical and composite scales, higher scores indicate more problem behaviors. For the adaptive scales, lower scores indicate more problems with adaptation to changes in the environment. The BASC is standardized for age and gender and provides *T*-scores with a mean of 50 (SD, 10). *T*-scores on the clinical scales from 60 to 69 and on the adaptive scales from 31 to 40 are considered At-Risk, indicating the presence of significant problems. Mothers completed the parent-report form of the BASC at up to three follow-up visits between ages 4 and 9. The scores did not systematically vary across visits; therefore, we averaged the BASC *T*-scores for those children who returned for more than one visit (Engel et al., 2010). The BASC has been well-validated as a tool for diagnosing ADHD, particularly in children who display both inattention and hyperactivity (Vaughn et al., 1997; Ostrander et al., 1998; Pineda et al., 2005; Jarratt et al., 2005). Boys in the general population score more poorly than girls on Attention Problems, Aggression, Conduct Problems, Hyperactivity clinical scales and the Externalizing Problems and Adaptive Skills Composite scales of the BASC; similarly, children diagnosed with ADHD consistently score poorer in the same domains (Reynolds and Kamphaus, 1998).

Statistical analyses

Data analysis was performed using SAS statistical software, version 9.2 (Cary, North Carolina). Multivariable linear regression was used to estimate the effect of the two-level genotype on each BASC behavioral domain, with the common variant as the referent. To estimate the joint effect of genotype and infant sex (girls as referent), we included an interaction term in the models (infant sex times two-level genotype, any variant allele vs. wild type homozygous as referent). To obtain the estimate for boys with a variant allele, we reversed the coding of the genotype. We also examined 3-level genotypes in the models for main effects only, and they were consistent with the dominant genotype models. The numbers, however, were insufficient to use the 3-level genotype in models testing for interactions.

The final model was adjusted for self-reported race/ethnicity (black, Hispanic, white) and for ancestral genetic structure using ancestry informative markers (AIMs), markers that have

large allele frequency differences among different populations and can distinguish between the ancestral founders of African-Americans and Hispanic populations (Shriver et al., 1997). We previously reported on a set of 35 AIMs in our population (Lee et al. 2010). A composite measure (i.e. the percentage of black and Hispanic race/ethnicity for each subject) was used in the statistical models. Self-reported race/ethnicity was presumed to include sociodemographic factors that may be linked to behavioral domains, whereas AIMs were included to account for differences in linkage disequilibrium resulting from population geographic origin. Consideration of other potential confounders such as education and marital status did not alter the estimates. BASC *T*-scores are age-standardized, therefore, no additional adjustment was made for child age in the model.

Sensitivity Analysis

We examined a subset of our study population (n=53) for which maternal genotype and child genotype were available. Along with the covariates in the final model, we included an interaction term for child sex and gene activity. Since none of the models with child genotype-sex interaction reached statistical significance, the term was removed. In a stepwise manner, we then examined the significance of the main effect for child genotype for each domain. The results of these analyses are summarized below.

Results

Table 2 displays the characteristics of the families who completed the BASC questionnaires when the child was 4, 6 or 7 years of age. (n = 150). Half of the study participants were Hispanic, and half were married or living with a partner at the time the questionnaire was completed. Two thirds of the mothers who returned for follow-up assessments had more than a high school degree at enrollment. There were slightly more boys than girls among the children who returned for a follow-up assessment. BASC mean scores (and standard deviations for the scales analyzed were as follows: Attention Problems 49.3±9.6, Hyperactivity 46.6±10.5, Aggression 46.5±9.3, Conduct Problems 48.0±9.2, Externalizing Problems Composite 46.6±9.8, and Adaptive Skills Composite 53.8±9.3.

Overall there were a number of suggestive interactions between child sex and maternal SNP on behavior. The more active variant alleles showed a consistent pattern of association with problem behaviors, i.e., higher *T*-scores on BASC scales, for boys compared to girls after adjusting for child's race, sex and AIMs (Fig. 1).

In multivariate adjusted models, all three variant polymorphisms were significantly associated with more Attention Problems (*CYP17*, beta = 4.93, p = 0.009; *CYP19*, beta = 6.43, p = 0.001; *CYP1B1*, beta = 5.40, p = 0.002) and poorer Adaptive Skills (*CYP17*, beta = 4.95, p = 0.009; *CYP19*, beta = 4.27, p = 0.03; *CYP1B1*, beta = 5.94, p = 0.001) in boys compared with girls (Table 4). The *CYP19* variant allele was significantly associated with worse BASC scores on the Attention Problems and Hyperactivity (beta = 4.47, p = 0.04) clinical scales as well as on the Externalizing Problems Composite (beta = 4.46, p = 0.03) and Adaptive Skills Composite scales (beta = 4.27; p = 0.03). Overall, associations of similar magnitude and direction were observed for all of the domains analyzed in boys whose mothers carried any of the more active variant alleles. There were no occasions in which the variant alleles were associated with poorer behaviors in girls as compared to boys (Table 4).

We examined the main effect of child genotype and of the interaction between child genotype and sex in a subset of the study population for which genotyping had been performed on cord blood (n = 53). After including maternal genotype and the maternal genotype-child sex interaction, the child genotype-sex interaction never reached statistical

significance and was dropped from the model. We next examined the importance of the child genotype main effect after accounting for the maternal genotype main effect and sex interactions. In the majority of cases, there was no significant child genotype main effect for any of the domains examined. In addition, excluding child genotype from the model did not materially change (i.e. < 10%) the effect of the maternal genotype in either stratum of child sex (i.e. no confounding by child genotype) in a nested model. The only exception was for *CYP1B1* in the domain of Hyperactivity; however, the magnitude and direction of the incremental effect on the Hyperactivity domain score by sex for the variant genotype was the same in both models, and the interpretation of the maternal genotype effect within child sex strata did not change.

Discussion

We report on polymorphisms in sex steroid synthesis and metabolism of maternal origin associated with problem behaviors in offspring. Both androgens and estrogens have been shown to influence the organization of neural structure and function (Arnold and Breedlove, 1985; Rubinow and Schmidt, 1996). The prenatal hormonal milieu influences the development of brain structures involved not only in sexual behaviors but also in cognition, memory, aggression and mood, resulting in a multitude of phenotypes that vary both within and between the sexes (McCarthy, 2009). The mechanisms underlying the sexual differentiation of the brain, however, are complex and incompletely understood (MacLusky and Naftolin, 1981). Sex steroids may act directly on sexually dimorphic regions of the brain; they may affect the spatial patterning of sex steroid receptors across brain regions; or they may impact the pituitary-gonadal axis, i.e., negative feedback from excess estradiol would result in decreased gonadotropin release and, subsequently, diminished testosterone serum levels (Arnold and Gorski 1984). The relative contributions of testosterone and estrogen to the sexual differentiation of the human brain are also unclear; however, just as the ratio of androgens to estrogens has recently been implicated in the etiology of prostate diseases (Ellem and Risbridger 2010), the brain also appears to be sensitive to the relative levels of estrogens and androgens during development (Sharpe 1998; Gonzales et al., 2007).

Although the literature on the relationship between genotype, hormone levels and hormone-related diseases is not entirely consistent, a few relevant studies support the hypothesis that the CYP17 variant allele is associated with increased estrogen levels in premenopausal women (Fiegelson 1998; Small et al., 2005; Jasienska et al., 2006) and postmenopausal women with BMI<25 (Onland-Moret et al., 2005). There are also several studies showing increased estradiol with CYP19 (Somner et al., 2004; Binder et al., 2005; Haiman et al., 2007; Olson et al., 2007), and there is limited evidence that carriers of the variant allele for the CYP1B1 gene have higher levels of estradiol in premenopausal and postmenopausal women (Garcia-Closas et al., 2002; D e VI et al., 2002). Interpretation of the findings of many of these studies is complicated by methodological difficulties in adequately characterizing hormones that vary over the menstrual cycle as well as by age, BMI and maternal behaviors such as tobacco smoking, alcohol consumption and physical activity (Sharp et al. 2004). Studies measuring serum hormone concentrations report that the polymorphisms we examined do not significantly affect androgen levels in women (Olson et al. 2007). It is not clear why prenatal estrogen exposure would preferentially influence males. One putative scenario for the effects of estrogen on male behavior involves the presence of estrogen receptors (ERs) in the developing male brain. In humans, as in rodent males, the hippocampus, prefrontal cortex and amygdala have ERs (Osterlund et al., 2000). ERs are also present in the developing human fetus (Takeyama et al. 2001). Sexually-dimorphic responses to estradiol-sensitive regions of these limbic structures may explain some of the sex-specific behaviors we observed (Gillies and McArthur 2010).

We did not measure sex hormone concentrations directly but rather hypothesized that genetic polymorphisms might alter hormone levels during critical windows of development. Furthermore, given the temporal variability in hormone levels, the genes for sex steroid hormone synthesis and metabolism may serve as more reliable markers of average hormonal activity. It remains to be seen whether endogenous maternal sex steroids directly impact fetal hormone levels, especially within the physiologic range observed throughout pregnancy. Studies looking at the CYPs we assessed in relation to serum estrogen concentrations during pregnancy have not been conducted. The potential mechanisms underlying the maternal hormonal affects on the fetus are further complicated by the fact that the fetus is normally protected from high levels of maternal estrogens during pregnancy. In addition to being preferentially metabolized in the maternal circulation, these estrogen compounds are bound to serum proteins in maternal circulation and blocked by the fetoplacental unit, preventing most of the active substances from reaching sensitive fetal target tissues (Albrecht and Pepe 1998). Our study findings rely on a single assessment instrument for childhood behavior problems obtained via parental report and are, therefore, subject to reporting bias; the differential bias resulting from parents being more likely to report certain problem behaviors in boys may also inflate our effect estimates. Similarly, the fact that we found increased problem behaviors in boys compared to girls may be biased by the restriction of our phenotype to ADHD behavior traits. Future studies should look into whether these associations are maintained over time and utilize several behavioral assessment tools, including more comprehensive scales, to enhance the validity of our findings. Our study would also benefit from a subset analysis of children clinically diagnosed with ADHD to assess whether their genotypic associations are consistent with the larger cohort. Non-sex hormone effects of the genes in our analysis and social experiences were not accounted for in our study but are known to contribute to behavioral outcomes in offspring (Murray et al., 2009; McCarthy et al., 2009; Szyf, 2009). The purpose of this specific study was to test for a relationship between polymorphisms involved in hormone production and metabolism and *a priori* selected sex-specific behaviors captured by the BASC. We did not apply any corrections for multiple comparisons, focusing instead on the aggregate pattern and magnitude of associations and the consistency of our results.

In conclusion, we found significant associations for maternal polymorphisms known to affect sex steroid synthesis and metabolism with several problem behaviors in male children. These gene-sex interactions appear to be driven by the maternal genotype and not the child genotype. Epidemiologic studies of environmental toxins such as bisphenol A (BPA), polychlorinated biphenyls (PCB), and diethylstilbestrol (DES) demonstrate that low-level exposures to estrogenic compounds during pregnancy can induce abnormal neurodevelopment in offspring (Braun Diamanti-Kandarakis et al., 2009; Braun et al. 2009; Gore 2010). We, therefore, posit that alterations in endogenous levels of estrogens and androgens during vulnerable windows of brain development may tip the estrogen to androgen balance or achieve a threshold level for hormonal effect on fetal tissues including the brain. Future studies should address whether these CYPs have additive effects, non-hormonal effects, or linkage disequilibrium to unidentified functional polymorphisms that may influence behavior. As an exploratory study, ours would be the first to examine behavioral outcomes in offspring in relation to maternal sex steroid polymorphisms. While the effects may be modest, even a 5-point shift in the distribution of BASC scores for all males could significantly increase the proportion of boys that fall beyond the cut-off for clinical disorders such as ADHD.

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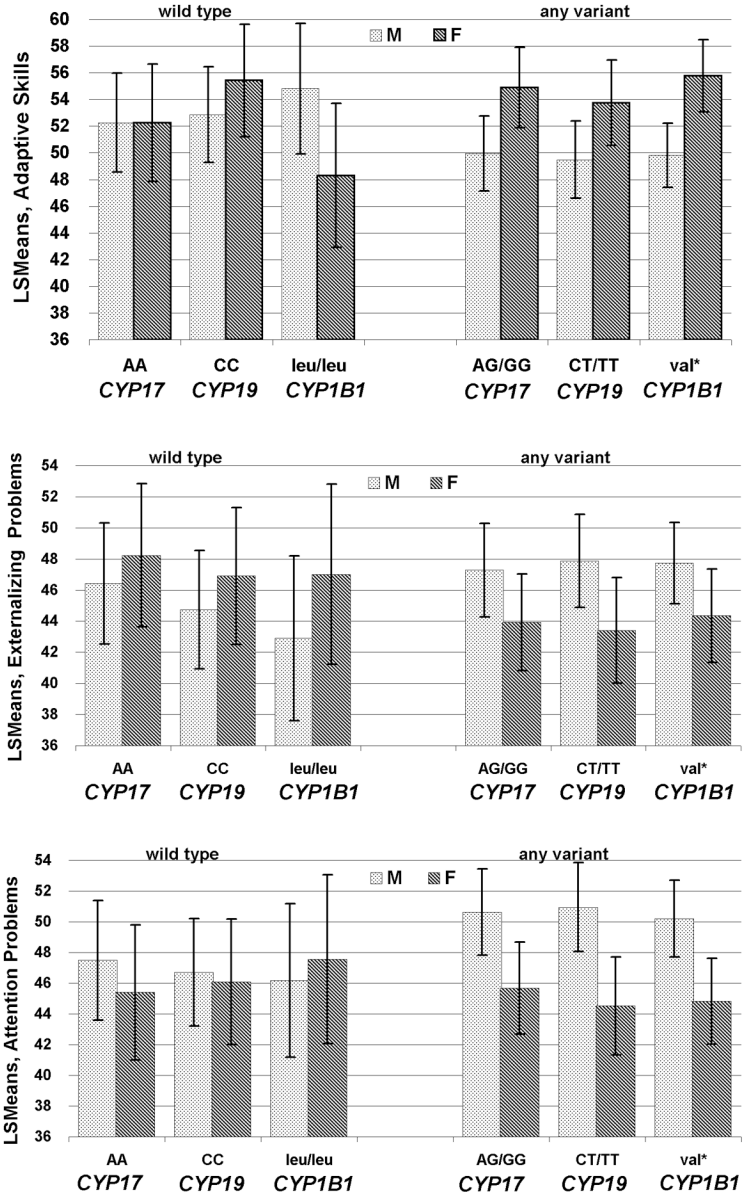

Figure 1.

Adjusted mean *T*-scores and 95% confidence intervals for three representative BASC scales by genotype and child sex. For Adaptive Skills Composite scale, higher scores are better. For Externalizing Problems Composite and Attention Problems scales, higher scores are worse. For children with any variant alleles (right side), boys scored worse than girls for all scales ($p<.05$) except Externalizing Problems Composite with *CYP17* and *CYP1B1* variants ($p<.10$). Means were adjusted for maternal race, child's sex, an interaction term for sex*genotype and ancestry informative markers.

val*= val/leu + val/val. †Adaptive Skills Composite: Adaptability, Social Skills and Leadership. ‡Externalizing Problems Composite: Hyperactivity, Aggression and Conduct Problems.

Table 1

Variant Allele Frequency by Race[†]

Variant Allele Frequency by Race	*CYP17* (AG + GG)	*CYP19* (CT + TT)	*CYP1B1* (Leu/Val + Val/Val)
Hispanic (n = 72)	0.44	0.37	0.46
Black (n = 43)	0.48	0.28	0.73
White (n = 32)	0.42	0.50	0.56

[†]All SNPs were in Hardy-Weinberg Equilibrium within self-reported racial subgroups.

Table 2

Population characteristics of the followed-up cohort, Mount Sinai Medical Center, New York, NY, 1998 – 2008.

Characteristics of Population	Children followed-up (N = 150)	
	N	%
Maternal Self-Reported Race/Ethnicity		
White	32	21.3
Black	43	28.7
Hispanic	72	48.0
Other	3	2.0
Child's Sex		
Male	83	55.3
Female	67	44.7
Marital Status at Enrollment		
Married/Living with a partner	77	51.3
Single/Divorced/Widowed	73	48.7
Maternal Education		
≤ High School degree	51	34.0
> High School degree	99	66.0

Table 4

The difference in adjusted mean BASC scores for boys vs. girls for any variant allele vs. the homozygous allele. Higher beta coefficients indicate worse behavior.

	CYP17 **(rs743572) A-34G N= 149**					***CYP19*** **(rs10046) 3′UTR C/T N =149**					***CYP1B1*** **(rs1056836) Leu432Val N= 150**				
BASC Domains	**genotype**	**Beta (boys vs. girls)**	**95% CI**	**p-value**	**Sex*gene Interaction p-value**	**genotype**	**Beta (boys vs. girls)**	**95% CI**	**p-value**	**Sex*gene Interaction p-value**	**genotype**	**Beta (boys vs. girls)**	**95% CI**	**p-value**	**Sex*gene Interaction p-value**
Attention Problems	AA	2.09	−3.32, 7.51	0.45	0.39	CC	0.60	−4.43, 5.64	0.81	0.07	Leu/Leu	−1.37	−8.18, 5.45	0.69	0.08
	AG/GG	4.94	1.27, 8.61	0.009		CT/TT	6.43	2.66, 10.2	0.001		Leu/Val +Val/Val	5.40	2.04, 8.76	0.002	
Hyperactivity	AA	−2.65	−8.78, 3.49	0.40	0.13	CC	−3.65	−9.38, 2.07	0.21	0.03	Leu/Leu	−3.77	−11.57, 4.02	0.34	0.15
	AG/GG	3.05	−1.10, 7.20	0.15		CT/TT	4.47	0.19, 8.75	0.04		Leu/Val +Val/Val	2.67	−1.18, 6.51	0.17	
Aggression	AA	−2.70	−8.01 2.60	0.32	0.07	CC	−1.77	−6.76, 3.22	0.49	0.09	Leu/Leu	−3.99	−10.71, 2.73	0.24	0.08
	AG/GG	3.15	−0.45, 6.74	0.09		CT/TT	3.69	−0.05, 7.42	0.053		Leu/Val +Val/Val	2.82	−0.49, 6.14	0.10	
Conduct Problems	AA	3.13	−2.45, 8.70	0.27	0.86	CC	2.26	−3.01, 7.54	0.40	0.75	Leu/Leu	−1.42	−8.36, 5.52	0.69	0.17
	AG/GG	2.51	−1.30, 6.32	0.19		CT/TT	3.32	−0.67, 7.31	0.10		Leu/Val +Val/Val	4.00	0.50, 7.50	0.03	
Externalizing Problems Composite[‡]	AA	−1.18	−1.72, 12.0	0.53	0.14	CC	−2.16	−7.49, 3.17	0.43	0.05	Leu/Leu	−4.11	−11.30, 3.08	0.26	0.07
	AG/GG	3.35	−0.51, 7.21	0.09		CT/TT	4.46	0.47, 8.44	0.03		Leu/Val +Val/Val	3.38	−0.16, 6.92	0.06	
Poor Adaptive Skills Composite[†]	AA	−0.02	−5.46, 5.41	0.99	0.14	CC	2.56	−2.53, 7.66	0.32	0.60	Leu/Leu	−6.51	13.18, 0.16	0.06	<0.01
	AG/GG	4.95	1.27, 8.63	0.009		CT/TT	4.27	0.46, 8.08	0.03		Leu/Val +Val/Val	5.94	2.65, 9.23	0.001	

[†]Adaptive Skills Composite: Adaptability, Social Skills and Leadership.

[‡]Externalizing Problems Composite: Hyperactivity, Aggression and Conduct Problems. Models are adjusted for maternal race, child's sex, ancestry informative markers (AIM). To obtain the estimate for boys with a variant allele, we reversed the coding of the genotype.