RESEARCH ARTICLE

Prenatal Stress Exposure, Oxytocin Receptor Gene (*OXTR*) Methylation and Child Autistic Traits: The Moderating Role of *OXTR* rs53576 Genotype

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LAY ABSTRACT

The gene encoding the oxytocin receptor (*OXTR*), localized on chromosome 3p25, is considered a promising candidate for explaining genetic vulnerability to autistic traits. Although several lines of evidence implicate *OXTR* SNP rs53576 (G/A) variation in social behavior, findings have been inconsistent, possibly because DNA methylation after stress exposure was eliminated from consideration. This study investigated the main and interactive effects of *OXTR* rs53576 genotype, stress exposure, and *OXTR* methylation on child autistic traits. Prenatal maternal stress exposure, but not *OXTR* rs53576 genotype and *OXTR* methylation, showed a main effect on child autistic traits. For child autistic traits in general and social communication problems in particular, we observed a significant *OXTR* rs53576 genotype by *OXTR* methylation interaction. More specifically, *OXTR* methylation levels were positively associated with social problems for *OXTR* rs53576 G-allele homozygous children but not for A-allele carriers. These results highlight the importance of incorporating epi-allelic information and support the role of *OXTR* methylation in child autistic traits.

SCIENTIFIC ABSTRACT

Findings of studies investigating *OXTR* SNP rs53576 (G-A) variation in social behavior have been inconsistent, possibly because DNA methylation after stress exposure was eliminated from consideration. Our goal was to examine *OXTR* rs53576 allele-specific sensitivity for neonatal *OXTR* DNA methylation in relation to (1) a prenatal maternal stress composite, and (2) child autistic traits. Prospective data from fetal life to age 6 years were collected in a total of 743 children participating in the Generation R Study. Prenatal maternal stress exposure was uniquely associated with child autistic traits but was unrelated to *OXTR* methylation across both *OXTR* rs53576 G-allele homozygous children and A-allele carriers. For child autistic traits in general and social communication problems in particular, we observed a significant *OXTR* rs53576 genotype by *OXTR* methylation interaction in the absence of main effects, suggesting that opposing effects cancelled each other out. Indeed, *OXTR* methylation levels were positively associated with social problems for *OXTR* rs53576 G-allele homozygous children but not for A-allele carriers. These results highlight the importance of incorporating epi-allelic information and support the role of *OXTR* methylation in child autistic traits.

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Keywords: DNA methylation; oxytocin receptor gene (OXTR); autistic traits; stress exposure

Introduction

Autistic traits manifest early in life and indicate impairments in social interaction and communication as well as patterns of restrictive, repetitive interests and behaviors [American Psychiatric Association, 2013]. Given the high heritability estimates for autistic traits [Hallmayer et al., 2011; Lichtenstein, Carlstrom, Rastam, Gillberg, & Anckarsater, 2010], much research has focused on unraveling their genetic underpinnings. Although previous studies have identified multiple genetic variants associated with autistic traits [Freitag, 2007; Geschwind, 2011; Liu et al., 2015; Persico & Napolioni, 2013], effect sizes are small and cannot explain the high heritability estimates derived from twin studies. Here we investigate the main and interactive effects of stress exposure, oxytocin receptor (*OXTR*) rs53576 genotype, and *OXTR* methylation on child autistic traits.

The gene encoding the *OXTR*, localized on chromosome 3p25, is considered a promising candidate for

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explaining genetic vulnerability to autistic traits [Yamasue, 2013]. Although several lines of evidence implicate *OXTR* SNP rs53576 (G/A) variation in social behavior [e.g., Liu et al., 2010; Park et al., 2010; Wermter et al., 2010; Wu et al., 2005], the results to date have been inconclusive. Whereas some studies indicated that the rs53576 A-allele is a "risk" allele for impaired social functioning in children and adolescents [Liu et al., 2010; Wermter et al., 2010; Wu et al., 2005], others reported that the A-allele is associated with better social cognitive ability [Park et al., 2010]. According to a recent meta-analysis, however, the *OXTR* rs53576 genotype is unrelated to social behavior or autistic traits [Bakermans-Kranenburg & Van IJzendoorn, 2014] However, the studies included in this meta-analysis did not examine the influence of epigenetic alterations.

A potential mechanism underlying the risk for autistic traits is the epigenetic process of DNA methylation. DNA methylation is involved in the transcriptional regulation of gene expression that can be influenced by environmental exposures [Szyf, 2011]. Higher levels of prenatal maternal stress exposure (e.g., maternal psychopathology, criminal behaviors, substance use) have been associated with higher methylation levels of the *OXTR* CpG island in neonates [Cecil et al., 2014]. Elevated methylation of the *OXTR* CpG island, in turn, has been associated with suppressed gene expression [Kusui et al., 2001] and lower levels of circulating oxytocin [Dadds et al., 2014]. Also of interest, Gregory et al. [2009] reported that elevated methylation of the *OXTR* CpG island decreased *OXTR* expression in the temporal cortex in autistic patients versus non-autistic controls. These findings suggest that *OXTR* methylation is functionally relevant to transcriptional regulation and possibly to the etiology of autistic traits.

It is increasingly recognized that DNA methylation patterns and associations may be allele-specific [Meaburn, Schalkwyk, & Mill, 2010]. For example, Van der Knaap et al. [2015] showed that stressful life events were positively associated with methylation of the serotonin transporter gene (*SLC6A4*) in the serotonin-transporter-linked polymorphic region (*5HTTLPR*) protective *ll* variant but not in the *sl*/*ss* variants. Van IJzendoorn, Caspers, Bakermans-Kranenburg, Beach, and Philibert [2010] reported that methylation of the *SLC6A4* gene at *5HTTLPR* was positively associated with risk of unresolved loss or trauma in the *5HTTLPR ll* variant but not in the *sl* and *ss* variants. Interestingly, elevated methylation of the *ss* variant was related to a decreased risk of unresolved loss or trauma. Elevated methylation of gene promoters is generally expected to decrease gene expression, and DNA methylation might (1) nullify the effect of the protective allele, resulting in a functionality equivalent to the risk allele or (2) mask the effect of risk alleles [Van der Knaap et al., 2015; Van IJzendoorn et al., 2010].

Recently, Ziegler et al. [2015] showed that *OXTR* methylation was predominant in social anxiety patients carrying the *OXTR* rs53576 A-allele. Similarly, Reiner et al. [2015] reported that, in their sample of clinically depressed women and healthy controls, *OXTR* rs53576 A-allele carriers exhibited significantly increased *OXTR* methylation levels. These studies provide suggestive evidence that *OXTR* methylation is allele-specific and might mask or reveal associations between *OXTR* rs53576 genotype and phenotype. However, it is not yet known whether carriers of the *OXTR* rs53576 G- and A-alleles are equally sensitive (1) to methylation after stress exposure and (2) to an increased risk for autistic traits by varying *OXTR* methylation.

The objective of the current study was to examine *OXTR* rs53576 allele-specific sensitivity for *OXTR* methylation in relation to (1) prenatal maternal stress exposure, and (2) child autistic traits at age 6. First, we investigated the extent to which prenatal maternal stress exposure interacted with *OXTR* rs53576 genotype in the prediction of *OXTR* methylation variation among neonates. Second, we investigated the extent to which prenatal maternal stress exposure and neonatal *OXTR* methylation combined either additively or interactively with *OXTR* rs53576 genotype to influence child autistic traits.

Method

Setting

The current study was conducted in a subsample of children participating in the Generation R Study, a population-based prospective cohort from fetal life onwards in Rotterdam, The Netherlands. The design and sample characteristics of the Generation R Study have been described in detail elsewhere [Jaddoe et al., 2012]. The study is in accordance with the guidelines proposed in the World Medical Association Declaration of Helsinki and has been approved by the Medical Ethics Committee of the Erasmus University Medical Center, Rotterdam. Written informed consent was obtained for all participating children. The subsample, known as the Generation R Focus Study, is ethnically homogeneous to exclude possible confounding or effect modification by ethnicity.

Study Population

DNA was collected from cord blood samples at birth. Information on autistic traits was obtained by two questionnaires when the children were 6 years of age. For 829 children, information on both *OXTR* DNA methylation and autistic traits was available. We excluded 51

Table 1. Sample Characteristics ($N = 743$)

	Oxytocin receptor gene (OXTR) rs53576 genotype	
	AA/AG ($n = 428$)	GG ($n = 315$)
Prenatal stress exposure, score (log)	0.15 ± 0.10	0.14 ± 0.10
OXTR methylation at birth, score	0.20 ± 0.05	0.20 ± 0.05
SRS total score at age 6, score (log)	0.07 ± 0.06	0.07 ± 0.07
Social communication, score (log)	0.06 ± 0.07	0.07 ± 0.08
Social cognition, score (log)	0.10 ± 0.09	0.11 ± 0.10
Autistic mannerisms, score (log)	0.04 ± 0.07	0.04 ± 0.07
Pervasive developmental problems at age 6, score (log)	0.37 ± 0.28	0.41 ± 0.29
Sex child (% boy)	53.0	48.6
Child nonverbal IQ, score	99.83 ± 15.38	100.23 ± 14.48
Maternal smoking during pregnancy (% yes)	11.0	12.7
Maternal postnatal depressive symptoms, score (log)	0.03 ± 0.07	0.04 ± 0.08

Note. Unless otherwise specified, values represent mean ± *SD*. No ant group differences were observed.

children with missing data on *OXTR* rs53576 genotype and an additional 35 children with missing data on prenatal maternal stress exposure. Overall, 743 children were included in one or more of our analyses. Sample characteristics are presented in Table 1.

Measures

Prenatal Stress Exposure

A prenatal cumulative stress composite had been previously created based on maternal reports [Rijlaarsdam et al., 2016], covering four stress domains: (1) life stress (e.g., death in family, illness, work problems), (2) contextual stress (e.g., financial difficulties, housing problems), (3) personal stress (e.g., psychopathology, substance abuse), and (4) interpersonal stress (e.g., family relationship difficulties, arguments with friends). For each domain, items were summed and divided by the number of completed items, allowing a maximum of 25% missing data. Intercorrelations between the risk domain scores were positive and statistically significant (all $P < 0.001$). We used confirmatory factor analysis (CFA) in Mplus [Muthén & Muthén, 1998–2012] to assess the internal reliability of the stress domains and to extract one cumulative prenatal stress composite, with higher scores indicating greater stress exposure. CFA showed good model fit (RMSEA; acceptable fit ≤0.08; CFI and TLI; acceptable fit ≥0.90) [Browne & Cudeck, 1993; Hu & Bentler, 1999]. The prenatal maternal stress exposure score was logarithmic (Log 10) transformed to approximate a normal distribution.

Genotyping

DNA from cord blood was genotyped on Illumina 610 K/660 W platforms. Basic quality checks for each SNP included sample call rates (≥97.5%), SNP call rates (≥98%), minor allele frequency (MAF) ≥0.1% and deviation from the Hardy Weinberg equilibrium ($P < 10^{-6}$). Samples were also checked for excess heterozygosity, gender accuracy, relatedness, and missing data. Following the quality control steps, phased genotype data were imputed to build 37 (hg19) of HapMap reference panel, using the MACH software [Li, Willer, Ding, Scheet, & Abecasis, 2010].

The distribution of rs53576 was: 42.4% GG, 47.1% GA, and 10.5% AA. There was no deviation of genotype frequencies from Hardy-Weinberg-Equilibrium [$\chi^2(1) = 1.77$, $P = 0.18$]. Due to the skewed distribution of rs53576 genotype, and in line with previous research [Reiner et al., 2015], we used a dominant model contrasting A-allele carriers (AA/AG genotype) versus GG homozygotes.

DNA Methylation Data

Five hundred nanograms of DNA from cord blood (birth) underwent bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, USA). Illumina Infinium HumanMethylation450 BeadChips (Illumina Inc., San Diego, USA) were run following standardized criteria. Quality control checks for each sample included status of bisulfite conversion, sample call rates, color balance, staining efficiency, extension efficiency, hybridization performance, and stripping efficiency after extension. This study included the 969 neonates who had DNA methylation data that passed quality control. Furthermore, all probes identified as having (1) a single nucleotide polymorphism in the single base extension site with a frequency of >1% in the GoNLv4 reference panel [Francioli et al., 2014] or (2) non-optimal binding (non-mapping or mapping multiple times to either the normal or the bisulfite-converted genome) were removed from the dataset. Samples were normalized using the Dasen method described by Pidsley, Cc, Volta, Lunnon, Mill, & Schalkwyk [2013] and dye bias corrected [Touleimat & Tost, 2012]. Normalized values are beta-values, which represent the methylation level at a CpG probe for each neonate. The current study was restricted to three probes (cg02192228, cg04523291, cg15317815; located within the *OXTR* CpG island; hg19; chr3:8808962–8811280), previously identified in the 450k HumanMethylation array, using a similar population and phenotype definition [Cecil et al., 2014]. These probes showed strong positive correlations (range $r = 0.64$–0.82, all $P < 0.001$) and their beta-values were averaged to represent an *OXTR* methylation score. Data inspection revealed three outliers (z-score > 3.29), which were winsorized (i.e., transformed to match the next highest value).

Child Autistic Traits

Child autistic traits were assessed via parental ratings using an 18-item short form of the Social Responsiveness Scale [SRS; Constantino, 2002; Constantino et al., 2003] when children were 6.0 ($SD = 0.29$) years of age. Specifically, children's social responsiveness in the past six months was rated on a 4-point scale, ranging from 0 (*never true*) to 3 (*almost always true*). In this study, the SRS total scale was used, as were the three subscales for further analysis. These subscales index social communication, social cognition, and autistic mannerisms. The SRS total scale correlated with the 13-item pervasive developmental problems (PDP) scale of the Child Behavior Checklist [CBCL; Achenbach & Rescorla, 2000] ($r = 0.50$, $P < 0.001$), which was included in a sensitivity analysis (mean age = 6.0 years, $SD = 0.20$). The child outcome scores were logarithmic (Log 10) transformed to approximate a normal distribution.

Covariates

We adjusted for several covariates, including family background characteristics (i.e., child sex, child age, and maternal smoking during pregnancy), technical covariates (i.e., the sample's array number and position on the array) and cell type proportions. Following the methods developed by Houseman et al. [2012], we included estimated proportions of cells in whole blood [proportion of CD8+ T-cells, CD4+ T-cells, natural killer (NK) cells, B-cells, monocytes and granulocytes] to adjust for cell type composition [Houseman et al., 2012].

Information on child sex was obtained from midwife and hospital registries at birth. Information on maternal tobacco smoking was obtained by postal questionnaires in early, mid- and late pregnancy. Maternal smoking was categorized on the basis of all three questionnaires into "never smoked during pregnancy or quit as soon as pregnancy was known" versus "continued smoking during pregnancy." Other family background characteristics, such as socio-economic status and prenatal maternal psychopathology, were already accounted for in the prenatal stress exposure score.

In follow-up analyses, we additionally adjusted for child nonverbal IQ and postnatal maternal depression. Child nonverbal IQ was assessed at age 6.0 years ($SD = 0.28$) using two subtests of the validated Dutch test battery "Snijders-Oomen Niet-verbale Intelligentietest–Revisie" [SON-R 2½–7; Tellegen, Winkel, Wijnberg-Williams, and Laros, 2005]; Mosaics (spatial visualization abilities) and Categories (abstract reasoning abilities). Raw test scores were converted into nonverbal IQ scores using norms tailored to exact age. Maternal depression was assessed using the Brief Symptom Inventory [BSI; De Beurs, 2004; Derogatis & Melisaratos, 1983] when children were 3.03 ($SD = 0.06$) years of age. The BSI is a validated 53-item self-report questionnaire, which is widely used in clinical and research settings. From this questionnaire, the 5-item subscale on depression was used. The depression score was logarithmic (Log 10) transformed to approximate a normal distribution.

Other potential confounders include maternal major depressive disorder (MDD) and medication (e.g., SSRI) use. This concerns rather small numbers ($n_{MDD} = 11$; $n_{SSRI} = 8$) in our population-based cohort, and we showed that offspring DNA methylation did not differ for maternal MDD, $t(669) = 0.53$, $P = 0.599$, or SSRI use, $t(666) = 0.30$, $P = 0.296$. Hence, these variables were not included as covariates.

Statistical Analysis

Linear regression analysis with product terms was performed in SPSS version 23 (IBM Corporation) to test our research questions. First, we examined *OXTR* rs53576 allele-specific sensitivity for *OXTR* methylation in relation to prenatal maternal stress exposure. In the first step of the regression equation, we entered prenatal stress exposure, *OXTR* rs53576 genotype, and covariates. In the second step, we entered the prenatal stress exposure x *OXTR* rs53576 genotype interaction.

Second, we examined *OXTR* rs53576 allele-specific sensitivity for *OXTR* methylation in relation to child autistic traits at age 6. In the first step, we entered *OXTR* methylation, *OXTR* rs53576 genotype, and covariates. We also accounted for prenatal stress exposure. In the second step, we entered the *OXTR* methylation × *OXTR* rs53576 genotype interaction. We also accounted for interactions of *OXTR* methylation and *OXTR* rs53576 genotype with prenatal stress exposure. Next to two-way interactions (step 2: *OXTR* methylation × prenatal stress exposure; *OXTR* rs53576 genotype × prenatal stress exposure), we also added a three-way interaction (step 3: *OXTR* methylation × *OXTR* rs53576 genotype × prenatal stress exposure). All independent variables were mean-centered prior to analysis.

Furthermore, we tested (1) the extent to which the observed findings were independent of child IQ and postnatal maternal depressive symptoms and (2) sex differences. Missing values on child IQ ($n = 120$) and postnatal maternal depressive symptoms ($n = 61$) were handled by use of the Markov Chain Monte Carlo multiple imputation technique with Predictive Mean Matching for continuous variables in SPSS. A total of five datasets were generated and parameter estimates were averaged over the set of analyses. Because we did not impute data of outcome measures, the study

Table 2. Associations of Oxytocin Receptor Gene (OXTR) Methylation, OXTR rs53576 Genotype, and Stress Exposure with Child Autistics Traits at age 6 years ($N = 680$)

	Social responsiveness scale (SRS)											
	SRS total			Social communication			Social cognition			Autistic mannerisms		
	B (95% *CI*)	*β*	*P*-value	*B* (95% *CI*)	*β*	*P*-value	*B* (95% *CI*)	*β*	*P*-value	*B* (95% *CI*)	*β*	*P*-value
OXTR methylation	0.01 (−0.11; 0.14)	0.01	0.819	0.08 (−0.07; 0.23)	0.05	0.279	−0.08 (−0.27; 0.11)	−0.04	0.411	0.01 (−0.13; 0.14)	0.01	0.907
OXTR rs53576 genotype	0.002 (−0.003; 0.01)	0.03	0.392	0.002 (−0.004; 0.01)	0.03	0.497	0.004 (−0.003; 0.01)	0.04	0.267	0.000 (−0.01; 0.01)	0.002	0.951
Prenatal stress exposure	0.10 (0.05; 0.15)	0.16	<0.001	0.10 (0.04; 0.15)	0.13	0.001	0.09 (0.02; 0.16)	0.09	0.018	0.10 (0.04; 0.15)	0.14	<0.001
OXTR rs53576 × methylation	0.11 (0.01; 0.21)	0.08	0.038	0.12 (0.030; 0.24)	0.08	0.044	0.14 (−0.01; 0.30)	0.07	0.068	0.03 (−0.08; 0.14)	0.02	0.614

Note. Test statistics are derived from the final block of the regression model. All analyses are adjusted for maternal smoking during pregnancy, technical covariates (i.e., the sample's array number and position on the array), cell type proportions, child sex and age at the assessment of outcome.
CI, confidence interval.

population differs per analysis ($N = 680$ in all primary analyses; $N = 721$ in the sensitivity analysis using the CBCL PDP score).

Results

OXTR Methylation

Overall prenatal maternal stress exposure was unrelated to *OXTR* methylation, $\beta = -0.002$, $P = 0.940$, across both *OXTR* rs53576 G-allele homozygous children and A-allele carriers, β for interaction $= -0.05$, $P = 0.122$. Similarly, the specific prenatal maternal stress domains (i.e., life stress, contextual stress, personal stress, and interpersonal stress) were unrelated to *OXTR* methylation. This finding argues against a mediating role of *OXTR* methylation in the association between prenatal stress exposure and child autistic traits. There was no main effect of *OXTR* rs53576 genotype on *OXTR* methylation, $\beta = 0.03$, $P = 0.318$.

Child Autistic Traits

Table 2 shows the final regression model of child autistic traits (SRS social total problem scale and subscales: social communication, social cognition, and autistic mannerisms). Step 1 explained a significant amount of variance in social total problem scores, $R^2 = 0.061$, $P < 0.001$, with a significant main effect of prenatal stress exposure, $\beta = 0.15$, $P < 0.001$, but not of *OXTR* rs53576 genotype, $P = 0.381$, or *OXTR* methylation, $P = 0.967$. Steps 2 and 3 produced non-significant increases in R^2 ($\Delta R^2 = 0.008$, $P = 0.134$ and $\Delta R^2 = 0.001$, $P = 0.409$, respectively). With the non-significant prenatal stress exposure × methylation interaction and the prenatal stress exposure × *OXTR* rs53576 genotype interaction excluded from step 2, however, the increase in R^2 ($\Delta R^2 = 0.006$) was significant, due to the significant *OXTR* methylation × *OXTR* rs53576 genotype interaction, $\beta = 0.08$, $P = 0.038$. Thus, the final, most parsimonious model as presented in Table 2 included all main effects (*OXTR* methylation, *OXTR* rs53576 genotype, and prenatal stress exposure) and the interaction effect of interest (*OXTR* methylation × *OXTR* rs53576 genotype).

As shown in Table 2, this *OXTR* methylation × *OXTR* rs53576 genotype interaction was specific to social communication problem scores, $\beta = 0.08$, $P = 0.044$. The association between *OXTR* methylation and communication problem scores was stronger for G-allele homozygous children ($\beta = 0.14$, $P = 0.068$) than for A-allele carriers ($\beta = -0.03$, $P = 0.639$). Of note, despite these numerical differences between the association of *OXTR* methylation with communication problem scores for G-allele homozygous children versus A-allele car[illegible] neither contrast was statistically significant.

observed interaction between *OXTR* methylation and *OXTR* rs53576 genotype remained significant after adjustment for child IQ, $\beta = 0.08$, $P = 0.038$, and postnatal maternal depressive symptoms, $\beta = 0.08$, $P = 0.028$, as well as after the inclusion of the sex × *OXTR* methylation, sex × *OXTR* rs53576 genotype, and sex × *OXTR* methylation × *OXTR* rs53576 genotype interactions (all $P > 0.05$), $\beta = 0.08$, $P = 0.045$. The observed interaction also held when we excluded children with the highest levels of autistic traits, based on cutoffs for screening in a population-based setting [SRS weighted scores >1.078 in boys ($n = 6$) and >1.00 in girls ($n = 1$)] [Constantino, 2002], $\beta = 0.09$, $P = 0.029$. Furthermore, a similar *OXTR* methylation × *OXTR* rs53576 genotype interaction emerged in the analysis of CBCL PDP, $\beta = 0.11$, $P = 0.004$ ($N = 721$), as well as in the analysis of averaged SRS and CBCL scores, $\beta = 0.12$, $P = 0.003$ ($N = 658$). Increased levels of methylation were statistically significantly associated with more CBCL PDP and higher averaged SRS and CBCL scores in *OXTR* rs53576 G-allele homozygous children ($\beta = 0.22$, $P = 0.004$ and $\beta = 0.21$, $P = 0.005$, respectively) but not in A-allele carriers ($\beta = -0.08$, $P = 0.200$ and $\beta = -0.09$, $P = 0.138$, respectively). Thus, according to these latter findings, *OXTR* rs53576 G-allele homozygous children with higher levels of *OXTR* DNA methylation had higher social problem scores.

As a follow-up analysis, we examined the three CpGs included in the *OXTR* DNA methylation score separately. The *OXTR* rs53576 genotype × DNA methylation interaction was statistically significant for cg15317815 ($\beta = 0.10$, $P = 0.010$) but not for cg04523291 ($\beta = 0.06$, $P = 0.131$) and cg02192228 ($\beta = 0.05$, $P = 0.219$).

Discussion

The objective of this prospective population-based study was to examine *OXTR* rs53576 allele-specific sensitivity for neonatal *OXTR* methylation in relation to (1) prenatal maternal stress exposure, and (2) child autistic traits at age 6. Our main finding was that *OXTR* rs53576 genotype and methylation of the *OXTR* CpG island contributed interactively, but not additively, to child autistic traits in general and social communication problems in particular. Specifically, the association between *OXTR* methylation and communication problem scores was stronger for G-allele homozygous children than for A-allele carriers. Prenatal maternal stress exposure was uniquely associated with child autistic traits but was unrelated to *OXTR* methylation across both *OXTR* rs53576 G-allele homozygous children and A-allele carriers.

The current findings extend those of others who have demonstrated that autistic traits may arise from genetic factors [Freitag, 2007; Geschwind, 2011; Liu et al., 2015; Persico & Napolioni, 2013] whose expression may be regulated by DNA methylation. Previous studies suggest, although not unequivocally, that the *OXTR* rs53576 A-allele is a "risk allele" for autistic traits [Liu et al., 2010; Wermter et al., 2010; Wu et al., 2005]. Elevated methylation of the *OXTR* CpG island is expected to decrease gene expression [Kusui et al., 2001] and levels of circulating oxytocin [Dadds et al., 2014]. Thus, *OXTR* methylation may decrease the expression of the otherwise protective *OXTR* rs53576 GG-allele and elevate the risk for autistic traits. Consequently, one would expect the social communication problems of G-allele homozygous children to resemble more closely those of A-allele carriers. Future research will be needed to establish the functional relevance of the observed findings to gene expression and modulation of oxytocin levels in the brain.

OXTR methylation and *OXTR* rs53576 genotype were not interrelated but combined interactively to influence child autistic traits. This finding of no allele-specific methylation is discordant with those of Reiner et al. [2015] and Ziegler et al. [2015], suggesting that *OXTR* rs53576 A-allele carriers exhibit significantly increased *OXTR* methylation levels. Furthermore, in contrast to the study by Cecil et al. [2014], *OXTR* methylation did not associate with prenatal stress exposure. Inconsistent findings may be explained, at least in part, by differences in sample composition. Ziegler et al [2015] showed that, when analyzing social anxiety patients and healthy controls separately, rs53576 allele-specific *OXTR* methylation was driven by the patient group. Furthermore, according to Cecil et al. [2014], the association between prenatal maternal personal stress and *OXTR* methylation was observed only for early-onset persistent conduct problems youth with low versus high internalizing problems.

Particular strengths of the current study are the prospective population-based design and the inclusion of a wide range of covariates (e.g., child and family characteristics, cellular heterogeneity of the blood cells). Including child IQ and maternal postnatal depressive symptoms as covariates, and excluding children with the highest autistic trait scores, did not change the results. Interestingly, the observed *OXTR* methylation × *OXTR* rs53576 genotype interaction did not differ between boys and girls and extended to child PDP. Of note, the observed interaction reflected shared variance rather than variance due to solely autistic traits or PDP.

Several limitations should also be considered. First, because DNA methylation was assessed only once (i.e., at birth), stress-induced changes in DNA methylation could not be examined. Longitudinal research is needed to more fully establish the relationships between stress exposure and DNA methylation in the prediction of

child autistic traits. Second, the magnitude of the observed associations was not large, and replication in larger epidemiological samples is warranted. Third, all measures except *OXTR* DNA methylation and genotype were based on maternal reports, raising the possibility of shared method variance between prenatal maternal stress exposure and child autistic symptoms. Fourth, our European-ancestry sample decreased generalizability. In a recent meta-analysis on oxytocin-related behavior, the combined overall effect size for rs53576 was heterogeneous in the total set of studies, but homogeneous in the studies with mainly European participants [Bakermans-Kranenburg & Van IJzendoorn, 2014]. Thus, although our ethnically homogeneous sample is informative, investigations in other ethnicities are warranted to address the generalizability of our findings. Finally, although some studies have shown that blood samples are adequate proxies of DNA methylation in other tissues such as the brain [Farre et al., 2015; Houtepen et al., 2016], the *OXTR* gene might be differentially expressed in different tissues. Therefore, it will be important to establish the extent to which our findings reflect associations in the brain. Also, there was no validation of the DNA methylation patterns using different techniques, such as pyrosequencing. The present results should be considered hypothesis-generating and in need of replication.

This candidate gene study focused on the specific hypothesis that *OXTR* rs53576 genotype and methylation of the *OXTR* CpG island contributed interactively to child autistic traits at age 6. A focused approach optimizes the statistical power of the methylation by genotype interaction analyses. Given that other *OXTR* variants [e.g., rs2254298, see Bakermans-Kranenburg & Van IJzendoorn, 2014] have also been suggested to be associated with child social behavior, rs2254298 allele-specific sensitivity for neonatal *OXTR* DNA methylation in relation to prenatal maternal stress exposure and child autistic traits may be one of the promising avenues for future research. Furthermore, the current study focused on offspring *OXTR* genotype and DNA methylation at birth, which are all independent of postnatal risk. It will be important to investigate not only maternal stress during pregnancy, but also pre-pregnancy [e.g., a maternal history of childhood abuse, see Heim et al., 2009] and postnatal [e.g., post-traumatic stress, see Eidelman-Rothman et al., 2015] stress exposure, as well as their relative, temporal contributions to offspring *OXTR* DNA methylation and autistic traits.

In conclusion, the current findings support previous research linking prenatal maternal stress exposure and child autistic traits, but additionally point to molecular genetic differences that may be implicated in gene expression as a factor contributing to autistic traits. We observed a significant *OXTR* rs53576 genotype x *OXTR* methylation interaction in the absence of main effects, suggesting that opposing effects on child social problems cancelled each other out. Indeed, *OXTR* methylation increased the risk for social problems in *OXTR* rs53576 G-allele homozygous children but not in A-allele carriers. These findings might point to a genetic differential susceptibility model [Bakermans-Kranenburg & Van IJzendoorn, 2015]. The importance of incorporating epi-allelic information had been previously demonstrated in the context of, for example, *SLC6A4* methylation, *5HTTLPR* genotype, stressful life events, and unresolved loss or trauma [Van der Knaap et al., 2015; Van IJzendoorn et al., 2010], but not yet in the context of *OXTR* methylation, *OXTR* rs53576 genotype and child autistic traits. The apparent inconsistency in the literature on *OXTR* rs53576 genotype and social functioning might be explained, at least in part, by varying *OXTR* methylation.

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Conflict of Interest

Dr. Frank C. Verhulst is the contributing editor of the Achenbach System of Empirically Based Assessment, from which he receives remuneration. The other authors declare that they have no conflict of interest.

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