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ARTICLE

Genetic and epigenetic associations of *MAOA* and *NR3C1* with depression and childhood adversities

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Abstract

Monoamine oxidase A (*MAOA*) harbours a polymorphic upstream variable-number tandem repeat (u-VNTR). The *MAOA*-L allele of the u-VNTR leads to decreased gene expression levels *in vitro* and has been found to increase the risk of conduct disorder in males with childhood adversities. Early-life adversities have been associated with hypermethylation of the glucocorticoid receptor (*NR3C1*). In this study, we first performed a genetic association analysis of the *MAOA* u-VNTR using individuals with depression (n=392) and controls (n=1276). Next, DNA methylation analyses of *MAOA* and *NR3C1* were performed using saliva samples of depressed and control subgroups. Adult *MAOA*-L females with childhood adversities were found to have a higher risk of developing depression (p=0.006) and overall *MAOA* methylation levels were decreased in depressed females compared to controls (mean depressed, 42% *vs.* mean controls, 44%; p=0.04). One specific childhood adversity [early parental death (EPD)] was associated with hypermethylation of *NR3C1* close to an NGFI-A binding site (mean EPD, 19% *vs.* mean non-EPD, 14%; p=0.005). Regression analysis indicated that this association may be mediated by the *MAOA*-L allele (adjusted R^2=0.24, ANOVA: F=23.48, p<0.001). Conclusively: (1) depression in females may result from a gene×childhood-adversity interaction and/or a dysregulated epigenetic programming of *MAOA*; (2) childhood-adversity subtypes may differentially impact DNA methylation at *NR3C1*; (3) baseline *MAOA*-genotypic variations may affect the extent of *NR3C1* methylation.

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Introduction

Depression is a psychiatric disorder that is more common in females than in males, with a female: male ratio of about 2:1 (Bromet et al., 2011). There is strong evidence that adverse childhood conditions are significant risk factors for the onset of depressive disorders (Fava and Kendler, 2000; Gilman et al., 2002). This is, among other ways, supported by the fact that sufficient parental attachment buffers the effects of negative life events (Armsden et al., 1990; Dekovic and Meeus, 1997). The heritability of depression is high (31–42%; Sullivan et al., 2000) and genetic factors are known to influence the risk of depression by affecting the sensitivity towards stressful life events (Kendler et al., 1995). Variations in the monoamine oxidase A (*MAOA*) gene and the glucocorticoid receptor (*NR3C1*) gene have been associated both with psychopathology and childhood adversities (Caspi et al., 2002; McGowan et al., 2009).

MAOA is the target of a class of antidepressants [monoamine oxidase inhibitors (MAOIs)] and codes for a mitochondrial enzyme that metabolizes neurotransmitters such as dopamine, serotonin and norepinephrine (Shih et al., 1999). *MAOA* is located on the X chromosome and contains a functional length polymorphism in its promoter region. This upstream variable-number tandem repeat (u-VNTR) affects

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transcriptional activity *in vitro*. More specifically, the u-VNTR's most frequent short allele (*MAOA*-L) is transcribed less efficiently than the most frequent long allele (*MAOA*-H; Sabol et al., 1998; Deckert et al., 1999; Denney et al., 1999). *MAOA*-L has been consistently associated with conduct behaviour in males exposed to childhood maltreatment (Caspi et al., 2002; Foley et al., 2004; Kim-Cohen et al., 2006; Nilsson et al., 2006; Widom and Brzustowicz, 2006; Frazzetto et al., 2007). While *MAOA*-L has also been associated with depressive symptomatology (Brummett et al., 2007; Aklillu et al., 2009; Doornbos et al., 2009; Huang et al., 2009), only one study has reported that childhood adversities may mediate this association. More specifically, Cicchetti et al. (2007) found increased depressive symptoms among *MAOA*-L youths who had experienced extensive child abuse and/or neglect.

NR3C1 encodes a protein that is involved in the inhibition of the hypothalamic–pituitary–adrenal axis and has been found to be decreased in post-mortem brain specimens from patients with depression (Webster et al., 2002). In many cases, promoter DNA methylation is associated with transcriptional silencing (Brenet et al., 2011). Childhood adversities in the form of abuse (McGowan et al., 2009), parental loss (Tyrka et al., 2012), exposure to maternal depression (Oberlander et al., 2008) and maternal separation (Weaver et al., 2004; McGowan et al., 2011), have been associated with DNA hypermethylation of a specific exon 1 splice-variant promoter of *NR3C1* (known as exon 1-F in humans). Exon 1-F is expressed in both the hippocampus and the immune system (Turner and Muller, 2005). DNA methylation at specific sites of this region interferes with the binding of a transcription factor [nerve growth factor-induced protein A (NGFI-A)] and leads to decreased *NR3C1* 1-F and total *NR3C1* mRNA-levels (McGowan et al., 2009).

In the present study we first hypothesized that the *MAOA* u-VNTR may interact with an exposure to childhood adversities and increase the risk of depression in adulthood. Such an interaction was found between the *MAOA*-L allele and the number of experienced childhood adversities, especially in females. As an epigenetic regulation (through DNA methylation) of *MAOA* has also been observed in females (Pinsonneault et al., 2006), we used individuals from this group to examine the putative association between *MAOA* methylation and depression. Next, we studied whether depression and different subtypes of childhood adversities were associated with the epigenetic status of the *NR3C1* 1-F region. The latter analysis revealed an association between early parental death (EPD) and *NR3C1* hypermethylation. Finally, since the action of serotonin through the 5-HT$_7$ receptor has been linked to the expression of *NR3C1* (Hackman et al., 2010) and as *MAOA* determines the availability of this neurotransmitter, we examined whether *MAOA*-L could also act as a mediator of the association between EPD and *NR3C1* hypermethylation.

Method

Origin of the study group

The cases and controls derived from the PART study; a longitudinal population-based study of mental health, work and relations in Stockholm County, Sweden (Hällström et al., 2003). PART includes data from 8613 randomly-selected Swedish nationals who have responded to an extensive questionnaire twice (in two waves with a 3-yr interval). The questionnaire included questions on adverse childhood experiences, demographic characteristics, financial status, social network, negative life events, somatic health, use of drugs and screening instruments for psychiatric disorders: the Major Depression Inventory (MDI; Bech and Wermuth, 1998); the Sheehan Patient-Rated (Panic) Anxiety Scale (Sheehan, 1983); the Yale-Brown Obsessive-Compulsive Scale (Goodman et al., 1989); screening for symptoms of social phobia and agoraphobia according to Marks and Mathews (1979); screening for eating disorders according to Beglin and Fairburn (1992); the World Health Organization Short Disability Assessment Schedule (Janca et al., 1996); screening for harmful alcohol use according to the Alcohol Use Disorder Identification Test (Saunders et al., 1993). Detailed attrition analyses using official registries assured that relationships between psychiatric disorders and living conditions were likely to be identified accurately (Lundberg et al., 2005; Bergman et al., 2010). The ethical committee of Karolinska Institutet approved the study and written consent was obtained from all the participants.

Definition of cases and controls

Individuals characterized as having depression (cases) were those diagnosed with major depression, mixed anxiety depression or dysthymia, in at least one of the two PART waves. Depression was defined according to DSM-IV and was identified using the MDI. Validation studies for the use of MDI in making DSM-IV-based diagnosis of depression have been performed in population-based settings (Forsell, 2005), clinical settings (Bech et al., 2001; Olsen et al., 2003) and outpatient settings (Cuijpers et al., 2007). For the

Table 1. *MAOA* upstream variable-number tandem repeat genetic association study: demographic, diagnostic and childhood characteristics of the study groups

	Females (n=993)			Males (n=675)		
	Mdn	Pctl (25th, 75th)	r	Mdn	Pctl (25th, 75th)	r
Age (yr)	56	43, 64	29–74	58	44, 64	29–74
	n	%		n	%	
Diagnosis						
Controls	708	71.3		568	84.1	
Depression	285	28.7		107	15.9	
No. of childhood adversities						
None	424	42.7		345	51.1	
One	281	28.3		170	25.2	
Two	204	20.5		126	18.7	
Three or four	81	8.2		32	4.7	
Type of childhood adversities						
Familial constraints	390	39.3		200	29.6	
Financial problems	340	34.2		194	28.7	
Parental divorce	153	15.4		86	12.7	
Parental death	52	5.2		38	5.6	

Mdn, Median; Pctl (25th, 75th), 25th and 75th percentiles; r, range.

diagnosis of mixed anxiety depression, additional information was used from the Sheehan Patient-Rated (Panic) Anxiety Scale (Sheehan, 1983). Controls were individuals with no psychopathological symptom of depression, anxiety, social phobia, agoraphobia, obsessive-compulsive disorder, eating disorder, use of illicit drugs or social disability due to psychological problems, in either of the two PART waves. Additionally, controls had never received health care for a psychiatric disorder or nervous discomfort.

Definition of childhood adversities

Adverse childhood events were assessed based on self-reported answers provided in the PART questionnaire. In particular, individuals had been asked to respond whether they had experienced (1) EPD, (2) parental divorce, (3) financial problems or (4) other familial constraints, which had occurred during childhood (i.e. before age 18 yr).

Saliva DNA collection

PART's DNA-collection wave has been described elsewhere (Sjoholm et al., 2009; Melas et al., 2010). In brief, individuals who had responded to both PART waves were asked to contribute saliva samples using a whole-saliva collection device (Oragene•DNA sample collection kit; DNA Genotek Inc., Canada), which is different from buccal cell collection techniques. Previous studies have suggested that the majority of DNA extracted from whole saliva may actually originate from blood leukocytes. More specifically, DNA extracted from mouthwashes after allogeneic blood stem cell transplantation have been shown to contain up to ~100% of donor DNA; i.e. presumably of leukocyte origin (Endler et al., 1999; Thiede et al., 2000).

MAOA u-VNTR study groups, genotyping procedure and determination of the number of MAOA u-VNTR repeats

MAOA u-VNTR genotyping was performed on saliva DNA from 458 cases and 1482 controls, randomly selected from the collected samples. Genotyping was successful for 392 of the cases (86%; 285 females and 107 males) and 1276 of the controls (86%; 708 females and 568 males). The demographic, diagnostic and childhood-adversity characteristics of the successfully genotyped individuals are shown in Table 1. To amplify the *MAOA* u-VNTR, the following primer pair was used: forward; 5′-ACAGCCTGACC-GTGGAGAAG-3′ (Fam), reverse; 5′-GAACGGACGC-TCCATTCGGA-3′ (tailed; Applied Biosystems Inc., USA). PCR reactions contained 5–200 ng genomic DNA, 0.5 mM dNTP, 0.4 μM primer, 0.5 unit DyNAzyme II DNA polymerase (Finnzymes, Vantaa,

Table 2. *MAOA* methylation study: demographic, genotypic and childhood characteristics of the study groups

	Depressed (n=82)			Control (n=92)		
	Mdn	Pctl (25th, 75th)	r	Mdn	Pctl (25th, 75th)	r
Age (yr)	52	40, 63	23–74	57	42, 65	21–74
	n	%		n	%	
MAOA u-VNTR genotype						
MAOA-H or *MAOA*-H/*MAOA*-L	46	55		53	56	
MAOA-L	36	45		39	44	
Females	82	100		92	100	
Smokers	16	19.5		11	12	
No. of childhood adversities						
None	25	30.5		37	40.2	
One	19	23.2		24	26.1	
Two	26	31.7		18	19.6	
Three	12	14.6		13	14.1	
Type of childhood adversities						
Familial constraints	46	56.1		39	42.4	
Financial problems	36	43.9		38	41.3	
Parental divorce	15	18.3		20	21.7	
Parental death	10	12.2		2	2.2	

Mdn, Median; Pctl (25th, 75th), 25th and 75th percentiles; r, range; u-VNTR, upstream variable-number tandem repeat; *MAOA*-H, allele with 4.1, 4.5 or 5,5 repeats; *MAOA*-L, allele with 3.5 repeats.

Finland) and 1×buffer. The PCR amplifications were carried out on a DNA Engine Tetrad 2 Peltier Thermal Cycler (Biorad, Bio-Rad Laboratories Inc., USA) under the following conditions: initial denaturation step (95 °C for 5 min); 32 cycles of denaturation (95 °C for 1 min); annealing (55 °C for 1 min); elongation (72 °C for 1 min); final elongation step (72 °C for 10 min). Eight negative controls were included in each 384-well plate and 45% of the DNA samples were genotyped in duplicate. The sizes of the PCR products were determined using an ABI 3730 DNA Analyser (Applied Biosystems), the ABI GeneScan 500 LIZ size standard and the ABI GeneMapper software version 4.0 (Applied Biosystems).

To determine the number of *MAOA* u-VNTR repeats present in this Swedish cohort, we sequenced the u-VNTR of individuals homozygous for one of the four alleles (n=2 for alleles 1, 2, 3 and n=1 for allele 4; with the four alleles numbered from shortest to longest) using saliva DNA. DNA sequencing reactions were performed unidirectionally (reverse-primed reactions) on an ABI 3730 DNA Analyser (Applied Biosystems) using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and the ABI Sequencing Analysis software version 5.3.1 (Applied Biosystems).

MAOA and NR3C1 DNA methylation study groups

DNA methylation levels of *MAOA* and *NR3C1* were quantified in saliva DNA from female cases and controls. These individuals were randomly chosen based on the number of experienced childhood adversities, so that both case and control groups had approximately the same number of individuals with none, one, two or three/four adversities. The demographic, genotypic and childhood-adversity characteristics of the individuals for whom the analyses were successful are shown in Table 2 for *MAOA* and Table 3 for *NR3C1*.

DNA methylation quantification of the MAOA and NR3C1 regions

DNA methylation levels of the *MAOA* and *NR3C1* regions were quantified using the Sequenom EpiTYPER platform (Sequenom Inc., USA) as previously described (Wong et al., 2010). In brief, DNA (~375 ng) was bisulfite-converted using the EZ-96 DNA Methylation-Gold Kit (Zymo Research Corporation, USA) and PCR reactions (T annealing=58 °C; cycles=35 for *MAOA* and cycles=40 for *NR3C1*) were performed using HotStarTaq DNA polymerase (Qiagen, Qiagen GmbH, Germany) and 2 μM primers. The following primers were used for amplifying the *MAOA*

Table 3. *NR3C1* methylation study: demographic and childhood characteristics of the study groups

	Depressed (n=93)			Control (n=83)		
	Mdn	Pctl (25th, 75th)	r	Mdn	Pctl (25th, 75th)	r
Age (yr)	55	42, 63	23–74	56	42, 65	21–74
	n	%		n	%	
Females	93	100		83	100	
Smokers	16	17.2		11	13.3	
No. of childhood adversities						
None	28	30.1		31	37.3	
One	23	24.7		22	26.5	
Two	29	31.2		17	20.5	
Three or four	13	14		13	15.7	
Type of childhood adversities						
Familial constraints	54	58.1		38	45.8	
Financial problems	37	44.6		40	43	
Parental divorce	17	18.3		18	21.7	
Parental death	9	9.7		3	3.6	

Mdn, Median; Pctl (25th, 75th), 25th and 75th percentiles; *r*, range.

region of interest (NCBI reference sequence: NG_008957.1; +174 to +392 from gene start): forward primer 5′-GTTAAAGTATGGAGAATTAAGAGAAGG-3′; reverse primer 5′-CAAAATATAAAACCAAACCATAACTACA-3′. The following primers were used for amplifying the *NR3C1* region of interest (NCBI reference sequence: NC_000005.9; +31173 to +31575 from gene start): forward primer 5′-TTTAATTTTTTAGGAAAAAGGGTGG-3′; reverse primer 5′-CCCTAAAACCTCCCCAAAAA-3′. All primers were ordered with the standard Sequenom MassCLEAVE tails; F: aggaagagag, R: cagtaatacgactcactatagggagaaggct. Successful PCR amplifications were checked on a 1% agarose gel and the assays were tested with methylated and unmethylated genomic human DNA controls (Millipore, EMD Millipore, USA). For *MAOA*, a total of 10 CpG sites were analysed as part of nine CpG units (each unit may correspond to a fragment that contains more than one CpG residue) and for *NR3C1* a total of 47 CpG sites were analysed as part of 22 CpG units. Standard quality-control measures were used to remove uninformative measurements and based on the useable mass window (1500–7000 Da) the EpiTYPER software generated informative data for seven out of nine *MAOA* CpG units and 15 out of 22 *NR3C1* CpG units.

Statistical analyses

The Hardy–Weinberg equilibrium for the four *MAOA* u-VNTR alleles was verified in the control group using a χ^2 test. Based on their genotypes, female individuals were divided into two main groups: *MAOA*-L (subjects homozygous for the shortest u-VNTR allele, i.e. L/L); *MAOA*-H (subjects homozygous for long u-VNTR alleles, i.e. H/H, together with subjects heterozygous for a long and the shortest allele, i.e. H/L). Genetic analyses in males were restricted to L *vs.* H, as *MAOA* is located on the X chromosome.

Odds ratios (OR) were calculated and χ^2 tests were performed to assess associations between depression and childhood adversities, depression and *MAOA* u-VNTR genotypes, and childhood adversities and *MAOA* u-VNTR genotypes, respectively. Thereafter, logistic regression was performed with depression as a dependent variable. The presence/absence of a childhood adversity was scored as 1/0 and the sum of the scores was used in the regression. Thus, a score of 0, 1, 2, 3 or 4 for the childhood-adversity item was possible for each subject. Due to few individuals with four adversities, those with three or four adversities were grouped together. For *MAOA* and *NR3C1* methylation data analyses, group comparisons were performed using non-parametric statistical analyses. Linear regressions were also performed to study the effect of putative predictors on methylation levels and to control for confounding variables. In order to present the methylation data in a simplified way, Figures were generated using mean percentages and standard error of means (S.E.M.). All analyses were performed using IBM SPSS Statistics version 20.0 (IBM

Table 4. *MAOA* upstream variable-number tandem repeat allele frequencies (n=1668)

Allele	Number of repeats	Allele frequency
1	3.5	38%
2	4.1	1.4%
3	4.5	59.6%
4	5.5	1%

Corporation, USA) and the statistical significance was set at $p<0.05$ for omnibus tests or was corrected accordingly based on the number of multiple tests. Supplementary S1 provides a more detailed description of the statistical analyses.

Results

Determination of the number of repeats in the MAOA u-VNTR

The *MAOA* u-VNTR repeat polymorphism was first described by Sabol et al. (1998) who reported the presence of 3, 3.5, 4 and 5 repeats in the u-VNTR and with the allele frequencies of the 3 and 4 repeats corresponding to 98% of the total. Deckert et al. (1999) reported the presence of 2, 3, 3.6, 4 and 5 repeats in the u-VNTR, while deviations between predicted and observed band lengths have also been observed (Denney et al., 1999). Our DNA sequencing results revealed the presence of 3.5, 4.1 (3.6+0.5), 4.5 and 5.5 repeats in the Swedish cohort (Supplementary S2). However, the frequency distribution of the two most common alleles in our population (the 3.5- and 4.5-repeat alleles; Table 4) is in accord with the frequencies of the two most common alleles reported elsewhere (i.e. the 3- and 4-repeat alleles; Sabol et al., 1998; Deckert et al., 1999; Caspi et al., 2002). Thus, the *MAOA*-L allele in the present study refers to the shortest allele of the studied population (i.e. the 3.5-repeat allele) and *MAOA*-H refers to the longer alleles (i.e. the 4.1-, 4.5- and 5.5-repeat alleles).

MAOA-L: gene×environment association with depression

The experience of childhood adversities *per se* was associated with depression both in adult females [OR =2.0, 95% confidence intervals (CI) 1.5–2.6; for having at least one childhood adversity compared to no adversities] and in adult males (OR=3.7, 95% CI 2.4–5.7).

The *MAOA* u-VNTR allele's distribution was in Hardy–Weinberg equilibrium (p=0.37) and *MAOA*-L females were found to be at a higher risk of having depression than *MAOA*-H females (OR=1.7, 95% CI 1.2–2.4; Table 5), while this difference was not seen in males (OR=1.0, 95% CI 0.8–1.4; Table 5).

The associations of (a) childhood adversities and (b) *MAOA*-L genotype with depression in females were confirmed in a combined logistic regression model with these two variables and age as covariates (*MAOA*-L genotype: OR=1.5, p=0.03; number of childhood adversities: Wald=31.0, d.f.=3, $p<0.001$; age: OR=1.0, p=0.1).

The *MAOA* u-VNTR was also found to interact with childhood adversities and increase the risk of depression, especially in one of the two sexes. More specifically, the logistic regression model in females showed a significant *MAOA*-L×number of childhood adversities interaction (Wald=16.4, d.f.=5, p=0.006), rendering the separate main effects of *MAOA*-L and childhood adversities non-significant (*MAOA*-L: OR= 1.3, p=0.6; number of childhood adversities: Wald= 1.7, p=0.2; age: OR=1.0, p=0.2). The effect of this interaction was significant even when only homozygous *MAOA*-H females (i.e. H/H and not H/H+H/L) were compared to *MAOA*-L (L/L) individuals (Wald=13.1, d.f.=5, p=0.02). Among *MAOA*-L female individuals, exposure to one childhood adversity was enough to increase the risk of depression and the risk increased gradually when more childhood adversities had been present (Fig. 1*a*). In males, this gene×environment interaction was less significant (Wald=13.4, d.f.=5, p=0.02) and there was no specific childhood-adversity group (based on the number of adversities) that displayed a different risk of depression when comparing *MAOA*-L with *MAOA*-H (Fig. 1*b*). When combining females and males (homozygosity for *MAOA*-H *vs.* *MAOA*-L), the effect of the gene×environment interaction on increasing the risk of depression was highly significant (Wald=26.8, d.f.=5, $p<0.001$).

Additionally, as the transcriptional efficiency of the longest reported u-VNTR (five repeats) has been inconsistent between *in vitro* studies (Sabol et al., 1998; Deckert et al., 1999), statistical analyses were performed grouping our sample's longest allele (5.5 repeats) with the *MAOA*-L allele. These analyses yielded similar results (data not shown).

Finally, there was no association between *MAOA* genotype and the number of childhood adversities (χ^2=0.7, d.f.=3, p=0.9 in females and males) or between *MAOA* genotype and the presence of a childhood adversity (OR=1.0, p=1.0 in females and males), suggesting that the *MAOA*-genotype×childhood-adversity

Table 5. *MAOA* upstream variable-number tandem repeat (u-VNTR) genotype distributions and associations with depression

	Genotypes				Statistics		
	H/H (*n*,%)	H/L (*n*,%)	L/L (*n*,%)	Total (*n*)	Model	OR (95% CI)	*p*
Females							
Depressed	107 (37.5%)	122 (42.8%)	56 (19.7%)	285	H/H+H/L *vs.* L/L	1.7 (1.2–2.4)	0.009**
Controls	279 (39.4%)	338 (47.7%)	91 (12.9%)	708	H/H *vs.* L/L	1.6 (1.1–2.4)	0.03*
	H (*n*,%)		L (*n*,%)	Total (*n*)	Model	OR (95% CI)	*p*
Males							
Depressed	64 (60%)		43 (40%)	107	H *vs.* L	1.0 (0.8–1.4)	1.0
Controls	347 (61.1%)		221 (38.9%)	568			

H/H, Homozygous for the longer *MAOA* u-VNTR alleles (H=4.1, 4.5 or 5.5 repeats); H/L, heterozygous for a long and the shortest *MAOA* u-VNTR alleles (H=4.1, 4.5 or 5.5 and L=3.5 repeats); L/L, homozygous for the shortest *MAOA* u-VNTR alleles (L=3.5 repeats); OR, odds ratio: ratio of depressed *vs.* controls with L/L (or L in males) relative to ratio of depressed *vs.* controls with H/H+H/L or only H/H (or H in males); 95% CI, 95% confidence interval.
* $p<0.05$, ** $p<0.01$.

interaction was not due to an increased exposure to childhood adversities in the *MAOA*-L, compared to the *MAOA*-H, group.

MAOA hypomethylation in females with depression

The DNA methylation levels of a region containing 10 CpG sites and spanning *MAOA*'s first exon/intron junction were quantified in female individuals with depression and female controls (Fig. 2*a*). Analysis of the overall methylation levels in this region (i.e. the average across CpG sites) revealed a hypomethylated pattern in depressed cases compared to controls (depressed mean=42% *vs.* controls mean=44%, Mann–Whitney $p=0.04$; Fig. 2*b*). The comparison of DNA methylation levels at individual CpG units showed that, after Bonferroni correction, the only significant reduction in depressed cases was at one unit belonging to *MAOA*'s first intron (CpG unit 8: depressed=30% *vs.* controls=34%; Fig. 2*b*). Detailed *MAOA* methylation data, for depressed and controls separately, and statistics are given in Supplementary Table S3. Linear regression analysis, with DNA methylation of CpG unit 8 as the dependent variable, was also performed to exclude confounding by the independent variables age, smoking, *MAOA* u-VNTR genotype and type of childhood adversities. The regression analysis verified the two-group comparison shown in Fig. 2*b* and showed that only depression diagnosis, and none of the other predictor variables, was associated with *MAOA* hypomethylation ($p=0.001$; Supplementary Table S4).

Early parental death is associated with NR3C1 hypermethylation

The DNA methylation levels of a region containing 47 CpG sites and spanning the *NR3C1* 1-F's promoter and exon were quantified in female individuals with depression and female controls (Fig. 3*a*). Only CpG units 10.11 and 35 were considerably methylated (median >10%); both of which are close to putative NGFI-A binding sites (Fig. 3*a*). Linear regression among all individuals showed that EPD and familial constraints were associated with an overall *NR3C1* hypermethylation [adjusted $R^2=0.101$, analysis of variance (ANOVA): $F=3.80$, $p=0.001$; Supplementary Table S5*a*]. Linear regressions were also performed for each one of the two *NR3C1* CpG units with the highest methylation (10.11 and 35) and showed that among the two significant predictors of overall methylation, only EPD was significantly associated with hypermethylation of only one CpG unit (CpG no. 35, $p=0.005$; Supplementary Table S5*a*). To confirm these results, non-parametric two-group comparisons between EPD and non-EPD individuals were also performed. In accord with the regression models, these comparisons (shown in Fig. 3*b*) also suggested that EPD was significantly associated with hypermethylation only at CpG 35 (EPD: 19.1% *vs.* non-EPD: 13.7%; Mann–Whitney $p=0.005$), even if there was still

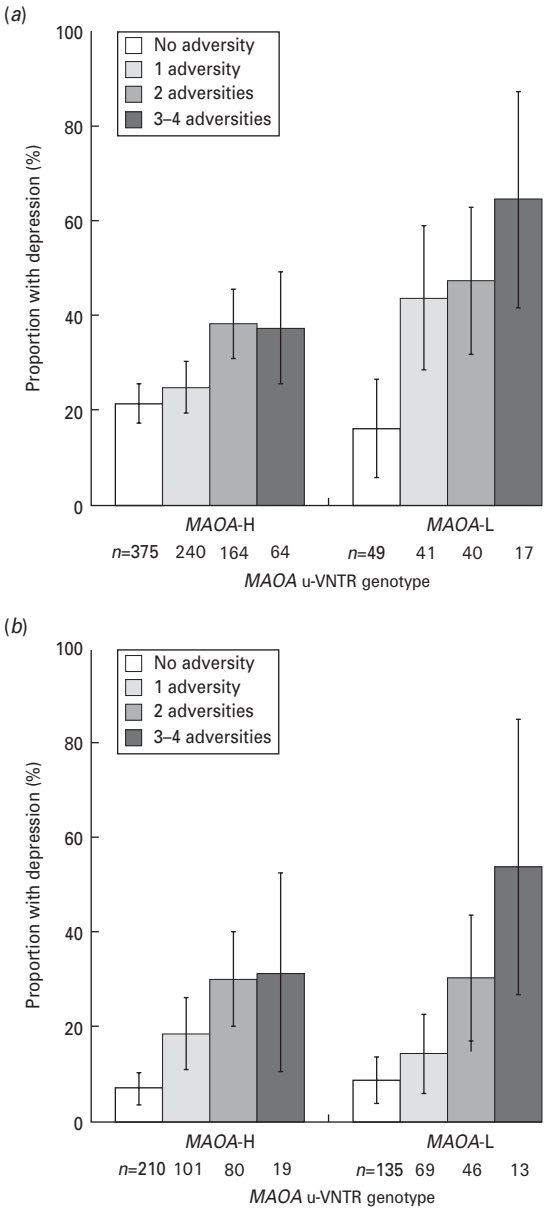

Fig. 1. (*a*) The proportion of depressed female individuals based on the number of childhood adversities and the *MAOA* upstream variable-number tandem repeat (u-VNTR) genotype. *MAOA* short-allele (*MAOA*-L) females with one childhood adversity and with three or four childhood adversities had a significantly higher risk of depression compared to *MAOA* long-allele (*MAOA*-H) female individuals with equal number of adversities. No such genotypic difference was found for those with no childhood adversity. Exposure to one childhood adversity was enough to significantly increase the risk of depression among *MAOA*-L females only. Odds ratios (with 95% confidence interval) for depression among females with childhood adversity/adversities *vs.* no adversity were: one adversity, *MAOA*-H 1.2 (0.83–1.8), *MAOA*-L 4.1 (1.6–10.3); two adversities, *MAOA*-H 2.3 (0.97–2.2), *MAOA*-L 4.6 (1.8–12.0); three or four adversities, *MAOA*-H 2.2 (1.2–3.8), *MAOA*-L 9.4 (3.0–30.0). (*b*) The proportion of depressed male individuals based on the number of childhood adversities and the *MAOA* u-VNTR genotype. There was no specific childhood-adversity group (based on the number of adversities) that had a different risk of depression when comparing *MAOA*-L with *MAOA*-H male individuals. Bars indicate proportion of depressed individuals. Error bars indicate 95% confidence intervals.

a tendency for hypermethylation overall (EPD: 7.4% *vs.* non-EPD: 6.2%; Mann–Whitney $p=0.073$) and at CpG 10.11 (EPD: 51.1% *vs.* non-EPD: 43.3%; Mann–Whitney $p=0.079$). Interestingly, CpG 35 belongs to the site that is closest to a canonical NGFI-A binding regions (McGowan et al., 2009). Supplementary Table S6 gives the detailed *NR3C1* methylation data, together with the non-parametric statistics for individuals with *vs.* without EPD.

Next, as the *MAOA* u-VNTR was shown to interact with childhood adversities and as MAOA determines the availability of serotonin which in its turn has been linked to the expression of *NR3C1* (Hackman et al., 2010), we investigated whether *MAOA*-L may also mediate the association between EPD and methylation of *NR3C1*. Linear regression analyses supported this hypothesis by generating a model in which EPD was associated with hypermethylation of *NR3C1* only among *MAOA*-L, and not *MAOA*-H, individuals (overall *NR3C1* methylation: adjusted $R^2=0.235$, ANOVA: $F=23.484$, $p<0.001$; Supplementary Tables S5*b* and S5*c*). Figure 3*c* depicts this finding for *NR3C1*'s CpG 35, and shows how only *MAOA*-L individuals with EPD seem to possess increased *NR3C1* methylated levels (*MAOA*-L with EPD, 24%; *MAOA*-L without EPD, 14%; *MAOA*-H with EPD, 15%; *MAOA*-H without EPD, 14%; Kruskal–Wallis $p=0.025$).

Finally, as EPD was the only variable significantly associated with *NR3C1* hypermethylation, we also examined whether this specific type of adversity is associated with depression in the gene × environment interaction model reported earlier in this study. Indeed, there was a weak tendency for increased frequency of depression among *MAOA*-L (L/L) females with EPD compared to *MAOA*-H (H/H+H/L) females with EPD (OR=2.9; 95% CI 0.8–10.6). This tendency was also present when only homozygous *MAOA*-H (H/H) females were studied (OR=2.6; 95% CI 0.6–10.9). The lack of statistical significance in these analyses might have been due to the low number of females with EPD ($n=52$; Table 1).

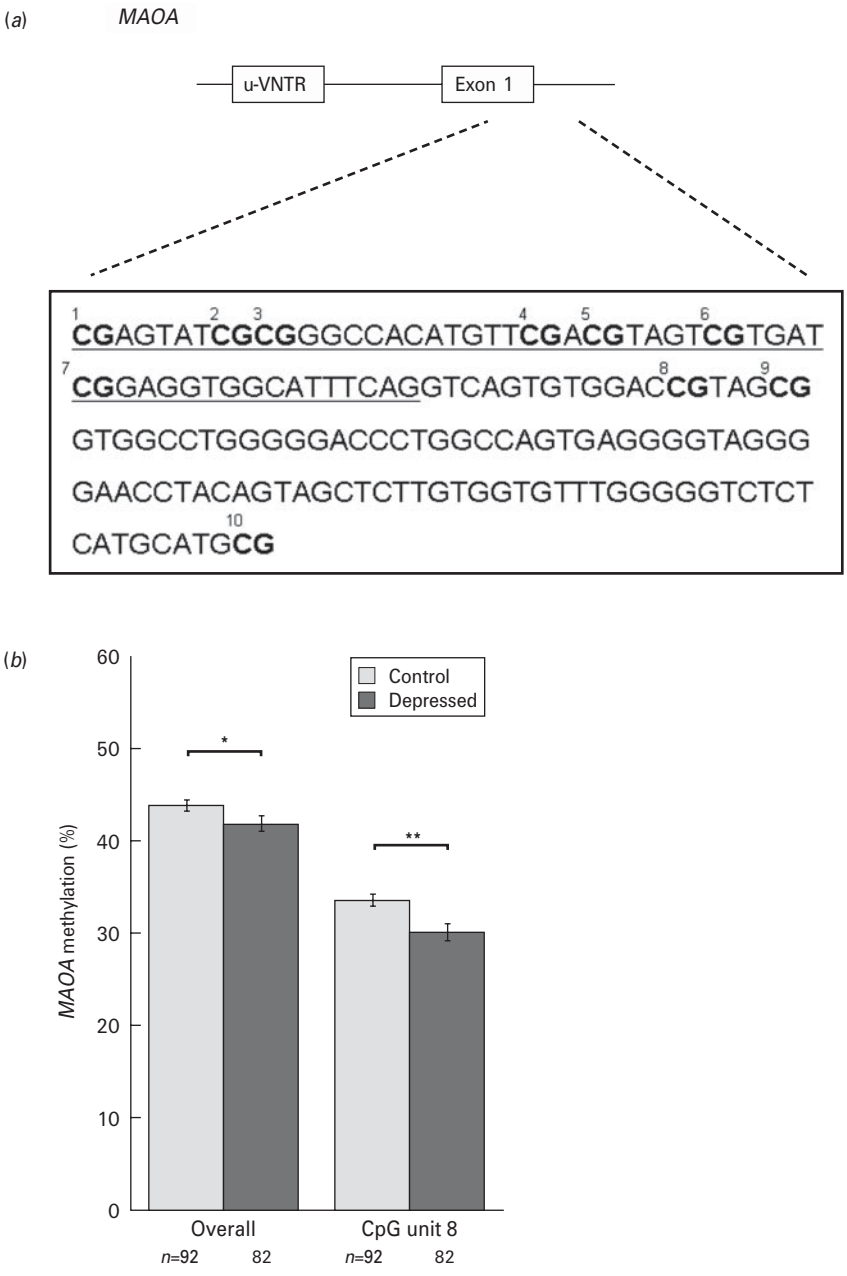

Fig. 2. (*a*) The *MAOA* sequence showing the CpG sites whose DNA methylation levels were quantified in the present study. The region's position in relation to *MAOA*'s upstream variable-number tandem repeat (u-VNTR) is also shown. The underlined sequence belongs to the downstream part of *MAOA*'s first exon and the non-underlined sequence belongs to the upstream part of the first intron. (*b*) The overall DNA methylation levels of the analysed *MAOA*-region were decreased in depressed cases compared to controls ($p=0.04$). Comparison analyses of individual CpG residues showed that the most pronounced hypomethylation in depressed cases was present at CpG unit 8 ($p=0.001$). Bars indicate mean DNA methylation. Vertical error-bars represent ±1 S.E.M. * $p<0.05$, ** $p<0.01$.

Discussion

Victims of early-life maltreatment are known to be at an increased risk for mood, anxiety and substance-use disorders (Scott et al., 2010). Genotypic variations leading to altered monoamine metabolism have been suggested to predispose to psychopathology by enhancing the effects of adverse childhood experiences (Buckholtz and Meyer-Lindenberg, 2008). Additionally, adverse environmental influences during

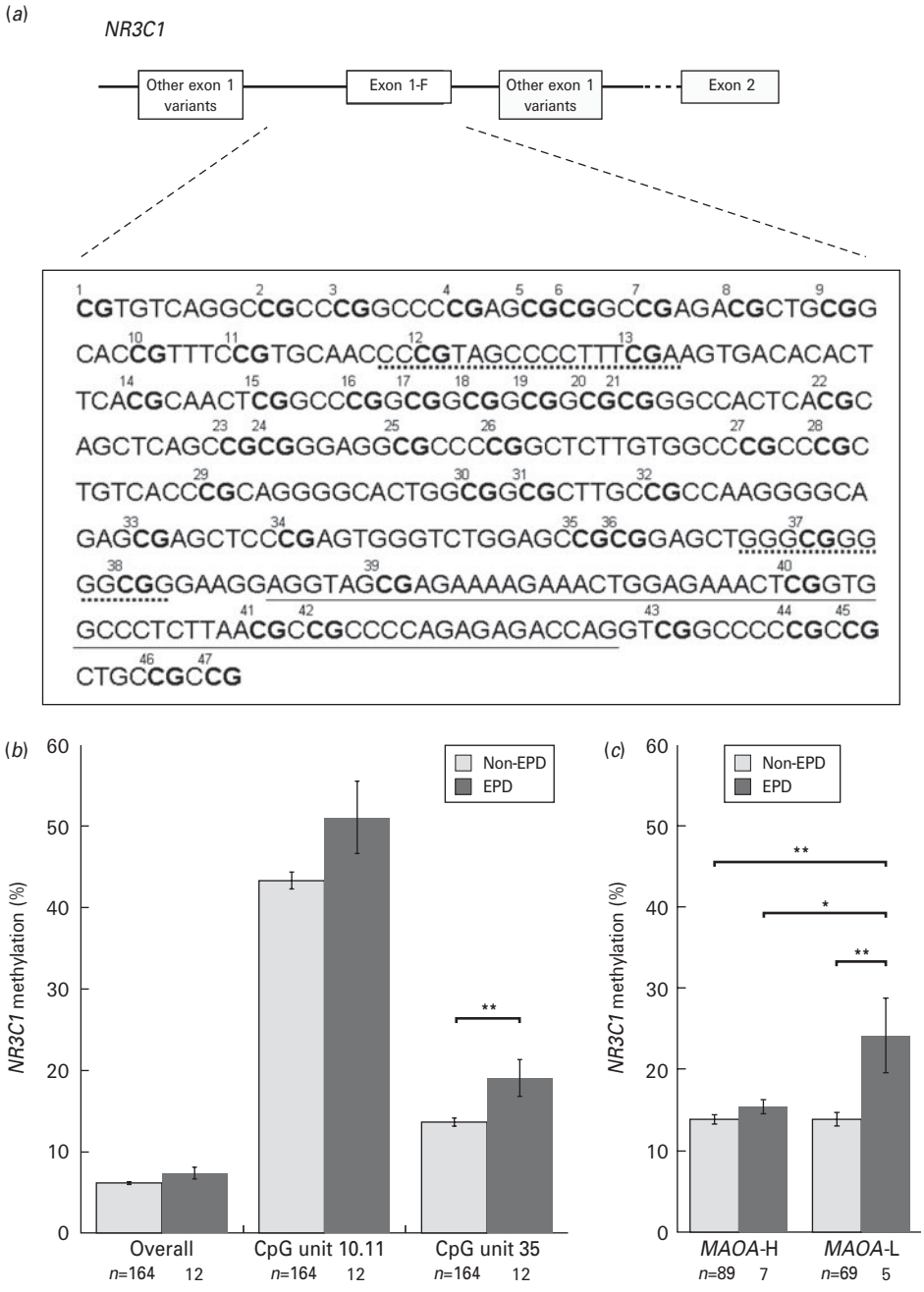

Fig. 3. (*a*) The *NR3C1* sequence showing the CpG sites whose DNA methylation levels were quantified in the present study. The region's position in relation to the other exon 1 variants of *NR3C1* is also shown. The first 38 CpG sites correspond to the CpGs analysed in the McGowan et al. (2009) study. The solid underlined sequence corresponds to the *NR3C1* 1-F exon. The broken underlined sequences correspond to nerve growth factor-induced protein A (NGFI-A) binding sites, as reported by McGowan et al. (2009). (*b*) The non-parametric two-group comparisons between individuals with or without early parental death (EPD) showed that EPD was significantly associated with hypermethylation only at CpG 35 ($p=0.005$), which is the residue closest to a canonical NGFI-A binding region according to McGowan et al. (2009). In addition, individuals with EPD showed a tendency for hypermethylation overall ($p=0.07$) and at CpG 10.11 ($p=0.08$). (*c*) A representative finding (for CpG 35) of the regression analyses that supported a putative function of *MAOA* short-allele (*MAOA*-L) as a mediator of the association between EPD and *NR3C1* methylation. This preliminary finding suggests that only *MAOA*-L individuals may be sensitive to the effects of EPD on *NR3C1* methylation. (Kruskal–Wallis $p=0.025$. Non-parametric two-group comparisons: *MAOA*-L EPD *vs.* *MAOA*-L Non-EPD: $p=0.004$, *MAOA*-L EPD *vs.* *MAOA*-H Non-EPD: $p=0.005$, *MAOA*-L EPD *vs.* *MAOA*-H EPD: $p=0.048$). Bars indicate mean DNA methylation. Vertical error-bars represent ±1 S.E.M. * $p<0.05$, ** $p<0.01$.

childhood have the capacity to alter the neuronal epigenome and give rise to a dysregulated stress–response system (Meaney, 2010). Despite the general consensus around these notions, little is known about: (1) how the *MAOA* u-VNTR may interact with childhood adversities to increase the risk of developing depression; (2) whether *MAOA* methylation signatures are different between depressed and control subjects; (3) whether different childhood-adversity subtypes have a differential impact on *NR3C1* methylation; (4) how allelic variants of one gene (in this case of *MAOA*) may affect the epigenetic status of a different locus (in this case of *NR3C1*) by modulating the effects of various types of early-life trauma.

Increased risk of depression in MAOA-L females with childhood adversities

The *MAOA*-L allele has low transcriptional efficiency *in vitro* (Sabol et al., 1998; Deckert et al., 1999; Denney et al., 1999) and has been consistently associated with conduct (e.g. aggressive and antisocial) behaviour in males exposed to childhood maltreatment (Caspi et al., 2002; Foley et al., 2004; Kim-Cohen et al., 2006; Nilsson et al., 2006; Widom and Brzustowicz, 2006; Frazzetto et al., 2007). However, to date, only one study has reported increased risk of depressive symptoms in *MAOA*-L individuals with childhood maltreatment (Cicchetti et al., 2007). Given that *MAOA* gene×environment interactions have not been extensively studied in depression, in combination with the fact that certain antidepressants exert their function by acting on this monoamine system, makes this type of investigations highly relevant.

In accord with these findings, and by using a much larger study group, we were able to show that *MAOA*-L females with childhood adversities had a higher risk of developing depression compared to those with the *MAOA*-H genotype. A trend for such an interaction was also present in males but did not reach an equally strong significance. A lack of association in males might either be due to an underpowered statistical analysis or may indicate that *MAOA*-deficiency within this gender is psychosomatically exhibited as aggression and antisocial behaviour (parameters which could not be studied with the data/instruments available in this study) rather than depression. Since *MAOA* is an X-linked gene which is regulated by sex hormones (e.g. oestrogen and androgen (Gundlah et al., 2002; Smith et al., 2004; Ou et al., 2006)), gender differences are highly likely both with regard to biochemical and behavioural phenotypes. However, what may seem counterintuitive based on the antidepressant action of MAOIs is that *MAOA*-L (which presumably leads to reduced MAOA levels *in vivo*; something also attained by MAOIs) is associated with psychopathology. A possible explanation for this is that *MAOA*-L carriers probably have increased levels of neurotransmitters during a critical period of brain development in early-life, which may then amplify the effects of childhood adversities (Buckholtz and Meyer-Lindenberg, 2008). Conversely, higher-than-normal MAOA levels later in life (i.e. higher than the levels achieved by the *MAOA*-H allele) may lead to a decrease in important neurotransmitters that are essential for maintaining mental health. Such increases in MAOA levels during adulthood could arise through epigenetic mechanisms, as discussed in the following section.

MAOA hypomethylation in depression

DNA methylation in a gene's promoter and first exon is inversely associated with transcriptional activity (Brenet et al., 2011). DNA methylation has also been shown to regulate *MAOA* specifically in females (Pinsonneault et al., 2006). In the present report, by analysing the DNA methylation levels of *MAOA*'s first exon-intron region, we were able to show that depressed female individuals were hypomethylated compared to controls. As DNA methylation of *MAOA* has been shown to be influenced by factors such as smoking and age (Philibert et al., 2010; Wong et al., 2010), we checked that these parameters did not significantly confound our findings. The *MAOA* hypomethylation observed in depressed adult females would theoretically lead to an abundance of MAOA that metabolizes its target neurotransmitters (e.g. dopamine, serotonin and norepinephrine) at a higher rate. This is in line with the mechanism by which MAOIs exert their therapeutic efficacy; in this case MAOIs would theoretically inhibit the 'excess' of MAOA that results from hypomethylation. However, MAOIs belong to antidepressants with more side-effects than newer classes of antidepressants (Mayo Clinic, 2010). Thus, examining the *MAOA* methylome may putatively serve as a biomarker in the future for identifying the group of patients which would benefit most from MAOI treatment. In line with our results, a recent study showed hypomethylation of *MAOA* in females with panic disorder (Domschke et al., 2012); a disorder that is highly co-morbid with depression (Gorman and Coplan, 1996). Nonetheless, it should be noted that the differences in percent methylation between depressed and controls were small and these types of small differences may also be a secondary effect of altered transcription

that is not likely to be a primary determinant of the level of *MAOA* expression.

Common link between the MAOA u-VNTR, parental loss and NR3C1 methylation

The *NR3C1* 1-F promoter is highly expressed in both the human hippocampus and the immune system (Turner and Muller, 2005) and this region's hypermethylation has been associated with childhood abuse in the form of sexual and/or physical abuse or severe neglect (McGowan et al., 2009), and also with parental loss in the form of death and/or prolonged desertion (Tyrka et al., 2012). In the present report we analysed the same region as in the study by McGowan and colleagues and found an association of EPD with hypermethylation of CpGs located near NGFI-A binding sites. In addition, when taking into account the *MAOA* u-VNTR genotype, we found that this association was significant in *MAOA*-L individuals only. To our knowledge, this is the first study to show both that EPD is associated with *NR3C1* hypermethylation and also that baseline *MAOA*-genotypic variations may affect the extent of *NR3C1*'s epigenetic programming as a result of this traumatic experience. However, due to the limited number of individuals within the EPD-groups (*MAOA*-L with EPD, $n=5$; *MAOA*-H with EPD, $n=7$) the *MAOA*-uVNTR-mediatory analyses warrant replication in larger cohorts and should be regarded as exploratory. If replicated, the mechanism for *MAOA* u-VNTR moderating the methylation of the *NR3C1* gene needs also to be elucidated. Nonetheless, the association of EPD only (and not of the other adversities; parental divorce, financial problems or familial constraints) gives support to the recent recommendation of not treating childhood maltreatment as a unitary construct when performing neurobiological investigations, but trying to distinguish and study each adversity subtype independently (Fisher and Pfeifer, 2011). Also, recall bias in this study should be low since EPD is a well-defined objective incident in a person's life. These results indicate the possibility of using the *NR3C1* methylation status as a childhood-adversity biomarker with a possible clinical significance. In particular, people suffering from depression and with a history of early traumatic experience are suggested to benefit more from a combinatorial treatment of psychotherapy and pharmacotherapy (Heim et al., 2010). Based on recent literature from rodents, one should however expect that epigenetic responses to early-life experiences will not only be confined to the *NR3C1* region but will probably involve regulatory regions of other genes (e.g. *Pcdh* and *Il-10*) also (McGowan et al., 2011; Schwarz et al., 2011). Finally, as parental loss during childhood (in the form of e.g. parental death or divorce) is strongly linked to an increased risk of depression in adulthood (Tennant et al., 1981, 1982; Tyrka et al., 2008; Coffino, 2009), future studies utilizing larger cohorts should also expect to find an association between depression and *NR3C1* hypermethylation.

Limitations

The present study has some main limitations. (1) In most analyses, heterozygous *MAOA*-H/*MAOA*-L female individuals were grouped together with homozygous *MAOA*-H subjects according to Doornbos *et al.* (2009). Convincingly, however, our genetic statistical analyses were verified by testing the alternative model; i.e. comparing homozygous *MAOA*-H with homozygous *MAOA*-L females. (2) When interpreting the *MAOA*-L association with depression, one should acknowledge the fact that this allele has been consistently associated with decreased gene expression levels *in vitro* only. There is however a recent study reporting that *MAOA*-L patients actually have higher-than-controls dopamine metabolite concentrations (Aklillu et al., 2009), suggesting that further *in vivo* investigations are needed to address this genotype/protein-levels relationship. (3) Self-reported childhood adversities other than EPD may have been confounded by the mental state of the responders. However, a comparative analysis between the two waves of the PART study (from which this population is derived) showed that there was not a bias towards reporting family problems during childhood when depressed (Y. Forsell and I. Lundberg, unpublished observations). (4) The number of individuals in the genotype × childhood-adversity interaction analysis was limited. (5) PART has not been tested for population stratification. However, it is known that only 11% of the PART individuals have a non-Swedish origin. Of those, the vast majority has a Nordic origin that is primarily Finnish (Hällström et al., 2003). The distribution of the *MAOA* u-VNTR is similar between Sweden and Finland (Saito et al., 2002). Also, even if Sweden is not entirely homogeneous, the current Swedish population has no strong internal genetic borders (Lappalainen et al., 2009) and especially the southern/middle parts of Sweden (from where the participants of this study are derived) are more genetically homogeneous (Humphreys et al., 2011). (6) In the *NR3C1* methylation analyses, due to the random process of selecting subjects with childhood traumas

(individuals were chosen based on the presence, and not on the specific type, of an adversity), the EPD-groups ended up being small in size. Replication of our findings in larger cohorts is thus warranted to confirm present associations. (7) In the DNA methylation analyses we only studied individuals of female gender and sex differences should therefore be addressed in the future. (8) DNA methylation is tissue specific and, inevitably, the most appropriate tissue for methylation studies in psychiatric disorders is the (inaccessible) brain. In this paper, DNA from whole-saliva samples was used for the epigenetic studies. The majority of DNA extracted from whole saliva has been shown to originate from blood leukocytes (Endler et al., 1999; Thiede et al., 2000) and previous studies on *NR3C1* methylation have generated similar results by utilizing DNA from brain (Weaver et al., 2004; McGowan et al., 2009, 2011) and leukocytes (Oberlander et al., 2008). These data indicate that adversities during a person's childhood may both be epigenetically reflected in peripheral tissues (like leukocytes) and are also stable enough to be measured much later in life. In support of this argument, a previous longitudinal study (using buccal epithelial cells) was able to demonstrate a correlation between parental stress during a child's first years and DNA methylation measured more than a decade later (Essex et al., 2013). (9) It was recently shown that methylated cytosine can be enzymatically converted to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine and 5-carboxylcytosine (Tahiliani et al., 2009; He et al., 2011; Ito et al., 2011). Interestingly, 5hmC has been found to be particularly abundant in the brain (Li and Liu, 2011) where it appears to mediate DNA demethylation (Guo et al., 2011). Since bisulfite-based methylation assays cannot distinguish between different forms of methylated CpG residues (Huang et al., 2010), it is also important to employ newly developed techniques to distinguish and investigate the contribution of 5hmC to the function of genes previously associated with DNA methylation changes.

Conclusion

Depression occurs more commonly in females than in males (Bromet et al., 2011) and the present study offers some new insights into the nature of potential underlying risk factors that are present in this group. First, depression may be the result of an interaction between an X-linked genotype (homozygosity for *MAOA*-L) and the number of adversities experienced during childhood. Additionally, depression may arise when *MAOA* is subjected to a dysregulated DNA methylation programming. The DNA methylation status of *NR3C1* has been recognized as a neurobiological hallmark of early-life trauma and this study showed both that individuals who lost a parent early in life had increased DNA methylation levels of *NR3C1* and also that *MAOA*-genotypic variants may mediate *NR3C1*'s methylation. Finally, this study shows the promise of using peripheral tissues (in this case saliva) for examining epigenetic marks. In the future, these marks could putatively serve as biomarkers of childhood traumas and dictate the most appropriate treatment interventions for individuals suffering from depression.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713000102.

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Statement of Interest

None.

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