Research report

Strong effects of environmental factors on prevalence and course of major depressive disorder are not moderated by 5-HTTLPR polymorphisms in a large Dutch sample

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ARTICLE INFO

ABSTRACT

Background: There is ongoing interest in the possible interaction of the serotonin-transporter-linked polymorphic region (5-HTTLPR) with environmental factors in determining Major Depressive Disorder (MDD). The current study contributes to this research area by comprehensively examining the interaction-effects and direct-effects of 5-HTTLPR and five environmental factors on MDD prevalence and course in a well-characterized longitudinal sample.
Methods: The sample consisted of 1625 patients with a CIDI-confirmed diagnosis of MDD and 1698 screened controls from the Netherlands. Four MDD outcomes were studied as dependent variables: one main MDD prevalence-outcome (all MDD), two more severe MDD prevalence-outcomes (suicidal and chronic MDD), and one MDD course outcome (chronic versus non-chronic MDD). Because SNP rs25531 modifies the effect of 5-HTTLPR, haplotypes of 5-HTTLPR and rs25531 were measured. For the four MDD outcome measures, we examined the direct effects of 5-HTTLPR/rs25531-haplotypes, five environmental factors (lifetime and recent stressful life-events, sexual abuse, low educational attainment, and childhood trauma) and their interaction in logistic regression models.
Results: The environmental factors had large and consistent effects on all four MDD outcomes, including course of MDD. The 5-HTTLPR/rs25531-haplotype had a suggestive effect on course of MDD, but not on presence of MDD. Gene-by-environment interaction was significant (< 0.05) for one of the 20 tests performed, which is not more than expected by chance.
Limitations: Environmental factors were not assessed before the onset of MDD.
Conclusions: Environmental factors had a strong impact on the presence and course of MDD, but no evidence for gene-by-environment interaction was found.

1. Introduction

Since the first findings of Caspi and colleagues in 2003 (Caspi et al., 2003), there has been ongoing interest in a possible interaction between the serotonin-transporter-linked polymorphic region (5-HTTLPR), which contains a repeat length polymorphism, and environmental factors in Major Depressive Disorder (MDD). Caspi et al. showed that individuals with at least one short allele of 5-HTTLPR experienced more depressive symptoms, diagnosable depression and suicidality following a stressful life-event. Many studies aimed to replicate these findings with contradictory results, and two meta-analyses published in 2009 by Munafò et al. (2009) and Risch et al. (2009) combining data of 5 and respectively 14 studies showed no evidence for this gene-by-environment interaction. However, an ensuing meta-analysis in 2011 by Karg and colleagues (Karg et al., 2011) used a different

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definition of stressful life events and a meta-analysis method that allowed inclusion of more studies (56 studies containing a total of 40,749 subjects). This meta-analysis supported Caspi's finding.

A major cause proposed for the conflicting results in studies on gene-by-environment interaction involving 5-HTTLPR lays in the different nature and measurement of the environmental factors that are considered (Duncan and Keller, 2011). Studies are not always comparable, because environmental factor-measurements differ both in content and in timing to the onset of MDD. For example, Karg et al. (2011) studied several environmental factors in their meta-analysis and found stronger gene-by-environment interaction for childhood trauma than for stressful life-events. Low educational attainment is another environmental factor that is strongly associated with MDD (De Graaf et al., 2011), but also to socioeconomic status (Bjelland et al., 2008), for which some studies found interaction effects involving 5-HTTLPR (Brummett et al., 2008; Uddin et al., 2010) and some did not (Uddin et al., 2011).

Another possible explanation for the inconsistent findings in studies on gene-by-environment interaction involves differences in the depression measures used. Although some studies used DSM- based diagnosis of MDD, others employed continuous scales of self-reported (often milder) depression symptoms. In the meta-analysis of Karg et al. (2011), studies with self-reported depression showed less evidence for gene-by-environment interaction than studies with interview assessed depression. Possibly, this was because self-reported depression measures are often state-measures neglecting remitted depression symptoms, whereas DSM-based depression measures might mark the more severe and clinically relevant depressed patients.

A final explanation for the inconsistent findings in studies on gene-by-environment interaction with 5-HTTLPR may be the measurement of the functional variants in the 5-HTTLPR. Functional characterization of the 5-HTTLPR has evolved and it has been shown that long alleles of 5-HTTLPR that form a haplotype with the G allele of rs25531 are functionally equivalent to short alleles, which are less expressed. (Hu et al., 2006; Wendland et al., 2006). Risch et al. and Karg et al. made no mention of rs25531 in their meta-analysis and few studies took rs25531 into account. However, 5-HTTLPR-L/rs25531-G haplotypes have a frequency of 6.5% (Wendland et al., 2006) and, therefore, it seems crucial to take rs25531 into account when aiming to study the functional length of 5-HTTLPR.

The present study contributes to the ongoing debate by comprehensively examining five environmental factors (lifetime and recent stressful life-events, sexual abuse, educational attainment and childhood trauma) and their interaction-effects with the functional length of 5-HTTLPR in a large and well-characterized study from the Netherlands. We studied patients with an interview-assessed and DSM-IV based diagnosis of MDD compared to carefully screened controls and, in addition to two more severe prevalence-outcomes (chronic MDD and suicidal MDD) to further increase the contrast between patients and controls. Since the 5-HTTLPR polymorphism has not only been linked to the onset of depression, but also to its chronicity (Zannas et al., 2012), we additionally examined gene-by-environment interaction in the course of MDD.

2. Methods

2.1. Subjects

Data from 1727 unrelated MDD patients and 1792 healthy controls from the Netherlands Study of Depression and Anxiety (NESDA) and the Netherlands Twin Registry (NTR) were analyzed. The NESDA study (Penninx et al., 2008) is an ongoing longitudinal cohort study of MDD and anxiety disorders and its subjects were recruited from mental health care settings, general practitioners, and the general population in the period from 2004 to 2007. The NTR study (Boomsma et al., 2006) has been collecting data on Dutch twin families since 1991 and comprises data on nearly 22,000 subjects who have been assessed longitudinally for depressive symptoms (multiple instruments), anxiety and neuroticism. Subjects from the NTR were included in this study based on longitudinal data up to 2005. Both studies (NESDA and NTR) were approved by the institutional Review Board and all their participants provided written informed consent. Most patients were from NESDA (1598 versus 129 from NTR) and patients were included in the current study when they were between 18 and 77 years of age and had a lifetime DSM-IV diagnosis of MDD. MDD diagnosis was assessed by specially trained clinical staff in a face-to-face interview using the Composite International Diagnostic Interview (CIDI, version 2.1). Persons who were not fluent in Dutch and those with a primary diagnosis of a psychotic disorder, obsessive compulsive disorder, bipolar disorder, or severe substance use dependence were excluded at NESDA study baseline. Most controls were from the NTR (1640 versus 152 from NESDA) and were included when they had no lifetime diagnosis of MDD, did not take any medication that may have been prescribed to treat MDD, had no known first-degree relatives with MDD and a low factor score based on a multivariate analysis of depressive complaints, anxiety, neuroticism, and somatic anxiety (Boomsma et al., 2000). The 152 controls from NESDA had no lifetime diagnosis of MDD or anxiety disorder, as assessed by CIDI, no other major psychiatric disorder and scored low (<4) on the Inventory of Depressive Symptoms scale (Rush et al., 1996). MDD patients and controls that were included had North-European ancestry, were matched for age and gender and were unrelated.

2.2. Assessment of environmental factors

2.2.1. Lifetime and recent stressful life-events

The number of various stressful life-events encountered in lifetime and those encountered in past year (recent stressful life-events) were assessed rather comparably in both studies. In NESDA, stressful life-events were assessed with the Brugha List of Threatening Experiences (Brugha et al., 1985). This assessment took place at the same day as the CIDI interview. Questionnaires from the NTR (Middeldorp et al., 2005; Middeldorp et al., 2010) were matched resulting in the following six stressful life-events encountered during lifetime up to assessment (combined prevalence in patients and controls between brackets): severe disease or victim of physical violence of self (35.2%); severe disease or victim of physical violence of close relative (68.9%); death of close relative (85.7%); forced dismissal from job (20.7%); ending of enduring intimate relationship (39.9%); and being robbed (33.8%). The number of different life-events encountered in this study ranged from 0 to 6.

2.2.2. Sexual abuse

The occurrence of lifetime sexual abuse was assessed in a slightly different way across studies. NESDA-subjects were asked if they were ever touched or forced to touch someone in a sexual way against their will. NTR-subjects were asked if they had ever been victim of a sexual misdeed, which was specified as being raped or assaulted. Because different wording across studies could have resulted in different prevalences, cohort-status (NESDA or NTR) was added as an additional covariate to all analyses focusing on sexual abuse. Although results in cohort-status unadjusted and cohort-status adjusted analyses differed slightly in estimated effect sizes, overall conclusions on the

importance of sexual abuse and their interaction with 5HTTLR-gene were very comparable in unadjusted and adjusted analyses.

2.2.3. Educational attainment

Educational attainment was defined as the years required to obtain the highest diploma attained.

2.2.4. Childhood trauma

Childhood trauma was measured in NESDA with the instrument of the Netherlands Mental health Survey and Incidence Study (De Graaf et al., 2002). Subjects were asked for emotional neglect, psychological abuse, physical abuse and sexual abuse.The definition of emotional neglect included lack of parental attention or support and ignorance of one's problems and experiences. Psychological abuse was defined as being verbally abused, undeserved punishment, subordinated to siblings and being blackmailed. Physical abuse was defined as being kicked or hit with hands or an object, beaten up or physical abuse in any other way. Sexual abuse was defined as being sexually approached against your will, meaning being touched or having to touch someone in a sexual way. Participants answered 'yes' or 'no' to each of the four forms of childhood trauma and were asked to give an indication about the frequency on a five-point scale, '1' once, '2' sometimes, '3' regular, '4' often and '5' very often. In the analyses, the frequencies were categorized into three groups (0: absent, 1: once or sometimes, 2: regular, often and very often). The number of different traumas encountered were combined with their frequencies, resulting in a sum score ranging from 0 to 8, as has been defined before (Hovens et al., 2010; Hovens et al., 2012; Wiersma et al., 2009).

2.3. MDD outcomes

Three MDD prevalence outcomes and one MDD course outcome were examined as dependent variables. The first MDD prevalence-outcome compared all MDD patients (defined by a lifetime DSM-IV based diagnosis) to healthy controls. Two additional MDD prevalence-outcomes were examined to further increase the contrast between patients and controls and compared suicidal MDD patients to healthy controls and chronic MDD patients to healthy controls. Suicidal MDD was defined as having ever attempted to commit suicide as assessed in the CIDI interview. Chronic MDD was defined as having a MDD diagnosis and symptom duration of more than two years. Symptom duration was obtained for NESDA only using baseline and 2-year follow-up data of those individuals with an MDD diagnosis one year prior to baseline ($n=997$). Symptom duration was assessed using the Life Chart Interview (LCI), which uses a calendar approach to assess the percentage of time that symptoms were present during the four years prior to and the two years following baseline. Computations with LCI data were described in more detail by Penninx et al. (2011). In the analyses on recent stressful life-events MDD patients with a past year diagnosis of MDD were included only, changing the main MDD and suicidal MDD measure, but not the chronic MDD measure, as chronicity could only be assessed for subjects with past year MDD.

In addition to comparing patients to healthy controls, chronic MDD patients were also compared to non-chronic MDD patients. This case-only analysis yielded the opportunity to examine gene-by-environment interaction on the course of MDD.

2.4. 5-HTTLPR and rs25531

Sample collection procedures and DNA isolation were harmonized between NESDA and NTR as previously described (Boomsma et al., 2008). The 5-HTTLPR/rs25531 haplotypes were assessed by the PCR protocol described by Wendland et al. (2006) at the Karolinska Institute in Stockholm (Sweden). In short, genomic DNA amounting to at least 10 ng was used for PCR amplification of the long (L) and short (S) promoter repeats (forward primer: 5′–TCCTCCGCTTTGGCGCCTCTTCC–3′, reverse primer: 5′–TGGGGGTTGCAGGGGAGATCCTG–3′). Half of the reaction product was digested with HpaII FastDigest (FD0514, Fermentas) at 37 °C for 10 min whereas half was left undigested. HpaII digests amplicons carrying the rs25531-G genotype, but leaves rs25531-A undigested. The digested and undigested amplicons were separated on a 3% Ultrapure agarose (Invitrogen) gel at 160 V for approximately 1 h. Due to the methylation sensitivity of HpaII, 160 samples were also digested using the methylation-insensitive MspI FastDigest (FD0544, Fermentas) and no discrepancies regarding digestion and interpretation of genotypes were discovered. Based on the length difference between the S and L amplicons, and the resulting digested amplicons caused by the presence of rs25531-G, the 5-HTTPR/rs25531 haplotypes could be resolved.

Quality control of genotypes was performed with additionally genotyped trios (30 trios) and duplicates (18 duplicates). There were no Mendelian errors or mismatches of duplicates. In addition, the 5-HTTLPR/rs25531 haplotypes were in Hardy Weinberg Equilibrium ($p=0.9$). The 5-HTTLPR/rs25531 haplotypes were used to define the functionality of the length polymorphism, with the 5-HTTLPR-long/rs25531-G haplotypes classified as short 5-HTTLPR alleles. The number of functional short alleles (0, 1 or 2) from all unrelated subjects were used to test for direct gene effects, and gene-by-environment interaction on the presence and course of MDD.

2.5. Statistical analyses

MDD patients were compared to healthy controls with respect to age, gender, and the four environmental factors using t-test and chi-square statistics. Associations between the number of functional short 5-HTTLPR alleles and environmental factors were examined using linear regression and linear-by-linear chi-square statistics to test for gene-environment correlation as this can influence tests of gene-by-environment interaction and lead to spurious results (Purcell, 2002).

The impact of the number of functional short 5-HTTLPR alleles (0,1, or 2), environmental factors and gene-by-environment interaction was examined for the three MDD prevalence-outcomes and MDD course-outcome. First, the direct effects of the number of functional short 5-HTTLPR alleles and the direct effects of the five environmental factors were assessed and, subsequently, their interaction effects were assessed. In the analyses of interaction effects, the main effects of the number of functional short 5-HTTLPR alleles and the concerning environmental factor were included. All analyses were conducted using logistic regression with age and gender as covariates. Analyses focusing on sexual abuse additionally included the subjects cohort-status (NESDA or NTR). All together 20 interaction effects (five environmental factors times four MDD outcomes) were examined and we, therefore, had to correct for multiple comparison. However, the MDD prevalence outcomes were correlated and Bonferroni-correction would have been too stringent. Therefore, the threshold for significance was set at 0.05 with the number of tests taken into account in the interpretation of the results.

Although the interaction model (MDD outcome $= b_0 + b_1 \cdot$ 5-HTTLPR $+ b_2 \cdot E + b_3 \cdot$ 5-HTTLPR $\times E + b_4 \cdot$ gender $+ b_5 \cdot$ age) provides tests for 5-HTTLPR (b_1) and the environmental factor (b_2), these coefficients do not represent the direct effects on the MDD outcome, but rather the effect of 5-HTTLPR when $E=0$ and the

effect of E when 5-HTTLPR=0 respectively (Jaccard and Turrisi, 2003). Therefore, we examined the direct effects of 5-HTTLPR, the direct effects of the environmental factors and their interaction-effects in separate models as described above.

In addition to the straightforward test for multiplicative interaction, additional tests were performed for interaction as a departure from additivity using the procedure described by Knol et al. (2007). In this procedure we used the outcome of a logistic regression model to estimate the Relative Excess Risk due to Interaction (RERI). A RERI smaller than or bigger than zero indicates evidence for additive interaction. The 95% confidence intervals of the RERI were estimated using bootstrap simulations. In this way we tested for additive interaction for the five main environmental factors (lifetime stressful life-events, recent stressful life-events, sexual abuse, educational attainment and childhood trauma) with 5-HTTLPR for the four main outcome measures (all MDD, suicidal MDD, chronic MDD and chronic versus non-chronic MDD).

Finally, in order to compare the impact of different environmental factors on MDD prevalence and course, the variation explained by the four environmental with age and gender as covariates, as estimated by Nagelkerke pseudo R-Squares, were compared. Nagelkerke R-Squares are used in logistic regression to approximate the R-Square known from linear regression. Analyses were conducted in SPSS (SPSS inc., 2009) and R (R Development Core Team, 2010).

3. Results

3.1. Sample characteristics

A total of 1625 MDD patients and 1698 healthy controls had valid data on their number of functional short 5-HTTLPR alleles, i.e. 5-HTTLPR/rs25531 haplotypes. Data on environmental factors were missing for 319 (stressful life-events), 308 (sexual abuse), 259 subjects (educational attainment). Data on childhood trauma

were only available for NESDA participants, leaving 1502 patients and 143 controls for these analyses.

The demographics of healthy controls ($n=1698$) and MDD patients ($n=1625$) are given in Table 1. Controls were slightly older (43.9 versus 42.6 years, $p=0.006$) and less often female (61.7% versus 69.7%, $p < 0.001$). Stressful life-events, sexual abuse, and childhood trauma were significantly more frequent and educational attainment was significantly lower in MDD patients compared to healthy controls. Out of all 1625 MDD patients, 123 (7.6%) had suicidal MDD. Out of all 837 patients with a current diagnosis of MDD one year prior to NESDA baseline and sufficient LCI data, 396 (47%) fulfilled criteria for chronic MDD.

Table 2 reports the associations between environmental factors with number of functional short 5-HTTLPR alleles. The occurrence of environmental factors was not significantly associated with the number of functional short 5-HTTLPR alleles, indicating the absence of gene-environment correlation.

3.2. Effects of 5-HTTLPR, environmental factors and their interaction on MDD prevalence measures

Table 3 displays the number of patients and controls and the effect of the number of functional short 5-HTTLPR alleles on the three MDD prevalence-outcomes (MDD, suicidal MDD and chronic MDD). In none of the analyses, the number of functional short 5-HTTLPR alleles had any effect on MDD outcome measures.

Effects of environmental factors on the three MDD prevalence-outcomes were strong. The effects of the number of lifetime stressful life-events (with ORs of 2.01, 2.59 and 2.11 per stressful life event; all $p < 0.001$), recent stressful life-events (with ORs of 1.29, 1.36 and 1.29 per stressful life event; all $p < 0.001$) and childhood trauma (with ORs of 1.98, 2.47 and 2.15 per increased score on the overall index; all $p < 0.001$) were strong and rather comparable across the MDD prevalence outcomes. In contrast, high educational attainment was protective for the three MDD outcomes with ORs of 0.94, 0.84 and 0.86 respectively (all $p < 0.001$). For sexual abuse, the OR was smallest for MDD versus controls (OR=2.79), higher for chronic MDD (OR=3.17), and very high for suicidal MDD (OR=6.46) (all $p < 0.001$). When we examined the effect of sexual abuse separately within NESDA and NTR—to prevent a potential impact of measurement differences across studies—strongly significant effects of sexual abuse on the prevalence of MDD were confirmed (in NESDA: OR=2.23 with 95% CI=1.41–3.52; and in NTR: OR=4.01 with 95% CI=2.20–7.33) indicating that measurement differences are not responsible for the described association.

The impact of the environmental factors on MDD versus controls were compared to each other in two steps. First, Nagelkerke R-Squares were compared in the sample who had data on lifetime stressful life-events, sexual abuse and educational attainment available (1587 MDD patients and 1370 controls). Lifetime stressful life-events explained more variation ($R^2=0.210$) than sexual abuse ($R^2=0.158$), and both explained more variation than educational attainment ($R^2=0.026$). In addition, including all these three environmental factors in one model, all effects remained significant with $p < 0.001$, which indicates that they had independent effects on MDD. Second, to compare childhood trauma to the three other environmental factors, the Nagelkerke R-Squares were also computed on subjects with data on all four environmental factors available (NESDA only with 1501 MDD patients and 143 controls). In this comparison, childhood trauma explained more variation ($R^2=0.145$) than the other three environmental factors ($R^2=0.046$, $R^2=0.029$ and $R^2=0.054$ respectively). When all four environmental factors were examined in one model, sexual abuse had no significant effect, but the other three environmental factors did. Including sexual abuse

Table 1
Sample characteristics by MDD status.

	MDD patients N=1625	Healthy controls N=1698	p-Value[a]
Sociodemographic variables			
Age, mean years ± SD	42.62 ± 12.56	43.91 ± 14.45	0.006
Women, %	69.66	61.66	< 0.001
Environmental factors			
Lifetime stressful life-event, number ± SD	3.30 ± 1.24	2.29 ± 1.19	< 0.001
N with data available	*1593*	*1411*	
Recent stressful life-event, number ± SD	0.60 ± 0.83	0.45 ± 0.68	< 0.001
N with data available[b]	*929*	*1411*	
Lifetime sexual abuse, %	33.27	6.06	< 0.001
N with data available	*1596*	*1419*	
Educational attainment, years ± SD	11.98 ± 3.25	12.62 ± 3.30	< 0.001
N with data available	*1597*	*1467*	
Childhood trauma, number ± SD	2.00 ± 2.24	0.35 ± 1.00	< 0.001
N with data available	*1502*	*143*	
Types of MDD			
Number suicidal MDD, N (%)	123 (75.69%)		
Number chronic MDD, N (%)[c]	396 (24.37%)		

[a] *p*-values based on t-test (continuous or > 5 ordered categories) and chi-square statistics (dichotomous variables).
[b] Patients are subjects with recent MDD only.
[c] Out of 837 MDD patients with a recent MDD diagnosis and data on chronicity.

Table 2
Correlation of 5-HTTLPR* to environmental factors.

Environmental stressor		5-HTTLPR*			Beta	p-Trend
		0	1	2		
Lifetime stressful life-events	Mean value (SE)	2.82 (0.05)	2.87 (0.03)	2.72 (0.05)	−0.03	0.16
	N	731	1547	726		
Recent stressful life-events	Mean value (SE)	0.51 (0.03)	0.51 (0.02)	0.47 (0.03)	−0.02	0.29
	N	729	1544	725		
Sexual abuse	Prevalence in %	17.76	21.48	21.02		0.12
	N	732	1555	728		
Educational attainment	Mean value (SE)	12.14 (0.12)	12.32 (0.08)	12.38 (0.12)	0.03	0.16
	N	746	1575	743		
Childhood trauma	Mean value (SE)	1.66 (0.11)	1.96 (0.08)	1.83 (0.11)	0.03	0.27
	N	402	859	384		

   * p-Trends are based on linear regression (continuous or > 5 ordered categories) and linear-by-linear chi-square tests (dichotomous variables). The effect size (beta) is displayed for linear regression results.

Table 3
Effects of 5-HTTLPR, environmental factors and their interaction on MDD, suicidal MDD and chronic MDD versus healthy controls.

MDD outcome measure	N		5-HTTLPR		E		5-HTTLPR × E	
	Patient	Control	OR (95%CI)	p-Value	OR (95%CI)	p-Value	OR (95%CI)	p-Value
Lifetime stressful life-events (E)								
MDD versus healthy control	1593	1411	0.96 (0.86–1.06)	0.39	2.01 (1.88–2.16)	< 0.001	1.00 (0.91–1.10)	1.00
Suicidal MDD versus healthy control	121	1411	1.04 (0.80–1.36)	0.75	2.59 (2.20–3.07)	< 0.001	1.32 (1.03–1.69)	0.03
Chronic MDD versus healthy control	395	1411	1.03 (0.88–1.21)	0.70	2.11 (1.91–2.33)	< 0.001	0.97 (0.84–1.12)	0.63
Recent stressful life-events (E)								
MDD versus healthy control	929	1411	0.91 (0.81–1.02)	0.11	1.29 (1.15–1.44)	< 0.001	1.05 (0.89–1.23)	0.59
Suicidal MDD versus healthy control	93	1411	0.88 (0.65–1.18)	0.40	1.36 (1.03–1.77)	0.03	1.16 (0.79–1.73)	0.45
Chronic MDD versus healthy control	395	1411	1.03 (0.88–1.21)	0.70	1.29 (1.10–1.49)	< 0.001	1.06 (0.86–1.31)	0.61
Sexual abuse (E)								
MDD versus healthy control	1596	1419	0.99 (0.82–1.20)	0.94	2.79 (1.90–4.13)	< 0.001	1.61 (0.92–2.79)	0.09
Suicidal MDD versus healthy control	121	1419	1.07 (0.77–1.47)	0.70	6.46 (3.61–11.80)	< 0.001	2.04 (0.94–4.51)	0.07
Chronic MDD versus healthy control	396	1419	1.06 (0.81–1.39)	0.68	3.17 (1.96–5.28)	< 0.001	1.31 (0.67–2.61)	0.43
Educational attainment (E)								
MDD versus healthy control	1597	1467	0.96 (0.87–1.07)	0.45	0.94 (0.92–0.96)	< 0.001	1.00 (0.97–1.03)	0.80
Suicidal MDD versus healthy control	121	1467	1.05 (0.81–1.36)	0.74	0.84 (0.79–0.90)	< 0.001	1.03 (0.95–1.12)	0.50
Chronic MDD versus healthy control	396	1467	1.04 (0.89–1.22)	0.64	0.86 (0.83–0.89)	< 0.001	0.98 (0.93–1.03)	0.50
Childhood trauma (E)								
MDD versus healthy control	1502	143	0.97 (0.76–1.25)	0.83	1.98 (1.66–2.44)	< 0.001	1.03 (0.78–1.37)	0.83
Suicidal MDD versus healthy control	115	143	1.04 (0.74–1.46)	0.81	2.47 (1.98–3.23)	< 0.001	1.19 (0.85–1.68)	0.30
Chronic MDD versus healthy control	396	143	1.06 (0.81–1.39)	0.67	2.15 (1.78–2.67)	< 0.001	1.09 (0.81–1.44)	0.57

The direct effects of the number of functional short 5-HTTLPR alleles (0, 1 or 2), five various environmental factors (E) and their interaction effects (5-HTTLPR × E) on the three MDD prevalence-outcomes. For every combination of E and MDD outcome measure, the direct effect of 5-HTTLPR, direct effect of E, and their interaction effect were estimated in separate logistic regression models including age, gender and, for the analysis focusing on sexual abuse, also cohort-status. The models estimating 5-HTTLPR × E included 5-HTTLPR and E in addition. Note that for recent stressful life-events only subjects with recent MDD were included.

subsequently into a model with one of the three other environmental factors, indicated that the original impact of sexual abuse was included in the effect of childhood trauma. This seems logical, because the childhood trauma measure included sexual abuse before the age of 16, which is also included in lifetime sexual abuse variable.

Gene-by-environment interaction with the number of functional short 5-HTTLPR alleles was tested for 5 environmental factors and 3 outcomes. Of these 15 tests, one interaction-effect had a p-value smaller than 0.05, namely, the interaction-effect with lifetime stressful life-event on suicidal MDD versus healthy controls (OR=1.32, p=0.03). The direction of this interaction effect is in line with the finding of Caspi: extra copies of the short allele and a higher number of stressful life-events contributing more to the risk on MDD than their additional risk. In the tests for interaction as departure from additivity, all 95% confidence intervals included 0, indicating no evidence for additive interaction.

3.3. Effects of 5-HTTLPR, environmental factors and their interaction on the course of MDD

Within patients, impact of environmental factors on the course of MDD was evaluated (Table 4). The effect of the number of functional short 5-HTTLPR alleles on the course of MDD was 1.24

Table 4
Effects of 5-HTTLPR, environmental factors and their interaction on chronic MDD versus non-chronic MDD.

Environmental factor	N		5-HTTLPR		E		5-HTTLPR x E	
	Chronic	Non-chronic	OR (95%CI)	p-Value	OR (95%CI)	p-Value	OR (95%CI)	p-Value
Lifetime stressful life-events (E)	395	441	1.24 (1.01–1.51)	0.04	1.12 (1.00–1.26)	0.04	1.03 (0.88–1.20)	0.74
Recent stressful life-events (E)	395	441	1.24 (1.02–1.51)	0.04	1.01 (0.86–1.20)	0.87	1.04 (0.82–1.32)	0.74
Sexual abuse (E)	396	441	1.24 (1.02–1.52)	0.03	1.69 (1.25–2.29)	<0.001	1.20 (0.78–1.84)	0.41
Educational attainment (E)	396	441	1.24 (1.02–1.52)	0.03	0.91 (0.87–0.95)	<0.001	0.95 (0.89–1.02)	0.15
Childhood trauma (E)	396	440	1.25 (1.02–1.53)	0.03	1.15 (1.08–1.23)	<0.001	1.03 (0.94–1.13)	0.52

The direct effects of the number of functional short 5-HTTLPR alleles (0, 1 or 2), five various environmental factors (E) and their interaction effects (5-HTTLPR × E) on course of MDD. For every E, the direct effect of 5-HTTLPR, direct effect of E, and their interaction effect were estimated in separate logistic regression models including age, gender and, for the analysis focusing on sexual abuse, also cohort-status. The models estimating 5-HTTLPR × E included 5-HTTLPR and E in addition.

($p=0.03$) indicating that the functional short allele increases the risk of a chronic course of MDD.

The environmental factors had direct effects on the course of MDD: number of lifetime stressful life-events (OR=1.12, $p=0.04$), sexual abuse (OR=1.69, $p<0.001$) and childhood trauma (OR=1.15, $p<0.001$) increased the risk of chronic MDD versus non-chronic MDD and educational attainment was protective (OR=0.91, $p<0.001$). Only the recent stressful life-events had no significant effect on the course of MDD (OR=1.01, $p=0.87$). Most variation was explained by childhood trauma and educational attainment, with Nagelkerke R-Squares of 0.046 and 0.049 respectively, and variation explained by lifetime stressful life-events (0.022) and sexual abuse (0.033) was considerably less. When these four environmental factors were included in a model together a Nagelkerke R-Square of 0.081 was found. No significant gene-by-environment interaction-effect on the course of MDD was found for any of the five environmental factors. In the tests for interaction as departure from additivity, no evidence was found for additive interaction.

As suicidality also selects a more severe subgroup of MDD patients, suicidal MDD patients were compared to non-suicidal MDD patients in post-hoc analyses, analogous to comparing chronic versus non-chronic MDD patients. These analyses showed that all environmental factors had a significant effect on the risk of suicide attempt for MDD patients in the expected direction: lifetime life-events (OR=1.41; 95%CI 1.21–1.65; $p<0.001$), sexual abuse (OR=2.69; 95%CI 1.82–4.00; $p<0.001$) and childhood trauma (OR=1.34; 95%CI 1.24–1.45; $p<0.001$) increased the risk on suicide attempts, whereas higher educational attainment reduced this risk (OR=0.90; 95%CI 0.85–0.96; $p<0.001$). The functional length of 5-HTTLPR had no effect on the risk of suicide attempts in MDD patients. Of the four environmental factors tested, only lifetime life-events showed significant interaction with 5-HTTLPR (OR=1.41; 95%CI 1.12–1.76; $p=0.002$).

3.4. Imputation of 5-HTTLPR

We checked whether it was possible to increase sample size by imputing 5-HTTLPR using the haplotype proxy of surrounding SNPs from Vinkhuyzen et al. (2011) This haplotype proxy consists of rs2129785-T and rs11867581-A and tags the short allele of 5-HTTLPR with an r^2 of 0.775. The frequencies of 5-HTTLPR/rs2129785/rs11867581-haplotypes given by Vinkhuyzen et al. were used to simulate a reference sample of 2823 subjects (the number of subjects in the discovery set of Vinkhuyzen et al.). With this simulated reference sample and the available SNP data, Beagle-software (Browning and Browning, 2007) was used to impute 5-HTTLPR. 5-HTTLPR was imputed with an r^2 of 0.72 and comparison of imputed to genotyped values of 5-HTTLPR showed a rather low accuracy of 87%. This limited accuracy does not justify imputation of 5-HTTLPR on an extended sample of

NESDA and NTR. Moreover, Vinkhuyzen et al. found that rs25531 could not be tagged by SNPs giving rise to an even greater inaccuracy of imputation with respect to the functional length of 5-HTTLPR.

4. Discussion

Since first reported by Caspi and colleagues in 2003, there has been a fierce controversy about the reproducibility of the interaction between 5-HTTLPR and environmental factors in MDD. This controversy can only be resolved by empirical data. To that aim, we tested the effects of five environmental factors (lifetime and recent stressful life-events, sexual abuse, educational attainment, and childhood trauma), the number of functional short 5-HTTLPR alleles and their interaction on MDD prevalence and course in a large sample from the Netherlands. We found that the environmental factors had large and consistent direct effects on both prevalence and course of MDD. Additionally, our results suggested that the environmental impact is stronger for the more severe outcomes (suicidal MDD patients versus controls and chronic MDD patients versus controls) than for the main outcome (all MDD patients versus controls). Comparison of Nagelkerke R-Squares showed that of all environmental factors, childhood trauma explained most variation in both the prevalence and course of MDD. We did not find a direct gene-effect of the number of functional short 5-HTTLPR alleles on the prevalence of MDD, but we did find some effect ($p=0.03$) on course of MDD, with short alleles contributing to chronic course. MDD course is a very different concept and outcome than the MDD prevalence outcome. Consequently, it may be too conservative to further adjust the course analyses for the number of associations tested for the prevalence outcomes. Nevertheless, it is clear that our finding for a direct effect on course of MDD deserves confirmation in future longitudinal studies. Out of all 20 tests conducted, we found one gene-by-environment interaction-effect with an uncorrected p-value smaller than 0.05, namely, in the test with stressful life-events for the suicidal MDD outcome ($p=0.03$). We argued the Bonferroni-correction to be too stringent, because the different MDD outcomes are correlated, but this single p-value of 0.03 is not more than expected by chance.

Our finding that the environmental factors have a large impact on MDD is in line with previous literature (Caspi et al., 2003; De Graaf et al., 2011). The absence of a direct effect of 5-HTTLPR on MDD prevalence-outcomes in our study is in line with the meta-analysis by Risch et al. (2009), but not with the meta-analysis by Clarke et al. (2010), where a direct effect of 5-HTTLPR on MDD was found. Because the direct gene-effect found by Clarke et al. was very small, the lack of a gene-effect in our study could also be caused by the limited sample size. The effect of environmental factors on course of MDD, and childhood trauma in particular, is in

line with a recent meta-analysis (Nanni et al., 2012). The suggestive effect of 5-HTTLPR on course of MDD found in our study is interesting, but more studies are needed, as the effect of 5-HTTLPR on course of MDD has not been examined in many studies. One other study found no such effect (Zannas et al., 2012), whereas another study did find an effect of 5-HTTLPR on the course of MDD (Myung et al., 2010), but in the opposite direction with long alleles contributing to chronic course. We found no evidence for gene-by-environment interaction, which is in line with the meta-analysis of Risch et al. (2009) and Munafò et al. (2009), but it is not in line with the meta-analysis of Karg et al. (2011).

Some studies found significant interaction-effects in tests different to the one published by Caspi et al. For example, Uddin et al. (2010) reported gene-by-environment interaction for males only and Brummett et al. (2008) reported gene-by-environment-by-gender interaction. We performed these tests on our sample to be comprehensive, but none of the interaction effects in male-only and female-only analyses nor results for gene-by-environment-by-gender interaction were significant, indicating that sex-specific results are not further providing evidence for the presence of gene-by-environment interaction. Uher et al. found that gene-by-environment interaction is stronger for childhood trauma in persistent depression (Uher et al., 2011), but in our study we found no interaction effect for childhood trauma in chronic MDD. Some have argued that biological interaction should better be tested as departure from additivity than as departure from multiplicativity, as in biological interaction both causes are needed for a disease to develop (Knol et al., 2007; Uher et al., 2011). Therefore, we additionally tested for interaction as departure from additivity, but these analyses also showed no evidence for interaction.

Our study is well sized with around 1600 patients and 1400 controls for the main MDD prevalence outcome. For comparison, only three of the 56 studies included in the meta-analysis of Karg et al. (2011) had larger sample sizes. Nevertheless, only interaction-effect sizes smaller than 0.87 and larger than 1.17 could be detected with a power of 0.8 for stressful life-events in our study, as computed with Quanto-software (Gauderman and Morrison, 2006). Thus, our study lacked power for possible smaller interaction effects. Therefore, and because we had additional subjects with genome-wide SNP data but without measurements of 5-HTLLPR, we checked whether it was possible to increase sample size by imputing 5-HTTLPR using the haplotype proxy of surrounding SNPs from Vinkhuyzen et al. (2011). However, limited accuracy of this imputation in our sample was found. Moreover, Vinkhuyzen et al. found that rs25531 could not be tagged by SNPs giving rise to an even greater inaccuracy of imputation with respect to the functional length of 5-HTTLPR. Therefore, we restricted our analyses to the subjects for which 5-HTTLPR/rs25531-haplotypes were genotyped.

The question remains whether we would have been able to detect interaction-effects when we would have had a larger sample size. However, of the 56 studies included in the meta-analysis of Karg et al., eight contained more than 1000 subjects and of these only two found positive results for gene-by-environment interaction. This phenomenon, that larger studies produce more negative findings than smaller studies, was explained by Duncan and Keller (2011) as an indication of publication bias amongst smaller studies.

In our study we performed analyses with 5-HTTLPR/rs25531-haplotypes (denoted as functional 5-HTTLPR alleles) instead of plain 5-HTTLPR alleles, because long alleles of 5-HTTLPR are functionally equivalent to short alleles when they form a haplotype with the G-allele of rs25531 (Shiroma et al., 2010). However, only few studies took rs25531 into account and, therefore, we also conducted the interaction analysis for the number of plain

short 5-HTTLPR alleles. These analyses showed two interaction effects with uncorrected p-values smaller that 0.05 out of the sixteen tests performed: for sexual abuse in the main MDD prevalence outcome and for educational attainment in the MDD course outcome. Thus, analyses with plain 5-HTTLPR yielded one more interaction-effect with a p-values smaller than 0.05 than the analyses with functional 5-HTTLPR. However, because two tests out of 20 with a p-value smaller than 0.05 is still not very convincing, we suspect this finding to be due to chance.

The role of genetic factors in the relation between educational attainment and MDD has been studied by Lopez-Leon et al. (2009), who found that shared genetic factors play a role in the co-occurrence of lower socioeconomic status and symptoms of depression. Educational attainment is an easy measure to test, has a strong direct effect on MDD (De Graaf et al., 2011), and has often been used as proxy for socioeconomic status, for which some evidence of gene-by-environment interaction was found (Brummett et al., 2008; Uddin et al., 2010). To the best of our knowledge we were the first to test educational attainment (as measurement for environmental stress) for gene-by-environment interaction with 5-HTTLPR. We found no correlation between 5-HTTLPR and educational attainment, suggesting that the gene does not contribute to the gene-by-environment correlation reported by Lopez-Leon et al. and that the interaction could be meaningfully tested. Nevertheless, low educational attainment, like the other sources of stressful life events did not support a gene-by-environment interaction for 5-HTTLPR.

Our study has several strengths. First, we analyzed a large sample of MDD patients and controls. As stated before, only three of the 56 studies included in the meta-analysis of Karg et al. had larger sample sizes. Second, we used DSM-IV based diagnoses of MDD which ensured we studied the clinically relevant MDD patients. Moreover, our controls were screened for lifetime MDD diagnosis and a low probability of developing MDD later on in life. Third, we studied four different environmental factors making it less likely that we missed any gene-by-environment interaction in our sample. Fourth, we not only studied MDD patients compared to controls, but also two subgroups of more severe MDD patients in order to increase the contrast. Finally, we studied 5-HTTLPR/rs25531-haplotypes instead of plain 5-HTTLPR which is in line with the latest insights into the function of 5-HTTLPR.

There also are some limitations, including the assessment of stressful life-events only after the onset of MDD. In addition, some MDD patients had a lifetime, but no current diagnosis of MDD and because we assessed the lifetime occurrence of stressful life-events and sexual abuse, some reciprocal causation may have occurred. Reciprocal causation for life-events and MDD was reported by Middeldorp et al. (2008) and describes the phenomenon that life-events do not only increases the chance of developing MDD, but that MDD also increases the chance of encountering stressful life-events. This reciprocal causation might have influenced the results for stressful life-events and sexual abuse, but not for childhood trauma and educational attainment and we found no gene-by-environment interaction for these environmental factors either. We used two different studies with not entirely equal time and measurement frames, and the controls in both studies were not screened for other psychiatric disorders (NTR) or for family history of MDD (NESDA), which all may have had a potential influence on our results. However, as we used stringent other selection criteria as described and only used the most comparable environment instruments in analyses, it seems unlikely that this has had a significant impact. An additional limitation is that a current depressive mood could influence the recall of environmental factors.

Our study has several potential clinical implications. It shows that environmental factors increase the risk on MDD, as well as

the risk on chronic MDD for already depressed patients. In addition, our study suggests that childhood trauma contributes more to the risk on MDD than stressful life-events, sexual abuse or education attainment. For the course of MDD, childhood trauma and educational attainment contribute comparable risks on developing a chronic course of MDD and more than stressful life-events or sexual abuse. In sum, our study shows the strong effects of environmental factors on both the prevalence and course of MDD. It also shows the larger impact of childhood trauma compared to stressful life-events, sexual abuse and educational attainment. However, no evidence for gene-by-environment interaction effects for the 5-HTTLPR gene was found.

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