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Interplay between stress response genes associated with attention-deficit hyperactivity disorder and brain volume

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The glucocorticoid receptor plays a pivotal role in the brain's response to stress; a haplotype of functional polymorphisms in the *NR3C1* gene encoding this receptor has been associated with attention-deficit hyperactivity disorder (ADHD). The serotonin transporter (5-HTT) gene polymorphism *5-HTTLPR* is known to influence the relation between stress exposure and ADHD severity, which may be partly because of its reported effects on glucocorticoid levels. We therefore investigated if *NR3C1* moderates the relation of stress exposure with ADHD severity and brain structure, and the potential role of *5-HTTLPR*. Neuroimaging, genetic and stress exposure questionnaire data were available for 539 adolescents and young adults participating in the multicenter ADHD cohort study NeuroIMAGE (average age: 17.2 years). We estimated the effects of genetic variation in *NR3C1* and *5-HTT*, stress exposure and their interactions on ADHD symptom count and gray matter volume. We found that individuals carrying the ADHD risk haplotype of *NR3C1* showed significantly more positive relation between stress exposure and ADHD severity than non-carriers. This gene–environment interaction was significantly stronger for *5-HTTLPR* L-allele homozygotes than for S-allele carriers. These two- and three-way interactions were reflected in the gray matter volume of the cerebellum, parahippocampal gyrus, intracalcarine cortex and angular gyrus. Our findings illustrate how genetic variation in the stress response pathway may influence the effects of stress exposure on ADHD severity and brain structure. The reported interplay between *NR3C1* and *5-HTT* may further explain some of the heterogeneity between studies regarding the role of these genes and hypothalamic–pituitary–adrenal axis activity in ADHD.

Keywords: Attention-deficit hyperactivity disorder, gene–environment interaction, glucocorticoid receptor, gray matter volume, HPA axis, serotonin transporter

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Long-term stress exposure may have harmful effects on body and brain and is involved in a range of psychiatric disorders (McEwen *et al.* 2015), including attention-deficit hyperactivity disorder (ADHD, Biederman *et al.* 2002). Inter-individual differences in activity of the components of the stress response pathway can lead to large differences in the effects of stressors (Kudielka *et al.* 2009), and thereby in the association of stress exposure with ADHD.

The glucocorticoid receptor (GR) plays a pivotal role in the stress response by binding to cortisol and other glucocorticoids released from the adrenal gland upon stressor-induced activation of the hypothalamic–pituitary–adrenal (HPA) axis. Upon ligand binding, the activated GR regulates the expression of a large number of genes (Buckingham 2006). It further has rapid effects on neuronal excitability (Groeneweg *et al.* 2011), and provides negative feedback to the HPA axis that leads to inhibition of the release of cortisol (Mizoguchi *et al.* 2003). Differential activity of the GR, and its main endogenous agonist cortisol, has been associated with attention, arousal, perception, memory and emotional processing (Erickson *et al.* 2003), functions frequently impaired in individuals with ADHD (Corbett & Glidden 2000; Shaw *et al.* 2014; Talbot & Kerns 2014). There is also significant, although heterogeneous, evidence of a relation between ADHD and cortisol (Scassellati *et al.* 2012); both higher and lower levels of circulating cortisol in individuals with ADHD have been reported, independent of comorbidities (for a review, see Corominas *et al.* 2012).

Given its central role in the stress response, functional variation in the *NR3C1* gene coding for the GR makes it a prime candidate to moderate the effects of stress exposure

and subsequent cortisol release on ADHD. A haplotype of single nucleotide polymorphisms (SNPs) in *NR3C1* known to influence GR activity (Claes 2009) has been associated with ADHD (Fortier *et al.* 2013). The risk haplotype differs from the other combinations by an SNP in the 3′ untranslated region of exon 9β (rs6198) (Fortier *et al.* 2013; van den Akker *et al.* 2006); this polymorphism has been found to stabilize the mRNA of the GR-9β splice variant, which may lead to increased expression of the GRβ receptor (Derijk *et al.* 2001). This GR variant does not bind cortisol, is transcriptionally inactive and is thought to be a dominant-negative inhibitor of the functional GRα variant (Bamberger *et al.* 1995; Yudt *et al.* 2003). The GR-9β stabilizing polymorphism has been associated with higher cortisol levels in response to acute stressors (Kumsta *et al.* 2007) and altered glucocorticoid-regulated gene expression (van den Akker *et al.* 2006). Long-term exposure to stress is known to lower expression of *NR3C1*, leading to reduced negative feedback of the HPA axis as measured by slower return of cortisol levels to baseline after an acute stressor (van der Knaap *et al.* 2015). The combined inhibitory effect of the GR-9β haplotype and stress exposure may reduce GR activity to a pathologically low level, contributing to ADHD-related behavior.

The effect of *NR3C1* on the stress response may be further moderated by variation in the serotonin transporter (*5-HTT*) gene. We have reported that individuals carrying the short variant (S-allele) of a polymorphism in the promoter region of this gene (*5-HTTLPR*) show a stronger relation between stress exposure and ADHD severity than those homozygous for the long variant (L-allele) (van der Meer *et al.* 2014). A meta-analysis has established that the *5-HTTLPR* S-allele is associated with higher cortisol levels in response to acute stressors than the L-allele (Miller *et al.* 2013), a difference further strengthened by long-term stress exposure (Alexander *et al.* 2009). Administration of dexamethasone, a GR-specific glucocorticoid, increases *5-HTT* expression (Glatz *et al.* 2003), and genetically conveyed high 5-HTT availability is associated with lower *NR3C1* expression after stress exposure in rats (van der Doelen *et al.* 2014). These findings suggest the presence of a feedback loop between the GR and 5-HTT, raising the possibility that genetic variation in *NR3C1* and *5-HTT* may moderate each other's effects on the brain's stress response.

The GR is involved in the regulation of brain development and neuronal plasticity (Buckingham 2006). The few studies employing neuroimaging to investigate the relation between *NR3C1* and brain measures have focused primarily on the hippocampus, amygdala and medial prefrontal cortex (Dedovic *et al.* 2009), driven by the large body of literature tying together glucocorticoid actions, stress, emotion and memory (Finsterwald & Alberini 2014). These three regions are also prominently featured in the literature on the relation between *5-HTTLPR* and stress (Caspi *et al.* 2010). However, even though the GR is highly expressed in many brain regions (Morimoto *et al.* 1996) and pivotal to the brain's stress response, to our knowledge no study to date has employed neuroimaging to investigate whether *NR3C1* moderates the effect of stress throughout the brain, nor to study the potential role of *5-HTTLPR* in this stress response.

Given their reported interplay, we examined the relation between variation in *NR3C1*, stress exposure and ADHD severity, as well as the potential moderating role of *5-HTT*. The analyses were carried out in a sample of adolescents and young adults (mean age: 17.2 years) consisting of individuals with ADHD and healthy controls, as well as individuals with some symptoms of ADHD but not enough to meet the diagnostic criteria, referred to as 'subthreshold'. This sample composition enabled analysis within a wide range of ADHD severity, in accordance with the continuous distribution of ADHD in the general population (Levy *et al.* 1997). We additionally employed mediation analysis to determine how these interactions might be related to gray matter volume, in order to unravel the potential neurobiological mechanisms linking them to ADHD. Given the widespread expression of *NR3C1* in the brain and the availability of a large sample size, we chose for a whole-brain approach to allow for the discovery of effects on previously possibly overlooked brain regions.

Materials and methods

Participants and protocol

Participants were selected from the NeuroIMAGE study, a follow-up of the Dutch part of the International Multicenter ADHD Genetics (IMAGE) study (von Rhein *et al.* 2015). NeuroIMAGE included 365 families with at least one child with ADHD and at least one biological sibling (regardless of ADHD diagnosis) and 148 control families with at least one child, without any formal or suspected ADHD diagnosis in any of the first-degree family members. The study was approved by the regional ethics committee (CMO Regio Arnhem, Nijmegen; 2008/163; ABR: NL23894.091.08) and the medical ethical committee of the VU University Medical Center. All participants signed informed consent (parents signed informed consent for participants under 12 years of age).

The 539 participants who met the inclusion criteria and had magnetic resonance imaging (MRI) data available came from 311 families; 225 participants from 174 families had a diagnosis of ADHD, 63 participants from 58 families had subthreshold ADHD (i.e. had ADHD symptoms without meeting the criteria for a full ADHD diagnosis) and 251 participants from 196 families were healthy controls. The ADHD diagnoses were made in accordance with diagnostic and statistical manual of mental disorders fourth edition Text revision (DSM-IV-TR) criteria on the basis of a combination of a semi-structured diagnostic interview, the Kiddie Schedule for Affective Disorders and Schizophrenia – Present and Lifetime version (Kaufman *et al.* 1997) and the Conners Rating Scales. More information on the NeuroIMAGE study, its diagnostic algorithm and its participants is presented in Appendix S1 (Supporting information) and in von Rhein *et al.* (2015).

ADHD outcome measures

We constructed a DSM-IV-TR-based ADHD symptom count from the Conners ADHD Rating Scales questionnaires (Conners *et al.* 1998). These questionnaires were filled in by the parents and either a teacher (for participants <18 years) or the participants themselves (for participants ≥18 years old). The symptom count ranged from 0 to 18 with an average of 5.4 and SD of 5.1.

Stress exposure

Two questionnaires were used to quantify the exposure to psychosocial stress. Parents filled in the Long-Term Difficulties questionnaire (Bosch *et al.* 2012; Oldehinkel *et al.* 2008), which contained 13 items measuring whether their children have been exposed to chronic stressors such as handicap, being bullied, having financial difficulties or other persisting problems at home or school. They were asked to

only report chronic, ongoing difficulties. In addition, participants themselves filled in a Stressful Live Events questionnaire (Bosch *et al.* 2012; Oldehinkel *et al.* 2008), which contained 11 items on exposure to specific major stressful events in the past 5 years, such as death or serious illness of a loved one, physical or sexual abuse or failure at something important to them. For the composite stress measure, the scores on the questionnaires were transformed to *Z*-values and averaged according to common practice for aggregating similar measures, as previously described (van der Meer *et al.* 2014).

Socioeconomic status

As a measure of socioeconomic status, the highest, successfully completed education level of the parents was recorded into a measure reflecting years of education. This scale contained nine levels, ranging from 0 (no formal education) to 17 (university) years of education (Buis 2010). The average of both parents was used, which, in this sample, ranged from 5 to 17 with an average of 12.0.

Genetic data

An extensive description of DNA extraction and genotyping in IMAGE has been published previously (Brookes *et al.* 2006), and is documented in Appendix S1.

We based our investigation of variation in *NR3C1* on a study reporting a significant association between a haplotype in this gene and ADHD (Fortier *et al.* 2013). The authors of that study combined four SNPs in *NR3C1* (rs6189, rs6195, rs41423247 and rs6198), of which the G:A:G:G haplotype showed an association with multiple ADHD-related behaviors. Given the combinations of SNPs actually present in the data, carriers of this risk haplotype could be distinguished from non-carriers based solely on rs6189 and rs6198, as previously described (Kumsta *et al.* 2007; van den Akker *et al.* 2006). We calculated whether participants were carriers of the haplotype using the HAPLOSTATS package in R (v3.1.1) (R Core Team 2012; Schaid *et al.* 2002) and compared carriers of the risk haplotype (rs6189G and rs6198G), referred to as 9β (haplotype) carriers and coded as '1', to all others coded as '0'.

For the *5-HTTLPR*, we used an S-allele dominant genetic model, wherein S-allele carriers were coded as '1' and L-allele homozygotes were coded as '0', as previously described (van der Meer *et al.* 2014). In addition, L-alleles with the rs25531 C-G SNP were recoded as a functional S-allele, in accordance with prior studies (Hu *et al.* 2006). This led to 18 L-allele homozygotes being recorded as S-allele carriers.

MRI data acquisition and preprocessing

Both scanning locations used identical 1.5-Tesla scanners. Of each participant, two high-resolution T1-weighted MP-RAGE anatomical scans were obtained (176 sagittal slices, repetition time = 2730 milliseconds, echo time = 2.95 milliseconds, voxel size = $1.0 \times 1.0 \times 1.0\,mm^3$, field of view = 256 mm). Only scans with no or mild motion artifact were selected for further analysis. To increase signal-to-noise, scans from the same participant were averaged if they both contained no or mild motion. Three participants were excluded for further analysis because of severe motion in both scans and 15 participants were excluded due to incidental morphologic abnormalities (e.g. enlarged ventricles).

Preprocessing of the structural (s)MRI data was carried out with Statistical Parametric Mapping (SPM8; Wellcome Department of Cognitive Neurology, London, UK; http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) implemented in MATLAB 7.9 (Mathworks Inc., Sherborn, MA, USA), using the VBM8 toolbox with standard settings. This included normalization to Montreal Neurological Institute (MNI) space, segmentation into tissue-specific maps, modulation by dividing the images through the nonlinear component of the Jacobian determinant of the warp, and smoothing with an 8-mm full width at half maximum Gaussian kernel.

Statistical analysis

All behavioral data was analyzed using R (v3.1.1) (R Core Team 2012). The primary model investigating the effect of the gene–environment interaction on ADHD symptom count consisted of *NR3C1* haplotype, stress exposure and their interaction, as well as age, sex, socioeconomic status and location as covariates. In a second model, we added a three-way interaction between *5-HTTLPR* genotype, *NR3C1* haplotype and stress exposure, as well as the accompanying lower order terms. All continuous predictors were mean-centered. To account for the within-family correlation because of the inclusion of siblings in the sample, we analyzed the data with linear mixed effects models with family as a random factor, estimating a random intercept. The *P*-values of the mixed models results were estimated through a Markov chain Monte Carlo algorithm, included in the LANGUAGER package. For the significant predictors, we calculated Cohen's f^2 as a measure of effect size. This measure obtains the individual effect size of a regressor of interest by comparing the proportion of variance accounted for by the full model, with that of a model where this regressor is not included (Selya *et al.* 2012).

We used the multilevel mediation and moderation toolbox (Wager *et al.* 2008) to determine the relationship between the gene–environment interaction, gray matter volume and ADHD symptom count. This analysis technique is based on a standard three-variable mediation model, as described in greater detail elsewhere (van der Meer *et al.* 2015). Our primary whole-brain mediation model consisted of *NR3C1* genotype, amount of stress exposure and their interaction as predictors, gray matter volume as a mediator and ADHD symptom count as outcome variable. Sex, age, socioeconomic status and scanner location were added as covariates. For the subsequent three-way interaction analysis, we added *5-HTTLPR* genotype and its two- and three-way interaction terms with *NR3C1* and stress to the model. All continuous predictors were mean-centered. As a mask, we used the average gray matter image of the sample with an absolute threshold value of 0.2 (number of voxels: 464 067). The toolbox performed a bootstrap test (5000 samples), to estimate the significance of the effect on each voxel included in the mask. Family-wise error correction was applied through the use of FMRIB (Functional Magnetic Resonance Imaging of the Brain) software library (FSL v5.0)'s EASYTHRESH, which carries out cluster-based thresholding. A *Z*-value of 2.6 was used to define contiguous clusters and subsequently, each cluster's significance level was estimated on the basis of Gaussian Random Field Theory. To enhance confidence in the findings, we report those clusters surviving a conservative significance threshold of $P = 0.001$. Localization was determined with the Harvard-Oxford atlas. All reported co-ordinates are in MNI-space and in millimeter.

To further probe the effects, as well as to correct for the non-independence of the data, mean gray matter volume from significant clusters was extracted and analyzed with linear mixed effects models in R, as described above for the behavioral data.

Sensitivity analyses

We conducted sensitivity analyses to ensure that the findings were not biased owing to methodological choices. We checked the direction of effects of each significant analysis within diagnostic subgroups, testing locations, age groups and those with low or high internalizing or externalizing symptoms. More information on the methods for these analyses is presented in Appendix S1.

Results

Sample characteristics

We found no significant differences in stress exposure, age, socioeconomic status, sex, testing location or *5-HTTLPR* genotype between *NR3C1* 9β carriers and non-carriers, as summarized in Table 1. Genotyping frequencies did not deviate from Hardy–Weinberg Equilibrium (rs6189 $P = 0.66$, rs6198 $P = 0.18$; *5-HTTLPR* $P = 0.16$).

ADHD symptom count

There was a significant interaction between *NR3C1* genotype and stress exposure on ADHD severity ($B = 1.73$, SE = 0.66,

Table 1: Study sample characteristics. Differences between genotypes in the categorical variables 'location' and 'sex' were analyzed with a chi-square test; for the other continuous variables, we performed an analysis of variance

Variable	9β carriers	SD	Non-carriers	SD	Test-statistic	DF	*P*-value
Participants	114		425				
Covariates							
Amsterdam location	58.8%		50.8%		$\chi^2 = 2.28$	1	0.13
Male sex	56.1%		56.7		$\chi^2 < 0.01$	1	0.99
Age in years	17.23	3.26	17.25	3.50	$F < 0.01$	537	0.96
Parents' years of education	12.01	2.22	12.00	2.50	$F < 0.01$	537	0.96
Stress *Z*-score	0.02	0.75	–0.004	0.82	$F = 0.11$	537	0.74
Number of stressful live events	2.20	1.47	2.05	1.58	$F = 0.84$	523	0.36
Number of long-term difficulties	1.18	1.44	1.19	1.45	$F = <0.01$	526	0.94
5-HTTLPR S-allele carriers	60.5%		66.6%		$\chi^2 = 1.20$	1	0.27

SD, standard deviation; DF, degrees of freedom.

$P = 0.009$, $f^2 = 0.011$), with the association between stress exposure and ADHD symptom count being stronger in 9β carriers than in non-carriers. In this model, there was an effect of stress exposure ($B = 0.78$, SE = 0.29, $P = 0.007$, $f^2 = 0.008$), but not of *NR3C1* on ADHD symptom count.

In the second model, including *5-HTTLPR* genotype, both gene–environment interaction terms significantly predicted ADHD severity (*5-HTTLPR* × stress $B = 1.86$, SE = 0.57, $P = 0.001$, $f^2 = 0.021$; *NR3C1* × stress $B = 3.39$, SE = 1.09, $P = 0.002$, $f^2 = 0.019$). In addition, there was a three-way interaction between the two genes and stress exposure ($B = -2.66$, SE = 1.34, $P = 0.05$, $f^2 = 0.009$). As illustrated in Fig. 1, the interaction between *NR3C1* and stress was driven by L-allele homozygotes.

See Appendix S1 for the full test statistics from these analyses.

Gray matter volume

NR3C1 moderated the association between stress and gray matter volume in the cerebellum and parahippocampal cortex, as shown in Fig. 2a. In these regions, 9β carriers showed a stronger negative correlation between stress and gray matter volume (see Fig. S1). Further information on the clusters is presented in Table 2. Given our focus on the gene–environment interaction, significant clusters from the conditional effects of *NR3C1* and stress exposure are presented in Appendix S1.

The three-way interaction analysis with *5-HTTLPR* resulted in two significant clusters, one in the intracalcarine cortex and one in the angular gyrus (see Fig. 2b and Table 2). In both clusters, only individuals carrying the *NR3C1* 9β carriers who were homozygous for the *5-HTTLPR* L-allele showed a negative relation between stress and gray matter volume, whereas the other three groups showed no relation between stress and gray matter volume (see Fig. S1).

For both the *NR3C1* by stress and three-way interaction analysis we did not find any mediation effects, i.e. the local effects of these interactions on gray matter volume did not significantly explain their association with ADHD severity.

Sensitivity analyses

Results from the sensitivity analyses are presented in Appendix S1. Briefly, the direction of effects for the two- and three-way interactions was the same across all subsamples.

Discussion

We investigated whether variation in the GR gene *NR3C1*, an important component of the brain's stress response system, explained differences between individuals in the association of stress with ADHD severity and brain structure. Individuals carrying the *NR3C1* 9β haplotype showed a significantly stronger positive relation between long-term stress exposure and ADHD severity than non-carriers, as well as a more negative relation between stress exposure and gray matter volume. These gene–environment interaction effects were further moderated by another gene known to influence the response to stress, *5-HTT* (Caspi *et al.* 2010), such that only *5-HTTLPR* L-allele homozygotes showed susceptibility to the stress-sensitizing effects of the *NR3C1* 9β haplotype.

The observed stronger relation between stress exposure and ADHD severity in *NR3C1* 9β haplotype carriers in the current study adds to evidence of HPA axis involvement in ADHD. This haplotype is thought to tag genetic variation which may inhibit GRα activity by increasing expression of the functionally inactive GRβ variant and inhibiting the functional GRα (Derijk *et al.* 2001), potentially contributing to the reported gene–environment interaction by sensitizing carriers to the effects of lower GR availability owing to long-term stress exposure. Both have been associated with lower negative feedback of the HPA axis (Kumsta *et al.* 2007; van der Knaap *et al.* 2015), which in turn has been tied to ADHD (Corominas *et al.* 2012). However, given that the GR has highly pleiotropic effects through its role in gene regulation and neuronal excitability (Buckingham 2006), more research is needed to uncover the mechanism underlying the relation between *NR3C1*, stress exposure and ADHD.

We found a stronger negative relation between stress exposure and gray matter volume in 9β carriers than in non-carriers in the cerebellum and parahippocampal cortex. These brain regions are among those with the highest GR

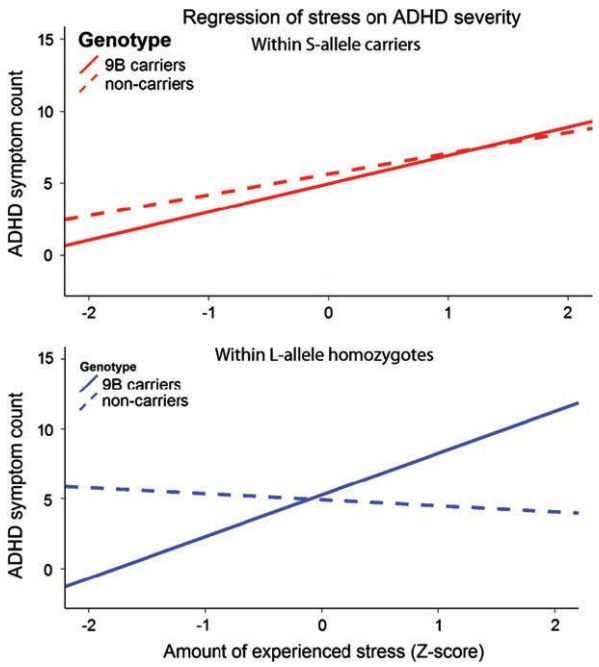

Figure 1: The association between stress exposure and ADHD severity, as a function of NR3C1 and 5-HTTLPR genotype. The stress score on the *X*-axis is a composite of two questionnaires asking about ongoing long-term difficulties and stressful live events experienced in the past 5 years. ADHD severity on the *Y*-axis was measured through Conners' questionnaires filled in by two informants for healthy controls and individuals with full or subthreshold ADHD alike, which ensured presence of the full range of ADHD symptoms in the sample, from 0 to 18. Among 5-HTTLPR S-allele carriers (red lines, top graph), both carriers and non-carriers of the NR3C1 9β haplotype show an effect of stress exposure on ADHD severity, whereas within L-allele homozygotes (blue lines, bottom graph) only those carrying the 9β haplotype (solid lines) show a positive association between stress and ADHD severity.

density (Morimoto *et al.* 1996), and are smaller in individuals exposed to stress (Hart & Rubia 2012). The negative effect of stress on these regions in 9β carriers may reflect decreased regulation of genes involved in neurodevelopment and plasticity because of lower GR activity (Buckingham 2006). Both regions are important for contextual learning and episodic, emotional memory retrieval (Andreasen *et al.* 1999; Desmond & Fiez 1998; Epstein & Kanwisher 1998), functions consistently associated with stress exposure, the glucocorticoid system and their interactions (Finsterwald & Alberini 2014). In addition, they are reliant on strong structural and functional connectivity with the hippocampus (Rochefort *et al.* 2013), the brain region most often reported to be sensitive to long-term stress exposure (McEwen *et al.* 2015). It should be noted that both regions have been associated with a diverse set of cognitive and affective functions (Aminoff *et al.* 2013; Stoodley 2012). Task-based studies are therefore needed to identify any specific behavioral correlates of the reported neuroanatomical effects.

The three-way interaction analysis indicated that carrying the 9β haplotype only strengthened the association between stress exposure and ADHD severity for L-allele homozygotes. Both animal and human studies have provided evidence of an inverse relation between *5-HTT* and *NR3C1* expression (Duman & Canli 2015; van der Doelen *et al.* 2014). One study has also directly investigated variation in these two genes together, and reported that individuals carrying both the *5-HTTLPR* S-allele and the *NR3C1* Bcl1 C-allele displayed higher cortisol reactivity in response to stress (Taylor *et al.* 2014). The authors did not look at the 9β haplotype so a direct comparison with the current study is not possible, but their findings do provide evidence that the effects of these two genes are intertwined. As the *5-HTTLPR* L-allele is associated with higher *5-HTT* mRNA after an acute stressor (Duman & Canli 2015), the reported lower GR activity in 9β haplotype carriers (Kumsta *et al.* 2009; van den Akker *et al.* 2006) could be further lowered by higher, stress-induced, 5-HTT activity. This may provide a mechanism whereby carrying the 9β haplotype enhances the relation between stress exposure and ADHD severity in L-allele homozygotes compared with S-allele carriers. The higher cortisol levels in response to stress conveyed by the S-allele compared with the L-allele (Miller *et al.* 2013) may be protective against the inhibitory effect of the 9β haplotype on GR activity. However, the presence of multiple feedback loops, as well as the likelihood of further interplay with other components of the stress response pathway, suggest that genetic variation in *NR3C1* and *5-HTT* does not influence HPA axis activity in a straightforward manner; rather, it may moderate both the initial release of cortisol as well as the return to baseline. Future studies should therefore carefully document the relation between the gene–environment interactions reported in this study and changes in cortisol levels over time, both basal and in response to (standardized) stressors.

The *5-HTT* moderated the interaction between *NR3C1* and stress exposure on gray matter volume in the intracalcarine cortex and angular gyrus; here, only L-allele homozygotes carrying the *NR3C1* 9β haplotype showed a negative relation between stress and gray matter volume. Both regions belong to the neural circuitry underlying social perception processes, which have been implicated in the association between psychosocial stress and psychiatric disorders (Meyer-Lindenberg & Tost 2012). The angular gyrus is important for direction of attention toward salient visual cues (Seghier 2013), while serotonergic projections to the intracalcarine cortex modulate the strength of affective visual stimuli (Keil *et al.* 2009; Kemp *et al.* 2004). Lower gray matter volume in these regions may therefore contribute to findings that *5-HTT* is associated with attentional biases for affective stimuli (Beevers *et al.* 2010), and that glucocorticoid activity modulates sensory perception (Fehm-Wolfsdorf & Nagel 1996) and processing of emotional information (Ellenbogen *et al.* 2010). Further, the angular gyrus allows us to use past information to infer others' intentions (Seghier 2013), which is in line with literature suggesting effects of both *NR3C1* and *5-HTT*, as well as stress exposure, on social cognition and memory (Roiser *et al.* 2007; van Goozen & Fairchild 2006). The clusters we found also partly overlapped with those of McLaughlin *et al.*

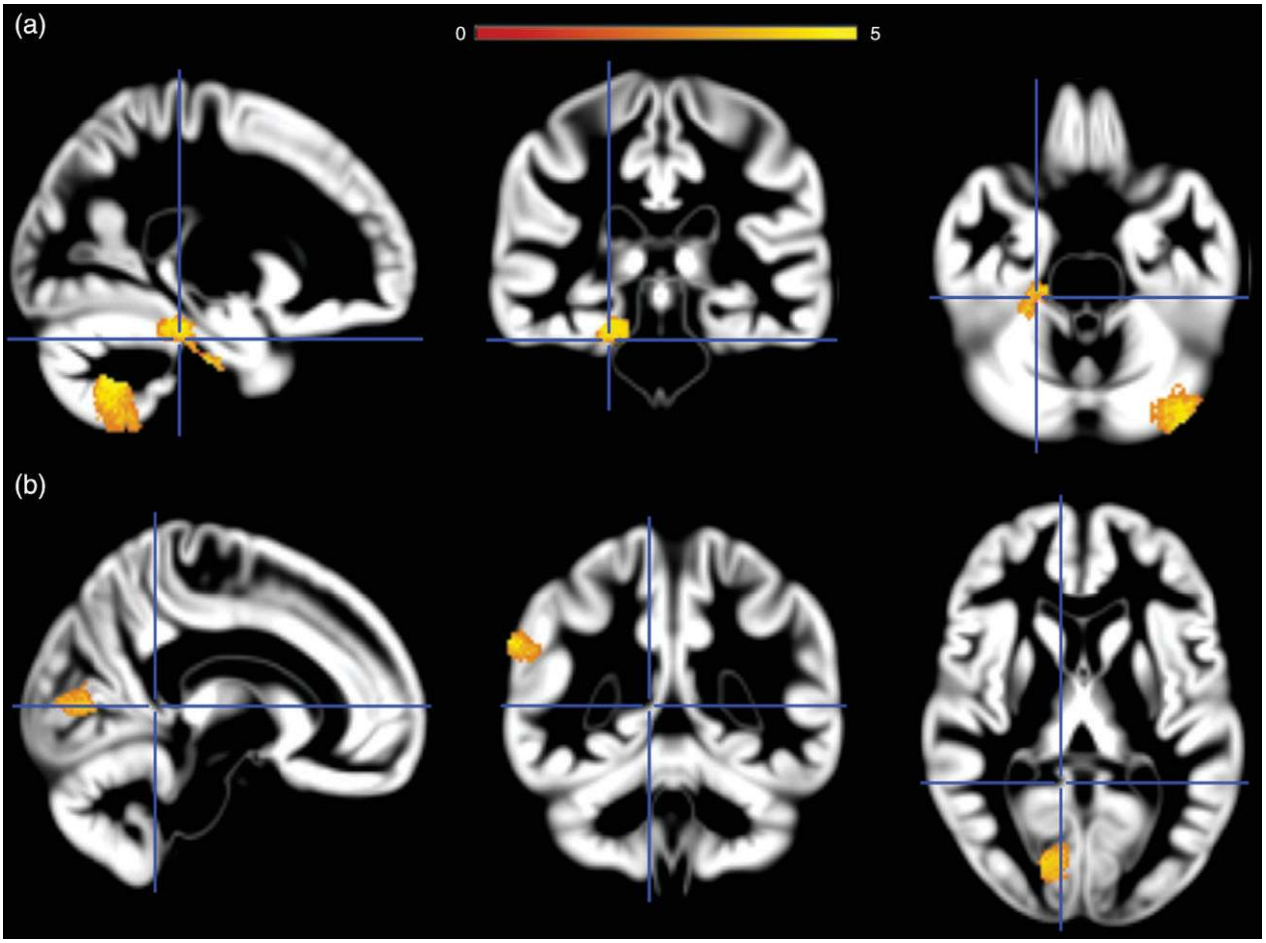

Figure 2: Results from the whole-brain analyses. Visualization of the location of the clusters where gray matter volume was significantly associated with the interaction between NR3C1 variation and stress exposure (a), and those significantly associated with the three-way interaction between NR3C1 haplotype, 5-HTTLPR and stress (b). The thresholded *Z*-value maps are overlaid on the sample's average gray matter image. The images are depicted in neurological convention, in MNI-space. Co-ordinates (in mm) (a): $X = 21$, $Y = -30$ and $Z = -26$, (b): $X = 9$, $Y = -45$ and $Z = 8$.

Table 2: Summary of the significant clusters found in the whole-brain analysis. The top part provides information on where NR3C1 significantly moderates the association between stress and gray matter volume, the bottom part displays this information for the three-way analysis of 5-HTTLPR, NR3C1 and stress

Predictor	Location (peak, other regions in cluster)	*X*	*Y*	*Z*	Cluster size	Coefficient	Cohen's f^2
NR3C1 × stress	Posterior parahippocampal gyrus, temporal fusiform cortex	23	−30	−21	542	−0.027	0.002
	Cerebellar Crus I	−38	−75	−23	769	−0.033	0.002
	Cerebellar VIIIb	23	−60	−44	1972	−0.043	0.009
5-HTTLPR × *NR3C1* × stress	Intracalcarine cortex	17	−77	6	483	0.074	0.005
	Angular gyrus, posterior supramarginal gyrus	66	−47	32	456	0.085	0.010

X, *Y* and *Z* co-ordinates are in MNI-space in mm, and represent the peak of the cluster. The anatomical labels are according to the Harvard-Oxford and Cerebellar MNI 152 atlases.

(2013) who reported that the relation between early-life deprivation and ADHD symptoms was mediated by reduced cortical thickness in the fusiform gyrus and supramarginal gyrus. These regions are thought to be central for recognizing facial expressions and for empathy (Saygin *et al.* 2012; Silani *et al.* 2013). A deficit in perceiving and understanding social cues has been weakly associated with ADHD (Humphreys *et al.* 2016; Petersen & Grahe 2012), although interaction effects raise the possibility that such a deficit may be more prominent in specific genetic subgroups. This illustrates the value of the gene–environment interaction approach, allowing for discovery of effects that may be specific to a subset of individuals, effects that would be overlooked when averaging over all groups.

The pattern of results found in the current study illustrates the complexity of the brain's stress response, and its intricate relation with ADHD. In addition to the three-way interaction, the independent contributions of variation in *5-HTT* and *NR3C1* to the stress response suggest that both have separable effects on behavior. These distinct contributions are also reflected in their neural correlates; while we previously found the interaction effect between *5-HTT* and stress on ADHD severity to be mediated by frontal brain regions involved in cognitive control (van der Meer *et al.* 2015), we found here that *NR3C1* moderates the effects of stress on more posterior brain regions involved in contextual learning, memory and, together with *5-HTT*, on regions that have a role in social perception. Therefore, while individuals with different combinations of these genetic factors may display a similar relation between stress exposure and ADHD severity, the underlying neural pathways appear to differ. Lack of mediation effects suggests that the interaction between *NR3C1* and stress exposure is related to ADHD through mechanisms not well captured by measures of gray matter volume, or through diffuse volumetric effects that do not reach our significance threshold. *NR3C1* is expressed in every cell of the body, and GR activity influences a very wide range of functions relevant for ADHD, such as attention, perception, memory and emotional processing (Erickson *et al.* 2003). Although based on the brain regions for which we found significant interaction effects, neuropsychological studies may investigate whether variation in *NR3C1* and *5-HTT*, and their interactions with stress exposure, influence performance on tasks measuring contextual learning and memory, attention biases to affective stimuli and cognitive control.

This study has made use of a relatively large sample size for neuroimaging studies, as well as extensive and carefully collected phenotypic information of its participants. This enabled us to find small effects, in accordance with what is known about the genetic architecture of ADHD (Banaschewski *et al.* 2010). However, the cross-sectional design of this study warrants caution with regard to causality, and lack of data on methylation and cortisol levels limits interpretation of the results. Research has shown that the relation between stress exposure and methylation patterns of *5-HTT* and *NR3C1* is highly complex (Alexander *et al.* 2014; Palma-Gudiel *et al.* 2015). Additionally, *NR3C1* contains several more functional polymorphisms that may influence GR activity (Bray & Cotton 2003), the effects of which require further study. For instance, a polymorphism in the promotor region of *NR3C1* has been found to be in high linkage disequilibrium with the 9β polymorphism. The authors found the minor allele was associated with lower transcriptional activity, which may act in concert with the inhibition of GR-9α activity by the 9β polymorphism (Kumsta *et al.* 2009), as well as serve as a target for stress-induced methylation to further lower *NR3C1* expression. Studies directly measuring 5-HTT and GR levels, as well as other indices of the stress response such as cortisol levels, should provide us with further insight into the mechanisms underlying the effects of these gene–environment interactions on ADHD and thereby resolve some of the heterogeneity present in the literature. Nonetheless, confidence in the current findings is strengthened by their biological plausibility and fit with a large body of literature describing the effects of glucocorticoids, 5-HTT and stress exposure on brain and behavior in both animals and humans.

In conclusion, we found that both *NR3C1* and *5-HTT* moderate the effect of stress on ADHD severity and gray matter volume. While in need of replication, the results from this study illustrate how the interplay between components of the stress response influences the effects of stress exposure on behavior, which may explain some of the large heterogeneity of findings in studies of ADHD. The reported effects also warrant further research into other genes associated with ADHD and HPA axis activity, such as *NR3C2* (Kortmann *et al.* 2013), *FKPB5* (Isaksson *et al.* 2015) and *MAP3K7* (Franke *et al.* 2009; Lasky-Su *et al.* 2008). Continued research into the stress response pathway, and its relation with ADHD, may generate information that can eventually be used to predict the consequences of stress exposure per individual.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supplementary information on the NeuroIMAGE, sensitivity analyses, and additional output from the main analyses.

Table S1. Results from the interaction between NR3C1 and stress exposure on ADHD symptom count.

Table S2. Results from the three-way interaction between NR3C1, 5-HTT and stress exposure on ADHD symptom count.

Table S3. Summary of the clusters where NR3C1, stress exposure and the gene-environment interaction (GxE) are significantly correlated with gray matter volume at $P = 0.001$, as determined by Random Field Theory.

Table S4. Direction of effects within the subsamples for the significant NR3C1 × stress analyses. The regression coefficients refer to that of the gene–environment interaction term for each subset.

Table S5. Direction of effects within the subsamples for the significant three-way interactions in the main analyses. The regression coefficients refer to that of the three-way interaction term for each subset.

Figure S1. The interaction effect between NR3C1, 5-HTTLPR and stress exposure on mean gray matter volume within each cluster identified in the whole-brain analyses. The stress score on the *X*-axis is a composite of two questionnaires asking about ongoing long-term difficulties and stressful live events experienced in the past 5 years. Gray matter volume on the *Y*-axis was measured by voxel-based morphometry. 5-HTTLPR S-allele carriers are represented by red lines, L-allele homozygotes by blue lines, NR3C1 9β carriers by solid lines and non-carriers of the NR3C1 9β variant by dashed lines.