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Epigenetic Changes of *FKBP5* as a Link Connecting Genetic and Environmental Risk Factors with Structural and Functional Brain Changes in Major Depression

Leonardo Tozzi*[,1,2], Chloe Farrell[1], Linda Booij[3,4], Kelly Doolin[1], Zsofia Nemoda[5], Moshe Szyf[5], Florence B Pomares[3,4], Julian Chiarella[3,4], Veronica O'Keane[1] and Thomas Frodl[1,2]

[1]Department of Psychiatry, Trinity College School of Medicine and Trinity College Institute of Neuroscience, Dublin, Ireland; [2]Department of Psychiatry, Otto von Guericke University Magdeburg, Magdeburg, Germany; [3]Department of Psychology, Concordia University, Montreal, Canada; [4]CHU Sainte-Justine Hospital Research Centre, University of Montreal, Montreal, Canada; [5]Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada

The gene for the glucocorticoid receptor regulator FK506 binding protein 5 (*FKBP5*) plays a role for risk, response to treatment, and changes in brain areas in major depressive disorder (MDD). Chronic stress is associated with lower methylation of *FKBP5*. Our aim was to investigate whether methylation of *FKBP5* reflected exposure to childhood adversity in MDD and controls and whether it was associated with structure and function of emotional processing regions. *FKBP5* intron 7 GR response element region methylation and rs1360780 allelic status were assessed from whole blood in 56 MDD adults and 50 controls. Using magnetic resonance imaging, we assessed gray matter concentration of selected areas and their function during valence recognition of emotional images. Childhood adversity was investigated using the Childhood Trauma Questionnaire. In MDD patients carrying the high-risk T allele of rs1360780, lower methylation of *FKBP5* was predicted by childhood adversity ($F = 4.95$, $p = 0.04$). In all participants, lower *FKBP5* intron methylation levels were associated with reduced gray matter concentration in the inferior frontal orbital gyrus bilaterally (Wald chi-square = 11.93, $p_{FDR} < 0.01$) and, in MDD, with its bilaterally higher activation during valence recognition (Wald chi-square = 5.58, $p = 0.02$). Activation of this region, regardless of side, was found to be lower in MDD compared to controls (Wald chi-square = 3.88, $p = 0.049$) and to be inversely correlated with depression severity (Wald chi-square = 4.65, $p = 0.03$). Our findings support the hypothesis that, in genetically predisposed individuals carrying a high-risk variant of the gene, childhood maltreatment might induce demethylation of FKBP5. This is in turn associated with structural and functional changes in the inferior frontal orbital gyrus, a relevant area for the clinical symptoms of MDD.
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INTRODUCTION

Many studies investigating the pathogenesis of major depressive disorder (MDD) have focused on dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and inflammation as key elements of the condition (Miller and Raison, 2016). Compatible with this hypothesis, many genes involved in HPA axis regulation have been examined to assess risk of developing MDD and as potential predictors of antidepressant treatment response (de Kloet *et al*, 2005).

The gene encoding the FK506 binding protein 5 (*FKBP5*), located on chromosome 6p21.31, is involved in steroid receptor sensitivity regulation creating an intracellular negative feedback loop (Scharf *et al*, 2011, p 20; Binder, 2009). In particular, increased intracellular levels of FKBP5 decrease the affinity of the glucocorticoid receptor (GR, Binder, 2009) after being upregulated by active corticosteroid receptors at the transcriptional level through GR response elements (GREs) (Wochnik *et al*, 2005; Grad and Picard, 2007). Compatible with its role in HPA axis regulation, variations of *FKBP5* have been investigated in several conditions that involve exposure to chronic stress, such as gastric cancer (Kang *et al*, 2012), childhood maltreatment (Gillespie *et al*, 2009), post-traumatic stress disorder (Binder *et al*, 2008).

How chronic stress might influence *FKBP5* gene transcription and its protein function is not known, but it has been suggested that their interplay might be mediated by epigenetic mechanisms (Tyrka *et al*, 2016). In support of this view, treatment of human hippocampal progenitor cells with glucocorticoids induced long-lasting demethylation of *FKBP5* regulatory intronic regions and increased its expression (Klengel *et al*, 2013). Recent work has expanded this finding to human populations by showing that exposure to

*Correspondence: Dr L Tozzi, University Hospital, Department of Psychiatry, Otto von Guericke University Magdeburg, Leipzigerstr. 44, Magdeburg 39120, Germany, Tel: +4915225191188, Fax: +493916714229, E-mail: leonardo.tozzi@med.ovgu.de
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chronically high cortisol levels in Cushing syndrome was associated with lower DNA methylation level of *FKBP5* introns assessed in white blood cells and smaller hippocampal volumes (Resmini *et al*, 2016). Decreased methylation was also observed in DNA taken from saliva samples of maltreated children (Tyrka *et al*, 2015) and in whole blood and saliva samples of adult victims of childhood trauma (Klengel *et al*, 2013). Therefore, current findings suggest that methylation levels at regulatory regions might constitute a link between *FKBP5* expression and both endocrine as well as environmental stress.

MDD is a multifactorial condition with complex inheritance, that is likely to involve the interaction between genetic predisposition and environmental triggers, which result in persistent dysregulation of the HPA axis (Tafet and Nemeroff, 2016). Therefore, it is not surprising that variants of *FKBP5* have been found to be associated with this disorder in several studies (see Rao *et al* (2016) for a recent meta-analysis). Furthermore, a role of *FKBP5* in mediating antidepressant treatment response has been highlighted (Horstmann *et al*, 2010; Binder *et al*, 2004).

It is still unclear which role *FKBP5* might play in the structural and functional brain differences detected between MDD patients and healthy controls (HC). Some magnetic resonance imaging (MRI) experiments have investigated gray matter (GM) volume, white matter integrity, and neural responses to stimuli in patients carrying high-risk allele variants of *FKBP5* rs1360780 SNP relative to individuals without a risk allele, highlighting structural and functional differences in brain areas involved in emotional processing. For instance, in patients carrying the minor (risk) *FKBP5* alleles, studies found volume changes in the amygdala and middle and inferior orbitofrontal gyri (Hirakawa *et al*, 2016) and dorsal anterior cingulate cortex (ACC) (Fujii *et al*, 2014). In these participants, white matter integrity was also found to be altered in the ACC (Fujii *et al*, 2014), insula, and inferior frontal gyrus (Tozzi *et al*, 2015). Functional studies, on the other hand, showed higher functional responses in the amygdala during an emotional face matching task which were also influenced by exposure to childhood trauma (White *et al*, 2012).

The first study investigating the interaction of MDD, *FKBP5* allele status, methylation, and GM changes in depressed patients has only recently been conducted (Han *et al*, 2017). In this work, the T allele of rs1360780 was associated with volume reduction of portions of the frontal and parietal cortex, but exclusively in patients. Interestingly, an effect of *FKBP5* methylation on right frontopolar gyrus GM thickness was detected in participants regardless of diagnosis, but dependent on rs1360780 allelic status. Overall, the authors provide further evidence that rs1360780 and MDD have interactive effects on GM volumes of cortical regions involved in emotion processing and mood regulation. In addition to this, *FKBP5* methylation might predict changes in the structure of these areas depending on rs1360780.

Childhood adversity has been shown to interact with rs1360780 and induce *FKBP5* demethylation (Binder *et al*, 2008; Klengel *et al*, 2013). Furthermore, in a previous study, we showed how MDD patients carrying the high-risk allele of rs1360780 showed reduced structural integrity and activation in the insula and inferior frontal gyrus depending on the amount of adversity endured (Tozzi *et al*, 2015). To our knowledge, the interaction between maltreatment, genetics, and epigenetics of *FKBP5* on both brain structure and function has not yet been comprehensively explored in MDD, as highlighted by Han *et al* as a limitation of their study (Han *et al*, 2017).

Our goal was therefore to investigate, in MDD patients and HC, the relationship between epigenetic modifications in *FKBP5* with allelic status of rs1360780, exposure to childhood adversity and structural as well as functional brain measures. First, we investigated the methylation of *FKBP5* CG-sites in whole blood DNA samples, expecting to find lower mean methylation levels in MDD patients expressing the T allele of rs1360780 and exposed to childhood abuse, compatibly with Klengel *et al* (2013). We then acquired structural MRI scans of our participants as well as functional ones during an emotion recognition task that consistently showed activation in emotional processing areas in the past (Tozzi *et al*, 2015, 2017). We hypothesized that lower whole blood *FKBP5* methylation would also be correlated with lower GM content and activation in emotional processing areas (Klengel *et al*, 2013; Han *et al*, 2017). Finally, we assessed if the regions whose activity and GM concentration were correlated with *FKBP5* methylation were also relevant for depression psychopathology.

MATERIALS AND METHODS

Sample Composition

We used data from two cohorts, which we collected independently between 2009 and 2015 at two imaging sites in Trinity College Dublin using the same Philips 3T MRI system: the Centre for Advanced Clinical Imaging (CAMI) and the Trinity College Institute of Neuroscience (TCIN). The data set comprised 53 HC and 60 MDD.

Exclusion criteria were age <18 or >65, history of neurological or any comorbid psychiatric disorders including alcohol or substance dependency (Axis I), personality disorders (Axis II), other severe medical illness, psychosis, head injury or current substance abuse. Demographic variables, inclusion and exclusion criteria were documented by a psychiatrist using a standardized questionnaire.

Written informed consent was obtained from all participants after being given detailed description of the study which was designed and performed in accordance to the ethical standards laid out by the Declaration of Helsinki and was approved by the ethics committee of St. James and Tallaght hospitals, Dublin and McGill University, Montreal, Canada.

Clinical Ratings

The Hamilton Depression Scale (HAM-D) 21-item version (Hamilton, 1986), Beck's Depression Inventory (BDI) (BECK *et al*, 1961) as well as the Childhood Trauma Questionnaire (CTQ) (Bernstein and Fink, 1998) were filled out for all participants included in the study. The CTQ measures emotional, physical and sexual abuse as well as emotional and physical neglect. A three-item minimization-denial subscale is also included to check for attempts by respondents to minimize their childhood abuse experiences

(Bernstein and Fink, 1998). The occurrence during his or her childhood of 28 items is rated by the participant on a 5-point Likert scale which ranges from 5 (no history of abuse or neglect) to 25 (extreme history of abuse or neglect). A total score is then obtained by adding all subscales (except the minimization-denial one).

Epigenetic Analysis

For a detailed description of the epigenetic analysis, see Supplementary Methods. Genomic DNA was extracted from whole blood samples, which had been collected into PAXgene Blood DNA Tubes. Since Resmini *et al* (2016) found more pronounced effects at CG-6 and CG-7 in the intron 7 region of *FKBP5*, we limited our analyses to these two sites, which are in or close proximity to a GRE (see supplementary figure 2 in Klengel *et al*, 2013).

Our region of interest (ROI) in the *FKBP5* gene intron 7 was amplified by PCR. The following sequencing primers were used: FKBP5_S3A: 5′-ATTTTTGTGAAGGGTA-TAATT-3′ and FKBP5-in7_S67: 5′-GTTGATATATAG-GAATAAAATAAGA-3′ (IDT Inc.) to assess CG-6 and CG-7 following the numbering of Resmini *et al* (2016), which correspond to Bin3, CG1-2 called by Klengel *et al* (2013). Finally, the average of CG-6 and CG-7 methylation was computed for each participant.

rs1360780 Analysis

For a detailed description of the rs1360780 analysis, see Supplementary Methods.

rs1360780 was genotyped from blood by the same laboratory for both sites using a Taqman SNP Genotyping Assay on a 7900HT Sequence Detection System (Applied Biosystems). Because homozygous TT samples were rare in our sample, we grouped them with heterozygous TC samples for analysis (T*) to define a binary rs1360780 variable.

Demographic and Clinical Measures

All statistical analyses were conducted using SPSS version 22 (IBM Corp).

Demographic variables and clinical test scores were compared between MDD and controls using independent *t*-tests (age, methylation percentage), chi-square tests (sex, rs1360780) and Mann–Whitney *U* tests (CTQ, HAM-D, BDI). The same tests were also conducted to compare participants based on the site and rs1360780 variables.

Predictors of FKBP5 Methylation

We entered methylation percentage as dependent variable in a full factorial general linear model (GLM) that had the following independent variables: diagnosis (binary: HC or MDD), site (binary: CAMI or TCIN), sex (binary: male or female), rs1360780 (binary: CC or T*), age (continuous), CTQ total score (continuous) as well as all four possible interactions between rs1360780 allele status, diagnosis, and CTQ total score. Upon identification of significant interactions, the *post-hoc* models were rerun splitting the data for the interacting factors. Within the MDD group, medication was added as a binary factor (medicated, unmedicated).

Magnetic Resonance Imaging

For a detailed description of MRI acquisition and analysis, see Supplementary Methods.

In summary, data were processed with Statistical Parametric Mapping 12 (SPM12, http://www.fil.ion.ucl.ac.uk/spm) and with the Computational Anatomy Toolbox 12 (CAT12, http://www.neuro.uni-jena.de/vbm/download/).

Functional MRI task. A Neurobehavioral Systems Presentation (https://www.neurobs.com/) experiment was run during the fMRI recording as described in detail in our previous publications (Tozzi *et al*, 2017). The task was event-related and consisted of 180 pseudo-randomized trials, evenly distributed in 3 emotion × 2 shape conditions. Each trial consisted of a fixation cross of jittered duration (mean = 1.5 s, range: 1–1.8 s), followed by positive, negative or neutral rectangular pictures from the International Affective Picture System database (IAPS) for 2 s. After seeing the picture, participants were either asked to focus on the emotion elicited by the picture and answer whether this was positive, negative or neutral or had to answer a question about its shape (horizontal or vertical). From here on, we will label the first type of trial emotional recognition trials (ERT) and the second shape recognition trials (SRT). The same amount (30) of ERT and SRT was delivered for each of the three valences (positive, negative, neutral).

During pre-processing, movement parameters of all subjects were inspected and four patients and three controls were excluded from the sample, resulting in a final sample of 50 HC and 56 MDD patients. Structural data were segmented and warped to MNI space using the default CAT12 pipeline. Functional data were co-registered with the structural one and also normalized using the warp fields obtained during the normalization of the structural data (see Supplementary methods for details).

A first-level GLM analysis was conducted on the normalized functional data, using a canonical HRF as response function and a high-pass filter of 128 s. Times at which the questions were presented were entered in the GLM along with the six motion parameters of each subject. T-tests were then run on ERT > SRT first-level contrasts for each emotional valence (neutral, negative and positive), thus representing brain response to the evaluation of each emotion elicited by the pictures in comparison to that of their shape.

ROI definition and extraction. We used an ROI approach, targeting parts of the brain that are known to be involved in emotion recognition and to be especially affected in MDD, in particular the medial and lateral prefrontal cortex, amygdala, insula, and hippocampus (Phillips *et al*, 2008; Drevets *et al*, 2008).

The automated anatomical labeling atlas (Maldjian *et al*, 2003) as provided in the CAT12 toolbox was used to identify the following emotional processing structures (left and right): superior frontal gyrus, superior frontal orbital gyrus, middle frontal gyrus, middle frontal orbital gyrus, inferior frontal operculum, inferior frontal gyrus, inferior frontal orbital gyrus, superior medial frontal gyrus, medial frontal orbital gyrus, insula, anterior cingulum, hippocampus, and

Table 1 Characteristics of Our Sample

	CAMI		TCIN		Test
	HC	MDD	HC	MDD	
N	29	31	21	25	
Age	38.28 (12.40)	40.42 (9.72)	34.00 (11.63)	37.76 (13.17)	$t=-1.20$, $p=0.23$
Sex (F/M)	17/12	21/10	13/8	15/10	Chi-square = 0.21, $p=0.69$
rs1360780 (CC/T*)	13/16	16/15	9/12	10/15	Chi-square = 0.06, $p=0.85$
FKBP5 methylation	60.74 (3.36)	62.11 (3.69)	59.36 (3.77)	60.75 (4.89)	$t=-1.74$, $p=0.08$
HAMD	2 (0–7)	29 (17–45)	0 (0–5)	23 (6–31)	$U=2797.50$, $p<0.01$
BDI	1 (0–12)	31 (5–59)	1 (0–13)	34 (17–51)	$U=2193$, $p<0.01$
CTQ total	30 (25–48)	40 (25–88)	28 (25–53)	36 (25–82)	$U=2084$, $p<0.01$
Medication type		9/12/4/3/3		4/14/3/2/2	

Abbreviations: BDI, Beck depression inventory; CAMI, Centre for Advanced Medical Imaging site; CTQ, childhood trauma questionnaire; HAMD, Hamilton scale for depression; HC, healthy controls; MDD, major depressive disorder patients; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCIN, Trinity College Institute of Neuroscience site.
For our HC and MDD groups, descriptives of the following measures are given: age, sex, rs1360780 allele status, peripheral mean intron 7 methylation percentage, clinical questionnaire scores, and medication. Parametric variables are shown as mean (SD), non-parametric ones as median (minimum-maximum). Medication is expressed as untreated/SSRI/SNRI/SSRI+SNRI/other (antipsychotic or agomelatine). Statistical tests for comparisons between MDD and HC and their results are also shown.

amygdala, for a total of 13 ROIs on each side (see Supplementary Table 1 for ROI details).

Mean activity change from the first level ERT > SRT contrasts for each emotional valence, as well as mean GM concentration, were extracted for each of the ROIs. These values and estimated total intracranial volume (TIV) were then entered into SPSS Statistics version 22 (IBM Corp.) for statistical analysis.

Effect of FKBP5 methylation on brain structure and function. Generalized estimating equations (GEE) as implemented in SPSS were employed for subsequent analyses, setting an exchangeable working correlation matrix, linear scale response, and subject variable as the subject ID.

First, we ran a model for each of our 13 ROIs as follows. GM concentration was set as dependent variable (continuous). To limit the number of models in analyses, since we did not expect strongly lateralized effects of blood *FKBP5* methylation percentage, we added the within-subject variable 'ROI side' (factor with two levels: left and right) to the model. Between-subjects independent variables were age, site (CAMI, TCIN), sex, rs1360780, diagnosis, *FKBP5* methylation percentage, and TIV. All main effects as well as all possible interactions between *FKBP5* methylation, diagnosis, and rs1360780 were tested using Wald Chi-Square tests, considering significant a $p<0.05$ false discovery rate (FDR) corrected for multiple comparisons (Benjamini, 2010).

We then assessed fMRI changes in our ROIs. First of all, to confirm that our ROIs showed significant BOLD activity in the experimental conditions, we conducted one-sample *t*-tests on their mean ERT > SRT response across all participants and excluded the ROIs for which there was no significant activation from our analysis ($p>0.05$). Then, we conducted another GEE analysis, defining activation as measured by our contrast values as dependent variable (continuous). Within-subject variables were ROI side and, since we did not expect correlates of blood *FKBP5* methylation to be valence-specific, emotional valence (factor with three levels: neutral, negative and positive). Between-subjects independent variables were site, age, sex, diagnosis, rs1360780, and *FKBP5* methylation percentage. As before, all main effects as well as all possible interactions between *FKBP5* methylation, diagnosis, and rs1360780 were tested.

Finally, since we found a main effect of diagnosis for inferior frontal gyrus pars orbitalis (IFGO) activation, we tested whether this was correlated to symptoms severity within the MDD group. To do so, we ran two Spearman correlations of the residuals from the GEE model (activation corrected for site, age, sex, medication, and rs1360780) with BDI total score and HAMD total score.

RESULTS

Demographic and Clinical Measures

Demographic information of our samples along with questionnaire scores and tests are summarized in Table 1. No differences were detected regarding mean age or sex and T allele distribution between patients and controls (all tests $p>0.05$). Methylation of the *FKBP5* intron 7 CG-6 and CG-7 sites were strongly correlated ($r=0.56$, $p<0.01$).

Data from the two sites did not significantly differ for age, mean *FKBP5* intron methylation, sex, rs1360780, CTQ, HAMD, and BDI. The same was true for T* and CC participants (all tests $p>0.05$).

MDD showed higher CTQ scores compared to controls in both data sets (CAMI: $U=676.50$, $p=0.001$; TCIN: $U=387.5$, $p=0.006$).

Predictors of FKBP5 Methylation

Our GLM analysis (see Supplementary Table 2 for model information) returned a significant effect for the triple interaction between diagnosis, rs1360780, and CTQ

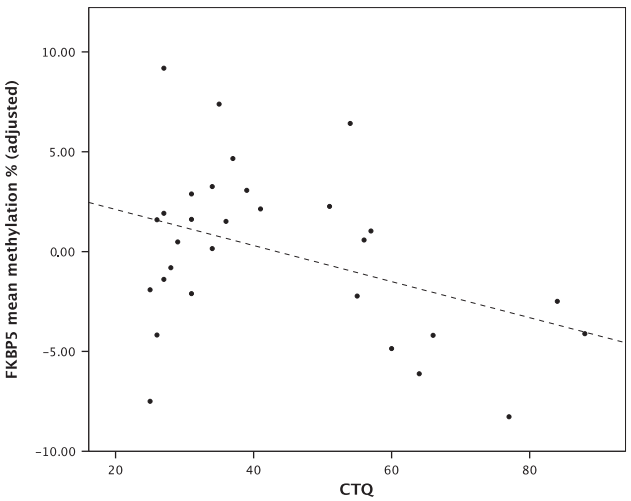

Figure 1 Correlation between peripheral mean *FKBP5* methylation in T* patients and CTQ scores. Lower peripheral DNA methylation of *FKBP5* intron 7 was associated to a higher exposure to childhood adversity in patients carrying the T allele of rs1360780 (F = 4.95, $p = 0.04$). Methylation values are adjusted for age, sex, site, and medication (residuals) and a least-squares fit line is shown. CTQ, childhood trauma questionnaire.

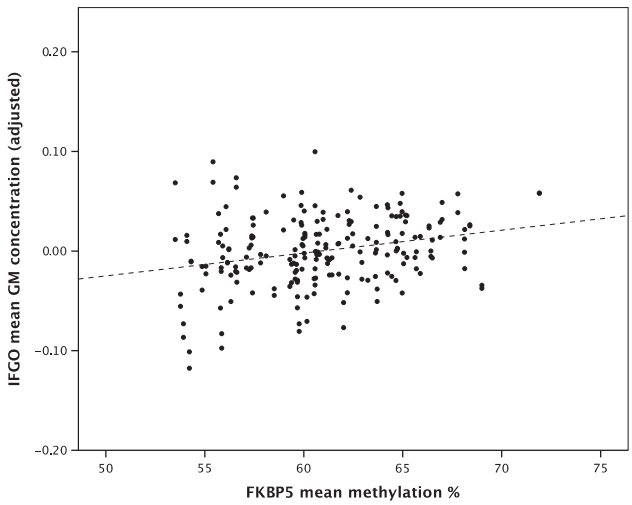

Figure 2 Correlation between IFGO mean GM concentration and peripheral mean *FKBP5* methylation across all participants. Lower peripheral DNA methylation of *FKBP5* intron 7 was associated to a lower mean gray matter concentration (Wald chi-square = 11.93, $p_{FDR} < 0.01$, to account for multiple regions). GM values are adjusted for site, age, TIV, diagnosis, sex, rs1360780 and side (residuals) and a least-squares fit line is shown. IFGO, inferior frontal gyrus pars orbitalis; GM, gray matter; TIV, total intracranial volume.

(F = 4.06, $p = 0.047$). *Post-hoc* testing revealed a significant role for higher CTQ in predicting lower mean FKBP5 intron methylation in the T* MDD group (F = 4.95, $p = 0.036$, Figure 1). Leverage values of the GLM were inspected: they were low, normally distributed (Kolmogorov–Smirnoff = 0.80, $p = 0.20$) and no outlier values (outside 1.5*IQR) in CTQ or FKBP5 were found. Residuals were also normally distributed (Kolmogorov–Smirnoff: 0.12, $p = 0.20$).

Effect of FKBP5 Methylation on Brain Structure and Function

Our GEE models investigating the role of mean *FKBP5* intron methylation in predicting GM concentration (see Supplementary Table 3 for models summary) returned a significant main effect for the IFGO across all participants (Wald chi-square = 11.93, $p_{FDR} < 0.01$, Figure 2).

All ROIs were significantly activated in the ERT > SRT contrast across all participants across all valences (all $p < 0.001$), with the exception of the inferior frontal gyrus pars opercularis ($t = -0.25$, $p = 0.80$), insula ($t = 0.36$, $p = 0.72$), middle frontal gyrus pars orbitalis ($t = -1.70$, $p = 0.09$) and superior frontal gyrus pars orbitalis ($t = -0.93$, $p = 0.35$), which showed no significant functional response to our task and were therefore excluded from the analysis.

In the IFGO, we found a significant interaction between diagnosis and FKBP5 intron methylation in predicting functional responses (Wald chi-square = 6.57, $p_{FDR} = 0.049$, see Supplementary Table 4 for models summary). To test whether this effect was valence-specific, we ran another model for this region including a methylation × valence term, as well as the main methylation effect. The interaction term was non-significant (chi = 0.318, $p = 0.853$) and the main effect of methylation remained significant (chi = 6.926, $p = 0.008$). *Post-hoc* investigation revealed that FKBP5 intron methylation showed a negative correlation with activation only in MDD regardless of valence and side (see Supplementary Table 5 for model summary, Wald chi-square = 5.58, $p = 0.02$, Figure 3).

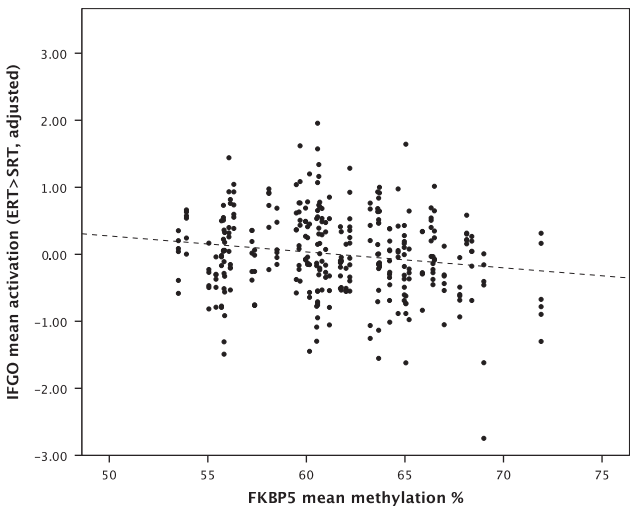

Figure 3 Correlation between IFGO mean activation and peripheral mean *FKBP5* methylation in MDD. Lower peripheral DNA methylation of *FKBP5* intron 7 was associated to a higher activation in ERT > SRT in patients (Wald chi-square = 5.58, $p = 0.02$). Activation values are adjusted for site, age, sex, medication, rs1360780, valence and side (residuals) and a least-squares fit line is shown. IFGO, inferior frontal gyrus pars orbitalis; ERT, emotional recognition trials; SRT, shape recognition trials; MDD, major depressive disorder patients.

GM concentration in the IFGO was not different between MDD and HC (Wald chi-square = 0.37, $p_{FDR} = 0.76$). However, its activation was lower in MDD compared to HC regardless of valence, age, side, and rs1360780 allele status (Wald chi-square = 3.88, $p_{FDR} = 0.049$). Its activation was also significantly negatively correlated with BDI (Spearman's

rho = − 0.143, $p = 0.009$) and HAMD (Spearman's rho = − 0.179, $p = 0.002$) in MDD (corrected for site, age, sex, medication, and rs1360780).

DISCUSSION

Firstly, our study supports the hypothesis that, in the subpopulation of MDD patients carrying the high-risk T allele of rs1360780, lower methylation of *FKBP5* introns is correlated to higher chronic stress exposure in early life. This finding is supported by numerous studies, ranging across several chronic stress conditions (Tyrka *et al*, 2016,2015; Provencal and Binder, 2015; Resmini *et al*, 2016; Klengel *et al*, 2013). We therefore expand to patients showing depressive symptoms the notion that *FKBP5* demethylation is a correlate of exposure to childhood adversity, highlighting the role of rs1360780 as a moderator of this effect.

Furthermore, across all participants, lower *FKBP5* intron methylation levels were associated with reduced GM concentration in the inferior frontal orbital gyrus. This region has been associated with response inhibition in general (Chikazoe *et al*, 2007) and, in particular, reappraisal and modulation of negative emotion (Goldin *et al*, 2008). Overall, structural changes in the orbitofrontal cortex in MDD are a common finding and have been confirmed at a meta-analytical level (Kempton *et al*, 2011). In rodents, exposure to chronic stress was associated with *FKBP5* gene demethylation and hyper expression in the prefrontal cortex (Guidotti *et al*, 2013; Lee *et al*, 2010). Also, in a recent study comparing youths with post-traumatic stress disorder and controls, a negative association between GM in the orbitofrontal cortex and evening cortisol levels across all participants was shown, regardless of diagnosis (Carrion *et al*, 2010). Taken together with these previous findings, our results suggest that *FKBP5* methylation might be related to GR function in chronic stress conditions and might influence the structural integrity of the inferior frontal orbital gyrus in both MDD and HC. Since cortisol measures were not available and childhood adversity was considerably more present in our depressed patients, we can provide supporting evidence for this theory only in our MDD T* group, in which *FKBP5* intron demethylation was specifically explained by exposure to childhood adversity.

We found activation of the IFGO to be reduced in MDD patients compared to HC, and to be inversely correlated with the self-reported intensity of symptoms. Interestingly, in MDD patients, *FKBP5* methylation was negatively correlated with IFGO hemodynamic response during our emotional recognition task regardless of valence. This finding is surprising, since after observing that lower *FKBP5* methylation was associated with lower GM in the IFGO, we expected it would rather mirror a reduction of function in this region. In addition, the lack of valence-specificity of the prediction of methylation on functional responses could hint at a change which affects the area's function in general. It is possible that the heterogeneity of our findings could reflect one of the underlying biological pathways leading to MDD. A subset of patients, for example, who could have developed it after enduring chronic stress exposure, might show lower *FKBP5* methylation, less symptoms and higher functional responses to emotional evaluation. The consequence for the clinical outcome of these patients would be interesting to know, but our cross-sectional data do not allow us to investigate this aspect. On the other hand, HPA axis dysregulation has been associated with more severe depressive symptoms intensity in the past (Burke *et al*, 2005; Guidotti *et al*, 2013). Since the effect of medication approached significance in predicting higher activity in the IFGO ($p = 0.07$), another possibility might be that *FKBP5* epigenetics could play a role in successful response to antidepressant therapy, although methylation was not different between treated and untreated patients in our sample, nor in Han *et al* (2017).

This study is not without limitations. First of all, it is necessary to address the fact that most of our patients were medicated and, although we corrected for medication use in all of our models, we cannot exclude that our results might be confounded by this factor. Secondly, we used data from two cohorts collected at two sites where MRI measurements were conducted using different sequences and receiver coils (although both sites used the same scanner model and, crucially, the TR for the functional acquisition was the same). Pooling data from different sites allowed us to increase our sample size and we tried to minimize differences between them by using the same processing pipeline and correcting for site in all our analyses. However, we cannot exclude that differences between sites might have confounded our results. Thirdly, our results differ from those reported by Han *et al* (2017) in a recent similar study. This might be due to several reasons, such as the use of different methods (VBM and fMRI compared to automated cortical segmentation using Freesurfer) and a different choice of ROIs and statistics. However, the present study addresses some of the limitations highlighted in Han *et al* (2017), for example the investigation of the role of childhood trauma in predicting *FKBP5* intron methylation, which we consider valuable for the interpretation of our findings. Furthermore, both studies are in agreement in highlighting the inferior frontal gyrus as a crucial site for the role of *FKBP5* on brain structure. Also, we did not have reliable measures of HPA axis function, such as cortisol levels. Another important point to be addressed is the still not well-known association between peripheral and central *FKBP5* methylation. The results of the present study suggest that peripheral *FKBP5* methylation is linked to CNS structure and function, but experimental research on this link is necessary. Finally, we did not detect any significant findings in the hippocampus. This region has been shown to be smaller in MDD (Schmaal *et al*, 2016; Frodl *et al*, 2002). Its volume was also positively correlated to FKBP5 methylation in Cushing's syndrome patients (Resmini *et al*, 2016) and its shape was different between PTSD carrying the T allele of rs1360780 (Fani *et al*, 2013). This might suggest that this structure does not show strong effects of FKBP5 regulation in MDD compared to other forms of stress exposure, such as Cushing's syndrome. The effects could also be weaker in this disorder and would need a larger sample size to be detected. To elucidate the matter, targeted studies should be conducted on this region, perhaps using dedicated segmentation methods to account for its structural complexity, such as those provided in the Freesurfer suite (https://surfer.nmr.mgh.harvard.edu/).

Overall, future studies might benefit from the investigation of the interaction between known genetic variants of *FKBP5*

and its methylation in large cohorts of medicated and unmedicated MDD patients, carefully controlling for childhood trauma levels, directly assessing HPA axis function and focusing on imaging the inferior frontal gyrus.

To summarize, in a subgroup of MDD patients carrying the high-risk allele of the *FKBP5* rs1360780 SNP, exposure to childhood trauma was inversely correlated to peripheral DNA methylation level of this gene. Across all participants, lower *FKBP5* methylation was associated with decreased GM in the inferior frontal orbital gyrus. In the MDD group, lower methylation correlated with its higher activation. This region is linked with emotional regulation and was functionally hypoactive in MDD in our sample, with its activation being negatively correlated with self-reported symptoms severity. Our findings suggest that epigenetic changes of *FKBP5* might be a link connecting the interaction of genetic and environmental risks with brain changes in an area relevant for the clinical symptoms of depression.

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