Grey matter volume in adolescent anxiety: an impact of the Brain-derived neurotropic factor Val^{66}Met polymorphism?

Sven C. Mueller, PhD, Aveline Aouidad, MD, PhD, Elena Gorodetsky MD, PhD, David Goldman MD, Daniel S. Pine, MD, Monique Ernst, MD, PhD.

Dr Mueller is with the Department of Experimental Clinical and Health Psychology, University of Ghent, Belgium and with the Section on Development and Affective Neuroscience, NIMH, NIH, Bethesda, MD, USA; Dr. Aouidad is with the Pierre & Marie Curie Faculty of Medicine, Sorbonne Université, Paris, France; Dr Gorodetsky is with the Mood and Anxiety Disorders Program, NIMH, Bethesda, MD, USA; Dr. Goldman is with the Laboratory of Neurogenetics, NIAAA, Rockville, MD, USA, and Drs. Pine and Ernst are with the Section on Development and Affective Neuroscience, NIMH, NIH, Bethesda, MD, USA

The corresponding author is Dr. Mueller, Ph.D., Department of Experimental Clinical and Health Psychology, Ghent University, Henri Dunantlaan 2, B-9000, Ghent, Belgium, Phone: +32-09-264.86.22, Fax: +32-09-264.64.89, Email: Sven.Mueller@UGent.be

Abstract word count: 188

Manuscript word count: 5857

Number of Tables / Figures / supplementary material: 2 / 3 / 0

Keywords**:** BDNF; VBM; adolescence; anxiety; insula

Acknowledgements

The study was funded by the intramural program of the NIMH and an NIMH Richard J. Wyatt memorial fellowship award to SCM. We acknowledge the assistance of Colin Hodgkinson, Pei-Hong Shen and Longina Akhtar (all National Institute on Alcohol Abuse and Alcoholism, NIH, Rockville, MD, USA).

Abstract

Objective: Minimal research links anxiety disorders in adolescents to regional gray matter volume (GMV) abnormalities and their modulation by genetic factors. Prior research suggests that a brain-derived neurotrophic factor (BNDF) Val^{66}Met polymorphism may modulate such brain morphometry profiles. **Method**: Using voxel-based morphometry and magnetic resonance imaging, associations of BDNF and clinical anxiety with regional GMVs of anterior cingulate cortex, insula, amygdala, and hippocampus were examined in 39 affected (17 Met allele carriers, 22 Val/Val homozygotes) and 63 non-affected adolescents (33 Met allele carriers, 41 Val/Val homozygotes). **Results**: Amygdala and anterior hippocampal GMVs were significantly smaller in patients than healthy adolescents, with a reverse pattern for the insula. Post-hoc regression analyses indicated a specific contribution of social phobia to the GMV reductions in the amygdala and hippocampus. Additionally, insula and dorsal-ACC GMVs were modulated by BDNF genotype. In both regions, GMVs were larger in the Val/Val homozygote patients than in those carrying the Met allele. **Conclusions**: These results implicate reduced GMV in the amygdala and hippocampus in pediatric anxiety, particularly social phobia. In addition, the data suggest that genetic factors may modulate differences in the insula and dorsal ACC.

Introduction

To date, few studies have examined gray matter volume (GMV) abnormalities in pediatric anxiety disorders[1-3] in comparison to the larger literature in adults.[4, 5] These pediatric studies mostly focused on the medial temporal lobe including the amygdala and hippocampus. Importantly, disagreement exists with regards to directionality of effects and diagnostic specificity. While some studies in pediatric generalized anxiety disorders (GAD) report enlarged amygdala volumes for the patient relative to the healthy comparisons,[1] other studies in pediatric mixed samples, with GAD, separation anxiety disorder, and social phobia, report reduced amygdala volume in the patient group relative to comparisons.[3] In addition to alterations of medial temporal structures, more recent evidence implicates the anterior cingulate cortex (ACC) and the insula in adults with clinical anxiety.[5, 6] These findings, however, are also mixed. [5] Critical to the present work, no reports have yet documented alterations in these latter structures in pediatric anxiety samples.

Some of the inconsistency in findings can be related to unstable data in small samples, but also to genetic modulation. For instance, recent work highlights a modulation by the common BDNFVal[66]Met polymorphism of brain structures relevant to anxiety.[7-9] In this single nucleotide polymorphism (SNP, rs 6265), a critical amino acid valine-to-methionine substitution at codon 66 (Val66Met) has been shown to reduce activity-dependent secretion of BDNF[9], a gene critically contributing to neuronal survival, migration, dendrite pruning and axonal growth.[10] Although BDNF is expressed throughout the cortex, high expression of this gene has been documented in the hippocampal formation, the prefrontal cortex and the ACC.[9, 11, 12] Notably, as a result of reduced BDNF expression in carriers with at least one Met allele, previous research has reported structural reductions of the amygdala, hippocampus, and ACC.[8, 13, 14] Furthermore, a recent functional imaging study in adolescents reported that the Val[66]Met polymorphism modulated amygdala and hippocampal function during emotional processing differentially in healthy and clinically-anxious adolescents.[15] These preliminary findings call for more work to further validate and better characterize associations among behavior, genetics, and brain structure in youth[16], given the known role of the BDNF gene in development.[10, 17] Specifically in the context of anxiety, the potential influence of the BDNF Val[66]Met polymorphism on brain structure has not yet been examined in youth.

The goal of the present study is to examine in healthy and clinically anxious adolescents 1) GMV abnormalities in pediatric anxiety, including diagnostic specificity, and 2) the influence of BDNF genotype on the four brain structures most commonly implicated in anxiety disorders, the ACC, hippocampus, amygdala, and insula.

With regards to neuroanatomical differences between patients and healthy comparisons, we hypothesize that significant GMV alterations of the amygdala and hippocampus would be found in anxious relative to comparison adolescents based on previous structural findings in adolescents[3] and adults.[4] With regards to effects of genotype, consistent with a role of the BDNFVal[66]Met polymorphism in modulating anxious behavior[7, 15], we expected volumetric reductions in patients with the risk Met-allele relative to patients with the Val/Val genotype. By contrast, based on a larger study in unaffected adolescents[18], we did not expect such volumetric reduction in healthy comparisons carrying the Met-allele relative to the Val/Val homozygotes.

**********Insert Table 1 about here please **********

Methods

Participants

Thirty-nine adolescents diagnosed with an anxiety disorder and 63 healthy control adolescents underwent structural neuroimaging (Table1). None were on medication at the time of testing. No morphometry data have been reported previously in these samples. Patients were recruited at the National Institute of Mental Health. Both patients and healthy comparison subjects completed a medical, physical and psychiatric assessment. Psychiatric status was assessed via a standardized, semi-structured psychiatric interview (Kiddie Schedule for Affective Disorders and Schizophrenia – Present and Lifetime version, K-SADS-PL).[19] This interview was performed by experienced clinicians, who had been trained to exceed a minimally acceptable inter-rater reliability of $k > .75$. Inclusion criteria for patients consisted of a diagnosis of an anxiety disorder and individual diagnoses were subsequently confirmed by expert review by a senior psychiatrist. To minimize confounding from other psychopathology, exclusion criteria consisted of a diagnosis of current obsessive-compulsive disorder, lifetime history of PTSD, mania, psychosis, substance use or pervasive developmental disorder. Healthy comparison subjects were recruited by advertisement in local newspapers with inclusion criteria defined by absence of psychiatric or neurological disorder, substance use, or medication as determined by the K-SADS. The Full-Scale IQ scores were prorated based on the Vocabulary and Block Design subtests of the Wechsler Intelligence Scales for Children[20] and

IQ > 70 for both groups. The Institutional Review Board of the NIMH approved the study. Parents signed consent forms and adolescents signed assent forms after being explained the study in detail.

Participants were characterized based on genotype and diagnostic status. Among the 39 patients, 15 were Val/Met heterozygotes, 2 Met/Met homozygotes, and 22 Val/Val homozygotes. Among the 63 healthy comparisons, 25 were Val/Met heterozygotes, 2 Met/Met homozygotes, and 36 Val/Val homozygotes (Table 1). Because of the rarity of Met homozygotes and following the grouping strategy used by previous investigators, [13] the Met/Met homozygotes were combined with the Val/Met heterozygotes to form a group of 17 Met carriers for the patient group and 27 Met carriers for the healthy comparison group. To assess whether the study sample size was sufficiently large to detect a reliable Group by Genotype interaction, a post-hoc power analysis using G*Power3[21] revealed an acceptable power level of .808. Power for the main group comparison was .894. Importantly, no differences emerged in genotypic distribution across patients and controls ($\chi 2(1)<.01$, p=.942), or genotype frequencies (Hardy–Weinberg equilibrium test, $\chi 2(1)=0.82$, p=.375). The between-group comparisons of demographic variables revealed trending differences on age (F(1,100)=3.78, p=.055) and IQ (F(1,100)=3.30, p=.072) between patients and comparisons, but no differences on sex distribution ($x^2(1) = 1.38$, p=.240), or socio-economic status (F(1,92)=1.15, p=.286) (SES, Hollingshead). Therefore, age and IQ were used as covariates of nuisance in all analyses. In addition, although total brain volume did not differ between patients and controls (F(1,100)=1.94, p=.167) or among the four genetic groups (F(3, 98)=0.83, p=.482), this factor was included as a covariate to account for inter-individual variability. Thus, individual variability in age, total brain volume and IQ was controlled for in all analyses. In addition, main effects of these variables as well as their potential influences on the findings were also assessed. Finally, patients in the Met and Val/Val group did not differ on any type of anxiety disorder (all p>.205)(Table1).

To detect potential effects of population origin, we relied on objective, genetic ethnic ancestry factor scores rather than self-report questionnaires. For each participant, seven ancestry factor scores were calculated in the context of worldwide ancestry based on 1052 individuals and 51 populations represented in the Human Genome Diversity Panel (HGDP) and genotyped for the same panel of 186 ancestry informative markers (AIMs). This AIMs panel yields a seven- factor solution for continental and certain sub-continental populations that has been robustly observed across multiple studies.[22] No significant differences in ancestry factor scores emerged between the patient and control group or the four BDNF genotype-by-diagnosis groups (all p >.05)(Table 1). In addition, full regression

analyses were run with the European and African ancestry factor scores included in the model, because these were the two principal ancestry components in this sample. Ethnic ancestry factor scores did not predict volumetric variation.

Single-Nucleotide Polymorphism (SNP) genotyping

Genomic DNA was extracted from EBV-transformed lymphoblastoid cell lines or untransformed white blood cells using Gentra Versagene Cell kits according to the manufacturer's protocol. Genotyping was performed using the Illumina GoldenGate SNP genotyping technology, determining haplotype coverage on the 1536 SNP NIAAA Addictions Array, with a 95% completion rate. Genotyping error was determined by replicating genotyping in 10% of the sample (error rate <0.005). BDNF $Val^{66}Met$ (rs6265) genotyping was confirmed using a Taqman Assay-on-Demand (Applied Biosystems, Foster City). Genotyping was performed according to the manufacturers protocol and genotype determined at end-point using an ABI 7900HT Sequence Detection System. Genotyping accuracy was determined empirically by duplicate genotyping of 25% of the samples selected randomly. The error rate was <0.005, and the completion rate was >0.95. These analyses showed 100% concordance across genotyping samples.

MRI data acquisition

Whole brain, high-resolution T1-weighted anatomical images were acquired on a 3 Tesla General Electric Signa Scanner (Waukesha, Wisconsin). For reasons independent of the present work, the Magnetic Resonance Imaging (MRI) data were collected under two acquisition sequences, but on the same scanner. A first set of subjects (n=78) were scanned using an MPRAGE sequence consisting of 124 1.2 mm axial slices (no-gap), Field of View (FOV) = 220 mm, matrix = 256 x 192, time to inversion (TI) = 725 ms, flip angle 6 deg, voxel size of 0.86 mm x 1.15 mm x 1.2 mm. A second set of subjects (n=24) were scanned using an FSPGR sequence consisting of 124 1.2 mm axial slices (no-gap), Field of View (FOV) = 240 mm, matrix = 256 × 256, flip angle 15 deg, voxel size 0.94 mm x 0.94 mm x 1.2 mm. To avoid the potential confound of using two different acquisition sequences, there was an equal distribution of scan sequences among the four genotype groups ($\chi^2(3)=1.68$, p=.642) and between patients and comparisons ($\chi^2(1)=0.76$, p=.381). Moreover, scan sequence was included as a covariate of no interest in the analyses.

MRI processing

Prior to processing, data were examined for motion artefacts (ghosting, blurring) and anatomical abnormalities. In case of presence of such artefacts, data were excluded from the study sample. MRI data was preprocessed and analyzed using SPM8 (http://www.fil.ion.ucl.ac.uk/spm) and the Diffeomorphic Anatomical Registration using Exponentiated Lie algebra (DARTEL) toolbox.[23] The image origin was set at the anterior commissure (AC). Structural imaging data were segmented into white matter, gray matter, cerebral spinal fluid, skull, and soft tissue classes outside the brain. These images were inspected for the quality of segmentation before a study-specific brain template was created from all individual tissue-class images of each subject using iterative realignments. Data were then normalized and warped to an isotropic voxel size of 1.5 mm x 1.5mm x 1.5 mm. Afterwards, the data were modulated by the Jacobian transformed tissue probability maps to obtain "volume" differences rather than "concentration" differences in gray matter.[24] Finally, data were checked for homogeneity across the sample, spatially normalized to the Montreal Neurologic Institute coordinate reference system and smoothed with a 10 mm (FWHM) isotropic Gaussian kernel.

Voxel-Based Morphometry (VBM) analysis

Based on strong *a priori* hypotheses of altered GMV volume in 4 regions associated with anxiety (amygdala, hippocampus, ACC, insula), an independent Region-Of-Interest (ROI) approach was adopted. We corrected for multiple comparisons using small volume correction (SVC) with a Gaussian random field threshold set at alpha $\alpha = .05$, corrected and an extent of at least 10 contiguous voxels. Coordinates are reported in MNI space in mm [x, y, z]. All anatomical ROIs were created using standardized anatomical criteria[25, 26] on the MNI template, which were then applied to the normalized brain, at the group level. The ACC was hand-traced by two experts with 100% agreement, while the amygdala and hippocampal ROI were drawn using a semi-automated computerized system with intraclass correlations between 2 operators reaching .80 to .97[25]. Finally, the insula ROI was created using the Anatomical Automatic Labeling (AAL) system[26]. The main analysis consisted of a full-factorial 2 x 2 Multivariate Analysis of Covariance (MANCOVA) with Group (healthy vs. patients) and BDNF Genotype (Val/Val vs. Met) as the between-subject factors, and age, IQ, scan sequence, and whole brain volume as covariates of nuisance. Due to potential problems of non-stationarity in VBM analyses, use of cluster-size p-values is not

recommended and individual peak voxels are reported instead.[24] These significant ($p<.05$, corrected) peak voxels were extracted and further analyzed with SPSS v.19 (Armonk, NY). However, to prevent spurious findings associated with outliers, we screened for and removed all outliers (2 standard deviations above or below the mean, range 1-4 outliers) from the analyses. To account for multiple testing in these follow-up analyses, multiple comparisons were corrected using the conservative step-down Holm procedure. Thus, only significant findings that survived the additional adjustment for multiple comparisons are presented. To assess the magnitude of a significant between-group effect, effect sizes (Cohen's *d*) were calculated. To examine diagnostic specificity on the GMV reductions in the amygdala and hippocampus, a regression analysis was performed, which coded for presence or absence of a specific anxiety disorder (Generalized Anxiety Disorder [GAD], Separation Anxiety Disorder [SAD], Social Phobia [SP]). To evaluate the potential impact of co-morbid depression on the findings, all analyses were re-run excluding the subjects with co-morbid MDD. In a third set of analyses, the potential impact of age, IQ, and sex was examined. To assess impact of symptom severity on the findings, regression analyses were conducted between the significant voxels and anxiety scores in the patient group.

***************Insert Figure 1 about here please *****************

***************Insert Table 2 about here please********************

Results

The MANCOVA revealed (1) a significant main effect of Group, and (2) a significant Group-by-Genotype interaction.

1) Neuroanatomic differences between patients and comparisons

Comparisons > Patients

Significant main effects of Group revealed reduced GMV in anxious adolescents relative to healthy adolescents in the right amygdala with a large effect size [28, 0, -18]($F(1,94)=9.84$, $p=.002$, $d=.876$) and right anterior hippocampus with a medium-to-large effect size [27, -4, -18]($F(1,95)=6.65$, $p=.011$, $d=.752$)(Figure 1).

********** Insert Figure 2 about here please **********

Patients > Comparisons

Significant volume increases were seen in two clusters in the left insula [-46, 12, -14] (F(1,94)=12.15, p=.001, *d*=0.203) and [-48, 27, 3](F(1,92)=4.11, p=.045, *d*=.326) and two clusters in the right insula [52, 11, -8](F(1,96)=8.98, p=.003, *d*=.19) and [40, 30, -5](F(1,94)=8.79, p=.004, *d*=.372), all with smaller effect sizes than for the medial temporal lobe (Figure2). No main effects in either direction were found in the ACC. Importantly, the insula and the ACC revealed significant Group-by-Genotype interactions.

**********Insert Figure 3 about here please**********

Diagnostic Specificity

Amygdala and Hippocampus

To investigate to what extent diagnostic specificity may have contributed to the amygdala and hippocampal GMV reductions in anxious vs. healthy adolescents, post-hoc regression analyses were run on these findings coding each type of anxiety disorder (GAD, SAD, SP) as a dichotomous variable (present, absent). Of these, 16 subjects were non-SP GAD patients, 16 non-GAD SP patients and 6 comorbid GAD/SP. Of the SAD patients, 7 were co-morbid with GAD, 3 co-morbid with SP, 1 had all three comorbidities and 1 had no other diagnoses. These analyses revealed that only diagnosis of SP was predictive of a smaller right amygdala [28, 0, -18](t=-2.96, p=.004), with no effects for GAD (p=.061) or SAD (p=.859). A similar picture emerged for the right anterior hippocampus cluster with a significant effect for SP [27, -4, -18] (t=2.58, p=.012), and no effects for GAD (p=.253) or SAD (p=.657).

Insula

To examine to what extent the GMV increases in the insula were driven by a specific diagnosis, regression analyses revealed that only one cluster in the right insula GMV [40, 30, -5] was associated with SP (t=2.25, p=.027) but not SAD (p=.312) or GAD (p=.464). No other associations with insula volume were found.

2) Impact of BDNF genotype: Group-by-Genotype interaction

*S*ignificant Group-by-Genotype interactions emerged in the right insula and right ACC (Figure 3, Table 2). Within the insula ROI, one cluster was significant in the posterior insula [48, -3, -12] ($F(3,90)=5.40$, $p=.002$) and one in the anterior insula [45, 17, 12]($F(3,91)=3.62$, $p=.016$). Within the ACC, a significant cluster emerged in the dorsal ACC (dACC) [3, 30, 27]($F(3,92)=4.24$, $p=.007$). To interpret these Group-by-Genotype interactions, we conducted two sets of follow-up analyses in these two ROIs. First, each diagnostic group was analyzed separately. Then, analyses were performed within genotype groups.

Insula ROI

Within the anterior insula cluster [45, 17, 12], anxious Met showed smaller GMV than anxious Val/Val ($F(1,32)=9.40$, $p=.004$, $d=.787$)(Figure 3). In the posterior insula cluster [48, -3, -12], GMV differed significantly between anxious Met and anxious Val/Val patients ($F(1,32)=7.93$, $p=.008$, $d=.569$). When GMV was compared within genotype, healthy Met carriers exhibited greater GMV than patient Met carriers ($F(1,37)=10.53$, $p=.002$, $d=.869$).

ACC ROI

GMV was larger in anxious Val/Val homozygotes when compared to healthy Val/Val-homozygotes in the dorsal ACC ($F(1,52)=10.77$, $p=.002$, $d=.455$). There were no significant between-group effects within the Met genotype (Figure 3).

In summary, the interaction of BDNF genotype and Group reflected (1) in the anxious group, smaller anterior and posterior insula volumes in the Met-carriers relative to Val/Val-homozygotes, and (2) no gene-related differences in the healthy group. Furthermore, within genotype, healthy Met-allele carriers had larger GMV in the posterior insula than patients with the Met allele.

<u>Additional exploratory analyses</u>

Effects of Age, IQ, or sex, anxiety symptom severity

To examine the potential influence of age, sex, IQ, or symptom severity on the current findings, main effects as well as interactions of these variables were examined in relation to group status and BDNF genotype. No significant main effects of any of these variables were detected (all $p>.05$). Importantly, no interactions with group or genotype were significant (all $p>.05$). Finally, regression analyses did not reveal a significant impact of severity of anxiety on the findings (all $p>.05$).

Psychiatric co-morbidity with MDD

To examine whether findings were driven by the patients with co-morbid MDD (n=8), the data were reanalyzed for all ROIs without these subjects. Findings remained significant for all comparisons, i.e., group effects on amygdala ($F(1,87)=7.03$, $p=.010$), hippocampus ($F(1,88)=4.64$, $p=.034$), and insula (all clusters: $F(1,84)>5.91$, all $p<.017$); and gene-by-group effects for all regions (all $F(3,82)>5.56$, all $p<.010$). This suggests that co-morbid depression did not significantly impact the findings.

Discussion

This structural MRI study of anxious and healthy adolescents examined GMV in four regions, insula, ACC, amygdala, and hippocampus, all previously implicated in anxiety disorders. In addition, preliminary evidence for a role of the BDNF Val66Met polymorphism in the modulation of anxiety-related structural differences was explored. Four main findings emerged. First, GMVs of the right amygdala and right anterior hippocampus were smaller in patients relative to comparisons, regardless of genotype. Second, additional regression analyses suggested some specificity of these GMV volume alterations to social phobia. Third, in contrast to the amygdala and hippocampus findings, insula volume was larger in the patient group relative to comparisons. Fourth, right-lateralized dACC and insula volume appeared to be modulated by the BDNF $Val^{66}Met$ polymorphism differentially in anxious adolescents and in comparisons. GMVs of the insula and ACC were smaller in the risk Met-allele carriers relative to the Val/Val homozygotes in the patient group, whereas comparisons showed the reverse effect.

To date, the few studies examining GMV abnormalities in pediatric anxiety report mixed results showing either GMV increases[1] or decreases[3] in patient relative to comparison groups. Moreover, these studies also differed in methodology using either VBM[3] or a manual-tracing approach[1] to measure volumetric changes. The first main

finding of the present study revealed reduced volumes of the amygdala and hippocampus in the anxious group relative to the comparison group. These data are consistent with, and form an independent replication of, an earlier study in a smaller group of clinically anxious adolescents (N=17; mean age 13 yo),[3] extending prior work in adults.[27-29] The current data also demonstrate reduced GMV of the hippocampus, in addition to the amygdala, a finding not reported previously in adolescents. One possible factor that may have contributed to the mixed findings in previous studies is the potential impact of diagnostic specificity on GMV alterations. Indeed, while amygdala GMV reductions were found in a mixed sample of pediatric anxiety disorders (GAD, SAD, SP),[3] amygdala and superior temporal gyrus volumetric increases were found in pediatric post-traumatic stress disorder[30] and GAD.[1,2] When diagnostic specificity was examined in relation to the current findings, the data suggested a specific relationship of the amygdala and hippocampal GMV reductions with SP, but not SAD or GAD. While these findings should be considered tentative, they suggest a contribution of SP to GMV reductions in the amygdala and hippocampus, at least in adolescents, and warrant replication in much larger samples of individuals with specific anxiety disorders.

The mechanisms that might produce structural abnormalities in the present study remain unclear. However, prior findings of functional changes in adolescent mood and anxiety disorders might provide clues.[31, 32] For example, a recent study in adolescent bipolar disorder reported an inverse relationship between amygdala function during emotion processing and amygdala structure.[33] Such findings are consistent with the suggestion that over-activation of the amygdala reflects glutamatergic excitotoxicity, which would lead to volume loss over time.[34] However, no direct evidence of excitotoxicity exists in available research on pediatric anxiety disorders and more work is needed in this area. Moreover, existing work focuses selectively on the amygdala, raising questions on the degree to which similar or different mechanisms might also explain anxiety-related gray-matter-volume increases in other regions.[2, 35] Enhanced interoceptive awareness has been postulated in anxiety[6] and is a key function of the anterior insula.[36] Underlying mechanisms of such disturbance in interoceptive awareness could manifest as increases in insula volume. Of note, current findings in the amygdala and hippocampus were right-lateralized. Such an effect is certainly consistent with prior reports of structural[1] and functional[32] perturbations in the right but not left amygdala associated with anxiety. Although left-lateral alterations in the current study were present at a sub-threshold level, the current data are also in-line with previous reports of larger perturbations in volume on the right

relative to the left side in other structures in pediatric anxiety.[2] These findings lend further support to laterality theories of emotion, which posit a special role of the right side of the brain in the processing of negative emotions.[37]

The third main finding suggests that BDNF genotype modulates GMV in the dorsal portion of the ACC (dACC) and insula differently in clinically anxious and healthy youth. Specifically, the regional selectivity of the dACC and insula suggest these regions may be particularly sensitive to the action of BDNF in adolescence, in a way that may harbor risk for anxiety. Regarding the dACC, studies in several cohorts of anxiety disorders (SP, specific phobia, GAD) document abnormalities in dACC function.[32, 38, 39] A previous pediatric functional MRI study reports elevated responses in this region [Talairach: 4 6 40] to fearful expression in youths with GAD relative to comparisons.[32] In addition, adult patients with SP show abnormal dACC responses to negative social conditions, such as negative comments[38] [Talairach: -13 3 44] or in anticipation of public speaking [Talairach: -24 24 24 or -20 4 28].[39] Finally, dACC response [Talairach*: 0 16 47] to implicit fear is modulated by BDNF polymorphism, with greater activation in Met carriers.[40] Our findings resonate with these studies, and provide further support for a role of dACC in anxiety disorders in adolescence, as well as a potential to be uniquely modulated by the BDNF Val[66]Met polymorphism in anxious adolescents.

The anterior insula is considered to be a primary contributor to anxiety in adults.[6] By comparison, functional neuroimaging studies of anxiety in adolescents have to date focused primarily on the amygdala.[15] The present finding further highlights the relevance of extending this focus to the insula in pediatric studies. Noteworthy, the Val[66]Met polymorphism modulated the volume of both the anterior and posterior insula regions in anxious, but not healthy adolescents. This functional link between the two insula regions is particularly relevant to anxiety, based on the hypersensitivity of anxious individuals to bodily signals.[6] The posterior insula integrates interoceptive signals,[41, 42] which in turn are carried to the anterior insula for integration, a process that may be excessively reinforced in anxiety.[36]

Collectively, these preliminary findings implicate BDNF in modulation of dACC and insula GMV in anxious adolescents. Such findings raise questions regarding the specific aspects of anxiety that are influenced by this genetic modulation, and the extent to which this modulation is specific to the adolescent period. Such genetic effect might be exploited therapeutically in the future, given recent evidence that a variant of the BDNF gene is associated with improved therapeutic outcome with serotonin reuptake inhibitors in anxious adult patients.[43]

Finally, this pediatric study may inform on developmental aspects of gene-brain associations. Our analyses failed to detect a genotypic modulation of amygdala or hippocampal volumes in healthy adolescents, in contrast to previous work in healthy adults.[8, 13] Yet, this discrepant finding is congruent with the study by Toro et al.[18] who found no significant impact of the Val[66]Met polymorphism on amygdala or hippocampal volume in 331 healthy adolescents. Similarly, Casey et al.[44] reported, in healthy children, the absence of BDNF genotypic effect on amygdala volume but reported a trend towards smaller hippocampal volume in the Met-allele carriers compared to the Val/Val homozygotes. Taken together, these studies suggest that the morphometry of the amygdala and hippocampus might start to be differentially affected by BDNF gene variants only in adulthood. This maturational change might be related to the ontogenic trajectory of BDNF levels,[35] or to changes in cellular receptivity to BDNF action with age. The fact that BDNF genotype affects brain structure in adolescents with psychopathology but not in healthy youths may be related to neurochemical changes associated with the pathology. For example, perturbations in BDNF levels may contribute to structural abnormalities in psychopathology by influencing critical protein (reelin) or receptor (TrkB) activity fluctuations in anxiety that further influences brain developmental processes such as neuronal differentiation or cortical lamination.[12] As a result of altered BDNF expression, possibly because of change in glutamate signaling,[12] individuals with anxiety or other psychopathology would then demonstrate perturbed structural neural development during the adolescent period. More research is needed to determine the influence of these molecular processes on brain maturation. However, given the absence of findings in several independent studies,[18, 44] an alternative explanation for the absence of an influence of the Val66Met polymorphism on healthy youths could be the potential presence of false positives in this prior line of work in adults. In any case, this study stresses the importance to understand the functional and clinical significance of the developmental effect of BDNF variant on brain morphometry, particularly in relation to the onset of psychiatric disorders.

Some limitations require consideration. One limitation concerns the relatively small number of subjects in the patient BDNF Met group (N=17). This limitation is mitigated by a few factors. Importantly, a power analysis revealed an acceptable level of power to detect reliable differences in the Group by Genotype interaction. In addition, the current study includes a relatively large number of patients, coupled with the fact that all patients were seeking treatment for acute, severe anxiety symptoms, and none were on medications. Medication-free, acutely ill subjects are difficult to recruit. In fact, small sample sizes have been the rule in prior research with such samples. Second, candidate gene studies need to be interpreted with caution. The association between a disorder and a gene is

complex due to a variety of possible influences including gene-gene and gene-environment interactions. Thus, replication in large samples is necessary to confirm findings and gain insight into the contribution of a gene to psychopathology. A third limitation relates to the use of two different scanning acquisition sequences. This concern is moderated by the fact that all groups were well-matched on scan sequence, and findings did not differ between scan sequences. Finally, we did not assess parental psychopathology, which was beyond the scope of the current study. However, future studies should include such measures as they could inform neurobiological predictors of at-risk individuals.

In summary, the current findings suggest that GMV reductions in the amygdala and anterior hippocampus are already present in adolescents with an anxiety disorder. Importantly, this is the first study to show in adolescents, that dACC and insula volume are modulated by the BDNF Val^{66}Met polymorphism, thus extending prior structural work in adults[8, 13] and functional work in adolescents.[15]

Note: * for easier comparison, MNI coordinates were converted to Talairach coordinates using WFU Pickatlas.

Clinical Guidance

- Few studies on gray matter volume changes in adolescent anxiety while genetic impact has not been examined
- BDNF is critically involved in developmental processes and has been linked to mood and anxiety disorders in adults
- Gray matter volume of patients with anxiety disorders was reduced in amygdala and anterior hippocampus relative to comparison adolescents while insula volume was slightly increased
- However, BDNF genotype modulated insula volume, which showed a smaller volume in anxious individuals with the Met allele relative to Val/Val homozygous patients

References

1 De Bellis MD, Casey BJ, Dahl RE, et al. A pilot study of amygdala volumes in pediatric generalized anxiety disorder. Biol Psychiatry. 2000;**48**(1):51-7.

2 De Bellis MD, Keshavan MS, Shifflett H, et al. Superior temporal gyrus volumes in pediatric generalized anxiety disorder. Biol Psychiatry. 2002;**51**(7):553-62.

3 Milham MP, Nugent AC, Drevets WC, et al. Selective reduction in amygdala volume in pediatric anxiety disorders: a voxel-based morphometry investigation. Biol Psychiatry. 2005;**57**(9):961-6.

4 Ferrari MC, Busatto GF, McGuire PK, Crippa JA. Structural magnetic resonance imaging in anxiety disorders: an update of research findings. Rev Bras Psiquiatr. 2008;**30**(3):251-64.

5 Shin LM, Liberzon I. The neurocircuitry of fear, stress, and anxiety disorders. Neuropsychopharmacology. 2010;**35**(1):169-91.

6 Paulus MP, Stein MB. An insular view of anxiety. Biol Psychiatry. 2006;**60**(4):383-7.

7 Chen ZY, Jing D, Bath KG, et al. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science. 2006;**314**(5796):140-3.

8 Bueller JA, Aftab M, Sen S, Gomez-Hassan D, Burmeister M, Zubieta JK. BDNF Val66Met allele is associated with reduced hippocampal volume in healthy subjects. Biol Psychiatry. 2006;**59**(9):812-5.

9 Egan M, Kojima M, Callicott J, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell. 2003;**112**(2):257-69.

10 Huang EJ, Reichardt LF. NEUROTROPHINS: Roles in Neuronal Development and Function. Annual review of neuroscience. 2001;**24**:677-736.

11 Gallinat J, Schubert F, Bruhl R, et al. Met carriers of BDNF Val66Met genotype show increased N-acetylaspartate concentration in the anterior cingulate cortex. Neuroimage. 2010 ;**49**(1):767-71.

12 Takahashi M, Shirakawa O, Toyooka K, et al. Abnormal expression of brain-derived neurotrophic factor and its receptor in the corticolimbic system of schizophrenic patients. Mol Psychiatry. 2000;**5**(3):293-300.

13 Montag C, Weber B, Fliessbach K, Elger C, Reuter M. The BDNF Val66Met polymorphism impacts parahippocampal and amygdala volume in healthy humans: incremental support for a genetic risk factor for depression. Psychol Med. 2009;**39**(11):1831-9.

14 Montag C, Weber B, Jentgens E, Elger C, Reuter M. An epistasis effect of functional variants on the BDNF and DRD2 genes modulates gray matter volume of the anterior cingulate cortex in healthy humans. Neuropsychologia. 2010;**48**(4):1016-21.

15 Lau JY, Goldman D, Buzas B, et al. BDNF gene polymorphism (Val66Met) predicts amygdala and anterior hippocampus responses to emotional faces in anxious and depressed adolescents. Neuroimage. 2010;**53**(3):952-61.

16 Pine DS, Ernst M, Leibenluft E. Imaging-genetics applications in child psychiatry. J Am Acad Child Adolesc Psychiatry. 2010;**49**(8):772-82.

17 Katoh-Semba R, Takeuchi IK, Semba R, Kato K. Distribution of brain-derived neurotrophic factor in rats and its changes with development in the brain. J Neurochem. 1997;**69**(1):34-42.

18 Toro R, Chupin M, Garnero L, et al. Brain volumes and Val66Met polymorphism of the BDNF gene: local or global effects? Brain Struct Funct. 2009;**213**(6):501-9.

19 Kaufman J, Birmaher B, Brent D, et al. Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL): initial reliability and validity data. Journal of the American Academy of Child and Adolescent Psychiatry. 1997;**36**(7):980-8.

20 Wechsler D. Wechsler Abbreviated Scale of Intelligence (WASI). San Antonio, Tx: Harcourt Assessment; 1999.

21 Faul. F, Erdfelder E, Lang A-G, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behavior Research Methods. 2007;**39**:175-91.

22 Hodgkinson CA, Yuan Q, Xu, K., Shen PH, et al. Addictions biology: haplotype-based analysis for 130 candidate genes on a single array. Alcohol Alcohol 2008;**43**:505-15.

23 Ashburner J. A fast diffeomorphic image registration algorithm. Neuroimage. 2007;**38**(1):95-113.

24 Ashburner J, Friston KJ. Voxel-based morphometry--the methods. Neuroimage. 2000;**11**(6 Pt 1):805-21.

25 Szeszko PR, Robinson D, Alvir JM, et al. Orbital frontal and amygdala volume reductions in obsessive-compulsive disorder. Arch Gen Psychiatry. 1999;**56**(10):913-9.

26 Tzourio-Mazoyer N, Landeau B, Papathanassiou D, et al. Automated anatomical labeling of activations in SPM using a macroscopic anatomical parcellation of the MNI MRI single-subject brain. Neuroimage. 2002;**15**(1):273-89.

27 Massana G, Serra-Grabulosa JM, Salgado-Pineda P, et al. Parahippocampal gray matter density in panic disorder: a voxel-based morphometric study. Am J Psychiatry. 2003;**160**(3):566-8.

28 Massana G, Serra-Grabulosa JM, Salgado-Pineda P, et al. Amygdalar atrophy in panic disorder patients detected by volumetric magnetic resonance imaging. Neuroimage. 2003;**19**(1):80-90.

29 Wignall EL, Dickson JM, Vaughan P, et al. Smaller hippocampal volume in patients with recent-onset posttraumatic stress disorder. Biol Psychiatry. 2004;**56**(11):832-6.

30 De Bellis MD, Keshavan MS, Frustaci K, et al. Superior temporal gyrus volumes in maltreated children and adolescents with PTSD. Biol Psychiatry. 2002;**51**(7):544-52.

31 Beesdo K, Lau JY, Guyer AE, et al. Common and distinct amygdala-function perturbations in depressed vs anxious adolescents. Arch Gen Psychiatry. 2009;**66**(3):275-85.

32 McClure EB, Monk CS, Nelson EE, et al. Abnormal attention modulation of fear circuit function in pediatric generalized anxiety disorder. Arch Gen Psychiatry. 2007;**64**(1):97-106.

33 Kalmar JH, Wang F, Chepenik LG, et al. Relation between amygdala structure and function in adolescents with bipolar disorder. J Am Acad Child Adolesc Psychiatry. 2009;**48**(6):636-42.

34 Siegle GJ, Konecky RO, Thase ME, Carter CS. Relationships between amygdala volume and activity during emotional information processing tasks in depressed and never-depressed individuals: an fMRI investigation. Ann N Y Acad Sci. 2003;**985**:481-4.

35 Kuhn S, Schubert F, Gallinat J. Structural correlates of trait anxiety: reduced thickness in medial orbitofrontal cortex accompanied by volume increase in nucleus accumbens. J Affect Disord. 2011;**134**(1-3):315-9.

36 Craig A. How do you feel? Interoception: the sense of the physiological condition of the body. Nature Reviews Neuroscience. 2002;**3**:655-66.

37 Davidson RJ. Cerebral asymmetry, emotion, and affective style. In: Davidson RJ, Hughdahl K, editors. Brain Asymmetry. MAssachusetts: MIT Press; 1995. p. 361-87.

38 Blair K, Geraci M, Devido J, et al. Neural response to self- and other referential praise and criticism in generalized social phobia. Arch Gen Psychiatry. 2008;**65**(10):1176-84.

39 Lorberbaum JP, Kose S, Johnson MR, et al. Neural correlates of speech anticipatory anxiety in generalized social phobia. Neuroreport. 2004;**15**(18):2701-5.

40 Mukherjee P, Whalley HC, McKirdy JW, et al. Effects of the BDNF Val66Met polymorphism on neural responses to facial emotion. Psychiatry Res. 2011;**191**(3):182-8.

41 Mesulam M-M, Mufson EJ. Insula of the Old World Monkey. III: Efferent Cortical Output and Comments on Function Journal of Comparative Neurology. 1982;**212**:38-52.

42 Shi C-J, Cassell MD. Cascade projections from somatosensory cortex to the rat basolateral amygdala via the parietal insular cortex. Journal of Comparative Neurology. 1998;**399**:469-91.

43 Real E, Gratacos M, Soria V, et al. A brain-derived neurotrophic factor haplotype is associated with therapeutic response in obsessive-compulsive disorder. Biol Psychiatry. 2009;**66**(7):674-80.

44 Casey BJ, Glatt CE, Tottenham N, et al. Brain-derived neurotrophic factor as a model system for examining gene by environment interactions across development. Neuroscience. 2009;**164**(1):108-20.

Figure captions

Figure 1. *Main effect of group (comparison > patients).* Figure displays the decreased gray matter volume in the amygdala [28 0 18] and anterior hippocampus for the patient relative to the comparison group regardless of BDNFVal^{66}Met polymorphism overlaid on the MNI (Montreal Neurological Institute) T1 brain template. Scatterplots for the amygdala cluster showing significant differences in the post-hoc tests. *p<.05. Black lines indicate group means. Units of Y-axis is in probability of grey matter tissue per mm^3.

Figure 2. *Main effect of group (patients > comparison).* Figure displays insula gray matter volume increases [52 11 -8] in the patients relative to the comparison group overlaid on the MNI (Montreal Neurological Institute) T1 brain template. Scatterplots showing significant differences in the post-hoc tests. *p<.05. Black lines indicate group means. Units of Y-axis is in probability of grey matter tissue per mm^3.

Figure 3. *Interaction of Brain Derived Neurotropic Factor polymorphism by group*. **Upper panel.** Significant gray matter volume changes in the dACC [30 3 27] overlaid on T1 MNI template with adjacent scatterplot for the significant interactions in the post-hoc tests. **Lower panel.** Significant gray matter volume changes in the anterior insula [45 17 12] overlaid on T1 MNI template with adjacent scatterplot illustrating the significant interactions in the post-hoc tests. *p<.0. Black lines indicate group means. Units of Y-axis is in probability of grey matter tissue per mm^3.

Table 1. Demographic information of the study sample separately for the patients and healthy controls split by Brain Derived Neurotropic Factor (BDNF) genotype.

Demographic information	Patients Met (N=17)	Patients Val (N=22)	Controls Met (N=27)	Controls Val (N=36)	*P-value*
Sex (female) (%)	8 (47.1)	14 (63.6)	14 (51.5)	14 (41.5)	.324
Age	11.3 (2.6)	13.7 (2.5)	13.5 (3.1)	13.9 (2.5)	**p<.05**
IQ*	109.1 (12.6)	110.4 (11.63)	116.0 (10.3)	113.3 (14.8)	.261
SES* Hollingshead	40.1 (23.7)	36.9 (16.1)	42.4 (17.8)	42.4 (16.1)	.704
STAI state*	32.79 (5.32)	33.38 (4.61)			.727
STAI trait*	37.85 (7.74)	41.50 (7.89)			.200
CDI*	52.86 (13.55)	56.00 (12.93)			.490
Ethnic ancestry factor scores (SD)					
Europe	.67 (.40)	.77 (.30)	.76 (.27)	.64 (.39)	.477
Africa	.00 (.01)	.10 (.25)	.03 (.12)	.15 (.32)	.094
Middle East	.11 (.24)	.07 (.12)	.09 (.16)	.08 (.14)	.894
America	.02 (.03)	.03 (.07)	.03 (.10)	.03 (.08)	.901
Asia	.13 (.30)	.02 (.03)	.04 (.05)	.04 (.07)	.084
Oceania	.00 (.01)	.00 (.00)	.00 (.00)	.01 (.01)	.688
Far East Asia	.06 (.24)	.00 (.00)	.05 (.19)	.05 (.18)	.730
Diagnosis: n (%)					
GAD*	7 (50)	14 (64)			.206[a]
Social Phobia	11 (65)	11 (50)			.517[a]
Specific phobia	1 (6)	5 (23)			.206[a]

Separation Anxiety Disorder	5 (29)	7 (32)	1.00[a]
Depression	2 (12)	6 (27)	.426[a]
ODD*	1 (6)	1 (5)	
ADHD*	1 (6)	3 (14)	
Tic disorder	1 (6)	-	
Enuresis	-	1 (5)	

Note: IQ = Intelligence Quotient; SES = Socio-Economic Status; STAI = State Trait Anxiety Inventory; CDI = Child Depression Inventory; GAD = Generalized Anxiety Disorder; ODD = Oppositional Defiant Disorder; ADHD = Attention Deficit Hyperactivity Disorder

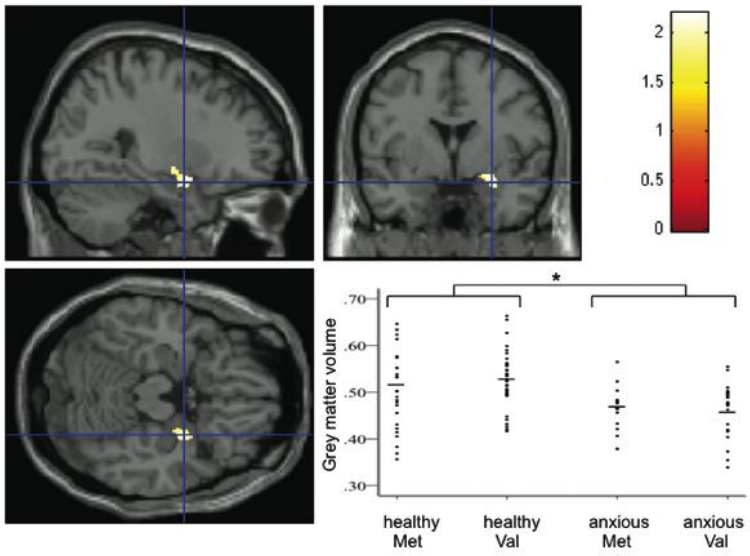
[a] Fisher's Exact Test

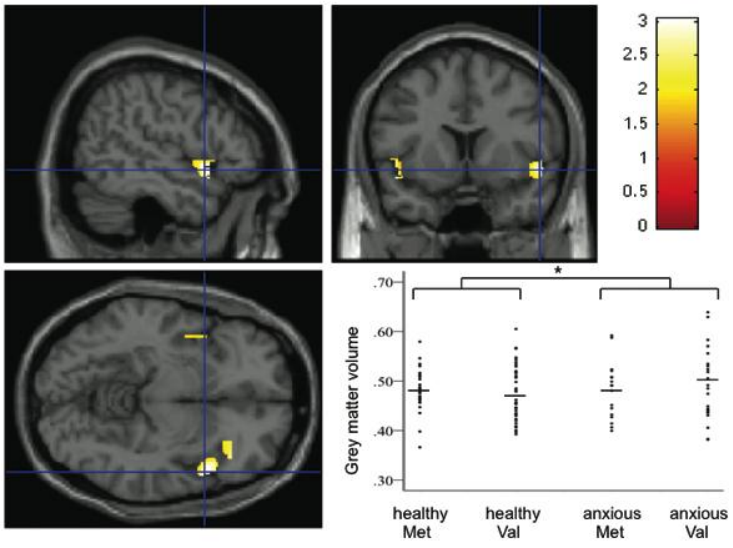
Table 2. Statistical Parametric Mapping results of Gray Matter Volume (GMV) differences in anxious vs. healthy adolescents split by main effect of Group and Brain Derived Neurotropic Factor (BDNF) genotype.

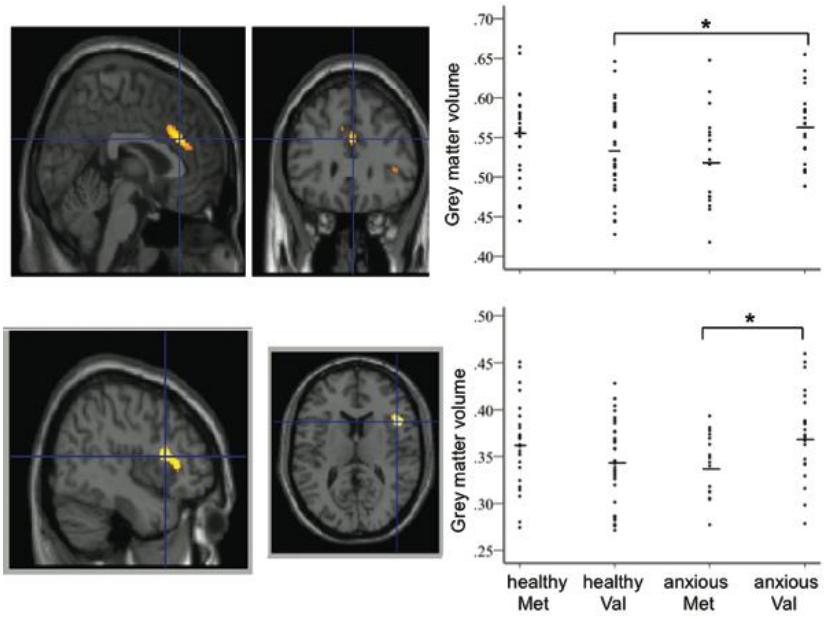
Region	***Side***	***Cluster Size mm^3***	***t-value***	***z-value***	***MNI coordinates***			***Talairach coordinates***		
					x	***y***	***z***	***x***	***y***	***z***
Main effect of group										
controls > patients										
Amygdala	R	243	2.20	2.17	28	0	-18	28	-1	-15
			2.07	2.05	25	-9	-8	25	-9	-6
ant. Hippocampus	R	48	1.89	1.88	27	-4	-18	27	-5	-15
			1.81	1.80	22	-7	-14	22	-7	-11
patients > controls										
Insula	L	219	3.04	2.96	-46	12	-14	-46	11	-12
	L	31	2.09	2.06	-48	27	3	-48	26	1
	R	438	3.00	2.93	52	11	-8	51	10	-7
	R	256	2.98	2.91	40	30	-5	40	29	-6
Interaction (Val (controls – patients) – Met (controls – patients))										
dorsal ACC	R	238	2.65	2.60	3	30	27	3	30	23
post. Insula	R	92	3.90	3.75	48	-3	-12	48	-3	-10
			3.16	3.07	46	-9	-8	46	-9	-6
			2.46	2.42	45	-15	-6	45	-15	-4
ant. Insula	R	135	3.21	3.11	45	17	12	45	17	10
			2.46	2.42	42	20	13	42	20	11
			2.35	2.32	43	27	4	43	26	2

Note: the printout was obtained using SPM8 (Wellcome Department of Cognitive Neurology, London, England; http://www.fil.ion.ucl.ac.uk/spm), small volume correction (SVC) applied for multiple comparisons at p<.05. MNI

(= Montreal Neurological Institute) coordinates were converted to Talairach coordinates using the Wake Forest University (WFU) Pickatlas tool. ACC = anterior cingulate cortex






Grey matter volume
.70
.65
.60
.55
.50
.45
.40
*
Grey matter volume
.50
.45
.40
.35
.30
.25
*
healthy Met
healthy Val
anxious Met
anxious Val