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GAD65 promoter polymorphism rs2236418 modulates harm avoidance in women via inhibition/excitation balance in the rostral ACC

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Abstract

Anxiety disorders are common and debilitating conditions with higher prevalence in women. However, factors that predispose women to anxiety phenotypes are not clarified. Here we investigated potential contribution of the single nucleotide polymorphism rs2236418 in *GAD2* gene to changes in regional inhibition/excitation balance, anxiety–like traits and related neural activity in both sexes. 105 healthy individuals were examined with high-field (7T) multimodal magnetic resonance imaging (MRI), including resting state fMRI in combination with assessment of GABA and Glutamate (Glu) levels via MR spectroscopy (MRS). Regional GABA/Glu levels in ACC subregions were assessed as mediators of gene–personality interaction for the trait harm avoidance and moderation by sex was tested. In AA homozygotes, with putatively lower *GAD2* promoter activity, we observed increased intrinsic neuronal activity and higher inhibition/excitation balance in pregenual ACC (pgACC), as compared to G carriers. The pgACC drove a significant interaction of genotype, region and sex, where inhibition/excitation balance was significantly reduced only in female AA carriers. This finding was specific for rs2236418 as other investigated SNPs of the GABA synthesis related enzymes (*GAD1*, *GAD2* and *GLS)* were not significant. Furthermore, only in women there was a negative association of pgACC GABA/Glu ratios with harm avoidance. A moderated–mediation model revealed that pgACC GABA/Glu also mediated the association between the genotype variant and level of harm avoidance, dependent on sex. Our data thus provide new insights into the neurochemical mechanisms that control emotional endophenotypes in humans and constitute predisposing factors for the development of anxiety disorders in women.

Significance statement

Anxiety disorders are among the most common and burdensome psychiatric disorders, with higher prevalence rates in women. The causal mechanisms are, however, poorly understood. In this study we propose a neurobiological basis that could help to explain female bias of anxiety endophenotypes. Using magnetic resonance brain imaging and personality questionnaires we show an interaction of the genetic variation rs2236418 in the *GAD2* gene and sex on GABA/Glutamate (Glu) balance in the pregenual anterior cingulate cortex (pgACC), a region previously connected to affect regulation and anxiety disorders. The *GAD2* gene polymorphism further influenced baseline neuronal activity in the pgACC. Importantly, GABA/Glu was shown to mediate the relationship between the genetic variant and harm avoidance, however only in women.

Introduction

Anxiety endophenotypes are considered as critical parameters for the disposition of psychopathologies including anxiety disorders (Mathews and Macleod, 2005). Epidemiological data report female bias in anxiety disorders (McLean et al., 2011; Donner and Lowry, 2013). Clarifying the neurobiological mechanisms underlying these endophenotypes and their sex specificity is crucial for the development of new treatments.

Investigations of neurobiological mechanisms of anxiety have highlighted the role of cortico–limbic circuits encompassing anterior cingulate cortex (ACC), ventromedial prefrontal cortex and amygdala (Dörfel et al., 2014) in anxiety development.

The ACC is characterized by a marked rostro–caudal division (Bush et al., 2000) reflected in the receptor distribution (Palomero-Gallagher et al., 2009) and metabolite composition (Dou et al., 2013). The anterior mid-cingulate (aMCC) is connected to salience detection and cognition networks (Menon and Uddin, 2010), whereas the pregenual ACC (pgACC) shows strong connections to the affective network (Yu et al., 2011) and is implicated in down–regulation of amygdala activity, modulating the processing of emotions and the formation of fear memory (Etkin et al., 2011; Giustino and Maren, 2015).

At neurotransmitter levels, emotion regulation through the pgACC depends on the balance of gamma–aminobutyric acid (GABA) and glutamate (Glu), which control neural excitability, plasticity and network stability (Cline, 2005). In general, prominent GABAergic system involvement in emotion regulation and anxiety disorders has been shown in pre-clinical and clinical studies (Nuss, 2015; Goddard, 2016). In the pgACC, which shows an adaptive response to anticipatory anxiety (Straube et al., 2009), dysregulation of inhibition/excitation balance has been reported for clinical populations (Phan et al., 2005; Long et al., 2013). Further evidence for a role of GABA in general affect regulation comes from a combined fMRI–MRS study that revealed a correlation between pgACC deactivation during emotion processing and local GABA concentrations (Northoff et al., 2007).

GABA is synthetized by two isoforms of glutamic acid decarboxylase (GAD65, encoded by *GAD2* and GAD67, encoded by *GAD1* gene), which differ in expression and activity–dependent regulation (Esclapez et al., 1994). GAD65 regulates phasic GABA release and couples to neuronal activity, whereas GAD67 is connected to cytoplasmic GABA production and metabolic activity (Soghomonian and Martin, 1998; Patel et al., 2006). GABA synthesis is subject to sex differences, modulated by gonadal hormones (Davis et al., 1999; Seney et al., 2013). The *GAD2* promoter region has been identified as a target for estrogen receptors (Hudgens et al., 2009), and estrogens modulate both GAD65 and GAD67 mRNA levels (McCarthy et al., 1995; Ikeda et al., 2015).

We therefore hypothesized genetic effects, via GABA synthesis, on the inhibition/excitation balance in the pgACC that would mediate its role in anxiety. To address this question, an A>G single nucleotide polymorphism (SNP) in the promoter region of *GAD2* (chromosome 10p, position –243, rs2236418) was chosen, which has been previously associated with a 6–fold increase in GAD65 transcription levels *in vitro* (Boutin et al., 2003). To validate the specificity of *GAD2* rs2236418 we tested additional candidate SNPs in the GABAergic pathway: in the GAD2 gene (position 26211729 in the intron region, rs10508715 A>G; Unschuld et al., 2009), in the sister gene *GAD1* (chromosome 2, position 170851590 in the intron region, rs3791850 C>T; Hettema et al., 2006, and position 170836945 in the intron region, rs769390, A>C; Marenco et al., 2010) as well as in the glutaminase gene (*GLS* chromosome 2, position 190964627, rs13035504 A>G; Yin et al., 2016).

The effects of polymorphism on the inhibition/excitation balance were studied with high–field (7T) multi–voxel ^{1}H MRS via GABA/Glu ratio, and on the brain activity using resting–state fMRI (rs–fMRI), measuring amplitudes of low–frequency fluctuations (ALFF) and regional homogeneity (ReHo). To test for the behavioral implications of the observed variations we determined individual proneness to anxiety as indicated by the harm avoidance scale of the Temperament and Character Inventory (TCI; Cloninger et al., 1994). The expected sex specificity was determined for all investigated measures and their interactions.

Methods

Study design

The study sample consisted of 105 healthy subjects (age= 27.09± 6.72, 44 females) pooled from three studies. Measurements included: genotyping, rs–fMRI and ^{1}H MRS at ultra–high field strength 7T. All three studies used identical protocols for recruitment and measurements: the scan order of the MRS voxels and rs–fMRI was the same. Some subjects were used as controls for patient studies, thus after the resting state scan, they performed additional fMRI tasks which differed according to the respective patient study, but this fMRI task did not affect prior MRS or resting state measurements. All subjects were in good physical condition and medication free as determined by relevant medical history. Subjects were asked about previous hospitalization (including psychotherapy), medical conditions and medication. Medication for neurological diseases (i.e. epilepsy), diabetes and hypo/ hyperthyroidism were considered as exclusion criteria. Medical history was acquired and approved by a study physician. Psychiatric disorders according to DSM–IV and ICD–10 were excluded using the German Version 5.0.0 of the M.I.N.I. Mini International Neuropsychiatric Interview (Sheehan et al., 1998). Further exclusion criteria were left–handedness as assessed with the short form (10 items) of the Edinburgh Handedness Inventory (Oldfield, 1971) and MRI contraindications, such as metallic implants or tinnitus. All three studies were approved by the Institutional Review Board of the University of Magdeburg, and all subjects provided written informed consent in accordance with the Declaration of Helsinki.

Genotyping

Whole–blood samples were collected from participants in EDTA–coated tubes (BD Vacutainer, K3E, 7.2 mg. REF 368884) and stored at 4°C. Genomic DNA was extracted from blood leukocytes using the GeneMole® automated system (Mole Genetics AS, Lysaker, Norway) according to the manufacturer's protocol. Genotyping was performed using PCR followed by allele–specific restriction analysis. Briefly, the DNA fragment on chromosome

10p12 containing the GAD2 –243 A>G polymorphism (NCBI accession number: rs2236418) was amplified using the primers GAD2–F: 5'–CGA AAG ACC AAA AGC CAG AG–3' and GAD2–B: 5'–TTC TAC CAA GGC GCT GAA AT–3' and standard Taq polymerase (Qiagen). The resulting PCR products were digested with DraI (Thermo Scientific #FD0224), yielding two fragments (279+ 575bp) for the A allele or a single fragment (854bp) for the G allele. DNA fragments were separated on a 1% agarose gel stained with Midori Green (Biozym Scientific, Hessisch Oldendorf, Germany) and visualized under UV light.

To control for specificity of effects related to the rs2236418 variant, we further performed genotyping for *GAD2* rs10508715 (Unschuld et al., 2009) as well as for rs3791850 (Hettema et al., 2006) and rs769390 (Marenco et al., 2010) in the *GAD1* gene and for glutaminase (*GLS*) rs13035504 (Yin et al., 2016). Details of additional genotyping protocols are available from the authors upon request.

Personality assessment

To asses self–reported harm avoidance, we used the corresponding scale from the Temperament and Character Inventory (TCI) (Cloninger et al., 1994). The TCI was designed as a tool to dissociate and predict clinical phenotypes and was built as a psychobiological model of personality based on temperaments– heritable and stable dimensions of "involuntary emotional processes" and characters– dimensions developed later in life of "voluntary rational processes". Temperament harm avoidance was connected to cognitive anxiety symptoms (Cloninger, 1987). It is composed of four subscales, which cover different aspects of anxiety propensity: Anticipatory worry, Fear of uncertainty, Shyness with strangers and Fatigability and asthenia (weakness). Harm avoidance is strongly positively correlated with Neuroticism and negatively with Extraversion domains from the NEO Personality Inventory and Eysenck personality dimensions (Stallings et al., 1996; De Fruyt et al., 2000) thus describing an anxiety-like trait. Moreover, the questionnaire and scales (in German version) display high internal consistency and factor structure (Richter et al., 2000) as well as long–term (Josefsson et al., 2013) and cross-cultural stability (Miettunen et al., 2006),

highlighting its transferability when exploring the underlying biological and environmental determinants of differences in heathy populations. From the entire sample, 72 subjects filled out the questionnaire. Internal consistency of Harm avoidance was good (for 69 subjects item by item scores available, Cronbach's alpha= 0.9, N= 35 items).

MRI data acquisition and analysis

MR images were acquired on a 7T scanner with a 32–channel head array coil (Siemens Healthineers, Erlangen, Germany). After automated shimming, we first acquired a high–resolution T1–weighted anatomical MR image, using a magnetization–prepared rapid gradient–echo (MPRAGE) sequence (TE= 2.73 ms, TR= 2300 ms, TI= 1050 ms, flip angle= 5°, bandwidth= 150 Hz/pixel, isotropic voxel size= 0.8 mm). The individual anatomical images were segmented and used for co–registration of the rs–fMRI data or to calculate partial volumes of the MRS voxels.

rs–fMRI: Subjects were instructed to lay still and awake with their eyes closed during the scanning session. Whole–brain T2*–weighted echo–planar images (EPIs) were acquired (280 time points, 62 axial slices, TE= 22 ms, TR= 2800 ms, flip angle= 80°, bandwidth= 2246 Hz, isotropic voxel size= 2 mm). Sequence parameters were optimized to circumvent intra voxel dephasing and loss of signal, particularly in lower mPFC. Online motion and distortion correction were applied (Speck et al., 2008) . The first 10 EPIs were discarded to allow for steady–state magnetization, and EPIs were visually inspected for data quality and scanning artifacts. Pre–processing was performed using Statistical Parametric Mapping (SPM12; Wellcome Trust Centre for Neuroimaging, London, United Kingdom) and DPARSFA toolbox V2.1 (Chao-Gan and Yu-Feng, 2010). EPIs were first temporally corrected for acquisition delay (slice timing), followed by a spatial correction for head motion (realignment). Subjects with head motion exceeding 2 mm were excluded from further analysis. The MPRAGE image was co–registered to the individual mean EPI image from realignment to improve the following spatial normalization into the MNI stereotactic reference frame (Montreal Neurological Institute). Normalized EPIs were smoothed using a double voxel length

Gaussian kernel of 4 mm full–width at half maximum (FWHM). Smoothing kernel size was optimized for strong local activations within the boundaries of cytoarchitectonic subregions, leading to a smaller than usual kernel size.

After the regression of mean white matter signal, mean CSF signal and six motion parameters (obtained from the realignment of the non–motion–corrected data), amplitude of low frequency fluctuations (ALFF) (Zuo et al., 2010) was calculated in the frequency band of 0.01 – 0.1 Hz. For the calculation of regional homogeneity (ReHo) (Zang et al., 2004), smoothing was omitted, and temporal filtering (0.01 – 0.1 Hz) and "scrubbing" via cubic spline interpolation were applied. Scrubbing was done for the time points which exceeded a frame–wise displacement threshold of 0.5 as calculated with the method described by Jenkinson (Jenkinson et al., 2002), as well as for adjacent time points. Subjects who had more than 13 volumes (5%) with >0.5 FD were excluded from further analysis. The similarity of time–series was estimated for 19 neighboring voxels in the calculation for ReHo. To test for between–group differences, ALFF and ReHo were converted to z maps using Fisher's r–to–z transformation (Chao-Gan and Yu-Feng, 2010). ReHo z maps were additionally smoothed with 4 mm kernel.

MRS: After region–specific automated shimming, a stimulated–echo acquisition mode (STEAM) sequence was used, and ^{1}H spectra were acquired from the pgACC (voxel size= 20 x 15 x 10 mm^3) and aMCC (voxel size= 25 x 15 x 10 mm^3; 128 averages, TE= 20 ms, TR= 3000 ms, TM= 10 ms, bandwidth= 2800 Hz) (**Figure 1**). Water signal with instance single average served as internal reference for quantification and eddy current correction. Spectral data (0.6 – 4.0 ppm) were analyzed with the LCModel (Stephen Provencher, Inc., Oakville, ON, Canada, V6.3.0) (Provencher, 2001). Absolute concentrations of target metabolites (GABA and Glu) with respective Cramér–Rao Lower Bound (CRLB) and FWHM values for spectral line–width estimation were obtained. Exclusion criteria for unreliable quantification were: CRLB > 20%, FWHM > 24 Hz, SNR < 20. Metabolite concentrations were expressed in institutional units (i.u.), due to the absence of individual correction for T1

and T2 relaxation differences between in vitro and in vivo metabolites. For each MRS voxel, the GABA/Glu ratio was calculated as an approximation of the inhibition/excitation balance. Metabolite ratios were residualized for individual grey matter partial volume of the respective voxel, as calculated from the segmented anatomical images using voxel–based morphometry with VBM8 (Structural Brain Mapping Group, University of Jena, Germany), implemented in the SPM8 (Wellcome Trust Centre for Neuroimaging, London, United Kingdom).

Figure 1 here

Statistical analyses

To control for possible confounds, full set genotype groups for *GAD2* rs2236418 were tested for age, sex, BMI, smoking, alcohol consumption or contraception use. All variables were checked for normality with Kolmogorov–Smirnov test ($p< 0.05$), and subsequently Mann–Whitney's U tests or χ^2 tests were conducted (**Table 1**). Datasets for respective analyses varied due to scanner artifacts or excessive head movement during scanning (27 subjects excluded), insufficient MRS quality (aMCC= 28; pgACC= 14; both= 37 subjects excluded), or incomplete questionnaires (33 subjects excluded). Therefore, each analysis was done with the maximum number of participants available for the modality or combination of them and as a nuisance variable, age was checked again for possible difference between the groups (**Table 2**).

Firstly, to determine the effects of genotype and sex on the local intrinsic neuronal activity we analyzed ALFF and ReHo z maps within the boundaries of the ACC subregions. ACC search volume was created following a previously established protocol (Li et al., 2016), using a 50% threshold for overlap of individual voxels. A two–way analysis of variance (ANOVA) was performed in SPM12 with genotype and sex as independent between–subject factors (46 AA, 22 females; 29 G carriers, 14 females). Because of a significant between–group difference in age (**Table 2**), it was included as nuisance covariate (Biswal et al., 2010). Statistical

significance was set at p< 0.05, peak level family–wise error FWE corrected, for the search volume.

The second ANOVA model was set up to assess region–specific (pgACC vs. aMCC) effects of *GAD2* rs2236418 genotype, sex, and their interaction on the GABA/Glu ratio (45 AA, 22 females; 21 G carriers, 6 females). First, to determine *GAD2* rs2236418 specificity, additional SNPs (*GAD2*: rs10508715; *GAD1*: rs3791850 & rs769390; and *GLS*: rs13035504) were tested with the same model (Bonferroni-corrected p< 0.05, equal to statistical threshold of p< 0.01). For significant interactions *post hoc* Student's t– or Mann–Whitney's U– tests were conducted. Second, to confirm metabolic specificity of the significant interaction on the ratio, we also compared GABA/Cr and Glu/Cr ratios, on an exploratory level (p< 0.05). The ANOVAs were computed using SPSS (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, USA) and included region as within–subject factor, and genotype and sex as between–subject factors. Age was included as a covariate.

To test for potential behavioral relevance of the inhibition/excitation balance in the pgACC with respect to anxiety–related traits, a non–parametric partial correlation of GABA/Glu ratio and harm avoidance scores was computed, controlled for age. Taking sex effects into consideration, this correlation was computed separately for males and females (35 males, 27 females), with p< 0.025, two–tailed, Bonferroni-corrected. Correlation coefficients were then compared for a significant difference with Fischer's Z–test with statistical threshold set at Z> 1.96 (equivalent to p< 0.05, two–sided), using VassarStats (Website for statistical computation, Poughkeepsie, NY, USA). To assess direct influence of sex on harm avoidance and/or pgACC GABA/Glu ratio *post hoc* Mann–Whitney's U-tests were calculated afterwards.

Lastly, to elucidate potential effects of the pgACC GABA/Glu ratio or baseline neuronal activity on the genotype–related prediction of harm avoidance, we performed mediation analyses using the SPSS extension PROCESS v2.15 (Preacher and Hayes, 2004). Mediation models are used to explain the indirect underlying influence of the predictor on the dependent variable through its interaction with a third–mediator variable. We included

genotype *GAD2* rs2236418 as predictor, harm avoidance as outcome and age as nuisance covariate. Firstly, we calculated models that took into account two mediator variables (GABA/Glu pgACC with ALFF or ReHo beta estimate), as well as their additive effect (N= 41) (Model 6 in PROCESS). Next, we estimated each mediator variable in a separate model (N= 48 for ALFF and ReHo; N= 62 for GABA/Glu pgACC). We furthermore added sex as a moderating factor to account for any sex–specific effects (Model 59 in PROCESS). For all models, heteroscedasticity–consistent standard errors were set and 95% confidence intervals (CI) were estimated via bootstrap resampling with 1000 repetitions.

Results

Sample characteristics

For the *GAD2* rs2236418 subjects were grouped into AA homozygotes (N= 65, 28 females, age= 27.58± 7.25) and G allele carriers (G carriers) (N= 40, 16 females, age= 26.28± 5.73; 31 AG and 9 GG). There were no differences in all demographic factors between the groups (**Table 1**). Further analysis–specific differences of subgroups can be found in the **Table 2**. There were overall no differences between genotype groups for other SNPs as well (reported in **Table 3**).

Table 1& 2& 3 here

Genotype differences in the local neuronal activity

To determine the effects of genotype and sex on the intrinsic activity within ACC subregions, we computed ALFF and ReHo z maps. Within the search area, there was an effect of genotype on both metrics with peak activation localized in the pgACC (**Figure 2**). Compared to AA homozygotes, G carriers exhibited a trend towards decrease in intrinsic neuronal activity as indicated by ALFF values ([–4 42 –2], t_{70}= 3.67, p= 0.059, FWE peak level corrected) (**Figure 2a**) and significant lower ReHo at the same location ([–2 42 –2], t_{70}= 3.80, p= 0.026, FWE peak level corrected) (**Figure 2b**). No significant genotype by sex interactions were found for either metric. However, a main effect of sex was present for ALFF in the pgACC ([6 38 -2], t_{70}= 4.62, p= 0.004, FWE peak level corrected), as well as marginally for ReHo in the aMCC ([-6 24 24, t_{70}= 3.43, p= 0.069, FWE peak level corrected), with lower activation found in women.

Figure 2 here

GAD2 rs2236418 and sex interaction on the GABA/Glu ratio in the pgACC

To test for potential effects of the *GAD2* rs2236418 polymorphism and sex on the regional inhibition/excitation balance in the pgACC and the aMCC, we analyzed the GABA/Glu ratio

from ^{1}H MRS as dependent variable in an ANOVA model. We observed a Bonferroni corrected significant two-way interaction of region and rs2236418 genotype for GABA/Glu ratios ($F_{1, 63}$= 7.53, p= 0.008, η^2= 0.11) and a three–way interaction (region by genotype by sex; $F_{1, 63}$= 8.66, p= 0.005, η^2= 0.12; **Figure 3**, **Table 4**). These effects were specific for the *GAD2* rs2236418 as other SNPs did not show any significant interaction (**Table 4**). *Post hoc* t–tests revealed that female G Carriers had significantly higher GABA/Glu ratios in the pgACC (t_{36}= –2.19, p= 0.035; **Figure 3**, **Table 5**).

Furthermore, in a follow–up exploratory analysis, for *GAD2* rs2236418, region by genotype by sex interaction was identified for GABA/Cr ($F_{1, 63}$= 4.92, p= 0.03, η^2= 0.072), and a trend level for region by genotype ($F_{1, 63}$= 3.57, p= 0.063, η^2= 0.054), which was not the case for Glu/Cr ratio (**Table 6**), suggesting GABA-levels as the driving force for the observed effect. From other tested SNPs, *GLS* rs13035504 showed significant region by genotype by sex interaction for Glu/Cr ($F_{1, 68}$= 4.18, p= 0.016, η^2= 0.082) and trend–level for GABA/Cr ($F_{1, 62}$= 3.56, p= 0.064, η^2= 0.054; **Table 6**).

Figure 3, and Table 4, 5& 6 here

Harm avoidance correlates with GABA/Glu ratio in women

In order to assess the functional relevance of the pgACC inhibition/excitation balance for anxiety–related traits, we computed non–parametric partial correlations of pgACC GABA/Glu ratios and harm avoidance, separately in male and female participants. A significant negative relationship between the pgACC GABA/Glu ratio and harm avoidance was observed in women (ρ_{24}= –0.549, p= 0.004, 95% CI= [–0.768, –0.214]), but not in men (ρ_{32}= 0.048, p= 0.79, 95% CI= [–0.289, 0.375]). Fischer's Z test confirmed a significant difference between the slopes (Z= 2.46, equivalent to p= 0.014) (**Figure 4)**. Women showed higher harm avoidance scores (U= 327.5, p= 0.039), although there was no difference between the sexes for the pgACC GABA/Glu ratio (U= 450, p= 0.75).

Figure 4 here

Mediation model

To clarify the relationship between GAD65 rs2236418 genotype, pgACC GABA/Glu ratio, baseline neuronal activity and harm avoidance, we performed mediation and moderated mediation analyses with genotype as predictor, harm avoidance as outcome, pgACC GABA/Glu or ALFF/ReHo as mediator variables, and sex as moderator variable.

The analyses revealed a significant effect only for the fully moderated model with the pgACC GABA/Glu as a mediator variable (index= –3.147, bootstrapped 95% CI= [–9.929, –0.478]). Specifically, a genotype–dependent influence of the pgACC GABA/Glu ratio on harm avoidance was observable in women (b= –3.088, boot 95% CI= [–9.896, –0.502]), but not in men (b= 0.059, boot 95% CI= [–0.334, 1.451]) (**Figure 5**). A direct effect of genotype on harm avoidance was not significant in either sex (women: b= 4.926, boot 95% CI= [–0.831, 10.684]; men: b= 1.726, boot 95% CI= [–3.026, 6.478]) (**Figure 5**). Other models did not show any significant effects. In short, the results are: additive mediation model with pgACC GABA/Glu and ALFF (total indirect effects: b= –0.641, boot 95% CI= [–3.656, 1.563]; direct effect: b= –0.903, boot 95% CI= [–6.258, 4.452]), additive mediation model with pgACC GABA/Glu and ReHo (total indirect effects: b= 0.114, boot 95% CI= [–1.966, 2.319]; direct effect: b= –1.658, boot 95% CI= [–6.991, 3.676]), moderated mediation model with ALFF (moderated mediation index= –1.291, boot 95% CI= [–5.369, 1.493]) and moderated mediation model with ReHo as mediator variable (moderated mediation index= 0.946, boot 95% CI= [–1.612, 4.937]).

Figure 5 here

Discussion

The importance of the GABAergic system for anxiety endophenotypes and anxiety disorders, and their sex-biased occurrence is well established. In this study, by taking advantage of a polymorphism rs2236418 in the promoter region of *GAD2*, we demonstrate a role of inhibition/excitation balance in the pgACC and its association to anxiety–related traits, specifically in women.

We found reduced ALFF and ReHo of local intrinsic resting state activity in the pgACC of G carriers (**Figure 2**). Remarkably, GABA/Glu ratio differences were also detected, with G carriers showing higher levels, indicative of a change in the inhibition/excitation balance in the pgACC (**Figure 3**, **Table 4**). Our results moreover showed metabolite (compared to *GLS*) and isoform specificity (compared to *GAD1*), as well as polymorphism specificity (**Table 4**). We speculate that polymorphism specificity may be due to transcription factor binding differences between two SNPs. The rs2236418 effects of the ratio were driven by the GABA/Cr ratio, however, with a smaller effect size (**Table 6**). Although Glu is a metabolic precursor of GABA, it is more abundant, so the presumed effect of the polymorphism on synthesis rates could have negligent effects on the total Glu concentration, while affecting GABA-levels. Nevertheless, the functional consequence becomes prominent only when we consider the entire metabolic milieu, as seen for the GABA/Glu ratio.

Our data are in line with previous reports showing highest ratios of GABA/Glu in pregenual- as compared to mid and caudal ACC compartments mirrored by high densities of $GABA_B$ receptors (Dou et al., 2013). We used rs–fMRI considering the previously shown influence of local GABA concentrations on resting-state activity (Kapogiannis et al., 2013). The pgACC is a key region for automatic emotion regulation, and its hyperactivation has been linked to affective pathologies (Etkin et al., 2011), with increased ALFF reported in anxious depression (Liu et al., 2015) and ALFF and ReHo being positively associated with trait anxiety (Tian et al., 2016). Following the same regional pattern, lower GABA levels and elevated Glu levels have been found in social anxiety disorder and panic disorder (Phan et al., 2005; Long et al.,

2013), indicating a shift of inhibition-excitation balance in various anxiety profiles. This regional behavioral congruity is also evident in healthy subjects as Hasler et al. (2010) described the connection between higher anxiety profiles and lower levels of GABA during anticipation of shock (Hasler et al., 2010). The inter-individual variability in the molecular response to acute stress, measured via MRS, points to its importance for successful affect regulation, and possibly to genetic predispositions for anxiety phenotypes.

The relevance of GAD65 gene activity for anxiety and stress responsiveness has become evident in animal research (Müller et al., 2014). GAD65-deficient mice display higher anxiety levels and lower GABA in cortico–limbic structures (Stork et al., 2000) and are more prone to develop post–traumatic stress disorder–like behavior upon fear stress (Bergado-Acosta et al., 2008; Sangha et al., 2009). Based on functionality and previous findings in animals, we did not expect a GAD65 effect in the aMCC, which is implicated in cognitive control and salience detection rather than emotion regulation (Menon and Uddin, 2010; Hoffstaedter et al., 2014). Furthermore, pgACC and aMCC differ in cell types and cortical layers (Vogt et al., 1995). Moreover, GAD65 and GAD67, although expressed in the vast majority of GABAergic neurons in the brain, differ in expression levels and regulation. Hence the GAD2 polymorphism is likely to act in a subregion–specific and cell-type specific manner. In line, we could not observe an effect GAD1 rs769390 on GABA levels in spite of previously reported GABA differences in the larger mid–cingulate area (Marenco et al., 2010). Since the previous report of a SNP-related 6 fold change in the GAD65 expression levels refers to expression in a pancreatic cell line (Boutin et al., 2003) it needs to be further examined how the polymorphism affects gene expression in particular cell types in the pgACC.

Notably, genotype–dependent difference in the pgACC GABA/Glu ratio was most evident in women (**Figure 3**, **Table 4**), suggesting that the rs2236418 promoter polymorphism might affect the regulation of GAD65 expression levels, and consequentially levels of GABA, by sex hormones (Hudgens et al., 2009). This is in line with previously reported sex and hormonal effects on the GABAergic system (Seney et al., 2013; Barth et al., 2015). It is

appealing to consider that the interaction between sex hormones and the GABAergic system might contribute to the higher prevalence of anxiety disorders in women. A previous study linking SNPs in the *GAD1* gene, coding for the second GAD isoform, GAD67, to panic disorder only in women (Weber et al., 2012), further supports this idea. It is thus of critical interest to explore sex–related variance in both preclinical and human research (Blanchard et al., 1995; Zakiniaeiz et al., 2016) (Blanchard et al., 1995; Zakiniaeiz et al., 2016), also in terms of genetic variation.

Personality associations of the GABA/Glu ratio with harm avoidance were also present only in women (**Figure 4**). Harm avoidance is considered a personality dimension tightly coupled with anxiety proneness (Cloninger et al., 1994) and in a healthy population reflects an endophenotype that might convert to disease phenotypes in patient populations (Zohar et al., 2003). In the light of the negative association of harm avoidance and GABA/Glu ratios, we suggest that at least in women, increased inhibition/excitation balance in the pgACC could denote protective mechanisms towards increased anxiety.

The relationship of gene and behavior in our study is best accounted for within the interaction of sex and metabolite mediators; in women the effect of the *GAD2* rs2236418 variation on harm avoidance became evident only when GABA/Glu levels were considered (**Figure 5**). This was specific for the metabolites as rs–fMRI estimates did not show any significant mediation. In the mediation model, it was delineated that female G carriers potentially have higher harm avoidance scores within the same inhibition/excitation levels. This finding suggests a complex interplay between genes encoding components of the GABAergic system and sex hormones in anxiety endophenotypes. The functional consequence of genetic polymorphisms must be viewed in terms of their plasticity potential which can be either protective or disadvantageous depending on the environment (Meyer-Lindenberg and Weinberger, 2006). Consequently, female G carriers might be conservative for anxiety phenotypes in healthy young subjects. This might not hold true for other situations such as during childhood stress or trauma and it will be interesting to determine how GAD65

463 genotype affects clinically relevant changes in affect regulation, including trauma resilience
464 or vulnerability.
465 This multimodal study has several constraints that need to be taken into account. Due to
466 quality exclusion criteria and incomplete measurements some genotype/ sex subgroups have
467 modest number of participants. However, it should also be noted that a previous meta-
468 analysis of the well-known COMT Val108/158Met polymorphism has shown that
469 neuroimaging phenotypes are more strongly associated with gene variants compared to
470 behavioral or disease phenotypes (Mier et al., 2010). The authors argued that this was most
471 likely due to the neural activity and the resulting BOLD response being more proximal to the
472 cellular effects of genetic variations. Analogously, it should be noted that the metabolic ratio
473 may associate even more closely with the genotype difference, whereas, in a healthy
474 population, baseline BOLD might be compensated by other factors such as vascular
475 responsiveness. The high-field 7T allowed us to analyze high-resolution data (2 mm
476 acquisition voxel), as well as implement specific MRS sequence to obtain both GABA and
477 Glu (Dou et al., 2013) at high quality in multiple regions, yielding neurochemical measures
478 that should be sufficiently close to the immediate biological effects of the genotype. This
479 limited us to scan at a single site in Magdeburg, which offered the requested technical
480 conditions, instead of using protocols which would allow replication or pooling from other
481 centers. We nevertheless recognize that study warrants future replication, preferably with
482 sufficient power to detect group differences at the level of behavior. Given the results of our
483 study, one could decide to return to lower field strengths and focus on one region or
484 specifically on GABA. Moreover, we did not control for the menstrual cycle in our female
485 participants which might have brought an additional dimension. Functional associations
486 between genes, metabolites and harm avoidance were only present in women implying
487 differential modulation of gene effect either through transcription activity, or through
488 hormones (Seney et al., 2013). Additionally, it was reported that menstrual cycle can
489 influence MRS (Epperson et al., 2002; Batra et al., 2008). Therefore, we cannot exclude the

effect of cycle on GABA/Glu ratio, but one can expect similar distribution of menstrual phases between genotypes in randomly picked sample from a healthy young population.

In conclusion, our findings suggest that a *GAD2* genotype–by–sex interaction shapes GABAergic inhibition in the pgACC, and its control of anxiety–related traits. Our results provide new insight into the complex and region–specific regulation of GABAergic inhibition and the development of transitional phenotype particularly in women, which is highly relevant for the development of affective pathologies.

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Table 1 Study sample characteristics with respect to GAD2 rs2236418: Mann–Whitney test was conducted to test for difference in age and BMI (mean± SD), and χ^2 test of independence to test for distribution of genotype by sex, smoking status, alcohol consumption or contraception use. All values were p> 0.05, two–sided. Allele frequencies of the whole data set (N = 105) were within the Hardy–Weinberg equilibrium.

Variable	***AA***	***G carriers***	***Statistical test***
Whole sample	65 subjects	40 subjects	χ^2 (1) = 3.208, p = 0.073 [#]
Age	27.58 ± 7.25	26.28 ± 5.73	U = 1102, p = 0.189
Sex	28 females	16 females	Pearson χ^2 value (1) = 0.096, p = 0.756
BMI *	23.49 ±2.88 (N= 59)	24.46 ± 3.20 (N= 36)	U= 887, p= 0.179
Smoking status *	21 yes (N= 62)	10 yes (N= 38)	Pearson χ^2 value (1) = 0.629, p = 0.428
Alcohol consumption * [1]	39 yes (N= 56)	20 yes (N= 33)	Pearson χ^2 value (1) = 0.759, p = 0.384
Contraceptive use *	13 yes (N= 27)	9 yes (N= 16)	Pearson χ^2 value (1) = 0.264, p = 0.607
GAD2 rs10508715	58 AA	6 AA	Pearson χ^2 value (1) = 59.404, p < 0.001
GAD1 rs3791850	37 CC	20 CC	Pearson χ^2 value (1) = 0.359, p = 0.549
GAD1 rs769390	35 AA	17 AA	Pearson χ^2 value (1) = 0.945, p = 0.331
GLS rs13035504	48 AA	33 AA	Pearson χ^2 value (1) = 0.580, p = 0.446

* The data was not available for all subjects, total number is written in brackets for the respective genotype group

[1] yes= at least one drink per week – daily; no= rarely - never

[#] 0.1 > p > 0.05

Table 2 Analyses sample characteristics with respect to GAD2 rs2236418: for each analysis number of subjects differed; indicated are genotype groups with age (mean ± SD) and sex frequencies. Mann–Whitney test was conducted to test for difference in age, and χ^2 test of independence to test for distribution of genotype by sex. All values are p> 0.05, two–sided.

Analysis	*Groups*	*Subjects, N*	*Age (mean ± SD)*	*Mann–Whitney test*	*Sex, N*	*Chi square, interaction genotype * sex*
Local intrisic activity	AA	46	28.07 ± 7.53	U = 474, p = 0.035 *	22 females	Pearson χ^2 value (1) = 0.001, p = 0.970
	G carriers	29	24.86 ± 3.92		14 females	
Inhibition/ excitation balance	AA	45	27.87 ± 7.59	U = 403 p = 0.136	17 females	Pearson χ^2 value (1) = 3.267, p = 0.071 #
	G carriers	23	25.30 ± 4.48		6 females	
Behavioral correlates	Males	35	26.91 ± 6.44	U = 441, p = 0.653		
	Females	27	26.63 ± 6.69			
Mediation model 6 (M= pgACC GABA/Glu and ALFF/ReHo beta estimate)	AA	20	29.20 ± 9.29	U= 140, p= 0.066 #	12 females	Pearson χ^2 value (1) = 0.631, p= 0.427
	G carriers	21	24.52 ± 4.09		10 females	
Mediation model 59 (M= ALFF/ ReHo beta estimates)	AA	26	28.62 ± 8.29	U = 190.5, p= 0.047 *	15 females	Pearson χ^2 value (1) = 0.284, p= 0.594
	G carriers	22	24.77 ± 4.16		11 females	
Mediation model 59 (M= pgACC GABA/Glu)	AA	36	27.89 ± 7.45	U = 347, p = 0.083 #	16 females	Pearson χ^2 value (1) = 0.028, p = 0.867
	G carriers	26	25.27 ± 4.61		11 females	

*** p< 0.05; # 0.1 > p > 0.05**

738 **Table 3 Study sample characteristics for additional polymorphisms:** Mann–Whitney test
739 was conducted to test for difference in age (mean± SD), and χ^2 test of independence to test
740 for distribution of genotype by sex. All values were p> 0.05, two–sided.
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Polymorphism	*Groups*	*Age (mean ± SD)*	*Mann–Whitney test*	*Sex, N*	*Chi square, interaction genotype * sex*
GAD2 rs2236418	AA	27.58 ± 7.25	U = 1102, p = 0.19	28 females	Pearson χ^2 value (1) = 0.1, p = 0.76
	G Carriers	26.28 ± 5.73		16 females	
GAD2 rs10508715	AA	27.36 ± 7.02	U = 1073, p = 0.43	22 females	Pearson χ^2 value *(1) = 2.80, p = 0.094* [#]
	G Carriers	26.65 ± 6.31		19 females	
GAD1 rs3791850	CC	27.09 ± 6.17	U = 1162, p = 0.53	21 females	Pearson χ^2 value (1) = 1.21, p = 0.27
	T Carriers	27.14 ± 7.48		21 females	
GAD1 rs769390	AA	27.19 ± 6.91	U = 1250, p = 0.74	25 females	Pearson χ^2 value (1) = 2.09, p = 0.15
	C Carriers	26.92 ± 6.60		17 females	
GLS rs13035504	AA	27.11 ± 6.53	U = 795, p = 0.44	32 females	Pearson χ^2 value (1) = 0.25, p = 0.62
	G Carriers	26.41 ± 6.80		10 females	

[#] 0.1 > p > 0.05

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744 **Table 4 Main analyses of the GABA/Glu levels within the ACC sub–regions for 5**
745 **investigated SNP-s**: In the ANOVA model region was within–subject factor, genotype and
746 sex between–subject factors, and age nuisance covariate.

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Polymorphism	***Effect***	***GABA/Glu***
***GAD2* rs2236418**	Region x Genotype	**$F(1, 63) = 7.53$, $p = 0.008$, $\eta^2 = 0.11$ ****
	Region x Sex	$F(1,63) = 0.52$, $p = 0.47$, $\eta^2 = 0.008$
	Region x Genotype x Sex	**$F(1, 63) = 8.66$, $p = 0.005$, $\eta^2 = 0.12$ ****
GAD2 rs10508715	Region x Genotype	$F(1, 61) = 0.25$, $p = 0.62$, $\eta^2 = 0.004$
	Region x Sex	$F(1, 61) = 0.29$, $p = 0.60$, $\eta^2 = 0.005$
	Region x Genotype x Sex	$F(1, 61) = 0.81$ $p = 0.37$, $\eta^2 = 0.013$
GAD1 rs3791850	Region x Genotype	$F(1, 60) = 0.01$, $p = 0.93$, $\eta^2 < 0.001$
	Region x Sex	$F(1, 60) = 0.48$, $p = 0.49$, $\eta^2 = 0.008$
	Region x Genotype x Sex	$F(1, 60) = 0.82$, $p = 0.37$, $\eta^2 = 0.013$
GAD1 rs769390	Region x Genotype	$F(1, 61) = 1.71$, $p = 0.19$, $\eta^2 = 0.027$
	Region x Sex	$F(1, 61) = 0.38$, $p = 0.54$, $\eta^2 = 0.006$
	Region x Genotype x Sex	$F(1, 61) = 0.15$, $p = 0.69$, $\eta^2 = 0.002$
GLS rs13035504	Region x Genotype	$F(1, 62) = 0.17$ $p = 0.66$, $\eta^2 = 0.003$
	Region x Sex	$F(1, 62) = 1.41$ $p = 0.20$, $\eta^2 = 0.027$
	Region x Genotype x Sex	$F(1, 62) = 0.78$, $p = 0.38$, $\eta^2 = 0.012$
Bonferroni corrected threshold equal to ** p< 0.01		

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Table 5 Post hoc analyses of the GABA/Glu levels within the ACC sub–regions: Student's t–test or Mann–Whitney's U–test (two–sided), were conducted to assess directionality for the significant ANOVA interactions, region by *GAD2* rs2236418 by sex for the GABA/Glu ratios.

Region	***Sex***	***Genotype***	***Subjects, N***	***Statistics***
aMCC	Males	AA	27	U = 211, p = 0.458
		G carriers	18	
	Females	AA	24	U = 82, p = 0.564
		G carriers	8	
pgACC	Males	AA	30	U = 317, p = 0.615
		G carriers	23	
	Females	AA	24	**t = –2.19, df= 36, p = 0.035 ***
		G carriers	14	

*** p< 0.05**

Table 6 Exploratory analysis of GABA/Cr and Glu/Cr levels within the ACC sub–regions: In the ANOVA model region was within–subject factor, genotype and sex between–subject factors, and age nuisance covariate, statistical threshold was set at $p < 0.05$.

Polymorphism	***Effect***	***Glu/Cr***	***GABA/Cr***
***GAD1* rs2236418**	Region x Genotype	$F(1, 69) = 0.51$, $p = 0.48$, $\eta^2 = 0.007$	***F(1, 63) = 3.57, p = 0.063, η² = 0.054*** #
	Region x Genotype x Sex	$F(1, 69) = 0.002$, $p = 0.97$, $\eta^2 < 0.001$	**F(1, 63) = 4.92, p = 0.03, η² = 0.072 ***
GAD1 rs10508715	Region x Genotype	$F(1, 67) = 2.50$, $p = 0.12$, $\eta^2 = 0.036$	$F(1, 61) = 0.001$, $p = 0.98$, $\eta^2 < 0.001$
	Region x Genotype x Sex	$F(1, 67) = 1.55$, $p = 0.22$, $\eta^2 = 0.023$	$F(1, 61) = 0.09$, $p = 0.76$, $\eta^2 = 0.002$
GAD2 rs3791850	Region x Genotype	$F(1, 66) = 0.44$, $p = 0.51$, $\eta^2 = 0.007$	$F(1, 60) = 0.27$, $p = 0.61$, $\eta^2 = 0.004$
	Region x Genotype x Sex	$F(1, 66) = 0.99$, $p = 0.32$, $\eta^2 = 0.015$	$F(1, 60) = 0.20$, $p = 0.66$, $\eta^2 = 0.003$
GAD2 rs769390	Region x Genotype	$F(1, 67) = 0.009$, $p = 0.93$, $\eta^2 < 0.001$	$F(1, 61) = 2.06$, $p = 0.16$, $\eta^2 = 0.033$
	Region x Genotype x Sex	$F(1, 67) = 0.84$, $p = 0.36$, $\eta^2 = 0.012$	$F(1, 61) = 0.57$, $p = 0.45$, $\eta^2 = 0.009$
***GLS* rs13035504**	Region x Genotype	$F(1, 68) = 2.29$, $p = 0.14$, $\eta^2 = 0.033$	$F(1, 62) = 0.89$, $p = 0.35$, $\eta^2 = 0.014$
	Region x Genotype x Sex	**F(1, 68) = 4.18, p = 0.016, η² = 0.082 ***	***F(1, 62) = 3.56, p = 0.064, η² = 0.054*** #

*** p < 0.05; # 0.1 > p > 0.05**

Figure 1 MRS voxel: Position of the 7T magnetic resonance spectroscopy (MRS) voxel (yellow box) in the pgACC (a) and the aMCC (b), for a single subject.

Figure 2 Difference in resting–state fMRI values: for Amplitude of Low–Frequency Fluctuations (ALFF; blue) and Regional Homogeneity (ReHo; green) between AA homozygotes and G carriers of the *GAD2* rs2236418 in left pgACC (view z = –2), with the pgACC voxel mask of 25% overlap (yellow), 50% overlap (red) and 75% overlap (pink) of subject's individual single voxel location, dotted line represents transverse section; **2a** Contrast estimates of ALFF at x = –4, y = 42, z = –2; **2b** Contrast estimates of ReHo at x = –2, y = 42, z = –2. *p< 0.05, $^{\#}$ 0.1> p> 0.05, error bars represent ± 2 standard error means (SEM) of the contrast estimates.

Figure 3 Differences in the GABA/Glu ratio: Post hoc results for the significant interaction of region by *GAD2* rs2236418 by sex [$F_{1, 63}$ = 8.66, p = 0.005, η^2 = 0.12] for GABA/Glu ratio residuals, controlled for age. Depicted are GABA/Glu residuals in the pgACC (left) and aMCC (right) split by genotype (AA = blue, G carriers = green) and sex: Only in pgACC and only in females, G carriers showed significantly increased GABA/Glu (t_{36} = –2.19, p = 0.035); *p< 0.05, error bars represent ± 2 standard error means (SEM).

Figure 4 Harm avoidance and GABA/Glu ratio in the pgACC: correlation slopes of harm avoidance and pgACC GABA/Glu residuals differed significantly (p = 0.014) between women (ρ_{24} = –0.549, p = 0.004) (orange) and men (ρ_{32} = 0.048, p = 0.79) (purple). Confidence intervals depict 95% of the mean.

Figure 5 Mediation model moderated by sex: index = –3.147, bootstrapped 95% CI = [–9.929, –0.478]; in both sexes the direct effect of GAD65 genotype on harm avoidance was not significant. However for the indirect effect, the GABA/Glu ratio (curved arrow), in the pgACC significantly mediated the relationship between GAD65 and harm avoidance in women (b = –3.088, boot 95% CI = [–9.896, –0.502]), bold), but not in men; M = males (purple), F = females (orange), b = effect, CI = confidence interval.