



Specifically altered brain responses to threat in generalized anxiety disorder relative to social anxiety disorder and panic disorder



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ABSTRACT

Background: Despite considerable effort, the neurobiological underpinnings of hyper-responsive threat processing specific to patients suffering from generalized anxiety disorder (GAD) remain poorly understood. The current functional magnetic resonance imaging (fMRI) study aims to delineate GAD-specific brain activity during immediate threat processing by comparing GAD patients to healthy controls (HC), to social anxiety disorder (SAD) and to panic disorder (PD) patients.

Method: Brain activation and functional connectivity patterns to threat vs. neutral pictures were investigated using event-related fMRI. The sample consisted of 21 GAD, 21 PD, 21 SAD and 21 HC.

Results: GAD-specific elevated activity to threat vs. neutral pictures was found in cingulate cortex, dorsal anterior insula/frontal operculum (dal/FO) and posterior dorsolateral prefrontal cortex (dlPFC). Defining these effects as seed regions, we detected GAD-specific increased functional connectivity to threat vs. neutral pictures between posterior dlPFC and ventrolateral prefrontal cortex, between cingulate cortex and amygdala, between cingulate cortex and anterior insula, as well as decreased functional connectivity between dal/FO and mid-dlPFC.

Conclusion: The findings present the first evidence for GAD-specific neural correlates of hyper-responsive threat processing, possibly reflecting exaggerated threat sensitivity, maladaptive appraisal and attention-allocation processes.

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1. Introduction

Generalized anxiety disorder (GAD) is characterized by excessive and uncontrollable worry across a variety of domains (American Psychiatric Association, 2000; Duval et al., 2015). GAD is associated with increased health care costs (Lieb et al., 2005), often comorbid with other anxiety and mood disorders (Newman et al., 2013) and remains to have the lowest rate of remission after treatment in comparison to other anxiety disorders (Kinney et al., 2016). According to recent reviews and established cognitive models, GAD patients are suggested to be hypervigilant to threat: they are biased towards worry-related information in their environment, struggle with disengaging from that information, and tend to appraise information as threatening under ambiguous conditions (Behar et al., 2009; Hayes and Hirsch, 2007; Mennin et al., 2009; Newman et al., 2013). Favoring the processing of threat serves among other factors to maintain high levels of anxiety and initiates as well as maintains pathological worrying in GAD patients (Behar et al., 2009; Newman et al., 2013). Consequently,

hyper-responsive threat processing seems to play a key role in the etiology and maintenance of GAD pathophysiology (Hayes and Hirsch, 2007). Gaining a comprehensive understanding of threat processing in GAD is of utmost importance for conceptualization and treatment development of the disorder.

Despite considerable effort, underlying neural circuits of immediate threat processing in GAD have remained elusive. Previous functional magnetic resonance imaging (fMRI) studies have primarily compared GAD patients to healthy controls (HC), yielding no consistent pattern of immediate threat-related blood oxygen level-dependent (BOLD) responses in GAD. Depending on the paradigm, GAD patients responded with increased, decreased or no differentiating activity in areas such as amygdala or prefrontal cortex (PFC) (Ball et al., 2013; Blair et al., 2012, 2008; Etkin et al., 2010; Etkin and Schatzberg, 2011; Fonzo et al., 2015, 2014; Hölzel et al., 2013; Moon and Jeong, 2015; Nitschke et al., 2009; Palm et al., 2011; Price et al., 2011; Whalen et al., 2008). To elucidate GAD-specific threat-related BOLD response and to identify shared neural circuits underlying threat processing in anxiety disorders in general, it may be promising to follow the line of the Research Domain Criteria project (RDoC) (Kozak and Cuthbert, 2016). RDoC provides a framework to study functions and deficits across a spectrum of

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psychopathology and across the health-illness dimension. To date, few fMRI studies investigating brain activity have followed this approach by comparing GAD patients not only to HC but also to other anxiety disorder patients (Ball et al., 2013; Blair et al., 2012, 2008; Fonzo et al., 2015). Two studies used affective facial stimuli and compared brain responses in GAD patients to HC and additionally to patients with social anxiety disorder (SAD) and/or panic disorder (PD) (Blair et al., 2008; Fonzo et al., 2015). One study detected a GAD-specific reduced amygdala activity in response to fearful facial stimuli comparing GAD patients relative to SAD patients and to HC (Blair et al., 2008). Yet, in another study no GAD-specific brain activity pattern was detected in response to facial stimuli relative to HC, PD and SAD patients (Fonzo et al., 2015). It appears that the relevance of affective facial stimuli might be limited to GAD patients and not sufficient to induce disorder-specific threat processing. Other studies used affective scene pictures presented in an explicit or implicit emotion-regulation context and compared GAD patients either to PD or SAD patients, and to HC (Ball et al., 2013, 2012). GAD patients displayed reduced activity of parietal/occipital cortex and subcortical structures to threat compared to PD patients or HC (Ball et al., 2013), while no differences emerged between GAD and SAD patients (Blair et al., 2012). Taken together, clear evidence of GAD-specific threat-related brain activity is lacking, at least under instructed and/or demanding picture presentation conditions (Ball et al., 2013; Blair et al., 2012).

Similarly, clear evidence of GAD-specific functional connectivity (FC) patterns in response to threat relative to HC and other psychiatric patients is lacking (Andreescu et al., 2015; Chen and Etkin, 2013; Etkin et al., 2010; Etkin and Schatzberg, 2011; Makovac et al., 2015): Worry-induction studies comparing GAD patients to HC showed altered FC in amygdala-PFC circuitry associated with monitoring the salience of interoceptive and external events (salience network [SN]) and with emotion regulation (executive control network [ECN]) (Andreescu et al., 2015; Makovac et al., 2015). Another study detected a negative correlation between activations in amygdala and anterior cingulate cortex (ACC) in GAD patients relative to HC in response to affective facial stimuli in an emotional conflict task (Etkin et al., 2010). Following that, aberrant engagement of the amygdala-PFC circuitry has been suggested as a key factor underlying GAD pathophysiology (Makovac et al., 2015). Yet, when comparing GAD patients not only to HC, but additionally to patients with major depressive disorder (MDD) or post-traumatic stress disorder (PTSD) patients, using the same emotional conflict task or a similar attention-demanding task, no GAD-specific FC was detected (Chen and Etkin, 2013; Etkin and Schatzberg, 2011). This highlights the importance of investigating FC across disorders to reveal specific and unspecific alterations in GAD. Overall, current findings render an investigation of threat-related brain activity and FC in GAD relative to SAD and PD patients necessary.

The aim of the present fMRI study was to identify neural circuits underlying immediate threat processing in GAD patients relative to HC and other anxiety disorder patients. In the present study brain responses of GAD patients were compared to SAD and PD patients, because the disorders are highly prevalent, often comorbid and are marked by widely generalized fear/anxiety (Fonzo et al., 2015). Threat-related brain activity was investigated by presenting threatening and neutral pictures in an event-related design. Threat relative to neutral pictures was expected to engage an emotion-processing network in all groups comprising amygdala, insula, prefrontal and occipital cortex, and thalamus. Due to the inconsistent picture regarding immediate threat processing in GAD (Ball et al., 2013; Blair et al., 2012, 2008; Etkin et al., 2010; Etkin and Schatzberg, 2011; Fonzo et al., 2015, 2014; Hölzel et al., 2013; Moon and Jeong, 2015; Nitschke et al., 2009; Palm et al., 2011; Price et al., 2011; Whalen et al., 2008), our hypotheses were based on worry induction studies. These studies revealed a rather consistent finding of elevated activity in medial PFC (mPFC) and ACC, suggesting both areas to play cardinal roles in GAD pathophysiology (Andreescu et al., 2011; Paulesu et al., 2010). Following the suggested important role of

ACC/mPFC in GAD pathophysiology, GAD patients were expected to show altered ACC/mPFC activity to threat (Etkin et al., 2011; Paulesu et al., 2010). If observing GAD-specific threat-related brain activations, we were interested in whether these brain regions additionally showed GAD-specific FC to other areas. We expected positive correlations within SN, reflecting exaggerated threat processing, and reduced FC within ECN, and between SN and ECN, reflecting deficient emotion regulation (Mennin et al., 2009).

2. Methods and materials

2.1. Subjects

Sixty-three patients (GAD: $n = 21$, PD: $n = 21$, SAD: $n = 21$) and 21 HC were recruited through public advertisements and an outpatient clinic. Data of one GAD patient was excluded from analysis due to misunderstanding of task instructions. The final sample consisted of 20 GAD, 21 PD, 21 SAD patients and 21 HC matched for age, education and gender (see Supplement Table S3).

An experienced clinical psychologist diagnosed participants by means of the Structured Clinical Interview for DSM-IV axis-I Disorders (Wittchen et al., 1997). Patients met the criteria for the corresponding anxiety disorder as primary diagnosis and were excluded if they had a comorbid anxiety disorder from one of the other two disorder groups. Patients presented with the following axis I comorbidities: specific phobia (GAD: $n = 1$, PD: $n = 1$, SAD: $n = 4$), major depressive disorder (recurrent) (GAD: $n = 1$, PD: $n = 3$, SAD: $n = 5$), dysthymic disorder (PD: $n = 1$), obsessive-compulsive disorder (PD: $n = 1$, SAD: $n = 2$), eating disorder (GAD: $n = 1$, SAD: $n = 1$), posttraumatic stress disorder (GAD: $n = 1$), somatization disorder (PD: $n = 1$). HC were free of any current or past axis-I disorder. Disorder-related questionnaires given to the corresponding disorder group supported the diagnosis (GAD: Penn State Worry Questionnaire (Meyer et al., 1990) ($M = 65.95$, $SD = 8.46$); PD: Panic and Agoraphobia Scale (Bandelow, 1997) ($M = 21.90$, $SD = 6.83$); SAD: Liebowitz Social Anxiety Scale (Fresco et al., 2001) ($M = 70.81$, $SD = 17.57$)). Patient groups did not differ with regard to Beck Depression Inventory-II scores (BDI) (Beck et al., 1996) or Anxiety Sensitivity Index (ASI) (Reiss et al., 1986) (see Supplement Table S3).

Six to seven patients per group took long-term medication (antidepressive medication, one GAD patient used Pregabalin) (see Supplement Table S3), and had been stabilized on such medication for at least four weeks prior to study participation. All subjects had normal or corrected-to-normal vision and gave written informed consent. Exclusion criteria were neurological disorders, presence or history of psychotic or bipolar disorder, current drug abuse or dependence, or fMRI contraindications. The study has been approved by the ethics committee of the University of Muenster and is in compliance with the latest declaration of Helsinki.

2.2. Stimuli

Fifty threat and 50 neutral pictures were chosen from the International Affective Picture System (Lang and Bradley, 2007) (threat pictures: $n = 48$, neutral pictures: $n = 14$) and the Emotional Picture Set (Wessa et al., 2010) (threat pictures: $n = 2$, neutral pictures: $n = 36$). Threat pictures showed for example motor vehicle accidents, violence, threatening animals or injuries. Neutral pictures showed for example animals or objects. Picture sets were matched for color scheme, luminance and complexity (see Supplement Table S4).

2.3. Experimental design

Scanning took 8 min and 32 s. Pictures were presented in pseudo-randomized order controlled by Presentation Software (v17.2, Neurobehavioral Systems, Albany, California, USA). Each picture was

presented for 800 ms, followed by a fixation cross (with a jittered duration of 1280–18,960 ms, $M = 3,890$ ms; determined using the optseq algorithm [<http://www.surfer.nmr.mgh.harvard.edu/optseq/>]). To ensure sufficient attention to the pictures, participants were instructed to press a button in response to blurred pictures, which were presented five times during the run. After scanning, participants rated the stimuli on 9-point Likert scales for arousal (1 = *not arousing at all*, 9 = *highly arousing*), valence (1 = *very negative*, 5 = *neutral*, 9 = *very positive*), and anxiety induction (1 = *not anxiety-inducing*, 9 = *highly anxiety-inducing*). Participants received standardized instructions as well as training outside and inside the scanner. Training blocks consisted of seven trials comprising five positive and two blurred pictures.

2.4. Analysis of sociodemographic data, clinical questionnaire and rating data

Sociodemographic data, clinical questionnaires, and rating data were analyzed using IBM SPSS software (v22, Armonk, New York, USA). Rating data for anxiety, valence and arousal were subjected to separate 2 (*picture valence*: threat picture, neutral picture) by 4 (*group*: GAD, PD, SAD, HC) mixed model analysis of variance (ANOVA). A probability level of $p \leq 0.05$ was considered statistically significant. Post-hoc pairwise Bonferroni-corrected comparisons resolved the main effects for *group*, and Bonferroni-corrected *t*-tests resolved interaction effects (corrected significance level $p \leq 0.008$).

2.5. fMRI acquisition and analysis

Anatomical and functional data were collected with a 3 Tesla magnetic resonance scanner (“Magnetom PRISMA”, Siemens, Erlangen, Germany) using a 20 channel head-neck coil. After acquiring a high-resolution T1-weighted anatomical scan with 192 slices, functional data were recorded with a T2-weighted echo-planar sequence (TE = 30 ms, flip angle = 90°, matrix = 92 × 92 voxels, FOV = 208 mm², TR = 2080 ms). 255 volumes consisting of 36 axial slices (thickness = 3 mm, 0.3 mm gap, in plane resolution = 2.26 mm × 2.26 mm) were acquired.

fMRI data were preprocessed and analyzed using BrainVoyager QX (BVQX, Version 2.8, Brain Innovation, Maastricht, Netherlands). To ensure steady-state tissue magnetization, the first 10 volumes were discarded. Data were corrected for slice time errors and controlled for excessive head movement artifacts (>3 mm in any direction). Anatomical and functional data were co-registered and normalized to Talairach space (Talairach and Tournoux, 1988). Subsequently, data were smoothed spatially (6 mm full-width half maximum [FWHM] Gaussian kernel) and temporally (high pass filter: 10 cycles per run; low pass filter: 2.8 s; linear trend removal). Volumes were resampled to 2 × 2 × 2 mm voxel size.

Statistical analysis comprised a multistage approach: A canonical double-gamma hemodynamic response function (HRF) modeled the expected BOLD signal for each predictor. Predictors of interest were threat and neutral pictures, while blurred pictures were defined as predictors of no interest. First, predictor estimates based on z-standardized time course data were calculated, with adjustment for autocorrelation following a global AR(1) model. Second, a random-effects general linear model was computed and subjected to a 2 (*picture valence*: threat pictures, neutral pictures) by 4 (*group*: GAD, PD, SAD, HC) regions of interests (ROI, see below) ANOVA. In the next step, interactions were resolved by planned *t*-tests (see below). Disorder-specific brain activity to threat was defined as differential brain activity to threat vs neutral pictures relative to each other group.

In case of disorder-specific brain activity to threat vs. neutral pictures, we were also interested in disorder-specific FC patterns. As such, GAD-specific brain activity clusters derived from interaction effects of the 2 × 4 ROI ANOVA (see above) were used as seed regions in

psychophysiological interaction (PPI) analyses. PPI analyses were conducted per seed region within ROI (see below) (Friston et al., 1997). An interaction regressor, which was the product of the HRF-convolved task regressor (psychological factor) and the seed region time course (physiological factor), was subjected to a one-way ANOVA with *group* (GAD, PD, SAD, HC) as between-subjects factor. We only report clusters for which planned comparisons revealed GAD-specific FC alterations (as compared to the other groups).

Analyses were performed for a priori defined ROIs. ROIs were based on the current literature on GAD pathophysiology (Ball et al., 2013; Blair et al., 2012, 2008; Etkin et al., 2010; Etkin and Schatzberg, 2011; Fonzo et al., 2015, 2014; Hölzel et al., 2013; Moon and Jeong, 2015; Nitschke et al., 2009; Palm et al., 2011; Price et al., 2011; Whalen et al., 2008) and pictorial stimuli processing (Sabatinelli et al., 2011): cingulate cortex (anterior, middle and posterior), insula, PFC (medial and lateral), thalamus, amygdala, occipital cortex (OCC) and fusiform gyrus (FG). ROIs were defined based on the Automated Anatomical Labeling (AAL) atlas (Maldjian et al., 2004, 2003; Tzourio-Mazoyer et al., 2002) and transformed into Talairach space (Lancaster et al., 2007) using ICBM2TAL in Matlab (v8.2, The MathWorks Inc., Natick, Massachusetts, USA). The anatomically driven subdivision for cingulate cortex was used (Vogt, 2014). The widely used and functionally defined term of dorsal anterior cingulate cortex (dACC) refers to the same region as anterior mid cingulate cortex (amCC).

Statistical parametric maps resulting from voxel-wise analyses were considered significant for clusters that survived cluster-based correction for multiple comparisons. For ROI and PPI analyses, the voxel-level threshold was set to the voxel-level threshold of $p < 0.005$ in order to balance between Type I and II error rates (Lieberman and Cunningham, 2009).

Using the cluster-level statistical threshold estimator plugin for BVQX (Goebel et al., 2006), a mask consisting of all predefined ROIs (comprising cingulate cortex, insula, PFC, thalamus, amygdala, OCC, FG) was applied to the thresholded maps. ROI-specific correction criteria were based on the estimates of the maps' spatial smoothness and on an iterative procedure (Monte Carlo simulation) applied to estimate cluster-level false-positive rates (Forman et al., 1995). This procedure yielded after 1000 iterations a minimum cluster size to generate a map-wise corrected false positive rate of $p < 0.05$. To account for multiple testing in PPI analyses a Bonferroni-corrected threshold ($p \leq 0.01$) was used. Analysis of covariance (ANCOVA) in ROI and PPI analyses tested whether results were maintained after inclusion of BDI level, ASI score, medication intake, and subjective ratings as covariates.

To facilitate interpretation of the ROI main (*picture valence*) and interaction effects (*picture valence* × *group*) and PPI effect (*group*), the average percent signal change across all voxels within each cluster was extracted for each regressor per subject and resolved through SPSS.

3. Results

3.1. Rating data

Rating data were available for 82 participants (see Supplement Fig. S4). Main effect *group* (arousal: $F_{[3,78]} = 4.69$, $p = 0.005$; valence: $F_{[3,78]} = 6.55$, $p = 0.001$; anxiety: $F_{[3,78]} = 6.78$, $p < 0.001$) displayed that threat pictures were rated as more negative ($M = 2.92$, $SD = 0.85$), more arousing ($M = 5.66$, $SD = 1.79$) and more anxiety-inducing ($M = 4.53$, $SD = 2.26$) than neutral pictures (valence: $M = 5.88$, $SD = 0.84$; arousal: $M = 2.27$, $SD = 1.14$; anxiety: $M = 1.48$, $SD = 0.84$) by all participants. Main effect *picture valence* (arousal: $F_{[1,78]} = 456.74$, $p < 0.001$; valence: $F_{[1,78]} = 522.47$, $p < 0.001$; anxiety: $F_{[1,78]} = 226.05$, $p < 0.001$) showed that all stimuli were rated as more negative by GAD patients vs. HC ($p = 0.014$), SAD patients vs. HC ($p = 0.001$) and SAD vs. PD patients ($p = 0.036$). Moreover, stimuli were rated as more arousing by GAD patients vs. HC ($p = 0.004$) and as more anxiety-inducing by GAD vs. PD patients ($p = 0.015$), GAD

patients vs. HC ($p = 0.001$), and SAD patients vs. HC ($p = 0.026$). The significant interaction effect *group* by *picture valence* for anxiety ratings ($F_{[3,78]} = 4.56, p = 0.005$) showed that threat pictures were rated as more anxiety-inducing by GAD patients vs. PD ($t_{[38]} = 3.24, p = 0.003$) and vs. HC ($t_{[39]} = 3.92, p < 0.001$). All other effects failed to reach significance ($p > 0.05$).

3.2. ROI analysis

3.2.1. Main effects picture valence (across participants)

Threat vs. neutral pictures resulted in increased activity in amygdala, thalamus, OCC, ventrolateral PFC (vlPFC), dorsolateral PFC (dlPFC), mPFC, FG, anterior insula (al), pregenual anterior cingulate cortex (pgACC), aMCC, posterior mid cingulate cortex (pmCC), and posterior cingulate cortex (PCC) (Table 1; Fig. 1). Two clusters in vlPFC and one in dlPFC showed decreased activity to threat vs. neutral pictures.

3.2.2. Interaction effects picture valence by group

ROI analysis revealed six brain regions in which there were significant interactions between *picture valence* by *group*: left aMCC, left

pmCC, right dorsal PCC (dPCC), left posterior dlPFC, left vlPFC and left dorsal anterior insula/frontal operculum (dal/FO) (Table 1; Fig. 2). The influence of medication intake, subjective ratings, ASI score and BDI level were tested by means of ANCOVAs and revealed no significant effects of the covariates (all $p > 0.05$) and maintained *group* effects (all F -values ≥ 3.83 , all $p < 0.05$).

Interaction effects in aMCC, pmCC, dPCC, dlPFC and dal/FO derived from increased activity to threat vs. neutral pictures in GAD patients relative to SAD or PD patients or to HC (Table 1). As such GAD patients showed a GAD-specific brain activity pattern in these brain regions. Comparisons of SAD patients vs. HC, SAD patients vs. PD patients or PD patients vs. HC in aMCC, pmCC, dPCC, dlPFC and dal/FO revealed no significant effects, underlying that neither SAD or PD patients showed a disorder-specific brain activity responding in these regions in response to threat vs. neutral pictures. In addition, there was another interaction effect in one cluster in left vlPFC in which SAD and PD patients displayed reduced activity in response to threat vs. neutral pictures. Left vlPFC was the only brain region in which a significant deviating brain response was detected when comparing SAD patients vs. HC or PD patients vs. HC (Table 1).

Table 1

ROI analysis: Significant main and interaction effects of the *picture valence* (threat, neutral pictures) by *group* (GAD, PD, SAD, HC) ANOVA.

Region	Lateralization	x	y	z	F	mm ³
Main effect: picture valence						
Increased activity to threat > neutral pictures						
pgACC/aMCC/pMCC	L/R	-2	0	31	16.15	4920 ^a
PCC	L/R	-14	-39	36	14.69	3112 ^a
Anterior insula ventral/dorsal	L	-25	13	-10	19.19	2843 ^a
	R	28	21	-6	16.54	3201 ^a
Amygdala	L	-19	-5	-10	24.75	1560 ^a
	R	16	-6	-11	23.35	1872 ^a
OCC	L	-39	-55	-10	30.43	21302 ^a
	R	37	-71	-6	21.87	16734 ^a
Thalamus	L	-13	-29	0	13.04	2207 ^a
	R	1	-13	8	12.57	1305 ^a
FG	L	-39	-55	-11	29.17	6888 ^a
	R	38	-47	-10	24.70	7345 ^a
	L	-36	-12	-21	13.88	648
vlPFC to dlPFC	L	-47	29	10	20.53	20896 ^a
	R	41	5	30	23.17	22171 ^a
dlPFC	R	40	-2	52	9.49	160
mPFC	R/L	-3	49	34	14.91	13984 ^a
Reduced activity to threat > neutral pictures						
vlPFC	L	-23	48	1	11.87	560
	R	27	54	7	9.84	976
dlPFC	R	28	14	56	11.38	2600
Interaction effect: picture valence by group						
aMCC	L	-9	28	20	6.00	192 ^b
pmCC	L	-14	-13	43	6.22	184 ^b
dPCC	R	10	-42	34	5.29	88 ^b
dal/FO	L	-32	15	17	6.14	160 ^b
posterior dlPFC	L	-31	27	48	5.22	472 ^b
vlPFC	L	-38	33	6	4.92	56

Dissolving the interaction effects (*picture valence* by *group*) in ROI analysis in response to threat versus neutral pictures

	aMCC	pmCC	dPCC	dal/FO	posterior dlPFC	vlPFC
GAD vs. SAD	$t_{[39]} = 5.03, p < 0.001$	$t_{[39]} = 5.08, p < 0.001$	$t_{[39]} = 3.12, p = 0.003$	$t_{[39]} = 4.95, p < 0.001$	$t_{[39]} = 3.05, p = 0.004$	$t_{[39]} = 2.52, p = 0.016$
GAD vs. PD	$t_{[39]} = 2.84, p = 0.007$	$t_{[39]} = 3.88, p < 0.001$	$t_{[39]} = 3.64, p = 0.001$	$t_{[39]} = 3.68, p = 0.001$	$t_{[39]} = 3.36, p = 0.002$	$t_{[39]} = 2.23, p = 0.031$
GAD vs. HC	$t_{[39]} = 3.53, p = 0.001$	$t_{[39]} = 2.71, p = 0.010$	$t_{[39]} = 2.94, p = 0.005$	$t_{[39]} = 2.95, p = 0.005$	$t_{[39]} = 2.42, p = 0.020$	n.s.
SAD vs. PD	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SAD vs. HC	n.s.	n.s.	n.s.	n.s.	n.s.	$t_{[40]} = 2.79, p = 0.008$
PD vs. HC	n.s.	n.s.	n.s.	n.s.	n.s.	$t_{[40]} = 2.52, p = 0.016$

Note. ROI, region of interest; ANOVA, analysis of variance; GAD, generalized anxiety disorder; SAD, social anxiety disorder; PD, panic disorder; HC, healthy controls; pgACC, pregenual anterior cingulate cortex; aMCC, anterior mid cingulate cortex; pmCC, posterior mid cingulate cortex; PCC, posterior cingulate cortex; dPCC, dorsal posterior cingulate cortex; OCC, occipital cortex; al, anterior insula; FG, fusiform gyrus; mPFC, medial prefrontal cortex; vlPFC, ventrolateral prefrontal cortex; dlPFC, dorsolateral prefrontal cortex; dal/FO, dorsal anterior insula/frontal operculum L, left; R, right; x,y,z, Talairach coordinates of maximally activated voxel (activation threshold: $p < 0.05$ corrected).

^a Depicted in Fig. 1.

^b Depicted in Fig. 2.

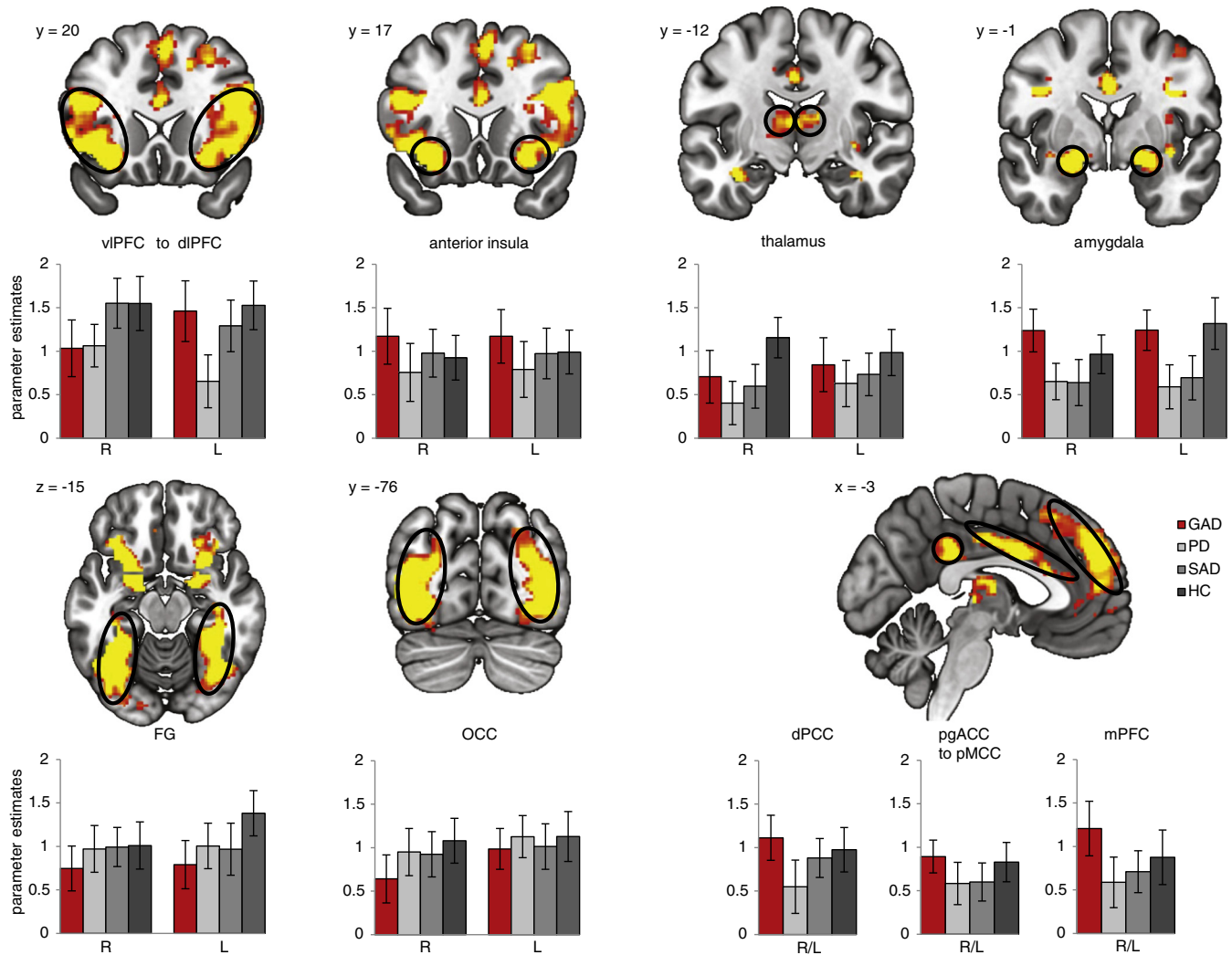


Fig. 1. Increased activation to threat vs. neutral pictures across all groups (GAD, generalized anxiety disorder; SAD, social anxiety disorder; PD, panic disorder; HC, healthy controls). The 2 (picture valence) by 4 (group) region of interest analysis of variance (ROI ANOVA) revealed significant brain activation clusters for the main effect *picture valence* in the following regions: amygdala, thalamus, pregenual anterior cingulate cortex (pgACC), anterior mid cingulate (aMCC), posterior mid cingulate cortex (pMCC), posterior cingulate cortex (PCC), medial prefrontal cortex (mPFC), ventrolateral prefrontal cortex (vIPFC), dorsolateral prefrontal cortex (dIPFC), occipital cortex (OCC), fusiform gyrus (FG) (Table 1). To visualize brain responses of each group within main effect clusters of *picture valence*, graphs display contrasts of parameter estimates (threat > neutral pictures [mean \pm standard error for activation cluster]) per group. Statistical parametric maps are overlaid on an averaged T1 scan (radiological convention: left = right).

3.3. PPI analyses

3.3.1. Main effects group

One-way ANOVAs per seed region (aMCC, pMCC, dPCC, dIPFC, dal/FO) revealed *group* effects in several regions. GAD patients showed increased FC for threat vs. neutral pictures relative to each group between left pMCC and right amygdala, between pMCC and right ventral al and between left posterior dIPFC and right anterior vIPFC, as well as decreased FC between left dal/FO and right mid-dIPFC (Table 2; Fig. 3). All results remained highly significant after including medication intake, BDI level, ASI score and subjective ratings as covariates (all F -values ≥ 5.19 , all $p < 0.05$), although the covariate itself was significant in three cases (for medication intake: FC between pMCC-amygdala, $F_{[1,78]} = 5.25$, $p = 0.025$; for BDI level: FC between dal/FO-dIPFC, $F_{[1,78]} = 7.44$, $p = 0.008$; and for ASI score: FC between dal/FO-dIPFC $F_{[1,78]} = 7.94$, $p = 0.006$).

4. Discussion

In order to identify GAD-specific neural circuits underlying immediate threat processing, threat and neutral pictures were presented in an

event-related design and GAD brain activity was compared between GAD, SAD and PD patients as well as HC. Shared elevated brain responses to threat in GAD, SAD, PD and HC were found in amygdala, al, thalamus, lateral and medial PFC, cingulate and visual cortex. GAD-specific threat-related increased brain responses emerged in aMCC, pMCC, dPCC, posterior dIPFC and dal/FO. In addition, GAD-specific enhanced activity to threat in pMCC, dIPFC and dal/FO showed a GAD-specific FC pattern to other regions, too: increased FC was found between posterior dIPFC and vIPFC, between pMCC and amygdala, between pMCC and al, and decreased FC was found between dal/FO and mid-dIPFC. With regard to RDoC, we provide evidence of shared neural networks underlying threat processing across anxious patients and HC. Crucially, GAD patients were also marked by threat-related disorder-specific brain responses.

To our knowledge, the present study is the first to report GAD-specific cingulate cortex involvement during threat processing. Previous studies (Ball et al., 2013; Blair et al., 2012, 2008; Fonzo et al., 2015) failed to reveal such GAD-specificity, possibly due to experimental designs with instructed, demanding stimulus presentation or facial expressions as stimuli. In the present study, GAD patients shared elevated activity in pgACC/aMCC/pMCC and in PCC to threat with SAD and PD patients, as

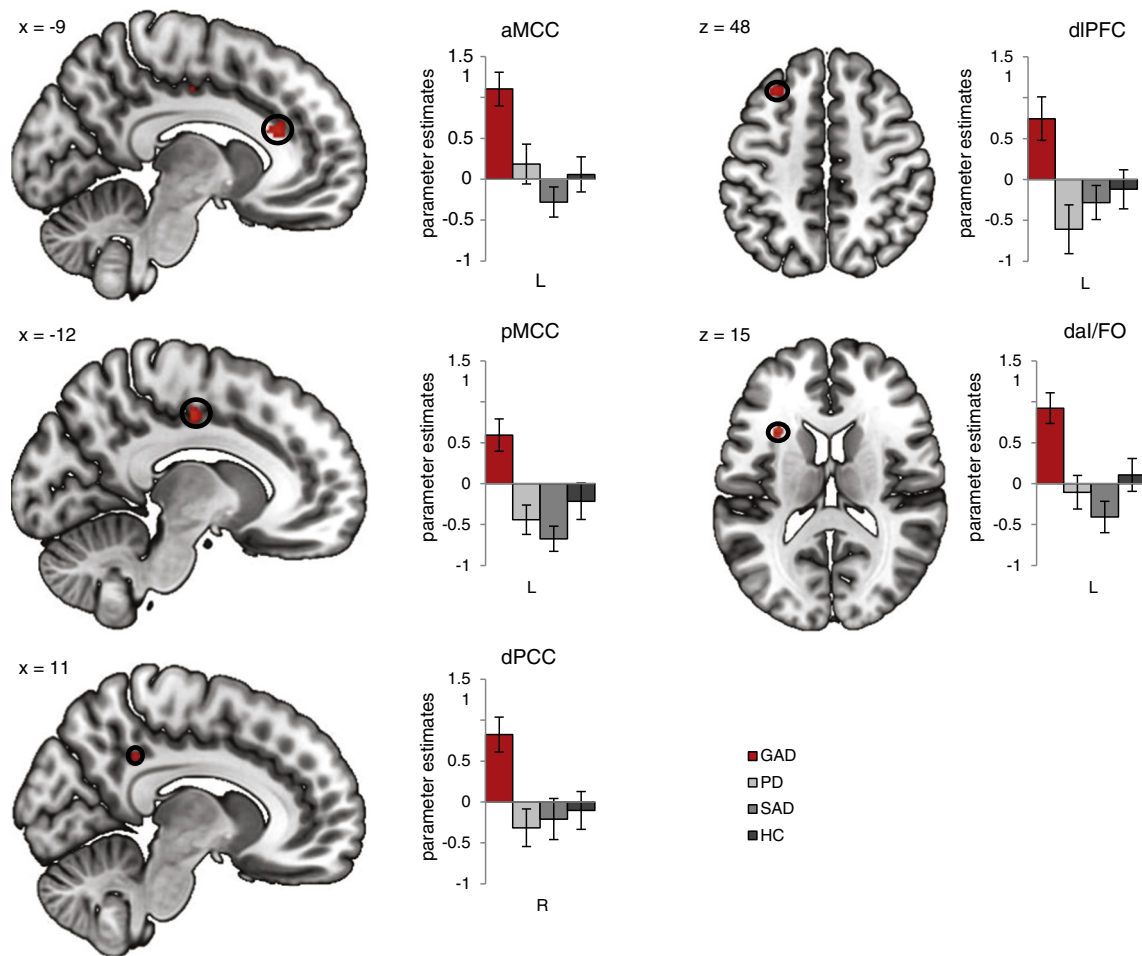


Fig. 2. Generalized anxiety disorder (GAD)-specific brain activations to threat. The 2 (*picture valence*) by 4 (*group*) region of interest analysis of variance (ROI ANOVA) revealed significant brain activations for the interaction effect *picture valence* \times *group* in which GAD patients showed significant disorder-specific responding relative to each group (vs. panic disorder patients [PD], vs. social anxiety disorder patients [SAD], vs. healthy controls [HC]; all $p < 0.05$) in the following regions: anterior mid cingulate cortex (aMCC), posterior mid cingulate cortex (pMCC), dorsal posterior cingulate cortex (dPCC), dorsolateral prefrontal cortex (dlPFC), dorsal anterior insula/frontal operculum (dal/FO) (Table 1). Statistical parametric maps are overlaid on an averaged T1 scan (radiological convention: left = right). Graphs display contrasts of parameter estimates (threat > neutral pictures [mean \pm standard error for activation cluster]) per group.

well as with HC. PgACC, aMCC, pMCC and PCC activities were shown to be associated with the elicitation of negative affect, threat appraisal, expression of learned fear/action selection and control of attentional focus, respectively (Etkin et al., 2011; Kalisch and Gerlicher, 2014; Leech and Sharp, 2014; Maier et al., 2012; Mechias et al., 2010; Stevens et al., 2011; Vogt, 2014). In addition to shared neural responses to threat, GAD patients showed increased activity in aMCC, pMCC and dPCC. Thus, GAD patients appeared particularly sensitive to threat, as they may have recruited additional appraisal, attention allocation and response preparation processes, possibly resulting in overestimation or over-interpretation of threat (Hirsch and Mathews, 2012). Previous studies suggested cingulate cortex to play a key role in the pathophysiology of GAD: Elevated aMCC activity was observed in GAD patients vs. HC, triggered by negative pictures (Blair and Blair, 2012; Blair et al., 2012) or in worry-induction tasks (Andreescu et al., 2011; Paulesu et al., 2010). In addition, reduced anterior cingulate cortex/ mid cingulate cortex activity was associated with medication-treatment response (Hoehn-Saric et al., 2004), which was even predictive of the magnitude of reduction (Nitschke et al., 2009; Whalen et al., 2008). Thus, GAD patients are marked by deviating aMCC recruitment in diverse contexts, possibly reflecting exaggerated negative appraisal. Concordantly, maladaptive appraisal is treated in cognitive behavioral therapy for GAD, to procure a more elaborate and adaptive threat appraisal (Kalisch and Gerlicher, 2014).

Furthermore, GAD patients displayed disorder-specific increased dal/FO activity and shared elevated ai activity with SAD, PD and HC in response to threat pictures. Shared ai activity was detected in both ventral and dorsal parts, replicating previous findings (Fonzo et al., 2015). AI is assumed to represent processing of interoceptive sensations and to signal information to brain areas critical for attention allocation and action execution, such as cingulate cortex (Lindquist and Barrett, 2012). Activity in dal/FO has been associated with focal attention regulation (Higo et al., 2011; Lindquist and Barrett, 2012; Nelson et al., 2010). Along these lines, all participants appeared to show similar neural representations of interoceptive sensations in response to threat. Yet, it seems that interoceptive sensations may have been more salient to GAD patients, indicated by the GAD-specific dal/FO activity (Higo et al., 2011; Lindquist and Barrett, 2012; Nelson et al., 2010).

Moreover, GAD patients displayed disorder-specific posterior dlPFC activity and shared PFC activity in medial and lateral PFC in response to threat. Activity in medial and lateral PFC regions is linked to evaluative and regulative aspects of emotions (Cohen et al., 2013; Phan et al., 2005). Posterior dlPFC is associated with attentional top-down processes underlying the processing of task-relevant information (Warren et al., 2013). GAD patients may have experienced more difficulty in shifting attention away from threat pictures (irrelevant task information), resulting in exaggerated attention regulation through posterior dlPFC. Hence, threat-related GAD-specific posterior dlPFC activity

Table 2
PPI analyses: Defining GAD-specific interaction effects of the ROI analysis as seed regions (dal/FO, pMCC, dlPFC) revealed significant FC differences between GAD patients and each other group in response to threat vs. neutral pictures.

Seed region	Finding region	x	y	z	F	mm ³
Left dal/FO	Right mid-dIPFC	24	49	16	5.58	296
Left pMCC	Right amygdala	22	1	-19	5.70	152
	Right ventral anterior insula	23	20	-8	5.98	144
Left posterior dlPFC	Right vIPFC	30	26	-13	6.30	144

Planned comparisons revealed GAD-specific FC patterns in response to threat vs. neutral pictures (seed–finding region)

	pMCC - amygdala	pMCC - al	dal/FO - mid-dIPFC	dlPFC - vIPFC
GAD vs. SAD	$t_{[39]} = 2.25, p = 0.030$	$t_{[39]} = 2.64, p = 0.012$	$t_{[39]} = 3.78, p = 0.001$	$t_{[39]} = 4.28, p < 0.001$
GAD vs. PD	$t_{[39]} = 3.82, p < 0.001$	$t_{[39]} = 3.69, p = 0.001$	$t_{[39]} = 2.63, p = 0.012$	$t_{[39]} = 2.40, p = 0.021$
GAD vs. HC	$t_{[39]} = 4.05, p < 0.001$	$t_{[39]} = 4.80, p < 0.001$	$t_{[39]} = 4.41, p < 0.001$	$t_{[39]} = 4.67, p < 0.001$

Note. PPI, psychophysiological interaction analyses; FC, functional connectivity; GAD, generalized anxiety disorder; SAD, social anxiety disorder; PD, panic disorder; HC, healthy controls; pMCC, posterior mid cingulate cortex; vIPFC, ventrolateral prefrontal cortex; dlPFC, dorsolateral prefrontal cortex; dal/FO, dorsal anterior insula/frontal operculum; L, left; R, right; x, y, z, Talairach coordinates of maximally activated voxel (activation threshold: $p < 0.05$ corrected).

seems to corroborate GAD-specific exaggerated attention regulation processes in response to threat.

FC analyses displayed that brain areas with GAD-specific threat-related responding (aMCC, pMCC, dal/FO, dlPFC) showed a GAD-specific FC pattern to other emotion generating and attention regulating regions. In line with our expectation of increased FC within SN, pMCC activity was positively connected to amygdala and ventral al activity. Amygdala activity is linked to faster threat processing and ventral al activity to the representation of interoceptive sensations (Lindquist and Barrett, 2012). Following that, heightened threat sensitivity in GAD seems supported. In line with our expectation of reduced SN and ECN connectivity, activity in dal/FO was negatively connected to mid-dIPFC activity in GAD patients. Mid-dIPFC is suggested to be involved in attention shifting processes (Warren et al., 2013). Thus, GAD patients may have focused strongly on interoceptive sensations in response to threat together with deficient attention shifting away from threat (Warren et al., 2013). Contrary to the hypothesized reduced FC within ECN, we detected GAD-specific positive coupling of dlPFC with vIPFC activity. vIPFC is suggested to be involved in regulation of unpleasant emotions (Cohen et al., 2013; Phan et al., 2005). Increased dlPFC activity may reflect heightened effort to ignore task-irrelevant information and increased connectivity to vIPFC may point towards heightened attempts of down-regulating unpleasant emotions in GAD patients (Cohen et al., 2013; Phan et al., 2005). The current FC results may indicate that GAD patients displayed exaggerated threat processing with heightened attempts to regulate attention and failure to resist distraction. However, other studies failed to detect GAD-specific FC alterations in amygdala-PFC circuitry relative to MDD or PTSD patients (Chen and Etkin, 2013; Etkin and Schatzberg, 2011). One explanation may be that our design presented a variety of very different threat pictures that are possibly more relevant to GAD patients, rather than facial expressions only and threat pictures were presented in a non-demanding task. Overall, FC results of the present study are in line with previous suggestions of alterations within amygdala-PFC circuitry and with reduced FC between insula and dlPFC (Andreescu et al., 2015; Etkin et al., 2010; Makovac et al., 2015) and support the suggestion of heightened threat sensitivity and altered attention regulation processes in GAD.

The authors of a recent review stated that there is currently no reason to believe that GAD patients show heightened threat processing (Blair et al., 2012). This tentative conclusion was derived based on studies using facial stimuli or sounds, as well as threat pictures presented in a cued task design (Blair et al., 2008; Grillon et al., 2009; Hoehn-Saric et al., 1989; Nitschke et al., 2009; Palm et al., 2011; Whalen et al., 2008). Possible explanations for the divergent results may be that our design presented a variety of threat pictures which likely were more relevant to GAD patients than facial expressions or sounds, and that these were presented in a non-demanding context. Cued designs might have induced preparation for upcoming picture exposure, an effect prevented in the present experimental design.

However, some limitations need to be addressed. First, FC analyses were restricted to seed regions based on GAD-specific findings. Future studies could use a comprehensive ROI approach. Second, six to seven patients per group were medicated. Unfortunately, studies with large samples always face possible confounds, and analyses showed that medication intake did not influence effects. Last, patients had comorbidities. Given that comorbidities frequently occur in GAD, the present sample is likely to be representative of the GAD population (Newman et al., 2013). Nevertheless, it would be beneficial to investigate patients without medication or comorbidities in future studies, possibly also accounting for other variables such as personality, genotypes, and intelligence and to include further control groups, such as patients suffering from MDD.

Overall, the present results show that GAD patients share common brain responses to threat with SAD and PD patients and HC. Yet, GAD patients also displayed disorder-specific brain activity and FC patterns during immediate threat processing, suggestive of hyper-responsive

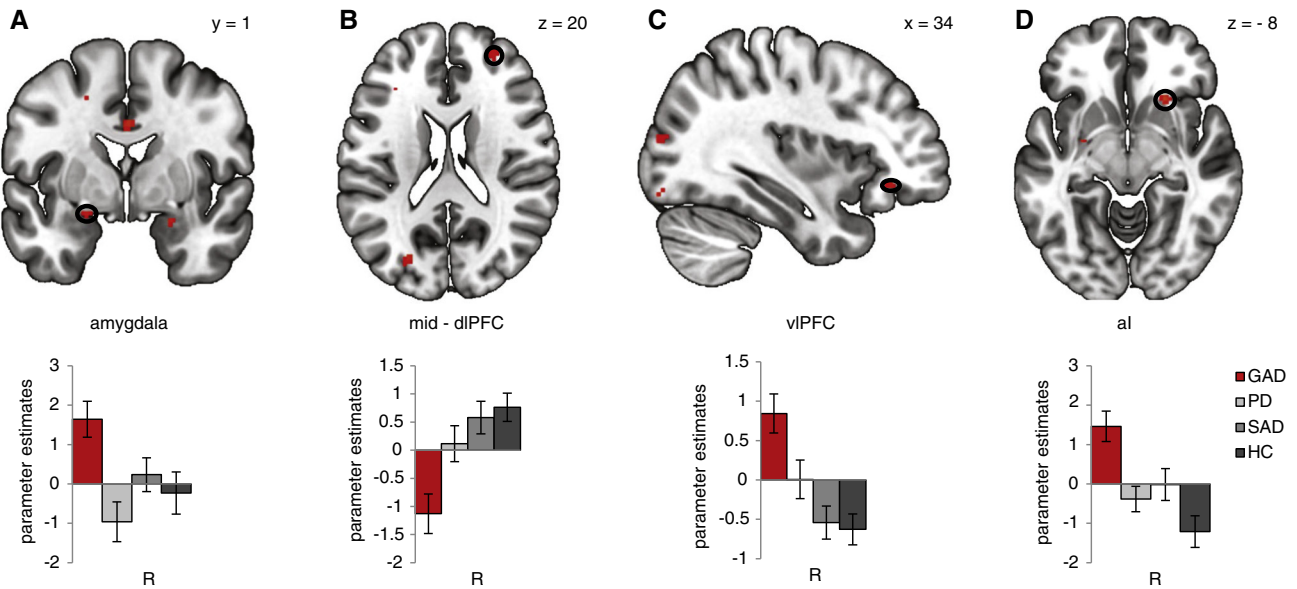


Fig. 3. Generalized anxiety disorder (GAD)-specific effects in psychophysiological interaction analyses (PPI). One-way region of interest analyses of variances (ROI ANOVAs) per seed region revealed significant functional connectivity (FC) effects for the main effect *group* in which generalized anxiety disorder (GAD) patients showed disorder-specific responding to threat vs. neutral pictures relative to each group (vs. panic disorder patients [PD], vs. social anxiety disorder patients [SAD], vs. healthy controls [HC]; all $p < 0.05$) (Table 2). A: Seeding left posterior mid cingulate cortex (pmCC) revealed significantly increased FC with right amygdala for GAD patients relative to healthy controls (HC), social anxiety disorder (SAD) patients or panic disorder (PD) patients. B: Seeding left dorsal anterior insula/frontal operculum (dal/FO) revealed significantly decreased FC with right mid-dIPFC for GAD patients relative to HC, SAD or PD patients. C: Seeding left posterior dorsolateral prefrontal cortex (dlPFC) revealed significantly increased FC with right ventrolateral prefrontal cortex (vlPFC) for GAD patients relative to HC, SAD or PD patients. D: Seeding left posterior mid cingulate cortex (pmCC) revealed significantly increased FC with right ventral anterior insula (ai) for GAD patients relative to HC, SAD or PD patients. Statistical parametric maps are overlaid on an averaged T1 scan (radiological convention: left = right). Graphs display contrasts of parameter estimates threat > neutral pictures [(mean \pm standard error for activation cluster)] per group.

threat processing in GAD primarily in frontal cortex. These findings support a neurobiological model of immediate threat processing in GAD, with increased threat sensitivity paralleled by maladaptive appraisal and exaggerated attention allocation, presumably resulting in over-interpretation and overestimation of threat, which distinguishes GAD from other anxiety disorders (Hölzel et al., 2013; Olatunji et al., 2011). Therewith, findings of the present study are in line with established cognitive models of GAD postulating patients to be hyper-responsive to threat and provide first neural evidence (Behar et al., 2009; Hayes and Hirsch, 2007; Mennin et al., 2009; Newman et al., 2013). Nevertheless, further investigations on immediate threat processing in GAD and replication of current findings will aid to conceptualization and treatment development of the disorder.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nicl.2016.09.023>.

Conflict of interest

The authors declare no conflict of interest.

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