Using novel imaging approaches in affective disorders : beyond current models

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Chapter 3
Resting-state functional connectivity abnormalities in limbic and salience networks in social anxiety disorder without comorbidity

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Abstract

The neurobiology of social anxiety disorder (SAD) is not yet fully understood. Structural and functional neuroimaging studies in SAD have identified abnormalities in various brain areas, particularly the amygdala and elements of the salience network. This study is the first to examine resting-state functional brain connectivity in a drug-naive sample of SAD patients without psychiatric comorbidity and healthy controls, using seed regions of interest in bilateral amygdala, in bilateral dorsal anterior cingulate cortex for the salience network, and in bilateral posterior cingulate cortex for the default mode network. Twelve drug-naive SAD patients and pair-wise matched healthy controls, all drawn from the Netherlands Study of Depression and Anxiety sample, underwent resting-state fMRI. Group differences were assessed with voxel-wise grey matter density as nuisance regressor. All results were cluster corrected for multiple comparisons ($Z > 2.3$, $p < .05$). Relative to control subjects, drug-naive SAD patients demonstrated increased negative right amygdala connectivity with the left middle temporal gyrus, left supramarginal gyrus and left lateral occipital cortex. In the salience network patients showed increased positive bilateral dorsal anterior cingulate connectivity with the left precuneus and left lateral occipital cortex. Default mode network connectivity was not different between groups. These data demonstrate that drug-naive SAD patients without comorbidity show differences in functional connectivity of the amygdala, and of areas involved in self-awareness, some of which have not been implicated in SAD before.

Introduction

Social anxiety disorder (SAD) is characterized by persistent fear of social or performance situations in which judgment or scrutiny by others and embarrassment can occur. Social situations are preferably avoided or otherwise experienced with extreme anxiety and discomfort (American Psychiatric Association, 1994, Furmark, 2002). The number of neuroimaging studies investigating SAD is rather modest, but these have provided some insight in the brain circuitry involved in this disorder. Structural neuroimaging studies have been scarce, and have used different approaches, reporting inconsistent results with some studies showing no abnormalities and others decreases in volume of the amygdala, right posterior right temporal gyrus and (para)hippocampus (Potts *et al.*, 1994, Irle *et al.*, 2010, Liao *et al.*, 2011). The number of functional studies is much greater, with most of these studies focusing on the processing of emotional faces or using symptom provocation designs (Etkin and Wager, 2007, Blair *et al.*, 2008a, Blair *et al.*, 2008b, Damsa *et al.*, 2009, Gentili *et al.*, 2009, Freitas-Ferrari *et al.*, 2010, Shin and Liberzon, 2010, Blair *et al.*, 2011a). A recent systematic review on neuroimaging studies in SAD by Freitas-Ferrari *et al.* (2010), mainly focusing on functional connectivity, found the amygdala and its connections in the emotional and fear circuitry to play a key role in SAD (Freitas-Ferrari *et al.*, 2010). However, other areas such as the insula (Shah *et al.*, 2009), anterior cingulate cortex (Amir *et al.*, 2005, Blair *et al.*, 2011a) and ventromedial prefrontal cortex have also been reported to be involved in SAD (Etkin and Wager, 2007, Damsa *et al.*, 2009, Freitas-Ferrari *et al.*, 2010).

Resting-state fMRI

Resting-state fMRI (RS-fMRI) enables the monitoring of brain activity and connectivity in the absence of externally controlled tasks or stimuli (Fox and Raichle, 2007). Consistently reported RS networks of functionally interconnected brain regions include the default mode network (DMN) (precuneus/posterior cingulate cortex, medial prefrontal cortex, and lateral parietal cortex) (Raichle *et al.*, 2001), and the salience network (Seeley *et al.*, 2007, Biswal *et al.*, 2010). The salience network is important in assessing the relevance of internal and external stimuli in order to guide behaviour (Seeley *et al.*, 2007). Its principal areas are the bilateral anterior insula and bilateral dorsal anterior cingulate cortex, two areas frequently

reported to be involved in SAD (Freitas-Ferrari *et al.*, 2010). So far, five RS-fMRI connectivity studies in SAD have been published, four of which were conducted by the same research group within overlapping cohorts of SAD patients (Liao *et al.*, 2010a, Liao *et al.*, 2010b, Hahn *et al.*, 2011, Ding *et al.*, 2011, Liao *et al.*, 2011). Using independent component analysis, Liao *et al.* (2010a) revealed altered connectivity in SAD patients in seven out of the eight RS networks resulting from their analysis, including the DMN and a self-referential network. Noteworthy, a limbic network was not among these net- works. Subsequently, Liao *et al.* (2010b) used a seed-based region of interest analysis and demonstrated altered amygdala RS connectivity in social anxiety patients (Liao *et al.*, 2010b). In their third study, Liao *et al.* (2011) combined voxel-based morphometry, RS functional connectivity (RSFC), and diffusion tensor imaging (Liao *et al.*, 2011), and found decreased volumes and altered connectivity of the right inferior frontal gyrus and hippocampal areas. Finally, a whole-brain resting-state analysis was also per- formed, showing abnormal connectivity of frontal and occipital lobes (Ding *et al.*, 2011).

The group of Hahn *et al.* (2011) examined in one study RSFC in SAD using a seed-based region of interest approach and found altered left amygdala connectivity. However, the investigated sample was heterogeneous, consisting of SAD patients as well as patients with comorbid panic disorder, and one panic disorder patient (Hahn *et al.*, 2011). It is therefore difficult to ascertain whether the differences were solely attributable to SAD. Additionally, in four out of the five published RS studies patients were not using medication at the time of research, but were not drug- naive, thus possible effects of recent pharmacotherapy cannot be ruled out. Since the effect of anti-anxiety medication on neuronal activity has been demonstrated in SAD patients (Van der Linden *et al.*, 2000, Furmark *et al.*, 2005), a drug-naive sample should preferably be used to rule out such effects.

Here, we set out to investigate RSFC in drug-naive SAD patients without psychiatric comorbidity using a seed-based correlation approach. Given the anatomical and functional abnormalities found in previous MRI studies in SAD, suggesting a key role for the amygdala (Kent and Rauch, 2003, Shin and Liberzon, 2010, Freitas-Ferrari *et al.*, 2010), and its key role in emotion generation, fear and anxiety (LeDoux, 2003), we hypothesised abnormalities in amygdala RSFC. Additionally, based on previous reports

of involvement of the dorsal anterior cingulate cortex in SAD and based on the functions of the salience network (Seeley *et al.*, 2007) and its relevance for anxiety disorders (Amir *et al.*, 2005, Etkin *et al.*, 2009, Etkin *et al.*, 2010, Freitas-Ferrari *et al.*, 2010, Blair *et al.*, 2011a) we also expected abnormalities in the connectivity of the salience network. As the DMN showed altered connectivity in many neuropsychiatric disorders such as depression (Greicius *et al.*, 2007, Broyd *et al.*, 2009), epilepsy, autism, attention deficit/hyperactivity disorder, anxiety disorders (Broyd *et al.*, 2009), and perhaps also in SAD where it is thought to play a role in social cognition (Gentili *et al.*, 2009, Liao *et al.*, 2010a), we also hypothesised abnormalities in the connectivity of the DMN in social anxiety patients.

Experimental procedures

Participants

Subjects were selected from the MRI study within the large-scale longitudinal multi-centre cohort Netherlands Study of Depression and Anxiety (NESDA). NESDA is designed to investigate the long-term course and consequences of depression and anxiety disorders. Participants in NESDA were recruited from the community, and through primary care and specialized mental health institutions. The rationales, methods and recruitment have been described in detail else- where; for an overview of diagnostics, inclusion and exclusion criteria see: (Penninx *et al.*, 2008, van Tol *et al.*, 2010).

After receiving written information, all subjects provided written informed consent. Participants underwent MR imaging in one of the three participating centres (Academic Medical Centre Amsterdam, Leiden University Medical Centre, and University Medical Centre Groningen) (van Tol *et al.*, 2010). The study was approved by the Medical Ethics Committees of all three centres and con- ducted in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

For the present study on SAD patients without comorbidity, MRI data were available from 12 right-handed SAD patients (5 male) and from 12 healthy controls matched for age, gender, education, handedness, and scan-location (Table 1). All subjects were new to this specific scanning situation. Patients were diagnosed with SAD and no other psychopathology using the DSM-IV-based Composite International Diagnostic Interview, lifetime version 2.1 (American Psychiatric Association, 1994). At the time

of scanning, anxiety and phobic symptoms were also assessed using the Fear Questionnaire (FQ) (Marks and Mathews, 1979) and depressive symptoms were rated with the Montgomery–Åsberg Depression Rating Scale (Montgomery and Asberg, 1979). Demographic and clinical characteristics were analysed using SPSS 17.0 (SPSS Inc, Chicago, Illinois) with significance set at $p<0.05$.

Table 1. Demographic and Clinical Characteristics of Patients with Social Anxiety Disorder and Healthy Controls

	Social Anxiety Disorder patients (N=12)		Healthy controls (N=12)		
Gender	5 male / 7 female		5 male / 7 female		
Scan location	2 AMC; 5 LUMC; 5 UMCG		3 AMC; 5 LUMC; 4 UMCG		
	Mean	SD	Mean	SD	*p*
Age	34.8	8.8 (24-53 years)	34.0	7.2 (21-47 years)	0.821
Education	11.8	3.2 (6-18 years)	13.7	2.4 (10-18 years)	0.123
FQ social phobia score	20.8	8.2 (8-36)	4.5	4.3 (0-13)	0.001**
MADRS score at scanning	7.8	8.6 (0-25)	1.2	2.0 (0-5)	0.001**

SD = standard deviation; AMC = Academic Medical Center Amsterdam; LUMC = Leiden University Medical Center; UMCG = University Medical Center Groningen; FQ = Fear Questionnaire; MADRS = Montgomery-Åsberg Depression Rating Scale.
** Mann-Whitney U Test.

Image data acquisition

Image acquisition took place at the Academic Medical Centre Amsterdam, the Leiden University Medical Centre, and the University Medical Centre Groningen. Images were obtained on Philips 3T magnetic resonance imaging systems (Philips Medical Systems, Best, The Netherlands), equipped with a SENSE-8 (Leiden University Medical Centre and University Medical Centre Groningen) or SENSE- 6 (Academic Medical Centre Amsterdam) channel head coil.

As part of a fixed imaging protocol, resting-state functional MRI data were acquired for each subject using T2*-weighted gradient- echo echo-planar imaging with the following scan parameters in Amsterdam and Leiden: 200 whole-brain volumes; repetition time 2300 ms; echo time 30 ms; flip angle 80°; 35 transverse slices; no slice gap; field of view 220 × 220 mm; in-plane voxel size 2.3 × 2.3 mm; slice thickness 3 mm; duration 7.51 min. Parameters in Groningen were identical, apart from: echo time 28 ms; 39 transverse slices; in-plane voxel size 3.45 × 3.45mm. In the darkened MR room participants were instructed to lie still with their eyes closed and not to fall asleep. After completion of the scan, subjects confirmed wakefulness during acquisition. A sagittal 3-dimensional

gradient-echo T1-weighted image was acquired for registration purposes and grey matter analysis with the following scan parameters: repetition time 9 ms; echo time 3.5 ms; flip angle 80°; 170 sagittal slices; no slice gap; field of view 256 × 256 mm; 1 mm isotropic voxels; duration 4.5 min.

No abnormalities were found upon inspection of the subjects' structural images by a neuroradiologist.

Preprocessing

FMRI data preprocessing and statistics were carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library, http://www.fmrib.ox.ac.uk/fsl) (Smith *et al.*, 2004). The following pre-statistics processing was applied: motion correction; non-brain removal; spatial smoothing using a 6 mm full-width at half-maximum Gaussian kernel; grand-mean intensity normalisation of the entire 4D dataset by a single multiplicative factor; high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with a 0.01 Hz cut-off). Registration of the RS data to high resolution T1-weighted, and the T1 to the 2 mm isotropic MNI-152 standard space image (T1 standard brain averaged over 152 subjects; Montreal Neurological Institute, Montréal, QC, Canada) was carried out (Smith *et al.*, 2004). The resulting transformation matrices were then combined to obtain a native to MNI space transformation matrix and its inverse (MNI to native space).

Statistical analysis

For the current study, a seed-based correlation approach (Fox and Raichle, 2007) was employed to study functional connectivity during rest (Roy *et al.*, 2009). The following seed regions-of-interest were selected: bilateral amygdala, bilateral dorsal anterior cingulate cortex (dACC) (for the salience network), and bilateral posterior cingulate cortex (PCC) (for the DMN). We created a mask in standard space for the amygdala based on the Harvard–Oxford Subcortical Structural Probability Atlas in FSL (Veer *et al.*, 2011). The coordinates for the dACC seeds were obtained directly from Table 1 of the study by Margulies *et al.* (2007), and PCC seed region coordinates were obtained from a study by Greicius *et al.* (2003). Table 2 contains the coordinates of the seed voxels. Spheres of 4 mm radius were created around the seed voxels. They were then transformed to native space by applying the inverse transformation matrix obtained from the

registration procedure, and spatially averaged time series were extracted for each seed and for each subject.

Table 2. MNI Coordinates of the Seed Regions

Seed region	MNI coordinates		
	x	*y*	*z*
Amygdala	+/- 22	-6	-16
dACC	+/- 6	18	28
PCC/precuneus	+/- 2	-52	26

MNI = Montreal Neurological Institute; dACC = dorsal anterior cingulate cortex; PCC = posterior cingulate cortex.

For each participant, and for each network of interest, we performed a multiple regression analysis using the general linear model (GLM) (as implemented in FEAT) (Smith *et al*., 2004). The time courses that were extracted from the voxels in all of our seed regions were entered as a regressor in a GLM for each network. Apart from the two regressors describing the left and right seeds, nine nuisance regressors were included in the model: signal from the white matter, cerebrospinal fluid signal, and the global signal, as well as six motion parameters (three translations and three rotations). The global signal was included to reduce the influence of artefacts caused by physiological signal sources (i.e., cardiac and respiratory) on the results (Fox and Raichle, 2007).

After reslicing the resulting parameter estimate maps and their corresponding within-subject variance maps into 2 mm isotropic MNI space, they were entered into a higher level within and between groups mixed effects analysis (one- and two-sample t-test).

As structural studies have indicated abnormalities in SAD, we used grey matter density information of each subject as a voxel-dependent covariate in our higher level model. By including structural information in the functional connectivity analysis, variance explained by potential differences in grey matter density and/or possible misregistrations are taken into account (Oakes *et al*., 2007). Lower level contrasts were analysed both within and between groups were analysed using the general linear model in which age and scan location were also entered as regressors. To correct for multiple comparisons, cluster correction was applied in all group analyses with significance set at a corrected $p<.05$ and an initial cluster-forming threshold of $Z>2.3$ (Worsley, 2001).

Results

Questionnaires

At the time of scanning, SAD patients scored significantly higher than controls on the FQ social phobia sub-scores, and on the Montgomery–Åsberg Depression Rating Scale (Table 1).

RSFC analysis

We first analysed amygdala RSFC. Within both groups the connectivity pattern largely overlapped with areas described to have functional and anatomical connections with the amygdala in previous studies (Stein *et al.*, 2007, Roy *et al.*, 2009). Areas showing positive connectivity with the amygdalae included the hippocampus, parahippocampal gyrus, insula, putamen, pallidum, thalamus, temporal pole, frontal orbital cortex, planum temporale, superior temporal gyrus, temporal fusiform gyrus, and brainstem. Areas showing a negative resting-state connectivity were the precentral gyrus, middle frontal gyrus, PCC/precuneus, supramarginal gyrus, angular gyrus, lateral occipital cortex, and cuneus.

In SAD patients we found increased negative right amygdala connectivity with the left middle temporal gyrus, left supramarginal gyrus and left lateral occipital cortex (Figure 1; Table 3). No group differences were found on left amygdala RSFC or when a contrast was made for the left and right amygdala combined.

Next, we explored RSFC of the dACC, probing the salience network. The connectivity pattern for this seed in both groups also corresponded with areas described in previous research (Margulies *et al.*, 2007). Areas showing positive connectivity in rest with the dACC included other parts of the ACC, the frontal pole, paracingulate gyrus, PCC, precentral gyrus, supramarginal gyrus, parietal operculum cortex, central operculum cortex, inferior frontal gyrus, and middle frontal gyrus. Areas showing negative connectivity in rest included the subcallosal cortex, precuneus, cuneus, lateral occipital cortex, occipital fusiform cortex, parahippocampal gyrus, and lingual gyrus.

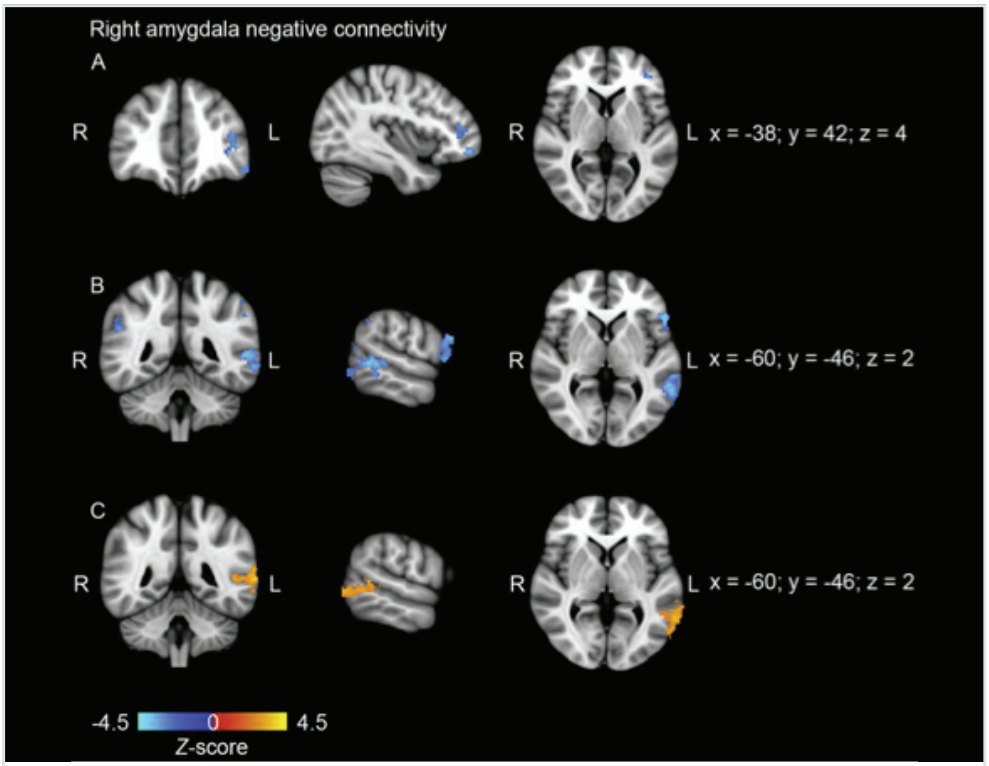

Figure 1.
Right amygdala negative connectivity. A = healthy controls; B = social anxiety disorder patients; C = group difference B > A. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

Table 3. Right Amygdala Resting-State Connectivity: Group Difference

MNI[a] coordinates			Side	*z*-value	*p*-value	Brain region
x	*y*	*z*				Cluster size: 536 voxels
-66	-44	4	L	3.78675	0.0002	Middle Temporal Gyrus, BA[b] 22
-62	-54	2	L	3.55479	0.0004	Middle Temporal Gyrus
-58	-68	0	L	3.50324	0.0005	Lateral Occipital Cortex, BA 19
-56	-48	8	L	3.42287	0.0006	Middle Temporal Gyrus
-62	-48	10	L	3.39525	0.0007	Supramarginal Gyrus, BA 21
-66	-46	14	L	3.32415	0.0009	Supramarginal Gyrus

[a]MNI = Montreal Neurologic Institute, coordinates of most significant voxels in cluster; [b]BA = Brodmann Area; voxel size 2 mm isotropic; *z*- and *p*-values of most significant voxel, cluster corrected.

The combined bilateral dACC showed increased connectivity in SAD patients with the left precuneus and left lateral occipital cortex (Figure 2; Table 4). Separate investigations of the left and right dACC did not produce any significant group differences.

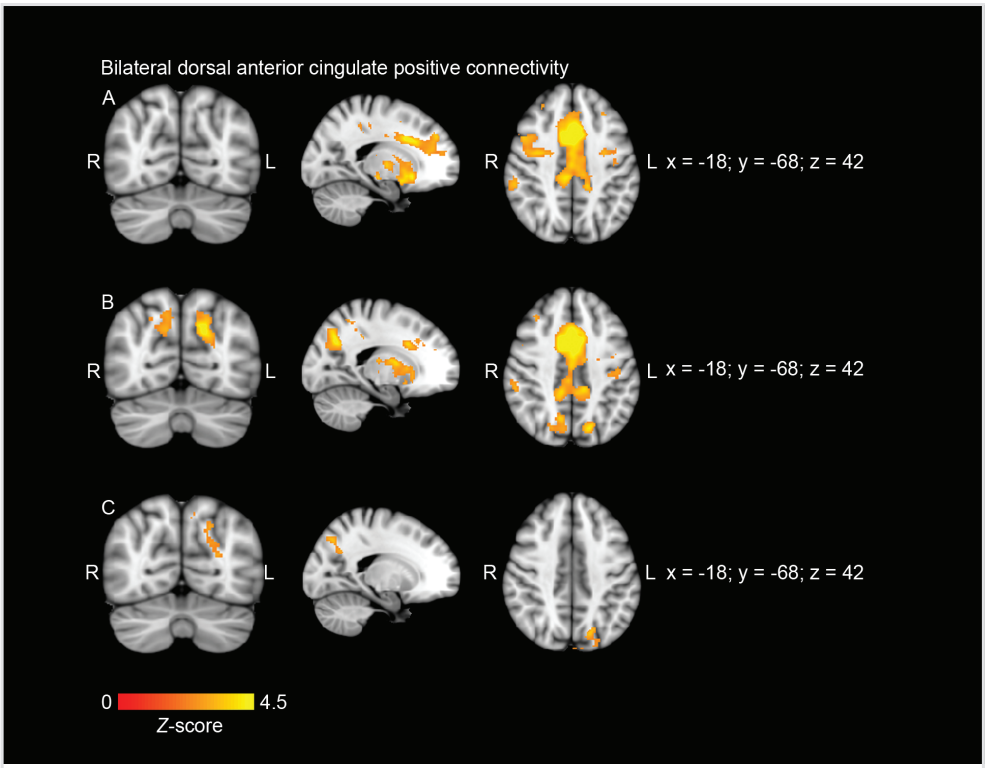

Figure 2.
Bilateral dorsal anterior cingulate cortex positive connectivity. A = healthy controls; B = social anxiety disorder patients; C = group difference: B > A. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

Table 4. Bilateral Dorsal Anterior Cingulate Cortex Resting-State Connectivity: Group Difference

MNI[a] coordinates			Side	*z*-value	*p*-value	Brain region
x	*y*	*z*				Cluster size: 410 voxels
-16	-72	40	L	3.64896	0.0003	Precuneus
-16	-64	32	L	3.34503	0.0008	Precuneus
-22	-84	40	L	3.28251	0.0010	Lateral Occipital Cortex
-26	-68	20	L	3.1207	0.0018	Lateral Occipital Cortex
-8	-88	40	L	3.0777	0.0021	Lateral Occipital Cortex
-12	-74	50	L	2.94138	0.0033	Lateral Occipital Cortex

[a]MNI = Montreal Neurologic Institute, coordinates of most significant voxels in cluster; voxel size 2 mm isotropic; *z*- and *p*-values of most significant voxel, cluster corrected.

We investigated DMN connectivity with a seed in the left and right PCC/precuneus. No group differences were found in DMN connectivity (Figure 3).

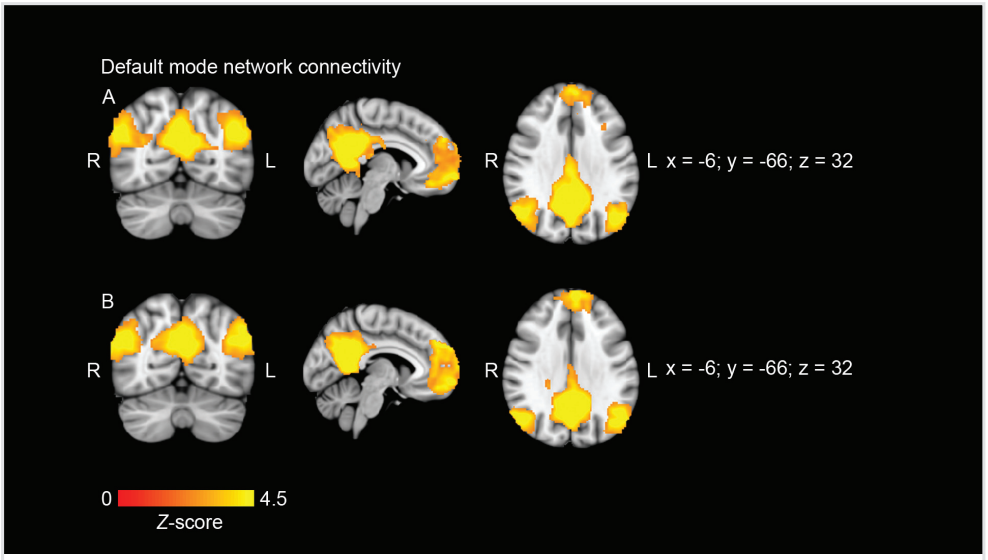

Figure 3.
Default mode network connectivity. A = healthy controls; B = social anxiety disorder patients. Results are cluster corrected at $p < .05$. Images are z-statistics, overlaid on the MNI-152 standard brain.

Correlation of clinical and RS fMRI data

Post-hoc, fMRI data were correlated with FQ scores using SPSS 17.0 (SPSS Inc., Chicago, Illinois, USA). If data did not meet the assumptions required to perform parametric analysis, and log-transforming did not resolve this problem, the appropriate non-parametric test was performed. The FQ contains a social phobia subscale that specifically focuses on feared situations that are associated with SAD. We created a mask of the resulting areas from our right amygdala analysis (the left middle temporal gyrus, left supramarginal gyrus and left lateral occipital cortex), and calculated the individual z-scores from these areas using Featquery, part of FSL (Smith *et al.*, 2004). Using non-parametric correlational analysis, we examined whether the strength of the functional connections was related to anxiety scores as measured with social phobia sub-scores on the FQ in the SAD group. We followed the same approach for the resulting areas showing aberrant connectivity in our bilateral dACC analysis (i.e., the left precuneus and lateral occipital cortex). No association was found between strength of the functional connectivity and anxiety scores in any of the identified regions.

Discussion

We investigated RSFC in drug-naive SAD patients without comorbidity and pair-wise matched healthy controls, using seed regions in the amygdala, the dACC and the PCC/ precuneus. As expected, we found abnormalities in SAD in RS connectivity for the amygdala seeds and the seeds in the salience network, but we found no differences in connectivity between patients and controls for the PCC/precuneus seeds probing the DMN. Specifically, in SAD we found altered RS connectivity between the right amygdala and the left middle temporal gyrus, left supramarginal gyrus, and left lateral occipital cortex. Increased connectivity between the bilateral dACC seeds (salience network) with both the left precuneus and left lateral occipital cortex was also found.

Our results are generally in agreement with previous findings of altered amygdala and anterior cingulate functional connectivity in SAD patients (Freitas-Ferrari *et al.*, 2010, Liao *et al.*, 2010b); however, our findings are not identical to the abnormalities in connectivity patterns found in other RS studies. Our results show some overlap with a previous study in finding involvement of the supramarginal gyrus (Liao

et al., 2010a) and middle temporal gyrus (Liao *et al.*, 2011), although these abnormalities concerned different networks. Whereas a previous functional MRI study (Gentili *et al.*, 2009) and a RS study (Liao *et al.*, 2011) in SAD have reported abnormalities in the DMN, we did not. These discrepancies could be explained by the different analysis method used (i.e., model-free analysis versus seed-based region-of-interest) and/or the drug-naive cohort without comorbidity that we used in the current study. Additionally, our relatively small sample size may be of influence, although lowering the threshold showed no significant group differences between social anxiety patients and healthy controls in DMN connectivity.

Another, admittedly speculative, explanation for the discrepancy in findings between our study and previous research is the difference in cultural backgrounds of the samples that were studied. As mentioned before, four of the five RSFC studies in SAD published to date used overlapping samples of subjects with an Asian cultural background. Several studies have compared the influence of cultural backgrounds in SAD and have found similarities as well as important differences in the phenomenology of social anxiety across Asian and Western cultures (Stein and Matsunaga, 2001, Furmark, 2002, Rapee *et al.*, 2011). Where Asian societies attribute a greater importance to concerns about offending others, the emphasis seems to be on embarrassing oneself in Western communities (Stein and Matsunaga, 2001).

We first analysed amygdala RSFC, using seeds in the bilateral amygdala. We found increased RSFC between the right amygdala, an area involved in fear and emotion processing, fear in particular (LeDoux, 2003), and the left middle temporal gyrus, left supramarginal gyrus, and left lateral occipital cortex, areas that have been linked to facial perception (Puce *et al.*, 1995, Grill-Spector *et al.*, 2004). Abnormal amygdala responses evoked by emotional faces have frequently been reported in SAD (Blair *et al.*, 2011a, Blair *et al.*, 2008b, Freitas-Ferrari *et al.*, 2010, Shin and Liberzon, 2010). The heightened RSFC of the amygdala in social anxiety patients may reflect an increased predisposition for the inaccurate interpretation of others' facial expressions.

We subsequently investigated bilateral dACC connectivity, probing the salience network. The salience network, comprising the bilateral dACC and bilateral anterior insula, is purportedly involved in identifying the most relevant internal and external stimuli, in order

to guide behaviour (Seeley *et al.*, 2007). We found increased positive dACC seed connectivity in SAD patients with the left precuneus and left lateral occipital cortex. The anterior cingulate, one of the structures of the salience network has, apart from its role in the salience network, also been associated with self- focused attention (Lemogne *et al.*, 2010). The precuneus is thought to play a central role in self-reflection and self-processing features, like mental imagery and episodic/ autobiographic memory retrieval (Cavanna and Trimble, 2006b, Cavanna, 2007). Another proposed function of the precuneus is the gathering of information on and representation of the self and the external world (Cavanna, 2007). Strong self-focus has been previously reported in depression, generalized anxiety disorder and SAD, specifically attributing a role to public self-focus in the latter (Lemogne *et al.*, 2010). The heightened connectivity between the bilateral dACC and the precuneus found in our study could underlie this raised awareness of the self and the environment, in particular of other people. More- over, since the salience network is thought to be essential in identifying the relevance of stimuli, a stronger connectivity between the dACC and the precuneus could reflect the tendency of social anxiety patients to attribute an exaggerated significance to possible self-relevant stimuli from the external world (Blair *et al.*, 2008a).

We observed that the strength of the RS functional connectivity was not related to anxiety scores as measured with the social phobia sub-scores of the FQ. Previous reports did show associations between abnormalities in connectivity and alterations in anxiety scores, as measured with the Liebowitz Social Anxiety Scale (Liao *et al.*, 2010a, Liao *et al.*, 2010b, Ding *et al.*, 2011, Liao *et al.*, 2011) and with the Spielberger State and Trait Anxiety Scores (Hahn *et al.*, 2011). Possibly, we did not find correlations due to methodological issues such as the characteristics of our sample, the sample size, or the use of the FQ social phobia sub- scores.

Overall, our results are in line with existing models of SAD. Stein (1998) reviewed several animal and human models of SAD and concluded that the neural circuitries of anxiety and self-consciousness were likely to be involved in the pathophysiology the disorder (Stein, 1998). Negative self-appraisal had been linked to SAD before in two models suggesting an abnormal tendency of SAD patients to retrospectively ruminate and to appraise themselves in a negative manner (Clark and Wells, 1995, Rapee and Heimberg, 1997). More recently, the role of circuitry underlying self-

referential processing has also been emphasized by Blair *et al*. (2008a), who found that SAD patients show amygdala and dorsal medial prefrontal cortex hyperresponsiveness to negative, self-referential comments (Blair *et al*., 2008a). This heightened propensity to focus on the self could possibly also be represented in the increased RSFC that we found between the bilateral dACC and the left pre- cuneus. Clearly, the interpretation of abnormalities in RSFC is more speculative and should be done with caution, as the relation between abnormalities in RFSC and abnormalities in task related functional connectivity in SAD has not been directly studied yet.

Our study has several strengths. This is the first study investigating RS functional connectivity in a group of drug- naive social anxiety patients without psychiatric comorbidity. There are also some noteworthy limitations to our study. Our sample size is relatively small and replication in other, larger samples is desirable in order to enable more conclusive interpretation. It should also be noted that subjects may engage in different forms of cognitive action during the resting-state condition, which can influence resting-state activity patterns. We emphasize that RSFC data should be interpreted cautiously, and stress that any interpretation refers only to functional connectivity between brain areas as opposed to the (dys)function of a distinct brain region. Also, by opting for a hypothesis-driven approach and thus only exploring specific networks, abnormalities in other networks might have been missed. On the other hand, the use of a seed-based region-of-interest approach allows easier replication of our findings. Another limitation of the current study was the possible influence of between-group differences in heart rate variability and breathing on the results. Since physiological activity was not monitored in the current study, it remains unclear if any difference between the two groups has influenced the results, although previous research shows that heart rate variability did not differ between anxiety patients and healthy controls in the larger NESDA sample (Licht *et al*., 2009). Additionally, regressing out global signal changes has shown to at least partly filter out the effects of cardiac and respiratory fluctuations (Birn *et al*., 2006, Fox and Raichle, 2007). Pooling data from different centres is another possible limitation. To account for this as much as possible, we have matched our groups based on scanning site and also added a confound regressor for site in our statistical model. A further limitation is the absence of a specific questionnaire for the assessment of social anxiety. Use of a specific social anxiety scale instead of

the more generalised Fear Questionnaire might prove to be more sensitive to identify an association between altered functional connectivity and extent of SAD symptoms. Finally, our RS fMRI data were acquired at the end of a fixed imaging protocol, including a facial expression task, which could potentially have influenced RS connectivity (i.e., a spillover effect) with SAD patients still showing aberrant connectivity in areas involved in the processing of emotional faces while the facial stimulus was no longer present.

In summary, we found altered RS connectivity in drug- naive SAD patients without comorbidity, between areas involved in the processing of fear and emotion and areas that contribute to facial perception. Additionally, we found aberrant connectivity between a key region of the salience network and areas involved in the processing of self-relevant stimuli, which has become a recent focus of research in SAD.