Using novel imaging approaches in affective disorders : beyond current models

Pannekoek, J.N.

Chapter 4
Investigating shared and unique abnormalities of resting-state functional connectivity in depression, anxiety, and their comorbid states

Justine Nienke Pannekoek, Steven J.A. van der Werff, Marie-José van Tol, Dick J. Veltman, André Aleman, Frans G. Zitman, Serge A.R.B. Rombouts, Nic J.A. van der Wee

Submitted for publication

Abstract

Depression and anxiety disorders are highly comorbid and share neurobiological characteristics. However, this is usually not explicitly addressed in studies on intrinsic brain functioning in these disorders. We investigated resting-state functional connectivity (RSFC) in medication-free patients with depression, anxiety, comorbid depression and anxiety, and a healthy control group. RSFC was investigated in 140 medication-free subjects: 37 major depressive disorder patients (MDD), 30 patients with one or more anxiety disorders (ANX), 25 patients with MDD and one or more anxiety disorders (COM), and 48 healthy controls (HC). RSFC networks were calculated using a probabilistic independent component analysis. Using a dual regression approach, individuals' timecourses were extracted and regressed to obtain subjects-specific spatial maps, which were used for group comparisons in four networks of interest (limbic, default mode, salience and sensory-motor networks). When compared to HC, the COM group showed increased RSFC of the limbic network with a cluster containing the bilateral precuneus, intracalcarine cortex, lingual gyrus, and posterior cingulate, and with a cluster including the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. This effect was specific for comorbid depression and anxiety. No abnormal RSFC of other networks or in the MDD and ANX groups was observed. No association was found between strength of RSFC and symptom severity. These results indicate that altered RSFC of regions in a limbic network could be specific for comorbid depression and anxiety.

Introduction

Major depressive disorder (MDD) is one of the most prevalent of all psychiatric disorders, and according to the National Comorbidity Study, 27.1% of all MDD patients presented with comorbid social anxiety disorder (SAD), 17.2% with comorbid generalised anxiety disorder (GAD), and 9.9% with comorbid panic disorder (PD) (Gorman, 1996, Kessler *et al.*, 1996). Comorbid depression and anxiety are associated with a greater societal burden, and with worse outcome (less response to treatment, longer illness duration, greater risk of suicide) (Gorman, 1996, Kessler *et al.*, 2005b).
MDD and anxiety patients not only show frequent comorbidity; they also respond to the same treatment strategies and it has been suggested that they share a similar etiology (Ressler and Mayberg, 2007). However, comorbidity of depression and anxiety disorders is usually not explicitly addressed in studies examining the underlying neurobiological characteristics of MDD and anxiety disorders. Instead, the separate disorders have been the focus of neurobiological studies and as a result have received considerable attention in recent years.

In depression, neuroanatomical and functional abnormalities have been reported for a range of brain regions including (subregions of) the anterior cingulate cortex (ACC), prefrontal cortex (PFC), hippocampus, amygdala, posterior cingulate cortex (PCC), thalamus, striatum, pallidum, and temporal cortical areas. Additionally, abnormalities have been reported in neural circuits such as limbic, prefrontal, sensory, motor, and default mode networks; for a review, see Drevets *et al.* (2008). In SAD, PD and GAD, the amygdala is the brain area most commonly reported to show abnormalities compared to healthy controls (Damsa *et al.*, 2009, Freitas-Ferrari *et al.*, 2010). Other brain areas that were frequently found to show aberrancies in anxiety include the PFC, ACC, insula, striatum, superior temporal gyrus, thalamus, and hippocampus (Damsa *et al.*, 2009). Although it is apparent that many brain areas and circuits are implicated in both depression and anxiety, their distinct and common roles in the disorders are still unclear.

So far, only five neuroimaging studies investigated the unique and shared neural properties of depression and anxiety in a design comparing three clinical groups of individuals with MDD only, anxiety only, and comorbid MDD and anxiety, with a group of healthy controls. In one task functional MRI (fMRI) study, depression and GAD were investigated in their unique as

well as comorbid states (Etkin and Schatzberg, 2011). The other four studies were conducted within the context of the Netherlands Study of Depression and Anxiety (NESDA), a large multi-centre longitudinal cohort designed to chart the long-term course and consequences of depression and anxiety (Penninx *et al.*, 2008). The anxiety disorders included in NESDA are the common anxiety disorders PD, SAD, and GAD. In one neuroanatomical and three task fMRI studies in the NESDA cohort, the unique and common profiles of depression and anxiety were investigated (Demenescu *et al.*, 2011, van Tol *et al.*, 2010, van Tol *et al.*, 2011, van Tol *et al.*, 2012). The results of these five studies point to shared as well as unique contributions of depression and anxiety to aberrancies in brain structure and function, with roles for the ACC, PFC, insula, amygdala, hippocampus, and the inferior, temporal and superior frontal gyri.

Functional MRI is not only widely employed to study functional activity and connectivity of brain areas within the context of task paradigms, but is also used to examine connectivity during the so-called resting-state, i.e. in the absence of externally controlled stimuli or tasks (Biswal *et al.*, 1995, Gusnard *et al.*, 2001). Coherent fluctuations in resting-state have consistently been identified across subjects and sessions, and are viewed as functional resting-state networks (Beckmann *et al.*, 2005, Damoiseaux *et al.*, 2006). Abnormalities in resting-state functional connectivity (RSFC) have been found in various neuropsychiatric disorders known to involve disturbed emotion regulation and self-processing, including depression and anxiety (Broyd *et al.*, 2009, Pannekoek *et al.*, 2013b, Pannekoek *et al.*, 2013c, Veer *et al.*, 2010, Wang *et al.*, 2012).

The default mode network (DMN) is a resting-state network containing the precuneus cortex, PCC, medial PFC (mPFC), lateral and inferior parietal cortex and ventral anterior cingulate cortex (vACC) (Greicius *et al.*, 2003, Gusnard *et al.*, 2001). Due to its critical role in self-referential processing (Broyd *et al.*, 2009, Gusnard *et al.*, 2001), this network has received substantial attention in research on depression, and abnormalities in depression were found (Broyd *et al.*, 2009, Wang *et al.*, 2012). Greicius *et al.* (2007) were among the first to investigate DMN RSFC with fMRI in depressed patients, finding greater connectivity with areas associated with depression including the subgenual ACC, thalamus, mPFC, and cuneus/precuneus. In addition, the effect in the subgenual ACC

correlated positively with length of the depressive episode (Greicius *et al.*, 2007). Various subsequent studies have also reported altered DMN RSFC, with areas such as the caudate (Bluhm *et al.*, 2009, Kenny *et al.*, 2010), precuneus (Andreescu *et al.*, 2011, Kenny *et al.*, 2010, Zhou *et al.*, 2010) and frontal cortical areas as the ACC (Andreescu *et al.*, 2011, Zhou *et al.*, 2010). In addition to altered RSFC of the DMN, aberrant RSFC of other brain networks has also been reported in MDD, such as altered cortico-limbic connectivity, especially between the PFC and the amygdala in an affective network (Anand *et al.*, 2005, Wang *et al.*, 2012, Veer *et al.*, 2010). Furthermore, altered RSFC of the frontal pole and of the lingual gyrus (Veer *et al.*, 2010), precuneus-caudate (Bluhm *et al.*, 2009), sgACC-insula (Cullen *et al.*, 2009, Horn *et al.*, 2010), thalamus (Greicius *et al.*, 2007, Kenny *et al.*, 2010), inferior frontal gyrus (Zhou *et al.*, 2010), dorsomedial PFC-precuneus (van Tol *et al.*, 2013), and cerebellum (Liu *et al.*, 2010) has been reported in MDD.

In anxiety disorders, RSFC abnormalities have been reported for a variety of networks, such as a limbic network and the salience network in PD (Pannekoek *et al.*, 2013b), and in a variety of networks in SAD (Liao *et al.*, 2010a, Pannekoek *et al.*, 2013c). Functional connectivity in the DMN was shown to be stronger between the PCC and mPFC in older GAD subjects relative to younger patients (Andreescu *et al.*, 2013). The presence of GAD, longer illness duration and more severe worrying were related to greater differences in DMN connectivity.

Evidence from neuroimaging literature indicates that many brain regions show similar abnormalities in MDD and in anxiety disorders. This is in agreement with a "common-disorder" model of depression and anxiety (Etkin and Schatzberg, 2011), although the few task fMRI studies and the neuroanatomical study that have addressed comorbidity also report unique characteristics of depression and anxiety (Demenescu *et al.*, 2011, Etkin and Schatzberg, 2011, van Tol *et al.*, 2012, van Tol *et al.*, 2011, van Tol *et al.*, 2010). In the present study, we aimed to investigate RSFC in depression, anxiety and their comorbid states by employing RS fMRI in three clinical groups and compare these with a healthy control group: MDD patients, anxiety patients (PD and/or SAD and/or GAD), and comorbid MDD and anxiety patients (MDD and PD and/or SAD and/or GAD), compared to healthy controls. Based on the current neurobiological models of depression and anxiety and the available literature, we expected abnormalities of RSFC of four networks

of interest previously associated with disturbances of emotion processing: the default mode network, the sensory-motor network, the salience network and a limbic network (containing brain regions such as the amygdala and hippocampus).

Method

Participants

Participants were recruited from a longitudinal, large-scale, multi-centre, observational cohort study: the Netherlands Study of Depression and Anxiety (NESDA) (Penninx *et al.*, 2008). This study was designed to chart the long-term course and consequences of depressive and anxiety disorders, including participants from different health care settings (i.e. the community, through primary care and specialized mental health institutions) and various developmental stages of illness.

The NESDA main sample consisted of 2981 participants, aged between 18 and 65 years. Participants aged between 18 and 57 years old were invited to participate in the NESDA neuroimaging study if they met the DSM-IV criteria for a half-year diagnosis of MDD and/or anxiety disorder (PD, SAD, and/or GAD), or no lifetime DSM-IV diagnosis (i.e. healthy controls). Participants were not screened for personality disorders, but individuals with a known personality disorder based on information from clinics or self-report were not included in the study. Patients were excluded based on the following criteria: presence of axis-I disorders other than MDD, PD, SAD, or GAD, and any use of psychotropic medication other than stable use of selective serotonin reuptake inhibitors (SSRIs) or infrequent benzodiazepine use (i.e. equivalent to 2 doses of 10 mg of oxazepam 2 times per week, or use within 48 hours prior to scanning).

Controls were currently free of, and had never met criteria for, depressive or anxiety disorders or any other axis-I disorder. They were not taking any psychotropic drugs. Overall, participants were excluded based on the following criteria: presence or history of major internal or neurological disorder, dependence or recent alcohol and/or drug abuse (past year), hypertension, and general MRI contraindications. The Composite International Diagnostic Interview (CIDI) lifetime version 2.1, administered by a trained interviewer, was used to diagnose depressive and anxiety disorders according to DSM-IV algorithms.

Overall, 301 native Dutch-speaking participants (233 patients and 68

controls) were included in the NESDA neuroimaging study and underwent MR imaging in one of the three participating centres (Academic Medical Centre Amsterdam, Leiden University Medical Centre, and University Medical Centre Groningen). After receiving written information, all subjects provided written informed consent. The study was approved by the Medical Ethics Committees of all three centres and conducted in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

For the present study, RS-fMRI data were available from 229 subjects. Ten patients were removed from the sample due to excessive head motion during scan acquisition (> 3 mm in any of the acquired volumes). Of the remaining 219 subjects, we only included participants that were medication-free at the time of the study (N = 140 in total, of which N = 114 medication-naïve). Our final sample consisted of 140 medication-free subjects: 37 patients with MDD, 30 patients with one or more anxiety disorders (PD, SAD and/or GAD) but no MDD (ANX group), 25 patients with comorbid MDD and one or more anxiety disorders (COM group: MDD and PD and/or SAD and/or GAD), and 48 healthy controls (HC group).

Image acquisition

Image acquisition took place at one of the three participating centres within on average 8 weeks after completion of the NESDA baseline interview (Penninx *et al.*, 2008). RS-fMRI data were acquired at the end of the fixed imaging protocol: after completion of three task-related functional MRI runs and the acquisition of an anatomical scan (scan sequence: Tower of London, word encoding, T1-weighted scan, word recognition, perception of facial expression, resting-state). 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.

RS-fMRI data were acquired for each subject using T_2^*-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.

Data preprocessing

The preprocessing of RS-fMRI images was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library) (Smith *et al*., 2004). The following processing steps were applied: motion correction (Jenkinson *et al*., 2002), removal of non-brain tissue, spatial smoothing using a Gaussian kernel of 6-mm full-width at half-maximum, grand-mean intensity normalization 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) and registration to the high resolution T1 and MNI-152 standard space images (T1 standard brain averaged over 152 subjects; Montreal Neurological Institute, Montreal, QC, Canada) (Jenkinson *et al*., 2002). Normalized 4D data sets were then resampled to 4-mm isotropic voxels to reduce computational burden in the following analysis steps.

Statistical analysis

Motion parameters (Table 1) did not differ significantly between groups for absolute displacement nor relative displacement. Standard group independent component analysis (ICA) was carried out using probabilistic ICA (PICA) (Beckmann and Smith, 2004) as implemented in FSL's Multivariate Exploratory Linear Decomposition into Independent Components (MELODIC). ICA attempts to split the 4D functional data into a set of spatial maps, each with an associated timecourse, by performing a linear decomposition of the original data. Time series of all participants were temporally concatenated into a single 4D time series, which was separated in 20 components using ICA in MELODIC. We selected three components based on spatial similarity to functional networks consistently described before (Beckmann *et al*., 2005, Damoiseaux *et al*., 2006): the default mode network; the sensory-motor system; and the salience network. In addition,

we selected one other component that was relevant for the present study: a limbic network (containing brain regions such as the amygdala and hippocampus).

Next, the subject-specific component maps were identified by extracting individual time series for each component, using the 20 component maps in a spatial regression against the individual data. In other words, the set of spatial maps from the group-average analysis was used to generate subject-specific versions of the spatial maps, and associated time series, using dual regression (Beckmann *et al.*, 2009). First, for each subject, the group-average set of spatial maps was regressed (as spatial regressors in a multiple regression) into the subject's 4D space-time dataset. This resulted in a set of subject-specific time series, one per group-level spatial map. Next, those time series were regressed (as temporal regressors, again in a multiple regression) against the same 4D dataset, resulting in a set of subject-specific spatial maps, one per group-level spatial map.

We segmented the 4 mm standard brain into grey matter, white matter, and cerebrospinal fluid (CSF) and created a mask of grey and white matter only. This mask was applied to the mask derived from the dual regression to ensure that the voxels tested in the voxel-wise nonparametric permutation test were all situated solely in brain tissue and not in the CSF.

For the main comparison, we performed a factorial analysis with one factor (group) at four levels (MDD, ANX, COM, and HC). We then used two-sample t-tests to investigate group differences for each of the selected functional networks. These tests were performed using voxel-wise nonparametric permutation in FSL (randomise; 5000 permutations) (Nichols and Holmes, 2002), including grey matter density information from each participant as a voxel-dependent covariate. This step was repeated without a voxel-dependent covariate to examine the potential influence of grey matter differences. 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).

Depression- and anxiety-related grey matter abnormalities were previously reported by our group in the NESDA neuroimaging sample (van Tol *et al.*, 2010). Three nuisance regressors describing scanner location and gender and age were added to the model, in addition to modelling regressors for each of the two groups. The resulting statistical maps for each

RS network were corrected for family-wise error using threshold-free cluster enhancement (TFCE) at a threshold of $p < .05$ (Smith and Nichols, 2009).

For between-groups effects present only in the contrast COM-HC, we planned post-hoc analyses with the contrasts COM-MDD and COM-ANX to further assess specificity for the comorbid depression and anxiety group. For these contrasts, we compared the RSFC in masks based on the effects found in COM-HC, using the FSL Randomise tool and TFCE corrected with a threshold of $p < 0.05$.

Demographic and clinical data were analysed using SPSS 20.0 (SPSS Inc, Chicago, Illinois). If data did not meet the assumptions required to perform parametric analysis, a non-parametric alternative was used. Significance was set at $p < .05$, and post hoc paired tests were Bonferroni corrected for multiple comparisons.

Results

Sample descriptives

Table 1 lists the clinical characteristics of the sample. The four groups were matched for age, sex, scan location, and handedness. However, they did differ on education as shown by the Kruskal-Wallis Test (Table 1); further exploration using the Mann-Whitney U-Test revealed that HC had more years of education than MDD patients ($U = 498$; $p < .001$), ANX patients ($U = 460$; $p = .006$), and COM patients ($U = 276$; $p < .001$).

As expected, HC showed lower scores on the Beck Anxiety Inventory (BAI) than MDD ($U = 1322$; $p < .001$), ANX ($U = 1288.5$; $p < .001$), and COM ($U = 1129.5$; $p < .001$). They also reported lower scores on the Montgomery-Åsberg Depression Rating Scale (MADRS) than MDD ($U = 1549$; $p < .001$), ANX ($U = 1270.5$; $p < .001$), and COM ($U = 1164.5$; $p < .001$). Furthermore, HC showed lower scores on the Inventory of Depressive Symptomatology (IDS) than MDD ($U = 1466.5$; $p < .001$), ANX ($U = 1288.5$; $p < .001$), and COM ($U = 1161.5$; $p < .001$). Additionally, COM scored significantly higher on the BAI compared to MDD ($U = 703.5$; $p < .001$), and on the MADRS compared to ANX ($U = 554.5$; $p = .002$). All these results survived Bonferroni correction for multiple testing.

Table 1. Demographic and Clinical Characteristics of the Sample (*N* = 140).

Characteristic	MDD	ANX[a]	COM[b]	HC	*H*	χ^2	*df*	*p*
Sample, *N*	37	30	25	48				
Sex, *N* (%)								
Male	19 (51.4)	8 (26.7)	6 (24)	18 (37.6)		6.48	3	.09
Female	18 (48.6)	22 (73.3)	19 (76)	30 (62.5)				
Age (years), Mean (SD)	35.7 (10.11)	33.1 (8.39)	34.8 (10.54)	40.0 (9.43)	9.89	3	.019	
Education (years), Mean (SD)	5.84 (1.44)	6.07 (1.84)	5.16 (2.01)	6.96 (1.64)	21.53	3	<.001	
Scan location, *N*								
AMC	7	6	5	18		10.93	6	.09
LUMC	15	9	10	21				
UMCG	15	15	10	9				
Handedness								
Left	4	2	2	4		0.39	3	.94
Right	33	28	23	44				
BAI score at scanning, Mean (SD)	8.78 (9.72)	14.17 (10.59)	16.8 (8.67)	2.09 (2.44)	63.89		3	<.001
Range	0–50	0–42	1–36	0–10				
MADRS score at scanning, Mean (SD)	11.73 (10.54)	10.4 (8.54)	19 (10.33)	1.11 (1.94)	73.97		3	<.001
Range	0–39	0–35	2–43	0–7				
IDS score at scanning, Mean (SD)	18.09 (12.41)	18.71 (10.67)	28 (11.54)	3.83 (3.8)75.83			3	<.001
Range	1–57	4–49	8–55	0–17				
Motion parameters in millimetres								
Absolute displacement, Mean (SD)	.28 (.213)	.28 (.218)	.32 (.249)	.27 (.173)				.823
Relative displacement, Mean (SD)	.09 (.038)	.08 (.030)	.09 (.060)	.08 (.034)				.572

Legend table 1

Abbreviations: MDD, major depressive disorder; ANX, anxiety disorder; COM, comorbid depression and anxiety; HC, healthy controls AMC, Academic Medical Centre Amsterdam; LUMC, Leiden University Medical Centre; UMCG, University Medical Centre Groningen; BAI; Beck Anxiety Inventory; MADRS, Montgomery-Åsberg Depression Rating Scale; IDS, Inventory of Depressive Symptomatology; *H*, Kruskal-Wallis non-parametric multiple independent-samples test; χ^2, chi-square test; *df*, degrees of freedom.

[a] 9 patients had panic disorder (PD); 12 patients had social anxiety disorder (SAD); 6 patients had PD and SAD; 2 patients had PD, SAD, and generalized anxiety disorder (GAD); 1 patient had PD and GAD.

[b] 8 patients had MDD and PD; 2 patients had MDD and SAD; 3 patients had MDD, PD, and SAD; 4 patients had MDD and GAD; 4 patients had MDD, SAD, and GAD; 4 patients had MDD, PD, and GAD.

Resting-state functional connectivity results

Twenty functional connectivity networks were generated during the independent component analysis and entered into a dual regression; four of which were selected for further analysis (Figure 1). These networks have been previously described in studies using similar analysis techniques, showing stable spatial similarity across participants and over time (Beckmann et al., 2005, Damoiseaux et al., 2006). All functional networks were present in ANX, COM, HC and MDD, all $p < .05$, family-wise corrected, based on the TFCE statistic image.

Between-group differences were examined by contrasting HC against the three clinical groups (i.e. HC versus ANX, HC versus COM, and HC versus MDD). Therefore, an additional correction for multiple comparisons was applied with a more stringent $p < .017$ (.05 / 3). Between-group differences were only revealed for the limbic network, for which patients with comorbid depression and anxiety (the COM group) showed increased RSFC of the limbic network with a cluster containing the bilateral precuneus, intracalcarine cortex, lingual gyrus, and posterior cingulate, as well as with a cluster including the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus compared to HC (Figure 2). This effect was not found in the ANX and MDD groups when compared to HC.

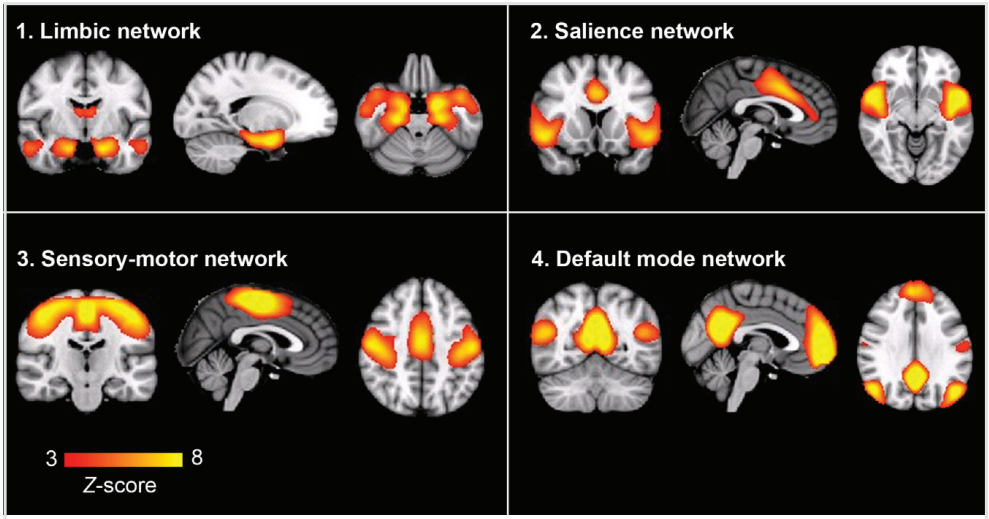

Figure 1. Independent component analysis functionally relevant resting-state networks.
Depicted are the four functional resting-state networks that were selected for further analysis, resulting from the group probabilistic independent component analysis that was carried out on the concatenated datasets from all groups (ANX, COM, MDD, HC). Images are z-statistics, ranging from 3 to 8, overlaid on the MNI-152 standard brain. The left hemisphere of the brain corresponds to the right side in this image.

Post-hoc, we investigated whether this specific increase in RSFC found in COM in comparison to HC was shared with DEP and ANX, or whether it was indeed unique to COM. Within the effect for the limbic network (i.e. increased RSFC with a more posterior and a more frontal cluster), COM showed significantly stronger RSFC compared to ANX as well as MDD, indicating that the increased RSFC of the limbic network with these two clusters was indeed specific for patients with comorbid depression and anxiety (Figure 3).

Given the grey matter differences found in the structural NESDA MRI study (van Tol *et al.*, 2010), we investigated whether the observed functional connectivity differences were influenced by grey matter density. We therefore repeated the analysis without a voxel-dependent covariate, which yielded the same results as analysis with the correction.

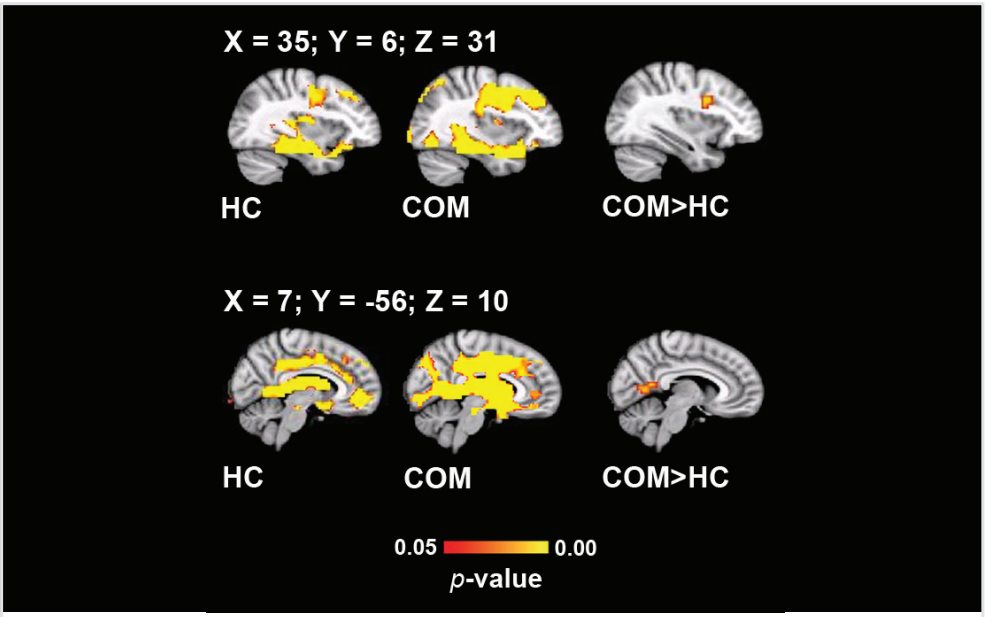

Figure 2. Group main effects and between-group effects of COM and HC. Depicted on the left side are the group main effects and the between-group difference of COM>HC for the limbic network. The top images show the group difference in an anterior cluster including the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. The bottom images show the group difference in a posterior cluster containing the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus. Images are threshold-free cluster enhancement corrected *p*-statistics with $p < .05$, overlaid on the MNI-152 standard brain.
COM, patients with comorbid depression and anxiety; HC, healthy controls. The left hemisphere of the brain corresponds to the right side in this image.

To investigate the relation of clinical scores on the BAI, MADRS, and IDS with the aberrant functional connectivity in the COM group, a hierarchical multiple regression was performed while controlling for age and gender. Following previous work of our group in which 'mood/cognition' and 'anxiety/arousal' factors of the IDS were identified (Wardenaar *et al.*, 2010), these two IDS subscales were also addressed separately in post hoc tests in addition to the IDS total score. As a measure of the functional connectivity strength, the individual z-scores obtained from the affected areas within the corresponding individual component maps were extracted and exported to SPSS. No association was found between strength of RSFC and symptom severity.

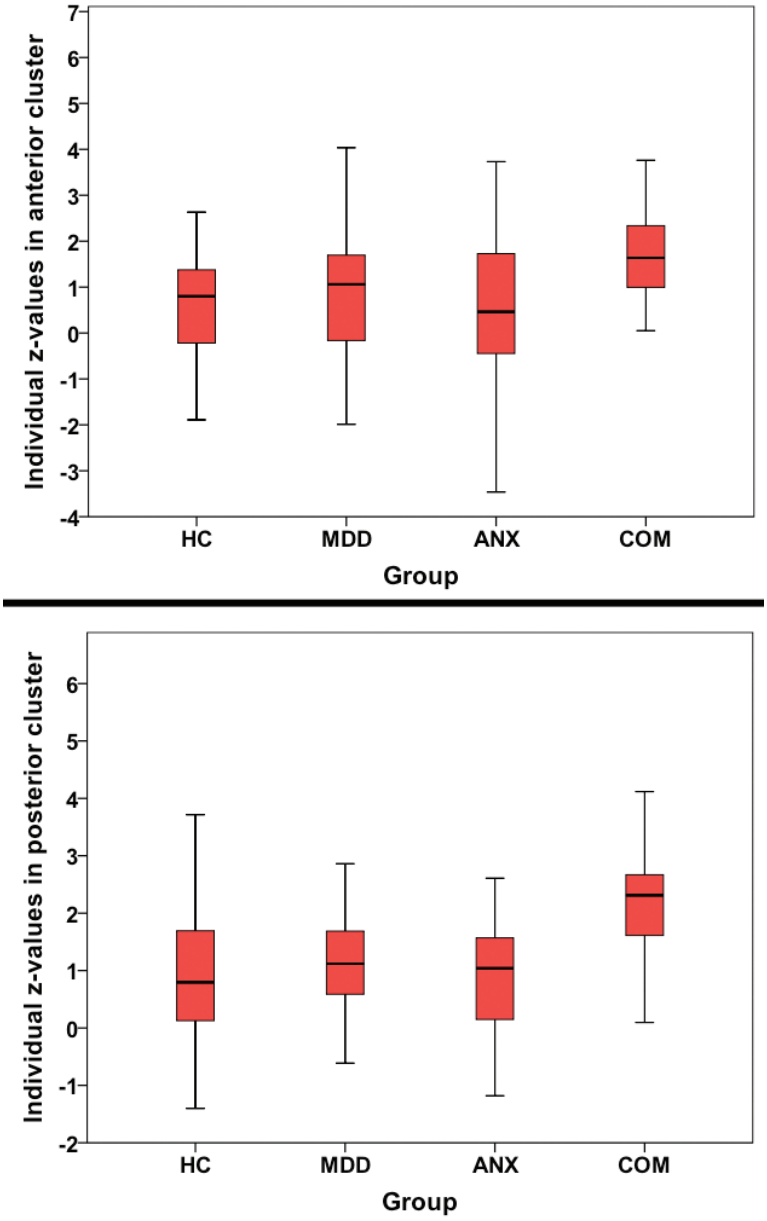

Figure 3. Graphic representation of the individual z-scores for all four groups. Displayed are the distributions for all groups of the mean individual z-scores of the effect in the anterior cluster (top plot), and of the effect in the posterior cluster (bottom plot). HC, healthy controls; MDD, patients with major depressive disorder; ANX, patients with anxiety; COM, patients with comorbid depression and anxiety.

Discussion

In this study we investigated RSFC in medication-free patients with depression, anxiety, and comorbid depression and anxiety compared to healthy controls. We used a data-driven dual regression approach to examine four resting-state networks of interest. In the comorbid group, we found increased RSFC of the limbic network with the bilateral precuneus, intracalcarine cortex, lingual gyrus, and posterior cingulate, as well as with the right precentral gyrus, inferior frontal gyrus, and middle frontal gyrus, compared to healthy controls. Post-hoc analyses showed that this effect was unique to the comorbid state of depression and anxiety. Contrary to our hypothesis, we found no abnormalities in the other networks of interest.

Recent RS studies have indicated involvement of the PCC/precuneus in depression (Greicius *et al.*, 2007) as well as in anxiety disorders (Pannekoek *et al.*, 2013b, Pannekoek *et al.*, 2013c, Strawn *et al.*, 2012). Using a seed-based region-of-interest approach, our group found increased RSFC between the limbic network and the bilateral precuneus in PD patients without comorbidity, using the amygdala as a seed (Pannekoek *et al.*, 2013b). As the precuneus has been related to a range of cognitive functions, namely visuo-spatial imagery, episodic memory retrieval, self-awareness, and consciousness (Cavanna and Trimble, 2006a), the increased RSFC of the precuneus with limbic regions in PD patients could be related to anxiety symptoms such as depersonalization and loss of control, for example during panic attacks (Pannekoek *et al.*, 2013b). In another seed-based study, in SAD patients without comorbidity, we observed an increased RSFC between the dACC and the precuneus within the context of the salience network, which could be associated with heightened self-awareness in SAD (Pannekoek *et al.*, 2013c). In depression, increased RSFC with the precuneus was also reported, albeit within the context of the DMN (Greicius *et al.*, 2007). In contrast to these RSFC findings, Strawn and colleagues (2012) demonstrated that adolescents with GAD exhibited a decrease in functional connectivity between the left amygdala and the precuneus during an attentional task involving strong negative emotional and neutral emotional distractors (Strawn *et al.*, 2012). Differences with the RSFC findings may be due to the task-related design and the adolescent sample.

The right precentral gyrus and inferior frontal gyrus also showed increased RSFC with the limbic network in COM patients compared to healthy controls. Reduced inferior frontal gyrus volumes were previously reported in MDD patients by our group, and it was suggested that this could represent a neuroanatomical basis for MDD (van Tol *et al.*, 2010). Also, in a group of clinically depressed adolescents, most of which presented with comorbid anxiety, increased RSFC was found between the amygdala and inferior frontal gyrus using seeds probing the limbic network (Pannekoek *et al.*, 2014a). In addition, increased activation of the right inferior frontal gyrus was found during an emotional word recognition task in anxiety patients (van Tol *et al.*, 2012). One study investigated emotion regulation of social situations in healthy subjects, and showed increased activation of the inferior frontal gyrus during negative appraisal of others' intentions. This region was part of a circuit acting as a modulator in socially induced emotions (Grecucci *et al.*, 2013). The inferior frontal gyrus has been implicated in coping with distracting emotions and amygdala-inferior frontal gyrus connectivity has been described as a system involved in emotion detection (Dolcos *et al.*, 2006), which is clearly relevant to mood and anxiety disorders.

We did not find an association between symptom severity scores and strength of RSFC. A possible explanation could be that the RSFC abnormalities are more trait than state dependent. However, an alternative explanation may be that the included patients mostly presented with mild to moderate symptomatology, as NESDA participants were recruited through general practitioners and outpatient clinics.

To the best of our knowledge, this is the first study to investigate RSFC in medication-free MDD patients, anxiety patients, and patients with comorbid depression and anxiety compared to healthy controls. We were able to include a large sample of patients who were all medication-free or even medication-naïve. We were therefore able to rule out any possible influence of current medication use on resting-state fMRI. However, several limitations are of note. The interpretation of abnormalities in RSFC in a cross-sectional observational design should be done with caution, and any interpretation of the current results refers to functional connectivity between brain areas and does not allude to dysfunction of specific brain regions. Additionally, no associations were found between strength of RSFC and depression and

anxiety scores as measured with the MADRS, IDS, and BAI. This suggests that there is no relation with symptomatology, but we did not use a specific instrument to assess severity of comorbidity. Although the differences did not reach statistical significance, the HC group was slightly older than the patient groups. We controlled for potential effects caused by age by including this variable as a covariate in our analyses. However, we cannot rule out that the absence of effects could be due to age differences between the HC and patient groups.

It is of note that results from previous resting-state research based on monodiagnostic subsamples of the NESDA cohort were not replicated in the present study (Pannekoek *et al.*, 2013b, Pannekoek *et al.*, 2013c, Veer *et al.*, 2010). One explanation could lie in the composition of the samples selected for each previous study. PD without comorbidity (Pannekoek *et al.*, 2013b) and SAD without comorbidity (Pannekoek *et al.*, 2013c) were investigated in smaller samples in former studies, but the current, larger sample of anxiety patients consisted of patients with PD, SAD, and GAD, or any combination of these disorders. Similarly, although there was overlap, the composition of the MDD group in the previous study by our group (Veer *et al.*, 2010) was also different from the larger MDD group in this study. In contrast to the present study, in the previous studies healthy controls were pair-wise matched for age and scanning location. In our current study, the distribution of scanning centre was different between groups, although not statistically significant. Also, the healthy controls were slightly older than the patients in the other groups and we therefore controlled for age in our analyses. However, by controlling for age, we could also have regressed out variance that was related to both group and age,

Taken together, the present RSFC study on patients with depression, anxiety, comorbid depression and anxiety, and healthy controls demonstrated an increased RSFC of a limbic network with the bilateral precuneus and the right precentral gyrus only in patients with comorbid depression and anxiety compared to healthy controls, which therefore may be specific to the presentation of comorbid depression and anxiety. This finding adds to literature suggesting that a distinctive pathophysiology is involved in comorbid depression and anxiety (Craske *et al.*, 2009, Etkin and Schatzberg, 2011). Our findings also further implicate connectivity of the limbic network with the precuneus in the pathophysiology of different affective disorders.