Altered functional connectivity in posttraumatic stress disorder with versus without comorbid major depressive disorder: a resting state fMRI study [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract
Posttraumatic stress disorder (PTSD) is an anxiety disorder that is often diagnosed with comorbid depressive disorder. Therefore, neuroimaging studies investigating PTSD typically include both patients with and without comorbid depression. Differences in activity of the anterior cingulate cortex (ACC) and insula have been shown to differentiate PTSD patients with and without major depressive disorder (MDD). Whether or not comorbid MDD affects resting state functional connectivity of PTSD patients has not been investigated to our knowledge. Here, resting state functional connectivity of PTSD patients with (PTSD+MDD; n=27) and without (PTSD-MDD; n=23) comorbid MDD was investigated. The subgenual ACC and insula were investigated as seed regions. Connectivity between the subgenual ACC and perigenual parts of the ACC was increased in PTSD+MDD versus PTSD-MDD. Reduced functional connectivity of the subgenual ACC with the thalamus was found in the PTSD+MDD group versus the PTSD-MDD group. These results remained significant after controlling for PTSD severity. In addition, the PTSD+MDD group showed reduced functional connectivity of the insula with the hippocampus compared to the PTSD-MDD group. However, this cluster was no longer significantly different when controlling for PTSD severity. Thus, resting state functional connectivity of the subgenual ACC may distinguish PTSD+MDD from PTSD-MDD. As PTSD patients with comorbid MDD are more treatment resistant, this result may be important for treatment development.

Keywords
posttraumatic stress disorder, PTSD, major depressive disorder, MDD, insula, ACC, resting state, functional connectivity, comorbidity, fMRI
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Introduction

Posttraumatic stress disorder (PTSD) is an anxiety disorder that can develop after a traumatic event. It is characterized by re-experiencing the traumatic event, avoidance of trauma reminders and emotional numbing symptoms, and increased arousal\(^1\). PTSD frequently co-occurs with other Axis I psychiatric disorders, such as major depressive disorder (MDD)\(^2\). Patients with both PTSD and depression were found to have more psychological distress and are also more treatment resistant than patients with PTSD or depression alone\(^3-5\). About 48% of PTSD patients were found to have comorbid MDD in a large national survey in the United States\(^6\). Therefore, studies investigating the neurobiology of PTSD often comprise patients with and without comorbid MDD. Neuroimaging studies have demonstrated dysfunction of similar brain regions in both PTSD and MDD. That is, PTSD and MDD are both associated with alterations in structure and function of the medial prefrontal cortex (mPFC), amygdala, insula, and anterior cingulate cortex (ACC)\(^6-8\). To what extent comorbid MDD contributes to the reported neurobiological alterations of PTSD is yet to be determined.

Thus far, two neuroimaging studies have directly investigated differences in PTSD patients with and without comorbid MDD. First, reduced activity of the mPFC and amygdala was found in PTSD patients with comorbid MDD versus PTSD patients without MDD, when fearful faces were presented\(^9\). Second, during a symptom provocation paradigm PTSD patients with comorbid MDD had decreased activity in the insula, and increased ACC and posterior cingulate cortex (PCC) activation versus PTSD patients without MDD\(^10\). In addition, decreased insula activation remained significant after controlling for PTSD severity. One other study has investigated the effects of depressive symptoms in PTSD patients. A positive correlation between depressive symptoms and (para-) hippocampal and ventral ACC activity during an emotional memory task was observed in PTSD patients\(^11\). A fourth fMRI study involving PTSD patients versus both controls and MDD patients found increased activity in several brain areas of PTSD patients including the insula when emotional pictures were presented\(^12\).

However, these four studies were limited by small sample sizes (8 PTSD-MDD, 8 PTSD+MDD, 11 PTSD-MDD and 15 PTSD+MDD\(^9,10\), 21 PTSD+MDD and 12 PTSD-MDD\(^11\), 16 PTSD and 16 MDD\(^12\)). In addition, these studies investigated neurobiological alterations during emotional tasks, potentially inducing PTSD (and/or depressive) symptoms. It is expected that PTSD and/or MDD symptom provocation induces an altered state in PTSD with or without MDD, which is reflected by alterations in brain activity. Whether regular functioning of the brain in the absence of symptom-inducing stimuli deviates in PTSD with versus without comorbid MDD remains unclear. To our knowledge, functioning of the brain during resting state, without presenting stimuli or requiring task performance, has not been investigated in PTSD patients with and without comorbid MDD. Thus, the effect of comorbid MDD on brain functioning at baseline of PTSD patients deserves further investigation.

Here, we investigate the effects of comorbid MDD on resting state functional connectivity in PTSD patients. Since the studies described above indicated that functioning of the ACC distinguishes PTSD with and without MDD during emotional tasks\(^8,11\), this brain area was chosen as a region of interest. MDD has been associated with alterations in structure\(^13\), function\(^14\), structural connectivity\(^9\), and reduced resting state functional connectivity\(^10-13\) of the subgenual ACC in particular, which is a subdivision of the ventral ACC. In addition, subgenual ACC activation and cortical thickness have been associated with symptom improvement in PTSD\(^14-16\). Therefore, the subgenual ACC was selected as a more specific region of interest. Second, alterations in activation of the insula also differed between PTSD patients with and without PTSD, even when controlling for PTSD severity\(^15\). Furthermore, insula activation distinguished PTSD patients from MDD patients\(^16\). Thus, the insula was chosen as a second region of interest. As increased ACC activity was found in PTSD with comorbid MDD, as well as a positive correlation of ACC activity with depressive symptoms, we hypothesize that functional connectivity of the subgenual ACC is increased in PTSD with versus without comorbid MDD. Since insula activity is increased in PTSD versus MDD and insula activity was reduced in PTSD with comorbid MDD versus PTSD without MDD, we expected to find lower insula functional connectivity in PTSD with MDD as compared to PTSD without MDD. In summary, in order to provide more insights into the potential effects of MDD on the neurobiology of PTSD, the present study examined the effects of comorbid MDD on subgenual ACC and insula resting state functional connectivity in PTSD patients.

Methods

Participants

In total, 30 male veterans with PTSD with comorbid MDD (PTSD+MDD, mean age 34.2 ± 8.5), and 25 male veterans with PTSD without comorbid MDD (PTSD-MDD, mean age 37.4 ± 10.1) were included in this study. All patients were recruited from the Military Mental Health Care Center, the Netherlands. PTSD and MDD diagnoses were confirmed using the Clinician Administered PTSD scale (CAPS\(^22\)) and the Structural Clinical interview for DSM-IV (SCID\(^23\)). Several patients were medication naive (PTSD+MDD; n=13, PTSD-MDD; n=10), some patients were currently taking antidepressants (e.g. selective serotonin reuptake inhibitors; PTSD+MDD; n=6, PTSD-MDD; n=6), and some patients used benzodiazepines (PTSD+MDD; n=5, PTSD-MDD; n=2), or both antidepressants and benzodiazepines (PTSD+MDD; n=1, PTSD-MDD; n=3). One patient from the PTSD+MDD group used both antipsychotics and benzodiazepines. Most of the veterans had been deployed to Afghanistan (n=28) and to the former Yugoslavia (n=10). After receiving a complete written and verbal description of the study, all participants gave informed consent. Participants received financial compensation for their participation. The Medical Ethical Committee of the UMC Utrecht approved the study (protocol number NL29550.041.09), and the study was performed in accordance with the Declaration of Helsinki\(^23\).

Data acquisition

Functional and structural images were obtained using a 3.0 Tesla magnetic resonance imaging scanner (Philips Medical System, Best, the Netherlands). Before the resting state scan, a ten minute T1-weighted high-resolution imaging scanner (TR = 10 ms TE = 4.6 ms flip angle 8, 200 slices sagittal orientation, FOV 240 x 240 x 160, 304 x 299 matrix) was acquired. This image was utilized for co-registration and segmentation purposes and also allowed the participants to adapt to
the scanner environment. During the nine minute resting state scan participants were asked to relax, to let their mind wander and to focus on a fixation cross. Three hundred and twenty T2* echoplanar interleaved images were collected (TR = 1600 ms, TE = 23 ms, flip angle = 72.5°, 30 transverse slices, FOV 256 × 208 × 120, 64 × 51 matrix).

Image analyses
Pre-processing was conducted with SPM5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5/), which included slice-timing correction, realignment, co-registration with the anatomical scan, normalization, and spatial smoothing (8 mm FWHM). Five participants (2 PTSD+MDD, 3 PTSD-MDD) were excluded due to excessive motion (more than 2 mm displacement in any direction (x, y or z) or 2 degrees rotation (pitch, roll or yaw)).

The Data Processing Assistant for Resting-State fMRI (DPARSF) was utilized for further analyses (restfmri.net96), which is based on MRicroN (http://www.mricro.com), SPM5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5/), and the Resting-State fMRI Data Analysis Toolkit 24. Resting state images were band-pass filtered (0.08-0.01 Hz) to reduce low-frequency drift and high-frequency noise, and detrended to correct for general signal drift. In order to correct for physiological processes and motion, the motion parameters from the realignment step, mean global signal, white matter signal, and cerebral spinal fluid signal were included as covariates in the analysis. In addition, motion scrubbing was applied to scans that surrounded a minimum of 0.5 mm frame displacement (one scan before displacement, two scans after displacement), using nearest neighbour interpolation97. A minimum of approximately 5 minutes of resting state (183 unscrubbed resting state images) was set as a required threshold for correct scrubbing. One participant was excluded due to excessive scrubbing, resulting in the following groups: 27 PTSD+MDD, and 22 PTSD-MDD.

Functional connectivity analysis
For the subgenual ACC two spherical seeds (left and right, 3.5 mm radius) were created around two seed point coordinates, as previously described by Kelly et al. (2009)98. The anterior insula seed was created from two distinct anterior insula subdivisions that were described as the insula regions involved in emotion and cognition, as reported by Kelly et al. (2012)99. The mean time series for each of those seeds was extracted for all individuals and correlated with the time series of every voxel in the brain in order to create functional connectivity maps. These correlation maps were normalized using Fishers z-transform, resulting in a z-map for each ACC network per participant. The individual z-maps were used for second-level group analysis (full factorial design, SPM). A general effect of group (F-test) was investigated to determine group differences within the positive and negative network of the seed pairs.

Cluster-level multiple comparison correction was applied according to Gaussian Random Field theory20. A height threshold of p<0.001 was applied and combined with an extended cluster threshold of k>11, that corresponds to corrected p<0.05 (as determined with 1000 Monte Carlo simulations using Alphasim, implemented in the REST toolbox; FWHM 8 mm, cluster connection radius 7 mm).

Post-hoc analyses were performed including the total CAPS score as a covariate, in order to assess whether the results were due to differences in PTSD severity (F-test, height threshold p<0.001 extended threshold k>11, resulting in false discovery rate (FDR) corrected p<0.05). In addition, the positive affect (PA) score from the mood and anxiety questionnaire (MASQ), which has been reported to reflect a core feature of MDD, was also investigated as covariate. In addition, functional connectivity values (z-values) were extracted from the peak voxels of clusters of significant differences in order to perform post-hoc correlations with PTSD and MDD symptoms. Total CAPS scores, CAPS symptom cluster B, C, and D scores, as well as MASQ PA scores were investigated. Correlations between whole brain functional connectivity and CAPS and PA scores were calculated respectively.

Results
Participants
Groups did not differ significantly in age, handedness, the number of times they were deployed, the time since their last deployment, and educational level as measured with the international standard classification of education (ISCED:10). The PTSD+MDD group differed from the PTSD-MDD group in total PTSD severity (CAPS score; p=0.008), which appeared to be largely driven by differences in avoidance and emotional numbing symptom scores (cluster C; p=0.001). In addition, the PTSD+MDD group had lower PA scores versus the PTSD-MDD group (p=0.012), while negative affect and somatic anxiety did not differ between groups. In the PTSD+MDD group 10 patients were diagnosed with a comorbid anxiety disorder (n=10), and one patient had a comorbid somatoform disorder. In the PTSD-MDD group seven patients met the current diagnostic criteria for a comorbid anxiety disorder, one patient had a somatoform disorder only, and one patient was diagnosed with both a comorbid anxiety and somatoform disorder. An overview of demographical and clinical data is presented in Table 1.

Functional connectivity
Spatial connectivity maps. Figure 1 shows the positive and negative networks for the bilateral insula and the bilateral subgenual ACC. Positive functional connectivity of the subgenual ACC was found with the ventromedial PFC, temporal regions (including the hippocampus) and a posterior cluster comprising the PCC/precuneus. Positive functional connectivity of the insula was found around the insular lobe, extending into the temporal and parietal lobe. A medial cluster around the dorsal ACC showed positive functional connectivity with the insula.

Group differences
Subgenual ACC. Reduced functional connectivity of the PTSD+MDD group versus the PTSD-MDD group was found in functional connectivity of the subgenual ACC with the bilateral thalamus (Left thalamus; 29 voxels; peak value F=25.71; peak MNI-coordinates x=-12, y=-16, z=4). Right thalamus; 16 voxels; peak value F=34.37; peak MNI-coordinates x=20, y=-12, z=4). Increased functional connectivity was found between the subgenual ACC and perigenual regions of the ACC (peak in left perigenual ACC; 100 voxels; peak value F=25.71; peak MNI-coordinates x=-12, y=40, z=-4) in the PTSD+MDD group versus the PTSD-MDD group (see Figure 2, Figure 3 and Table 2).
Insula. Functional connectivity of the bilateral insula with the left hippocampus (17 voxels; peak value F=19.05; peak MNI-coordinates x=-28, y=-32, z=-8) was reduced in the PTSD+MDD group as compared to the PTSD-MDD group, which showed no functional connectivity between these regions (see Figure 2, Figure 3, and Table 2).

Post-hoc analyses: correlation with symptoms

Because of group differences in PTSD severity, we repeated the analyses after including total CAPS scores as a covariate. This did not affect significance of the subgenual ACC connectivity differences in the bilateral thalamus (cluster in left thalamus 12 voxels (p=0.022) and right thalamus 15 voxels (p=0.005)) and perigenual ACC (cluster increased to 124 voxels). However, the cluster of altered connectivity between insula and hippocampus was no longer significant after controlling for total CAPS scores (cluster size reduced to 4 voxels (p=0.813)).

Post-hoc correlation analyses of the peak voxel of significant difference with CAPS total, CAPS symptom cluster, and PA scores were performed within both groups separately. CAPS cluster C scores correlated negatively with connectivity of the subgenual ACC with the peak voxel of significant difference in the left thalamus (r = -0.523, p=0.012) within the PTSD-MDD group. Within the PTSD+MDD group CAPS cluster B scores correlated negatively with connectivity of the subgenual ACC with the peak voxel of significant difference in the perigenual ACC (r = -0.396, p=0.041). No correlations were found between CAPS cluster D scores or PA scores and the peak voxels of difference in subgenual ACC and insula connectivity.

Exploring the relation of whole brain subgenual ACC connectivity with CAPS and PA scores revealed a negative correlation of CAPS and PA scores with subgenual ACC-PCC/precuneus connectivity, amongst other regions (see Supplementary Figure S1). In addition, a negative correlation was found between CAPS and PA scores and negative functional connectivity of the insula with the PCC/precuneus (see Supplementary Figure S1).

Table 1. Demographic and clinical characteristics of the PTSD+MDD and the PTSD-MDD group.

<table>
<thead>
<tr>
<th>Measure</th>
<th>PTSD + MDD (mean ± SD)</th>
<th>PTSD - MDD (mean ± SD)</th>
<th>df</th>
<th>Sig. (two-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>27</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (range 21–57)</td>
<td>37.41 (±10.12)</td>
<td>33.87 (±8.43)</td>
<td>47</td>
<td>0.239</td>
</tr>
<tr>
<td>Education (ISCED level)</td>
<td>4.00 (±1.20)</td>
<td>3.65 (±1.23)</td>
<td>46</td>
<td>0.311</td>
</tr>
<tr>
<td>Handedness (Right/Left/Ambidexter)</td>
<td>(21/4/2)</td>
<td>(20/0/2)</td>
<td>2</td>
<td>0.169</td>
</tr>
<tr>
<td>Number of times deployed (range 1–15)</td>
<td>2.16 (±1.43)</td>
<td>3.18 (±4.22)</td>
<td>45</td>
<td>0.898</td>
</tr>
<tr>
<td>Time since last deployment (years)</td>
<td>8.00 (±8.537)</td>
<td>7.05 (±8.72)</td>
<td>45</td>
<td>0.706</td>
</tr>
<tr>
<td>Country of last deployment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afghanistan</td>
<td>13</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former Yugoslavia</td>
<td>6</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>8</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAPS total score</td>
<td>75.15 (±12.45)</td>
<td>65.09 (±12.87)</td>
<td>47</td>
<td>0.008*</td>
</tr>
<tr>
<td>Cluster B</td>
<td>22.67 (±5.61)</td>
<td>22.64 (±5.43)</td>
<td>47</td>
<td>0.985</td>
</tr>
<tr>
<td>Cluster C</td>
<td>27.48 (±8.76)</td>
<td>18.59 (±8.30)</td>
<td>47</td>
<td>0.001*</td>
</tr>
<tr>
<td>Cluster D</td>
<td>25.00 (±4.47)</td>
<td>23.86 (±4.97)</td>
<td>47</td>
<td>0.404</td>
</tr>
<tr>
<td>Negative Affect (MASQ)</td>
<td>52.12 (±14.91)</td>
<td>46.00 (±10.50)</td>
<td>42</td>
<td>0.130</td>
</tr>
<tr>
<td>Positive Affect (MASQ)</td>
<td>40.87 (±15.80)</td>
<td>51.70 (±10.50)</td>
<td>42</td>
<td>0.012*</td>
</tr>
<tr>
<td>Somatic Anxiety (MASQ)</td>
<td>44.75 (±13.52)</td>
<td>41.50 (±10.91)</td>
<td>42</td>
<td>0.392</td>
</tr>
<tr>
<td>Current comorbid disorder (SCID)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major depressive disorder</td>
<td>27</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety disorder</td>
<td>10</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety disorder &amp; somatoform disorder</td>
<td>-</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Somatoform disorder</td>
<td>1</td>
<td>1</td>
<td></td>
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</tbody>
</table>

*Significant differences between groups; p<0.05

Discussion

This study showed that resting state functional connectivity of the subgenual ACC and insula differs between PTSD patients with and without comorbid depressive disorder. PTSD+MDD patients had increased functional connectivity between the subgenual ACC and the left perigenual ACC compared to PTSD-MDD patients.
difference in self-referential processing. A negative correlation between re-experiencing symptoms and functional connectivity of the subgenual ACC and perigenual ACC was also found within the PTSD+MDD group. This may suggest that when more specific PTSD symptoms (re-experiencing, cluster B) are prevalent, the balance of symptoms is tilted towards a PTSD only state, and the more functional connectivity resembles the PTSD-MDD group.

The perigenual ACC is part of the default mode network, which is the network that is active during rest and deactivated during task performance. Connectivity of the perigenual ACC with default mode network regions (PCC/precuneus and medial PFC) has been negatively correlated with general symptom severity in PTSD, even when correcting for depression diagnosis and depression severity. This was confirmed in our post-hoc results, as we also found a negative correlation between total CAPS and PA scores and subgenual ACC connectivity with the PCC/precuneus. In addition, a negative correlation was found between total CAPS and PA scores and negative functional connectivity between the insula and PCC/precuneus (see Supplementary Figure S1).

Specific correlations between CAPS scores and subgenual ACC-PCC/precuneus connectivity were also present, whilst controlling for PA scores. Connectivity in these regions did not differ between the PTSD+MDD and PTSD-MDD groups. Thus, it seems that subgenual ACC connectivity with the perigenual ACC in particular has the capacity to distinguish PTSD with and without comorbid MDD. The importance of differences in connectivity of the perigenual ACC in particular in MDD patients has been previously described for the default mode network, the affective network, and the salience network. Instead, functional connectivity of other regions of the default mode network may be related to general (PTSD and MDD) symptom severity.

Reduced functional connectivity was found in PTSD+MDD between the subgenual ACC and the bilateral thalamus, and between the insula and left hippocampus. The subgenual ACC results remained significant after controlling for PTSD severity. This study complements previous task-based studies by showing that differences in the subgenual ACC/insula between PTSD patients with and without comorbid MDD are also apparent using resting state functional connectivity.

Increased subgenual ACC connectivity with the perigenual ACC was found in PTSD+MDD versus PTSD-MDD, which is in line with neuroimaging studies in MDD that demonstrated specific alterations in these regions. Furthermore, increased resting state functional connectivity between the subgenual ACC and perigenual ACC has been previously reported in MDD, while reduced functional connectivity of the medial PFC, including the subgenual and perigenual ACC, has been shown in PTSD patients versus controls. Our results complement these results, by showing increased connectivity of the subgenual ACC with the perigenual ACC in PTSD+MDD versus PTSD-MDD. In addition, the perigenual ACC has been related to self-referential processing, which underlies depressive symptoms such as helplessness, self-reproach and guilt rumination. During self-referential processing tasks a reduced negative blood oxygen level dependent (BOLD) response (thus an increase in BOLD signal) has been found in medial PFC regions, including the perigenual ACC, in MDD patients versus controls. On the other hand, reduced medial PFC activation, including the perigenual ACC, has been observed in PTSD versus controls during a self-referential processing task. It can thus be suggested that the increases in subgenual-perigenual ACC connectivity in the PTSD+MDD group versus the PTSD-MDD group reflect a difference in self-referential processing. A negative correlation between re-experiencing symptoms and functional connectivity of the subgenual ACC and perigenual ACC was also found within the PTSD+MDD group. This may suggest that when more specific PTSD symptoms (re-experiencing, cluster B) are prevalent, the balance of symptoms is tilted towards a PTSD only state, and the more functional connectivity resembles the PTSD-MDD group.

The perigenual ACC is part of the default mode network, which is the network that is active during rest and deactivated during task performance. Connectivity of the perigenual ACC with default mode network regions (PCC/precuneus and medial PFC) has been negatively correlated with general symptom severity in PTSD, even when correcting for depression diagnosis and depression severity. This was confirmed in our post-hoc results, as we also found a negative correlation between total CAPS and PA scores and subgenual ACC connectivity with the PCC/precuneus. In addition, a negative correlation was found between total CAPS and PA scores and negative functional connectivity between the insula and PCC/precuneus (see Supplementary Figure S1). Specific correlations between CAPS scores and subgenual ACC-PCC/precuneus connectivity were also present, whilst controlling for PA scores. Connectivity in these regions did not differ between the PTSD+MDD and PTSD-MDD groups. Thus, it seems that subgenual ACC connectivity with the perigenual ACC in particular has the capacity to distinguish PTSD with and without comorbid MDD. The importance of differences in connectivity of the perigenual ACC in particular in MDD patients has been previously described for the default mode network, the affective network, and the salience network. Instead, functional connectivity of other regions of the default mode network may be related to general (PTSD and MDD) symptom severity.
severity was added as a covariate. Thus, hippocampus-insula connectivity does not seem to be specifically related to depressive symptoms, but rather to PTSD severity. The hippocampus is a brain region often associated with PTSD and is involved in memory. Therefore, differences found in connectivity between the insula and hippocampus may be related to trauma-related memory. Thus, alterations in connectivity between the insula and hippocampus between the PTSD+MDD and PTSD-MDD group may be due to differences in PTSD severity and not to comorbid MDD diagnosis per se.

Limitations

This study has some limitations. First, no MDD only group was included in the current study. Thus, this study does not show whether subgenual ACC and insula connectivity differs from patients with MDD only. The results only give insights in the effects of comorbid MDD in the context of PTSD, and not on general effects of PTSD or MDD. Including this group in future research can provide more insights in PTSD, MDD, and their neurobiological overlap or differences. Second, both groups included participants that were currently using antidepressants. Further studies should investigate the effect of medication on the neurobiology of PTSD with or without MDD. Third, no validated measure of the severity of all MDD symptoms was included in the study. If MDD severity was measured, it would have been possible to determine common and distinct factors of PTSD symptom severity and MDD symptom severity.

Previous fMRI studies including resting state paradigms, have reported decreased thalamus connectivity with the ACC in both depression and PTSD versus healthy controls. The thalamus is the relay station of the brain, and can modulate attention and arousal. The thalamus is also implicated as a target for surgical treatment of severe MDD. In line with these findings we found that connectivity between the thalamus and subgenual ACC is more reduced in PTSD+MDD versus PTSD-MDD, suggesting that MDD effects add up to the PTSD effects in these regions. Furthermore, the results were not influenced by general PTSD severity, which may indicate that reduced thalamus connectivity is specific for comorbid MDD. In addition, functional connectivity between the subgenual ACC and thalamus was negatively correlated with cluster C symptoms in the PTSD-MDD group only. Thus, individuals with less avoidance and reduced interest, a shared PTSD and MDD symptom, have a stronger connection between the subgenual ACC and thalamus. A possible explanation of the correlation may be that the more the clinical image shifts toward more depressive symptoms, the more the connectivity pattern resembles PTSD+MDD. However, this interpretation needs further investigation, since cluster C symptoms both include avoidance and reduced interest symptoms.

Insula connectivity with the hippocampus was reduced in the PTSD+MDD group versus PTSD-MDD. However, the cluster was no longer significantly associated with comorbid MDD when PTSD severity was added as a covariate. Thus, hippocampus-insula connectivity does not seem to be specifically related to depressive symptoms, but rather to PTSD severity. The hippocampus is a brain region that is often associated with PTSD and is involved in memory. Therefore, differences found in connectivity between the insula and hippocampus may be related to trauma-related memory. Thus, alterations in connectivity between the insula and hippocampus between the PTSD+MDD and PTSD-MDD group may be due to differences in PTSD severity and not to comorbid MDD diagnosis per se.
presence of comorbid MDD per se. Unraveling the neurobiological features of MDD and PTSD during rest can provide insights in which specific brain areas could be targeted for effective treatments. For example, tasks or therapy methods that increase functional connectivity between the regions with dysfunctional connectivity may be effective. Future studies should investigate long-term effects of training that is associated with functional connectivity alterations. This is particularly relevant for treatment of PTSD patients with comorbid MDD, since patients with this combination of psychological problems tend to be more treatment resistant.

Author contributions
EG and AR have made a substantial contribution to the conception and design of the study. MK and SvR have made a substantial contribution to the acquisition of data. MK performed the analyses and prepared the first draft of the manuscript. EG, AR, SvR and RK were involved in the interpretation of the data, and critically reviewing the article. All authors have agreed to the final content of the article.

Table 2. Peak voxels of significant differences between PTSD+MDD and PTSD-MDD for the subgenual ACC and insula.

<table>
<thead>
<tr>
<th>Network</th>
<th>Number of voxels (k)</th>
<th>Peak value (F)</th>
<th>MNI coordinates x</th>
<th>y</th>
<th>z</th>
<th>Brain area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subgenual ACC</td>
<td>100</td>
<td>25.71</td>
<td>-12 -40 -4</td>
<td></td>
<td></td>
<td>Left Anterior cingulate cortex</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>23.79</td>
<td>-12 -16 4</td>
<td></td>
<td></td>
<td>Left thalamus</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>34.37</td>
<td>20 -12 4</td>
<td></td>
<td></td>
<td>Right thalamus</td>
</tr>
<tr>
<td>Insula</td>
<td>17</td>
<td>19.05</td>
<td>-28 -32 -8</td>
<td></td>
<td></td>
<td>Left hippocampus</td>
</tr>
</tbody>
</table>
Competing interests
No competing interests were disclosed.

Grant information
This study was funded by the Dutch Ministry of Defence.

Acknowledgements
In addition, we thank Jonathan van Leeuwen for his help with data acquisition and preprocessing.

Supplementary material

Supplementary Figure 1. Correlations of PTSD symptom severity and reduced positive affect with subgenual ACC (a, b) and insula (c, d) functional connectivity. Violet = positive correlation with both CAPS scores and reduced PA, cyan = negative correlations with both CAPS scores and reduced PA, red = positive correlations with CAPS scores, blue = negative correlations with CAPS, yellow = positive correlations with reduced PA, and green = negative correlation with reduced PA (p<0.001, k>11, resulting in FDR-corrected p<0.05).


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Onno Meijer
Medical Pharmacology, Leiden University, Leiden, The Netherlands

The paper shows convincing (to the technical non-specialist) differences in resting state functional connectivity between PTSD patients with or without comorbid major depression MDD.

There are clear effects on connectivity of the subgenual anterior cingulate cortex with the left ACC (increased with MDD) and with the thalamus (decreased with MDD).

The interpretations are hampered by the design, which ideally would be a 2 X 2, i.e. +/- MDD and +/- PTSD. Therefore sentences like 'we found that connectivity ... is more reduced in PTSD +MDD than in PTSD-MDD' cannot be made.

The authors determined that effects on connectivity of insula and hippocampus are related to PTSD symptom severity - this should probably be indicated in figure 3 somehow, as this may end up being used as a graphic summary of the data.

I missed whether the authors looked at the use of medication as a factor that affected connectivity, as it may interact with the condition.

Second the authors, at correlation between connectivity (thalamus - sgACC) and symptom severity in the PTSD-MDD, group in a post hoc correlation. A similar effect is reported specifically for the PTSD+MDD group. It is unclear why the authors did not look at the correlation in all patients? What is the reason to now limit to a single group?

Interpretation: I do not see how on p.6 the authors suggest the cluster B symptoms would tilt the balance towards a PTSD only state, based on correlation, when table 1 clearly shows no differences between the groups for cluster B symptoms.

In terms of interpretation, I agree with the first reviewer that the statement in the abstract that the findings have impact on treatment development need to be substantiated. As markers? As targets?
It also seems important whether or not PTSD and MDD interact in terms of connectivity, or whether the association of MDD and PTSD 'just' add up. For this a 2x2 design seems necessary, but the paper may benefit from actual meaning that is given to the data. Obviously, at present describing correlates is what is done in research like this, but an explicit sense of direction would be helpful to the non-specialist reader who is nevertheless interested in the subject matter.

Minor remarks:
- I am not certain that (as in: I do not think that) the second, long, sentence in the discussion accurately reflects the findings, please check.
- The term however in the third paragraph of the intro seems needlessly negative about previous research.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 07 Apr 2014
Mitzy Kennis, Ministry of Defence, 3584 CX Utrecht, The Netherlands

We thank the reviewer for his valuable suggestions. See below our response to the comments.

Design and interpretations:

We agree with the reviewer that our study is limited by design, and lacks an MDD only group and a healthy control group. We acknowledge that we can therefore not clearly interpret the effects described here, with respect to the nature of either one of these disorders or the interaction of the two. As the reviewer suggests, we have attempted to be more cautious with our interpretations and elaborated on the implications (sense of direction, specific implications for treatment development) of the results in the discussion and abstract.

Analyses:

As proposed, additional analyses on a subsample of medication naive PTSD patients and PTSD patients that did not use benzodiazepines at least 48 hours prior to scanning were performed to investigate the effect of medication.

It was also questioned why we did not correlate the peak voxel of functional connectivity of the clusters of significant differences with all participants symptom scores, but only within groups. As also pointed out in our response to Andrew Kemp: a correlation analysis with the cluster of significant differences over all participants is inappropriate, since groups differ on both these values (functional connectivity cluster peak and PTSD severity (total CAPS score)) and therefore misleading correlations can be induced. To avoid this problem, within group
correlations can be performed and seem more appropriate here. Finally, we have also added figures of the correlations with CAPS symptom clusters.

**Competing Interests:** No competing interests were disclosed.

Reviewers Report 30 January 2014

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Andrew Kemp

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2 Discipline of Psychiatry and School of Physiology, University of Sydney, NSW, Australia

Thank you for the opportunity to review this interesting study on the impact of disorder comorbidity on resting state functional MRI. This is an important and under-studied area of research. I have a number of comments that may help to improve the manuscript.

**Abstract:**

- The abstract is disappointing, dense and requires development. At present it is mostly comprised of statements relating to the findings obtained, rather than providing an interpretation of findings. Are all the findings reported key to the bottom-lines of the article? The authors need to add a brief interpretation after each of the KEY findings. What is the take home message; what are the bottom-lines? While the findings may indeed be “important for treatment development”, it is unclear how. Please elaborate.

**Introduction:**

- The first paragraph would benefit from a little more context on why comorbidity is important. For instance, studies have demonstrated that comorbidity between the mood and anxiety disorders increases risk for CVD and mortality (Philips et al., 2009). I would recommend the authors integrate this information to help build study rationale.

- While the rationale for focusing on subgenual ACC as a seed region in connectivity analysis is well-founded, I thought the rationale for focusing on the insula to be much weaker. I would have thought that the amygdala region would have been a more appropriate second choice, especially given the many past studies that have focused on the amygdala as a region of interest in patients with PTSD.

**Methods:**

- The Participants section: who administered the psychiatric evaluation; were they psychiatrists or research assistants? How many people conducted the evaluations? How was the
reliability and validity of this measurement across assessors determined? If research assistants, how much training did they receive? What was the value of the financial compensation received by participants?

○ re Data Acquisition: To what extent could this (incentivisation) have impacted on findings (i.e. group interaction) especially given the unrestricted participant instructions (i.e. relax and let the mind wander)? Was any questionnaire administered to assess mental state of participants during the resting state scan?

Results:
○ I recommend the authors to review the article by Miller & Chapman (2001) on the problems associated with ANCOVA when participants are not randomly allocated to a group, as in the present study. The use of total CAPS scores as a covariate in post-hoc analysis of covariance is problematic.

○ One potential solution to this problem that the authors could consider is post-hoc correlational analysis. Although the authors ran a series of correlational analyses, these need to be conducted on all participants regardless of group, rather than by each group individually. The authors would need to determine the unique activations associated with the PTSD, with versus without depression, a contrast that is not associated with confounding variables (i.e. CAPS, positive affect).

○ Another issue the authors need to consider is the impact of medication on their findings. While the authors note in their limitations that “future studies should investigate the effect of medication on the neurobiology of PTSD with or without MDD”, the authors have a sufficient sample of medication naive participants on which sub-analysis could be run.

○ Error bars need to be added to Figure 3.

Discussion:
○ The authors link increases in subgenual-perigenual ACC connectivity in the PTSD+MDD group versus the PTSD-MDD group to differences in self-referential processing. This is an interesting point which I feel could be further elaborated (after of course confirming that these findings are still present after adjusting for confounds -see points I made regarding this issue under the results section). The same could be said for the discussion on the relationship between the disorders and the default mode network.

○ I also feel the discussion section needs to be further developed. Currently, much of the discussion restates the findings without more detail on what the differences might mean (especially in the first paragraph of the discussion). This makes the discussion section a little underwhelming.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
Mitzy Kennis, Ministry of Defence, 3584 CX Utrecht, The Netherlands

We thank the reviewer for his valuable comments. See below our response to the comments per section of the manuscript.

Abstract and introduction:

We have attempted to make the abstract clearer, and added more context in both the abstract and introduction. We also acknowledge that the amygdala could have been another interesting seed to investigate PTSD with and without comorbid MDD. However, since the two previous neuroimaging studies that investigated PTSD and MDD with task paradigms reported alterations in the insula (Lanius et al., 2007; Whalley et al., 2009) while differences in the amygdala between PTSD and MDD patients were only reported by Whalley et al. (2009). Therefore, we argued that the insula is an appropriate candidate to investigate here.

Methods:

In order to address the concerns on the methods section, we provide more details on the administration of the interviews and financial compensation. Reliability and validity were qualitatively assessed by comparing interview scores and diagnosis between raters during interview training.

With respect to the resting state design, we do not expect any effects of the financial incentive on the pattern of functional connectivity. Every participant received a similar financial compensation for participating in the study. We assessed mental states of participants during resting state after the scan session, by asking whether they felt relaxed during the rest scan. Most participants confirmed feeling relaxed during the resting state scan, and ten participants reported having some difficulties to relax (five in each group). Furthermore, we investigated co-activation patterns of the subgenual ACC and insula. Although mind states are of influence on brain activation it is not expected that different mind states (e.g. individuals that tend to think of the incentive) directly alter the spatial co-activation patterns within these functional networks.

Results:

We agree with the reviewer that the use of ANOVA applied here is inappropriate. However, the suggested solution, a correlation analysis with the cluster of significant differences over all participants, is also inappropriate, since groups differ on both these values (functional connectivity cluster peak and PTSD severity (total CAPS score)). Therefore misleading correlations can be induced (see Lord's Paradox; Lord, 1967).

Unfortunately, as Miller and Chapman (2001) have shown, there is no simple solution to “adjust” or “control” for differences between groups in non-randomized groups.

On the other hand, we agree with the reviewer that correlations can be explored in order to
provide some insight in the relation between functional connectivity and symptom severity, but then within groups rather than over all participants (to avoid misleading correlations). Thus, we have explored correlations within PTSD+MDD and PTSD-MDD groups between symptom severity scores (total CAPS and reduced PA) and the peak voxels of significant differences. There were no significant correlations between PTSD severity and functional connectivity. This suggests that functional connectivity of the clusters of significant differences was not related to differences in total CAPS or reduced PA scores.

Additionally, for the correlations with the symptom clusters performed within groups (for the same reasons as described above), we plotted the data for all subjects and added a fitted line for the data for each group and for the whole group respectively (see Figure S2 and S3). This way, an objective overview of the data is presented and both whole group and subgroup correlations are visualized.

Finally, as requested we performed a post-hoc analysis to investigate the effect of medication by including medication naive PTSD patients and PTSD patients that did not use benzodiazepines at least 48 hours prior to scanning (PTSD+MDD n = 20, PTSD-MDD n = 15). This revealed similar results for the subgenual ACC seed, although the hippocampal cluster of difference in connectivity with the insula disappeared. This information has been added to the paper. Figure 3 now includes error bars.

**Discussion:**

We expanded the discussion section and added more detail on the interpretation of the results. Furthermore, we have nuanced the interpretation of the correlational analyses.

**Competing Interests:** No competing interests were disclosed.