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Alterations in Amygdala-Prefrontal Functional Connectivity Account for Excessive Worry and Autonomic Dysregulation in Generalized Anxiety Disorder

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**Running title:** Amygdala connectivity and symptoms of GAD

**Keywords:** Generalized anxiety disorder, Perseverative cognition, Heart rate variability, Amygdala, Functional connectivity, Functional magnetic resonance imaging
ABSTRACT

BACKGROUND: Generalized Anxiety Disorder (GAD) is characterized by the core symptom of uncontrollable worry. Functional magnetic resonance imaging (fMRI) studies link this symptom to aberrant functional connectivity between amygdala and prefrontal cortex. Patients with GAD also display a characteristic pattern of autonomic dysregulation. However, while frontolimbic circuitry is implicated in the regulation of autonomic arousal, no previous study combined fMRI with peripheral physiological monitoring in this population to test the hypothesis that core symptoms of worry and autonomic dysregulation in GAD arise from a shared underlying neural mechanism. METHODS: We used ‘resting-state’ fMRI, alongside the measurement of parasympathetic autonomic function (heart rate variability) in 19 GAD patients and 21 controls to define neural correlates of autonomic and cognitive responses before and after induction of perseverative cognition. Seed-based analyses were conducted to quantify brain-wise changes in functional connectivity with right and left amygdala. RESULTS: Before induction, patients showed relatively lower connectivity between right amygdala and right superior frontal gyrus, right paracingulate/anterior cingulate cortex, and right supramarginal gyrus than controls. After induction, such connectivity patterns increased in GAD and decreased in controls and these changes tracked increases in state perseverative cognition. Moreover, decreases in functional connectivity between left amygdala and subgenual cingulate cortex and between right amygdala and caudate nucleus predicted the magnitude of heart rate variability reduction after induction. CONCLUSIONS: Results link functional brain mechanisms underlying worry and rumination to autonomic dyscontrol, highlighting overlapping neural substrates associated with cognitive and autonomic responses to the induction of perseverative cognitions in GAD patients.
Excessive and uncontrollable worry is established as a central feature in the definition of Generalized Anxiety Disorder (GAD). Importantly, worry has to be accompanied by symptoms of negative affect and tension and perceived by the individual as ‘difficult to control’ (DSM-V). The high prevalence of GAD creates massive economic burden (1, 2), yet its core symptom remains poorly characterized from a neurobiological perspective. The ‘spontaneous’ nature of intrusive thoughts suggests that the neurobiological processes underpinning worry may be better examined over periods of free-thinking rather than during behavioural engagement with an external task. The use of a ‘resting-state’ neuroimaging approach therefore provides a useful tool for examining dysfunctional neural circuitry in GAD.

The few published functional connectivity studies of GAD focus largely on the amygdala and associated networks, following evidence for the amygdala’s central contribution to fear and threat processing (3). Resting state neuroimaging studies support the view that perturbed amygdala-prefrontal connectivity underlies the core features of GAD (4). Decreased connectivity between amygdala and lateral PFC is reported in adults (5) and adolescents with GAD (6, 7). More recently, aberrant amygdala connectivity with ventromedial prefrontal cortex (vmPFC) and insula is noted in youths with anxiety disorders (8). Interestingly, amygdala-based connectivity is found to be negatively correlated with anxiety ratings scores (9, 10).

Taken together, these findings point to a neural basis for emotion regulation deficits in GAD, centred upon reduced functional connectivity within this major frontolimbic pathway. Conversely, effective emotion regulation and anxiety control are predicted by efficient communication between amygdala and prefrontal cortex. For example, the positive reappraisal of negative emotional material strengthens connectivity between amygdala and medial prefrontal regions, with self-reported effectiveness of emotion regulation correlating positively with the degree of functional coupling (11). Moreover, effective emotion regulation evokes a selective increase in connectivity of amygdala with vmPFC and dorsolateral PFC (12).
Emotion dysregulation in GAD is expressed through both poor ‘prefrontal’ control of worrisome thoughts and chronic failure to down-regulate autonomic arousal (13). Medial prefrontal cortices and amygdala are implicated in states of autonomic arousal during mental and emotional stress. These states are characterised by shifts in parasympathetic to sympathetic balance in which baroreflex suppression manifests as increased heart rate (HR) and blood pressure and decreased HR variability (HRV) (14, 15).

Decreased HRV is a notable autonomic signature of worry states (16, 17). However, no detailed characterization of functional brain processes linking worry to measured changes in autonomic arousal has been conducted in GAD patients. Here, we combined resting state functional magnetic resonance imaging (rsfMRI) with concurrent autonomic measurement, focusing on HRV as a measure of vagally-mediated parasympathetic change. The simultaneous assessment of cognitive and physiological correlates of GAD is particularly relevant in light of a previous study associating self-reported experience of worry and autonomic arousal with distinct patterns of neural connectivity (18).

We used a seed-based approach to analyse our rsfMRI data, first to validate earlier findings of decreased amygdala-prefrontal connectivity in GAD patients compared to controls, second to test the hypothesis that a behavioural induction of perseverative cognition (i.e., worry and/or rumination) will alter (uncouple) amygdala-prefrontal connectivity. To our knowledge, only one study (focusing on elderly patients) compared the consequences of a worry induction on neural connectivity patterns in GAD (19). It is important to note that, the induction may place participants in a task-based state, therefore our use of the term resting state, motivated by the absence of direct instructions, should be taken with this caveat in mind.

In line with a dimensional view of psychopathology, we hypothesized that the induction will change the pattern of connectivity in healthy controls to the pattern more typically associated with GAD patients and that such changes will reflect the dispositional tendencies
(trait measures) of individuals to engage in perseverative cognition.

We anticipated that resting state amygdala connectivity reflects ongoing state measures of core GAD symptoms. Drawing on the theoretical model that the PFC down-regulates amygdala responses to (real or perceived) threat, we hypothesized that aberrant resting amygdala-PFC would predict increases in self-reported state worry.

Similarly, given the involvement of PFC regions and amygdala in autonomic control (14, 15, 20-24) and notably in HRV (25), we tested the relationship between amygdala connectivity and HRV changes in response to the induction. HRV is a positive marker for emotion regulation (26) and is diminished during maladaptive emotion regulation processes, including worry (27, 28). We therefore hypothesized that changes in amygdala-PFC caused by the perseverative cognition induction would correlate with reductions in HRV evoked by the same induction. Again, we expected these relationships to be amplified in GAD patients compared to controls (29).

METHODS AND MATERIALS

Participants

After excluding one participant who did not complete the full experiment, the sample encompassed 19 patients (17 women, 2 men; mean age = 29.58 (6.93) years) who met diagnostic criteria for GAD and 21 healthy controls (HC; 18 women, 3 men; mean age = 28.67 (9.45) years) (see Supplement 1 for more details). All participants provided written informed consent. The study was approved by the National Research Ethics Service (NRES) with local approval the Brighton and Sussex Medical School Research Governance and Ethics Committee.

Procedure
The Structured Clinical Interview for DSM-IV (SCID) was administered by a trained postdoctoral fellow (FM) to both patient and controls to confirm/exclude the diagnosis of GAD. To assess comorbid disorders, participants were asked if they currently or previously had a diagnosis of any other psychiatric disorder or had ever been treated by their GP for symptoms other than anxiety. None of our participants had a formal diagnosis of co-morbid major depressive disorder. Participants then completed a series of online socio-demographic and dispositional traits questionnaires. Participants were subsequently familiarized with the neuroimaging environment, connected to the physiological recording equipment, and then underwent the MRI protocol.

**Questionnaires**

All participants completed a set of questions accessing socio-demographic, and lifestyle information (nicotine, alcohol, and caffeine consumption, physical activity). To assess physical activity, participants were asked to report the type and amount (hours/week) of exercise they regularly did and how active they considered themselves compared to others of the same age and sex. Based on their responses, their perceived physical fitness was classified as low, medium or high.

Dispositional measures of 1) stress-reactive rumination (Stress-Reactive Rumination Scale, SRRS; 30), 2) depressive rumination (Ruminative Response Scale, RRS; 31), and 3) worry (Penn State Worry Questionnaire, PSWQ; 32) were also obtained.

**Experimental Design**

In the scanner, participants underwent a series of four 5-min resting state periods, each followed by a 6-min easy visuomotor tracking task (described elsewhere; 33). During resting state periods participants were instructed to rest with their eyes open without thinking of
anything and not falling asleep. After the second or third resting block, randomly, participants underwent a recorded verbal induction procedure designed to engender perseverative cognition (see Supplement 1). The induction occurred after the second resting-state block in 9 GAD and 11 HC (n = 20) and after the third block in 10 GAD and 10 HC (n = 20) and it has been proved to be particularly effective in evoking worrisome and ruminative thoughts that are prolonged over time (perseverative), and findings have been replicated in different experimental settings in both healthy and clinical samples (16).

At the end of each resting-state period, participants rated their thoughts over the preceding period using visuo-analogue scales (VAS).

Visual analogue scales (VAS)

To assess levels of perseverative cognition occurring prior to and following the induction, participants were asked to rate on three separate visual analogue 100-point scales: “how much, for the duration of the previous resting period, were you distracted by: 1) external stimuli?; 2) ruminating/worrying?; and 3) internal thoughts?

Physiological data processing

Heart rate was monitored using MRI-compatible finger pulse oximetry (8600FO; Nonin Medical) recorded digitally as physiological waveforms at a sample rate of 1000 Hz (via a CED power 1401, using Spike2 v7 software; Cambridge Electronic, Design CED). Inter-beat-intervals (IBI) values were visually inspected and potential artifacts were manually removed. To this pulse data, we applied the root mean square successive difference (rMSSD) which is a reliable parameter for assessing vagally-mediated HRV (34). RMSSD has been shown to be sensitive to changes in the parasympathetic arm of the autonomic nervous system and particularly suited to capture autonomic perturbation in anxiety disorders (35). RMSSD is
known to be stable over short recording intervals (36) and is relatively free of the influences of respiration (37, 38). RMSSD was derived using RHRV 4.0 analysis software (http://rhrv.r-forge.r-project.org/) for the duration of each resting state scanning period. Attention was given to measures before (Pre) and after (Post) the worry induction. 

MRI acquisition and preprocessing

MRI images were acquired on a 1.5-Tesla Siemens Magnetom Avanto scanner. Structural volumes were obtained using the high-resolution three-dimension magnetization-prepared rapid gradient-echo sequence (HiResMPRAGE). Functional datasets used T2*-weighted echoplanar imaging (EPI) sensitive to Blood oxygenation level dependent (BOLD) signal (TR = 2.52s, TE = 43 ms, flip-angle 90°, 34 slices, 3mm slice thickness, 192 mm FOV, voxel size 3 x 3 x 3 mm).

Data were pre-processed using Statistical Parametric Mapping (Wellcome Department of Imaging Neuroscience; SPM8, http://www.fil.ion.ucl.ac.uk/spm/), and in-house software implemented in Matlab (The Mathworks Inc, Natick, Massachusetts, USA) (see Supplement 1 for preprocessing details). As global signal removal can potentially change functional connectivity distributions and result in increased negative correlations (39), it was avoided in our pre-processing.

Statistical Analyses

Questionnaire, behavioural, and HRV analyses

All data are expressed as means (±SD). Differences at p = 0.05 are regarded as significant. Data analysis was performed with SPSS 22.0 for Windows (SPSS Inc, USA). To test for pre-existing group differences, a series of t and χ² tests were conducted on self-report socio-demographic, physiological, and personality measures.
To test for the effects of the induction on cognitive and autonomic variables, a series of Group (GAD vs. HC) × Condition (Pre vs. Post) General Linear Models (GLMs) were performed on each VAS, HR, and rMSSD. Pre-induction values were derived from the average of 2 or 3 VAS, HR, and rMSSD values (depending on when the induction took place; i.e., after the second or third resting state period). Similarly, post-induction values consisted of 1 or 2 averaged VAS, HR and rMSSD values.

Seed-Based fMRI Analysis
Anatomical ROIs were constructed using an anatomical toolbox in SPM (40) for bilateral amygdala. The average resting state fMRI time-series over the ROIs were extracted for each participant and for each scan. For each participant, only data obtained from the scan occurring immediately before and the one immediately after the induction were analyzed.

This time series were then used as a regressor in a 1st level SPM analysis, extracting the voxels in the brain showing a significant correlation with it. To test for group differences, second level analyses were performed, in which the first level contrasts images were submitted to a two-sample (GAD vs. HC) t-test model. A flexible-factorial design was used to evaluate the Induction x Group interaction. Grey matter volume was used as a covariate of no interest.

To test for the associations between amygdala connectivity and dispositional and autonomic measures, a t-test was run, with Group (GAD vs. HC) as factor, VAS, questionnaires, and HRV as covariates of interest, and order (i.e., induction after the second or third resting state period) as covariates of no interest, to adjust for potential confounds. Additionally, whole brain GM volume was also introduced as covariate of no interest, to correct for possible structural differences between the two groups, which might influence the functional connectivity (41). To investigate whether the neural impact of induction could be predicted by dispositional tendencies to ruminate and worry, we calculated the shift in connectivity after the induction
(subtracting connectivity pre induction to connectivity post induction) in the group of GAD and HC, and correlated this connectivity shift with SRRS, RRS, and PSWQ scores. Statistical threshold was set to \( p < 0.05 \) - FWE-corrected at cluster level (cluster size defined using uncorrected voxel-level threshold \( p < 0.005 \); a more liberal voxel-level threshold \( p < 0.01 \) was used occasionally in order to capture meaningful trends in our data).

RESULTS

There were no significant gender, age, years of education, body-mass index, physical activity, nicotine, alcohol, or caffeine intake differences between GAD and HC (Table 1).

Questionnaires, VAS, HR, and HRV data

The GAD group reported higher levels of dispositional rumination and worry (SRRS, RRS, and PSWQ) and had higher HR and lower HRV at baseline (i.e., pre-induction) compared to HC (Table 1).

A main effect of group was evident for ruminating/worrying (\( F_{1,38} = 6.19, p = 0.02 \)), with GAD engaging in perseverative cognition more than HC (GAD = 45.26 ± 19.41, HC = 27.05 ± 26). GAD were also more distracted by internal stimuli when compared to HC, irrespective of the induction (GAD = 78.34 ± 15.1; HC = 68.43 ± 17.46), but the difference only approached statistical significance (\( F_{1,38} = 3.63, p = 0.06 \)). Lastly, a main effect of Group (\( F_{1,38} = 6.29, p = 0.02 \)) and Induction (\( F_{1,38} = 15.68, p < 0.0001 \)) emerged for the VAS “Distracted by external stimuli”, with GAD being overall more distracted than HC (GAD = 58.34 ± 16.45, HC = 42.17 ± 23.34) and both groups being more distracted by external stimuli before the induction (58 ± 23.45) then after the induction (41.7 ± 27.14).

As regards HR, the GLM revealed main effects of Group, with GAD having higher HR compared to HC (67.35 ± 8.83 vs 61.65 ± 7.63 respectively; \( F_{1,38} = 5.72, p < 0.001 \)) and
Induction, with baseline HR being lower compared to HR after the induction (63.84 ± 9.3 vs 65.37 ± 8.63, respectively; $F_{1,38} = 5.11, p < 0.05$). No Group × Induction interaction effect emerged. As to HRV, a significant main effect of Group emerged ($F_{1,38} = 7.92, p < 0.001$), with GAD having lower HRV (GAD = 43.53 ± 17.99 vs. HC = 76.43 ± 46.14). No effects of Induction or Group × Induction interaction emerged. When we calculated the shift (post - pre) in HRV after the induction, 14 out of 19 GAD patients reported a negative shift (one-tailed sign test $p < 0.04$), whereas only 7 out of 21 HC reported a negative shift (n. s.).

**Effects of group and induction on amygdala connectivity**

When compared to HC, GAD individuals reported lower connectivity between right amygdala and right superior frontal gyrus, right paracingulate/anterior cingulate cortex (ACC), and right supramarginal gyrus (Figure 1).

A Group x Induction interaction was evident within these same areas, where the induction increased their connectivity with right amygdala in GAD, yet decreased the connectivity between the same areas in HC (Table 2, Figure 1). It has to be noted that these results did not survive FWE correction. Nevertheless, given the anatomical overlap with the areas of lower connectivity in GAD compared to HC, we deemed appropriate to describe this trend, as it meaningfully contributes to the interpretation of our data.

No differences between GAD and HC emerged for the post-induction connectivity of the right amygdala and the rest of the brain. No significant connectivity results emerged for the analysis using the left amygdala seed.

**Amygdala connectivity and correlations with trait measures**

As depicted in Figure 2, a negative correlation was observed between the connectivity of the left amygdala with posterior paracingulate gyrus and anterior paracingulate gyrus/frontal
medial cortex and dispositional worry (PSWQ). Similarly, a negative correlation was observed for connectivity of the right amygdala with thalamus and right middle frontal gyrus and the tendency to ruminate after the occurrence of a stressor (SRRS).

**Amygdala connectivity and correlations with state measures**

**Worry.** The baseline connectivity between right amygdala and the paracingulate cortex was positively associated with Δ state worry [post - pre induction] indicating that, in both groups, higher connectivity between right amygdala and the paracingulate cortex predicted higher self-reported levels of worry after the induction (Figure 3).

**Heart Rate Variability.** The baseline connectivity between right amygdala and subcallosal cortex and between left amygdala and left caudate nucleus was negatively correlated with Δ HRV [post – pre] across groups (see Figure 4A), where more negative Δ HRV values indicate a stronger HRV decrease after the induction. This result indicates that a higher degree of connectivity between the amygdala and subcallosal cortex/caudate nucleus predicted a stronger decrease in HRV after the induction.

At the same time, a Δ HRV [post – pre] x Group interaction was evident for the connectivity between bilateral amygdala and PFC/cingulum, driven by a positive correlation in the GAD group and no correlation in the HC group. The positive correlation showed that a higher baseline connectivity between bilateral amygdala and PFC/cingulum predicted less decrease in HRV, acting as a protective factor (Figure 4B).

**DISCUSSION**

We combined rsfMRI techniques with peripheral physiological monitoring to disentangle the interplay between core psychological and physiological expressions of GAD, i.e. excessive
worry and autonomic dysfunction. We drew on pre-existing evidence for the central role of the amygdala in GAD pathology (42), to quantify the relationship between amygdala connectivity and subjective and physiological correlates of worry, particularly following an induction of perseverative cognition.

As in previous studies, at baseline GAD individuals showed lower connectivity between right amygdala and right superior frontal gyrus, right paracingulate/ACC, and right supramarginal gyrus compared to controls, supporting the hypothesis that disruption within the amygdala-PFC and amygdala-paracingulate networks (43-45) underlies the core features of GAD. Lower baseline connectivity in GAD may reflect failure to recruit PFC in the regulation of an anxiety state, leading to increased amygdala activity and difficulties in emotion regulation (4), as also supported by the chronically lower baseline HRV found in our pathological group and reported by others (17).

It has to be noted that current findings implicate more superior regions of the right frontal lobe compared to the decreased connectivity between ventrolateral PFC and amygdala associated with greater anxiety found in previous studies and consistent with the inhibitory function of ventrolateral PFC. However, this is not an unexpected result if we consider that the superior frontal gyrus is involved in cognitive processes and effortful regulation of affect and its activity has been found to be negatively correlated with that of amygdala (46).

Following the induction, although only at a trend-level, the connectivity between the same areas and the right amygdala increased in GAD, yet decreased in HC. This is in line with a dimensional perspective of anxiety disorders, which led us to predict that the perseverative cognition induction would bring connectivity within the control group closer to that observed in the GAD patient group. Thus, controls showed a tendency to respond to the perseverative cognition induction becoming “neurally” more similar to the usual state of GAD patients. At rest, healthy controls habitually perceive the environment as safe, and their PFC exerts tonic
inhibitory control on the amygdala and sympathoexcitatory neural circuits (24, 25), as reflected in their functional integration between these neural structures. In these individuals, the perseverative cognition caused by the induction acts as a threat response that temporarily takes the regulatory role of PFC cortex “off-line” disinhibiting these circuits and being characterized by a reduction in amygdala-PFC connectivity.

On the other hand, the post-induction amygdala-PFC coupling in GAD patients may reflect the habitual engagement in cognitive strategies with the aim to regulate excessive arousal. According to the most prominent psychological model of worry, Borkovec’s avoidance theory (13), GAD patients use worry as a maladaptive cognitive avoidance strategy in an attempt to ‘keep under control’ physiological arousal associated with anxiety. Our results fit well with Borkovec’s model as only in GAD, increased connectivity between bilateral amygdala and PFC/cingulum presumably reflecting a stronger engagement of the PFC as consistently suggested by brain activation studies on this topic (47), was associated with the attenuation of dysregulated autonomic arousal, confirming that worry may be an effective coping strategy to suppress physiological arousal in this clinical population. This finding provides additional insight into how this maladaptive strategy is maintained in this psychopathological disorder. Nevertheless with time, worry becomes dysfunctional in GAD, ultimately recalibrating the effective PFC control over structures including amygdala (diminished functional connectivity), and downgrading autonomic regulation (decreasing tonic HRV). The present data suggests the potential utility of therapeutic interventions aimed at enhancing connectivity in high arousal states and to reducing connectivity in low arousal states. However, future studies are needed to consolidate the role of amygdala-PFC connectivity as a predictive or modifiable biomarker in GAD.

Our results of a reduced connectivity at baseline but enhanced connectivity during worry may help explaining inconsistent results on amygdala-PFC coupling found in anxiety (48).
Moreover, present findings partially support a recently proposed view of anxiety symptoms as subserved by different neural mechanisms such as reduced connectivity within a DLPFC-thalamo-striatal network associated with trait anxiety and increased DLPFC functional connectivity with default mode regions associated with worry (49).

Our results also suggest a functional lateralization of amygdala, with the functional connectivity between right amygdala and prefrontal cortex being preferentially involved in anxiety and state worry lending support to the idea of different roles for the left and right amygdala in emotional processing (50).

The only previous work that examined the effects of a worry induction on GAD studied elderly participants, using insula as a seed region. Keeping in mind these differences, that study also observed stronger connectivity between insula and orbitofrontal cortex in GAD participants during a worry induction, compared to reappraisal (19).

Coherently with our group difference and in line with a dimensional view of psychopathology, trait measures of stress-reactive rumination and worry were negatively associated with baseline connectivity between the amygdala and areas of the frontal and cingulate cortex, again presumably reflecting efforts to suppress arousal.

Consistent with the effects of induction in increasing functional amygdala-PFC connectivity in GAD, baseline connectivity between right amygdala and paracingulate cortex predicted the post induction shift in state worry, with higher connectivity being associated with stronger increases in worry after induction. Such association fits well also with data on patients with generalized social phobia displaying stronger connectivity between amygdala and dorsomedial PFC than controls during self-referential criticism (51). Similarly, more neurotic individual shows greater connectivity between right amygdala and right dmPFC when processing angry and fearful compared to neutral faces (52).
Intriguingly, alteration in the connectivity of the amygdala with paracingulate cortex resulted as a significant group effect, changed from pre- to post-induction, was associated with trait and state measures of perseverative cognition, and was implicated in the delta HRV x Group interaction, suggesting overlapping neural mechanisms for the expression of (dispositional and state) worry and the accompanying autonomic dysregulation in GAD.

When the examined network did not involve the PFC but encompassed connectivity of the amygdala with limbic and subcortical structures, a stronger coupling between these areas predicted a stronger decrease in HRV after the induction, indicating a threat mode response. The caudate nucleus is implicated in HRV regulation in both patients with social anxiety disorder (53) and healthy subjects (54, 55). Moreover, interactions between the striatum and amygdala are of particular interest in the context of reward processing (56), and the caudate nucleus is implicated in the processing of threatening face stimuli (57). Interestingly, a positive correlation has been reported between the activation of the caudate nucleus and HRV in patients with social anxiety disorder, while a negative correlation is observed in healthy individuals (58). Our results provide further novel insight into striatolimbic interaction relevant to perseverative cognition. Such interactions are reminiscent of observations in patients with obsessive-compulsive disorder, in whom caudate nucleus is implicated in the expression of repetitive obsessions evoked by contamination fears (59, 60).

Overall, our data provides important new insight into neural mechanisms through which emotional regulation and autonomic dysfunction interact in GAD. The study has a broader relevance also, shedding light into potentially opponent processes that contribute to the relationship between anxiety disorders and cardiovascular risk (a still unresolved debate; 61). Present findings suggest that the aberrant engagement of amygdala-PFC circuitry might be one of the key factors underlying the pathophysiology of GAD. Thus, resting state connectivity
could potentially be used as a biomarker of treatment response if the robustness of this prediction is confirmed in future research.
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Financial Disclosures

The authors report no biomedical financial interests or potential conflicts of interest.
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Tables

**Table 1.** Socio-demographic, lifestyle, and baseline differences between Generalized Anxiety Disorder (GAD) and Healthy Controls (HC).

<table>
<thead>
<tr>
<th></th>
<th>GAD (n = 19)</th>
<th>HC (n = 21)</th>
<th>p</th>
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<td>29.58 (± 6.93)</td>
<td>28.67 (± 9.45)</td>
<td>0.72</td>
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<tr>
<td>Gender (M/F)</td>
<td>2/17</td>
<td>3/18</td>
<td>0.72</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.32 (± 0.08)</td>
<td>0.30 (± 0.09)</td>
<td>0.51</td>
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<tr>
<td>Education (years)</td>
<td>13.10 (± 1.82)</td>
<td>12.14 (± 2.57)</td>
<td>0.18</td>
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<tr>
<td>Disease duration (years)</td>
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<td>§</td>
<td></td>
</tr>
<tr>
<td>Smoking status*</td>
<td>6Y, 13N</td>
<td>5Y, 16N</td>
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<tr>
<td>Cigarettes per day</td>
<td>1.39 (± 2.80)</td>
<td>0.95 (± 2.40)</td>
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<td>4.44 (± 3.59)</td>
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<td>Coffee/other caffeinated</td>
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<td>2.14 (± 1.85)</td>
<td>0.82</td>
</tr>
<tr>
<td>Perceived physical fitness</td>
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<td>11H, 9M, 1L</td>
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<td>RRS</td>
<td>52.84 (± 11.81)</td>
<td>37.48 (± 11.93)</td>
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<td>PSWQ</td>
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<td>41.81 (± 7.34)</td>
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<td>SRRS</td>
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<tr>
<td>Negative inferential style</td>
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<td>310.00 (± 143.32)</td>
<td>&lt; 0.0005</td>
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<tr>
<td>Hopelessness</td>
<td>199.47 (± 114.48)</td>
<td>94.76 (± 97.76)</td>
<td>0.004</td>
</tr>
<tr>
<td>Problem Solving</td>
<td>314.74 (± 88.78)</td>
<td>355.72 (± 109.39)</td>
<td>0.20</td>
</tr>
<tr>
<td>Total score</td>
<td>1378.95 (± 253.99)</td>
<td>092.86 (± 238.18)</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Baseline HR (bpm)</td>
<td>67.35 (± 8.41)</td>
<td>60.67 (± 9.10)</td>
<td>0.01</td>
</tr>
<tr>
<td>Baseline RMSSD (ms²)</td>
<td>47.29 (± 17.47)</td>
<td>77.93 (± 42.80)</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>VAS rating (pre induction)</td>
<td>1378.95 (± 253.99)</td>
<td>092.86 (± 238.18)</td>
<td></td>
</tr>
<tr>
<td>Rumination/Worry</td>
<td>40.84 (± 24.71)</td>
<td>26.38 (± 26.09)</td>
<td>0.08</td>
</tr>
<tr>
<td>Distraction</td>
<td>68.42 (± 17.60)</td>
<td>48.57 (± 24.42)</td>
<td>0.01</td>
</tr>
<tr>
<td>Distraction by internal thoughts</td>
<td>73.94 (± 19.75)</td>
<td>69.05 (± 18.89)</td>
<td>0.43</td>
</tr>
</tbody>
</table>
Note. $^\S$ Assessed by the question: “At what age did anxiety symptoms first appeared?”; * Y = YES, N = NO; $^\#$ H = High, M = Medium, L = Low.
Table 2. Brain areas showing significant connectivity alteration in Generalized Anxiety Disorder (GAD) versus Healthy Controls (HC), or correlation with behavioural and autonomic measures.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Cluster</th>
<th>Voxel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Side</td>
<td>k</td>
</tr>
<tr>
<td><strong>(1) GAD &lt; HC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right amygdala seed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal pole</td>
<td>R</td>
<td>374</td>
</tr>
<tr>
<td>Superior frontal gyrus</td>
<td>R</td>
<td>1330</td>
</tr>
<tr>
<td>Paracingulate/ACC</td>
<td>R</td>
<td>323</td>
</tr>
<tr>
<td><strong>(2) Group x Induction Interaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right amygdala seed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paracingulate/Superior frontal gyrus</td>
<td>R</td>
<td>332</td>
</tr>
<tr>
<td>Middle frontal gyrus</td>
<td>R</td>
<td>347</td>
</tr>
<tr>
<td><strong>(3) Negative correlation with SRRS and PSWQ scores</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right amygdala seed - SRRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle frontal gyrus</td>
<td>R</td>
<td>270</td>
</tr>
<tr>
<td>Thalamus</td>
<td>R</td>
<td>250</td>
</tr>
<tr>
<td>Left amygdala seed - PSWQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior cingulate cortex</td>
<td>R</td>
<td>276</td>
</tr>
<tr>
<td>Paracingulate cortex/medial frontal gyrus</td>
<td>R</td>
<td>482</td>
</tr>
<tr>
<td><strong>(4) Positive correlation with ΔWorry [post – pre] across both groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right amygdala seed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paracingulate gyrus</td>
<td>R</td>
<td>262</td>
</tr>
<tr>
<td><strong>(5) Negative correlation with ΔHRV [post – pre] across both groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right amygdala seed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcallosal cortex/ACC</td>
<td>L</td>
<td>619</td>
</tr>
<tr>
<td>Paracingulate gyrus/Frontal medial cortex</td>
<td>L</td>
<td>3.58</td>
</tr>
<tr>
<td>Left amygdala seed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudate/Accumbens</td>
<td>L</td>
<td>604</td>
</tr>
<tr>
<td>Frontal orbital cortex</td>
<td>L</td>
<td>3.88</td>
</tr>
<tr>
<td><strong>(6) Δ HRV [post – pre] x group interaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right amygdala seed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal orbital cortex</td>
<td>R</td>
<td>339</td>
</tr>
<tr>
<td>Superior frontal gyrus</td>
<td>R</td>
<td>773</td>
</tr>
<tr>
<td>Frontal pole</td>
<td>L</td>
<td>3.85</td>
</tr>
<tr>
<td>Cerebellum: Vermis</td>
<td>L</td>
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</tr>
<tr>
<td>ACC</td>
<td>R</td>
<td>937</td>
</tr>
<tr>
<td>Left amygdala seed</td>
<td></td>
<td></td>
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<tr>
<td>Middle frontal gyrus</td>
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<tr>
<td>Frontal pole</td>
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<tr>
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<td>3.48</td>
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<tr>
<td>Lateral occipital cortex</td>
<td>R</td>
<td>431</td>
</tr>
<tr>
<td>Supramarginal gyrus</td>
<td>R</td>
<td>5.07</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1.** Regions showing lower connectivity between the right amygdala and superior frontal gyrus, right paracingulate/ACC, and right supramarginal gyrus (red areas) in Generalized Anxiety Disorder (GAD) compared to Healthy Controls (HC). A Group x Induction interaction was evident in the same areas, due to an increase of the connectivity in GAD and a decrease of connectivity in HC (yellow areas) after the induction.

**Figure 2.** Correlations between dispositional measures of stress reactive rumination (SRRS) and worry (PSWQ) and functional connectivity of the left and right amygdala with the cortex.

**Figure 3.** Positive correlation between state worry change score [$\Delta = \text{post} - \text{pre}$] and connectivity of the right amygdala and paracingulate cortex across groups.

**Figure 4.** A) Negative correlation between $\Delta$HRV [post – pre] and connectivity of the right amygdala and subcallosal cortex (red areas) and of the left amygdala and left caudate nucleus (blue areas). B) $\Delta$HRV [post – pre] x Group interaction driven by a positive correlation between $\Delta$HRV [post – pre] and connectivity of bilateral amygdala and bilateral middle frontal gyrus (MFG) and frontal orbital cortex.
A) Negative correlation with $\Delta$ HRV [post – pre]

$\Delta$ HRV [post – pre] x group interaction

B) $\Delta$ HRV [post – pre] x group interaction