Amygdala functional connectivity as a longitudinal biomarker of symptom changes in generalized anxiety

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Abstract

Generalized anxiety disorder (GAD) is characterized by excessive worry, autonomic dysregulation and functional amygdala dysconnectivity, yet these illness markers have rarely been considered together, nor their interrelationship tested longitudinally. We hypothesized that an individual's capacity for emotion regulation predicts longer-term changes in amygdala functional connectivity, supporting the modification of GAD core symptoms. Sixteen patients with GAD (14 women) and individually matched controls were studied at two time points separated by 1 year. Resting-state fMRI data and concurrent measurement of vagally mediated heart rate variability were obtained before and after the induction of perseverative cognition. A greater rise in levels of worry following the induction predicted a stronger reduction in connectivity between right amygdala and ventromedial prefrontal cortex, and enhanced coupling between left amygdala and ventral tegmental area at follow-up. Similarly, amplified physiological responses to the induction predicted increased connectivity between right amygdala and thalamus. Longitudinal shifts in a distinct set of functional connectivity scores were associated with concomitant changes in GAD symptomatology over the course of the year. Results highlight the prognostic value of indices of emotional dysregulation and emphasize the integral role of the amygdala as a critical hub in functional neural circuitry underlying the progression of GAD symptomatology.

Key words: generalized anxiety disorder; longitudinal; heart rate variability; amygdala functional connectivity; perseverative cognition; emotion regulation

Introduction

Generalized anxiety disorder (GAD) is a chronic condition characterized by excessive and uncontrollable worry, alongside symptoms of autonomic dysregulation, restlessness and muscle tension (American Psychiatric Association).

The "emotion dysregulation model" (Behar *et al.*, 2009) explains GAD symptoms as persistent emotional hyperarousal

(Mennin *et al.*, 2005). Patients with GAD implement maladaptive emotion regulation strategies, making unsuccessful attempts to either minimize or over-control emotions. Within this scenario, worry plays a fundamental role as an ineffective strategy to cope with and manage emotions (Behar *et al.*, 2009).

A consequence of this persistent emotional hyperarousal is autonomic dysregulation, which is often observed in GAD (Chalmers *et al.*, 2014 for a meta-analysis) as a reduction in heart

rate variability (HRV). HRV indexes the parasympathetic regulation of the heart via the vagus nerve and reflects the degree to which cardiac activity can be modulated to meet changing behavioural and emotional demands (Thayer and Lane, 2000). Tonic HRV is closely linked to individual differences in emotional responsivity and capacity for self-regulation (Thayer *et al.*, 2009). Importantly, symptoms of worry and rumination, encapsulated within the transdiagnostic concept of perseverative cognition (Brosschot *et al.*, 2006) are associated with chronic (e.g., Brosschot *et al.*, 2007; Pieper *et al.*, 2010; Ottaviani *et al.*, 2015a, b) and phasic (e.g. Ottaviani *et al.*, 2009, 2014; Ottaviani and Shapiro, 2011) reductions in HRV in patients with GAD (Chalmers *et al.*, 2014) and in healthy individuals (Ottaviani *et al.*, 2016). To date, despite this intrinsic relationship between worry and autonomic dysregulation as core features of GAD, only one previous study tests for a common underlying neurobiology for these primary symptoms (Makovac *et al.*, 2016). This study combined neuroimaging (resting-state functional magnetic resonance imaging; rsfMRI) with peripheral physiological monitoring to endorse the hypothesis that disrupted functional connectivity across amygdala-prefrontal networks underlies core features of GAD. Shared neural correlates of (dispositional and state) worry and accompanying autonomic dysregulation, further points to a common mechanism underpinning these affective and physiological manifestations of GAD (Makovac *et al.*, 2016).

Other published studies that characterize the neurobiology of GAD mainly focus on the role of the amygdala (Etkin *et al.*, 2009), consistent with a large body of evidence implicating this brain centre in fear and threat processing (LeDoux, 2003) and also in autonomic regulation (LeDoux, 2000; Critchley *et al.*, 2002; Makovac *et al.*, 2016). In GAD, aberrant connectivity of the amygdala is observed with prefrontal (Monk *et al.*, 2008; Etkin *et al.*, 2009), insula and anterior cingulate cortices (ACC) (Etkin *et al.*, 2010; Blair *et al.*, 2012). Taken together, this literature suggests amygdala functional dysconnectivity as a promising biomarker for GAD.

Deficits in emotion regulation represent a phenotypic correlate of anxiety disorders (Amstadter, 2008) that helps explain the maintenance of both autonomic dysregulation and excessive worry in GAD. Given the likely contributing role of the amygdala, we first hypothesized that indices of emotion regulation capacity would predict changes in amygdala functional connectivity over time. In line with a dimensional view of psychopathology, we expected these effects to be present in both GAD and (to a lesser extent) in healthy individuals. To date, few longitudinal studies have been conducted on brain functional connectivity. Our hypothesis is supported by the observation in children that amygdala connectivity longitudinally predicts anxiety symptoms and emotion regulation skills (Pagliaccio *et al.*, 2015). In this study, we measured the cognitive (i.e. amount of worrisome and ruminative thoughts) and autonomic responses to a perseverative cognition induction as indices of the capacity for emotion regulation in patients with GAD and in healthy participants. The rationale for considering both perseverative cognition and autonomic dysregulation as indices of emotion regulation capacity originates from the first being recognized as a maladaptive emotion regulation strategy, particularly in GAD (Aldao *et al.*, 2010 for a review) and the second being associated with emotion regulation capacity for the reason that vagally mediated HRV represents an index of inhibitory control (Williams *et al.*, 2015). We tested whether these baseline responses to worry induction carried prognostic value in

predicting long-term changes in amygdala connectivity with cortical and subcortical centres apparent 1 year later.

The second hypothesis of this study was that functional connectivity changes would reflect alterations in anxiety symptomatology (levels of anxiety, worry and autonomic dysregulation). This hypothesis was driven by an established finding; that amygdala functional connectivity can predict shifts between the manic and depressed phases of bipolar disorder (Cerullo *et al.*, 2012). Moreover, the degree of amygdala connectivity predicts medication response and clinical improvement in paediatric bipolar disorder regardless of medication type (Wegbreit *et al.*, 2011), further supporting this as treatment-relevant biomarker of symptom expression.

The length of the follow-up has been chosen for specific reasons: (i) it is the same time-lag examined by the few existing longitudinal studies conducted on amygdala functional connectivity, based on which we formulated our hypotheses (Pagliaccio *et al.*, 2015; Cerullo *et al.*, 2012); (ii) it is usually considered as an adequate time frame to evaluate the test–retest reliability of questionnaires assessing anxiety symptoms (e.g. Knappe *et al.*, 2014) and (iii) it is a relatively standard time interval to monitor symptom changes after treatment in GAD (e.g. Bradford *et al.*, 2011).

To our knowledge, ours is the first longitudinal study to test the effect of emotion regulation capacity on functional changes in neural connectivity, combining rsfMRI with simultaneous autonomic monitoring and symptom rating to detail the role of amygdala connectivity as a neurofunctional marker of the progression of anxiety symptoms.

Methods

Participants

At the beginning (time 0), 40 individuals were recruited by public advertisement to take part in the study, which was approved by the National Research Ethics Service (NRES) for the UK National Health Service (NHS) with university sponsorship granted via the Brighton and Sussex Medical School Research Governance and Ethics Committee. After 1-year (time 1), eight participants had dropped-out of follow up (three GAD and five healthy controls; HC). Thus, the final sample undergoing both assessments encompassed 16 patients (14 women; mean age = 29.62 ± 7.51 years) who met diagnostic criteria for GAD and 16 HC (13 women; mean age = 28.12 ± 10.11 years). No significant differences emerged between participants who dropped out and those who did not in terms of gender ($\chi^2 = 0.14$; $P = 0.71$), BMI ($t = 0.09$; $P = 0.93$), years of education ($t = 1.51$; $P = 0.17$), physical fitness ($t = 2.00$; $P = 0.09$) and nicotine ($t = 0.43$; $P = 0.68$) and alcohol ($t = 0.76$; $P = 0.47$) consumption. However, participants who dropped-out were significantly older than those who did not ($t = 2.80$; $P = 0.03$).

All participants were right-handed, native English speakers and had normal or corrected-to-normal vision. Exclusion criteria were: age younger than 18 years, prior history of head injury, major medical neurological or psychiatric disorder (other than GAD for the patient group), cognitive impairment, history of substance or alcohol abuse or dependence, diagnosis of heart disease, obesity (body mass index > 30 kg/m^2), pregnancy, claustrophobia or other general MRI exclusions. None of our participants had a formal diagnosis of co-morbid major depressive disorder (see Makovac *et al.*, 2016 for further details). Two GAD participants were included who used long-term

medications (one citalopram, one pregabalin) at both sessions of the study. All other patients and controls were medication free.

Between the two fMRI scans, six participants (three HC and three GAD) reported to have started psychotherapy or other interventions that might have influenced their wellbeing. Among HC, one participant started counselling and two participants started mindfulness-based yoga. Among GAD, two participants started cognitive-behavioural therapy and one started mindfulness-based yoga. No significant differences emerged between participants who started such interventions and those who did not in terms of anxiety ($t = 0.11$; $P = 0.68$) and HRV ($t = 0.17$; $P = 0.74$). However, participants who started those interventions had significantly higher increases in levels of worry from time 0 to time 1 compared with those who did not ($t = 6.84$; $P = 0.01$).

All participants provided written informed consent.

Procedure

At time 0, the Structured Clinical Interview for DSM-IV (SCID) was first administered to both patient and controls to confirm/ exclude the diagnosis of GAD. Participants then completed a series of online sociodemographic and dispositional traits questionnaires. Participants were subsequently familiarized with the neuroimaging environment, connected to the physiological recording equipment and underwent the MRI protocol. Participants went through the same procedure about 1 year after the first scan (average time between sessions = 10.5 ± 2.2 months).

Questionnaires

At time 0, all participants completed a set of questions accessing sociodemographic, and lifestyle (nicotine, alcohol and caffeine consumption, physical activity) information. Core symptoms of GAD (i.e. levels of anxiety and worry) were assessed at both time points by the State-Trait Anxiety Inventory (STAI; Spielberger, 1983) and the Penn State Worry Questionnaire (PSWQ; Meyer *et al.*, 1990).

FMRI design

At time 0, participants underwent a series of four 5-min resting-state periods, each followed by a 6-min easy visuomotor tracking task. During the 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. At the end of each resting-state period, participants rated their thoughts over the preceding period using visuo-analogue scales (VAS). At time 1, participants underwent one 5-min block of resting-state acquisition, followed by three blocks of visuomotor tracking task, without any induction procedure. Data obtained during the task blocks at time 0 and time 1, go beyond the scope of this study and will not be presented here.

Perseverative cognition induction

"Next I would like you to recall an episode that happened in the past year that made you feel sad, anxious, or stressed or something that may happen in the future that worries you. Then, I would like you to think about this episode in detail, for example about its possible causes, consequences, and your feelings about it. Please keep thinking about this until the end of

the next tracking task. Thank you. Please take as much time as you need to recall the episode and press the button whenever you are ready".

Visual analogue scales (VAS)

At time 0, levels of perseverative cognition occurring prior to and following the induction were assessed. Participants were asked to rate on a visual analogue 100-point scale: "how much, for the duration of the previous resting period, they were distracted by ruminative or worrisome thoughts" (see Makovac *et al.*, 2016 for a full description of the VAS administered at time 0).

Physiological data processing

At both time points, HR 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 values were visually inspected and potential artefacts 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 (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). 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. At time 0, attention was given to measures before (pre) and after (post) the perseverative cognition induction. For correlational analyses, we calculated the shift in RMSSD from pre- to post-induction at time 0 (ΔHRV [post-induction− pre-induction]) and the shift in resting RMSSD from time 0 (i.e. pre-induction) to Time 1 (ΔHRV [time 1−time 0]).

MRI acquisition and pre-processing

MRI images were acquired on a 1.5-Tesla Siemens Magnetom Avanto scanner (Siemens AG, Munich, Germany). Functional datasets used T2*weighted echoplanar imaging (EPI) sensitive to Blood oxygenation level dependent (BOLD) signal (TR = 2.52 s, TE = 43 ms, flip-angle 90°, 34 slices, 3-mm slice thickness, 192 mm FOV, voxel size $3 \times 3 \times 3$ 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, MA, USA). For each participant, the first four volumes of the fMRI series were discarded to allow for T1 equilibration effects. The pre-processing steps included correction for head motion, compensation for slice-dependent time shifts, normalization to the EPI template in standard space (MNI) coordinates provided with SPM8, and, to better account for inter-subject variability, smoothing with a3D Gaussian Kernel with 8-mm³ full-width at half maximum. The global temporal drift was removed using a third-order polynomial fit. To remove other potential sources of bias, data were further filtered regressing against the realignment parameters, and the mean white matter and cerebrospinal fluid signal averaged over all voxels. Then, all images were filtered by a phase-insensitive band-pass filter (pass band 0.01–0.08 Hz) to reduce the effect of low frequency drift and high-frequency physiological noise.

As global signal removal can potentially change functional connectivity distributions and result in increased negative correlations (Saad *et al.*, 2012), it was avoided in our pre-processing.

Statistical analyses

Questionnaire, behavioural and HRV analyses

All data are expressed as means ($\pm$SD). Differences at $P \leq 0.05$ are regarded as significant. Data analysis was performed with SPSS 22.0 for Windows (SPSS Inc, USA).

Baseline differences between groups and the effectiveness of the induction in enhancing levels of perseverative cognition were tested elsewhere and will not be repeated here (Makovac *et al.*, 2016). Here, the post- minus pre-induction change scores of (i) levels of perseverative cognition (VAS: *worrying or ruminating?*) and (ii) HRV measures have been calculated and adopted in subsequent correlational analysis.

Seed-based fMRI analysis

Anatomical ROIs were constructed using an anatomical toolbox in SPM8 (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) for bilateral amygdala. The average resting-state fMRI time-series over the ROIs were extracted for each participant and for each scan at time 0 (pre-induction resting-state block) and time 1.

The difference in amygdala connectivity between time 1 and time 0 was estimated for each subject in a first level SPM analysis by regressing the fMRI data against the corresponding ROI time series, and extracting the [time 0 < time 1] contrast. The pre-induction resting-state block at time 0 and the only resting state block gathered at time 1 have been used for the analysis of resting state functional connectivity changes in the amygdala.

To test for associations between amygdala connectivity and cognitive and autonomic measures of emotion regulation, a *t*-test was run at second level on these contrast images, with Group (GAD *vs* HC) as the factor and ΔVAS and ΔHRV as covariates of interest. To determine significance within a priori regions (functional connectivity) for which we had strong hypotheses, we restricted our analyses to these regions using an anatomically derived (AAL atlas) partial brain mask of entire prefrontal cortex/frontal lobe. Statistical threshold was set to $P < 0.05$—FWE-corrected at cluster level (cluster size defined using uncorrected voxel-level threshold $P < 0.005$). For completeness, results outside our ROI which might be highly informative of the GAD condition are also reported.

For illustrative purposes, parameter estimates for regions that showed significant correlations with the cognitive and autonomic measures of emotion regulation were extracted and plotted.

Table 1. Means and correlations between the key variables of the study at time 0 and time 1 in GAD patients and healthy participants

	Mean $\pm$ SD	1	2	3	4	5	6
1. STAI T0	45.09 $\pm$ 13.10	1	0.88**	0.00	0.87**	0.79**	0.02
2. PSWQ T0	54.59 $\pm$ 17.27		1	−0.11	0.75**	0.86**	−0.52
3. HRV T0	65.17 $\pm$ 43.77			1	−0.15	−0.23	0.53**
4. STAI T1	44.44 $\pm$ 12.36				1	0.79**	0.14
5. PSWQ T1	53.25 $\pm$ 17.74					1	0.14
6. HRV T1	61.63 $\pm$ 34.19						1

STAI, State Trait Anxiety Inventory; PSWQ, Penn State Worry Questionnaire; HRV, Heart Rate Variability; T0, Time 0; T1, Time 1; ** = $P < 0.01$.

Results

Neither at time 0 (see Makovac *et al.*, 2016) nor at time 1 (see Table S1 in the Supplementary Results) group differences emerged for any of the assessed sociodemographic and lifestyle variables; therefore, these were not included as covariates in the subsequent analyses. Table 1 shows the correlations between the main variables of the study at the two time points.

Cognitive responses to the induction of perseverative cognition at time 0 predict changes in functional connectivity from time 0 to time 1

Changes in state levels of perseverative cognition (VAS) after the induction predicted longitudinal alterations in functional connectivity from time 0 to time 1 across both groups of GAD and HC. As illustrated in Table 2$_{(1)}$, two significant associations emerged:

1. A negative correlation between pre- to post-induction shift in state levels of perseverative cognition (post-induction− pre-induction) and the change in functional connectivity [time 1−time 0] between right amygdala and ventromedial prefrontal cortex (vmPFC) ($r = -0.60$, $P < 0.001$). This finding indicates that individuals who reported a stronger increase in levels of ruminative and worrisome thoughts after the induction at time 0 exhibited the strongest reduction in connectivity between these two regions after 1 year. The correlation was driven by a strong negative correlation in the group of GAD compared to HC (Figure 1A, left panel).

2. A positive correlation between pre- to post-induction changes in state levels of perseverative cognition and longitudinal changes in functional connectivity between left amygdala and ventral tegmental area (VTA) across both groups ($r = 0.51$, $P < 0.01$). This observation indicated that individuals with stronger increases in levels of perseverative cognition after the induction were characterized by increased connectivity between these two regions at follow-up (Figure 1A, right panel).

Physiological (HRV) responses to the induction of perseverative cognition at time 0 predict changes in functional connectivity from time 0 to time 1

A positive correlation was observed between the pre- to post-induction shift in HRV [post–pre] values and longitudinal changes in functional connectivity [time 1–time 0] between right amygdala and the temporal region of the thalamus ($r = 0.56$, $P < 0.001$). This observation indicates that individuals with the strongest decrease in HRV after the induction at time 0 showed the greatest loss in connectivity between right amygdala and thalamus at 1-year follow-up (Table 2$_{(2)}$, Figure 1B).

Changes in functional connectivity from time 0 to time 1 reflect changes in GAD symptomatology (anxiety, worry and autonomic dysfunction)

As to the core symptom of anxiety, a positive correlation emerged across both groups between the change in STAI score over time (ΔSTAI [time 1–time 0]) and the shift in connectivity [time 1–time 0] between right amygdala and dorso-lateral PFC (dlPFC) ($r = 0.61$, $P < 0.001$). This shows that individuals who reported an increase in trait anxiety after 1 year also manifested an increase in functional connectivity between these brain areas. A negative correlation was observed between the shift in

Table 2. Brain areas showing significant correlations between amygdala connectivity changes over time and cognitive (1) and autonomic (2) responses to the induction and longitudinal modifications in symptoms of anxiety (3a) and autonomic dysregulation (3b) in generalized anxiety disorder patients and healthy participants

Brain region	Seed	Side	Cluster		Voxel	
			k	P FWE	Z	MNI xyz
(1) ΔPC (post–pre induction) as predictor of FC longitudinal alterations [T1–T0]						
vmPFC	Right amygdala	L	430	0.001	3.94	−12 44 −12
VTA	Left amygdala		250	0.018	3.64	2 −10 −14
(2) Δ HRV (post–pre induction) as predictor of FC longitudinal alterations [T1–T0]						
Thalamus (temporal region)	Right amygdala	L	247	0.021	4.38	−10 −22 18
(3) Correlation between FC longitudinal alterations [T1-T0] and changes in anxiety symptoms						
(3a) Anxiety (ΔSTAI [T1–T0])						
Middle frontal gyrus	Right amygdala	R	438	0.000	4.39	38 10 62
dlPFC	Right amygdala	R	318	0.000	5.04	24 48 8
PCC	Right amygdala	L	292	0.000	3.94	0 −54 28
(3b) HRV (ΔHRV [T1–T0])						
Insula	*Right amygdala*	L	545	0.008	3.85	−38 8 −2
Temporal/angular gyrus	*Right amygdala*	R	224	0.032	3.72	62 −52 12

PC, perseverative cognition; STAI, State Trait Anxiety Inventory; PSWQ, Penn State Worry Questionnaire; HRV, Heart Rate Variability; T0, Time 0; T1, Time 1.
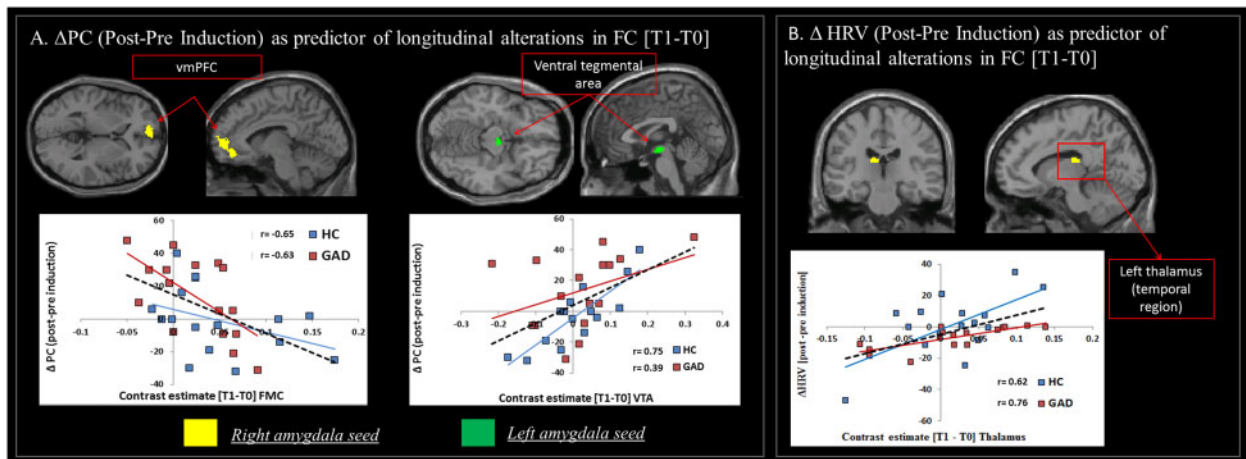

Fig. 1. Cognitive and autonomic responses to the induction of perseverative cognition at time 0 predict changes in functional connectivity from time 0 to time 1. (**A**) Associations between Δ perseverative cognition (ΔPC) (post–pre induction) and the shift in functional connectivity [Time 1–Time 0] between: right amygdala and vmPFC (negative correlation; left panel) and left amygdala and VTA (positive correlation; right panel). (**B**) Positive correlation across groups between ΔHRV (post–pre induction) and the shift in functional connectivity [Time 1–Time 0] between right amygdala and temporal thalamus. *Note.* Correlational plots are for illustrative purposes only. The dashed black line indicates the regression line across both groups; coloured (blue and red) lines indicate the regression line for the each group, separately, and are represented for illustrative purposes only (i.e. to elucidate the influence of each group individual slope on the overall regression).

STAI scores over time and the change in functional connectivity between right amygdala and the posterior cingulate cortex (PCC) ($r=-0.45$, $P<0.01$), indicating greater disconnection between these two regions in individuals reporting increases in anxiety symptoms after 1 year (Table 2$_{(3a)}$, Figure 2A).

Given the overlap, results for the PSWQ are provided in S1, Table S2, and Figure S1 in the Supplementary Results.

With respect to autonomic dysregulation, a group × ΔHRV [time 1–time 0] interaction was significant for functional connectivity alterations [time 1–time 0] between right amygdala and left insula. In patients with GAD, a longitudinal decrease in HRV was associated with an increase in connectivity between right amygdala and left insula ($r=-0.51$, $P<0.05$). The opposite pattern was evident in HC ($r=0.68$, $P<0.01$). Both groups showed a positive correlation between Δ HRV and change in the functional connectivity between right amygdala and right temporal/angular gyrus ($r=0.50$, $P<0.001$) (Table 2$_{(3b)}$, Figure 2B).

Here, HRV reductions from time 0 to time 1 were associated with decreased connectivity between these areas.

Additional analyses

For completeness, additional analyses on baseline levels of subjective and physiological responses to the induction and amygdala functional connectivity (time 0) as potential predictors of symptoms progression after 1 year were performed. In summary, results confirmed the role of HRV (S2, Supplementary Results) and baseline amygdala functional connectivity (S3, Figure S2, Supplementary Results) in predicting changes in levels of anxiety and autonomic dysregulation over the course of the year.
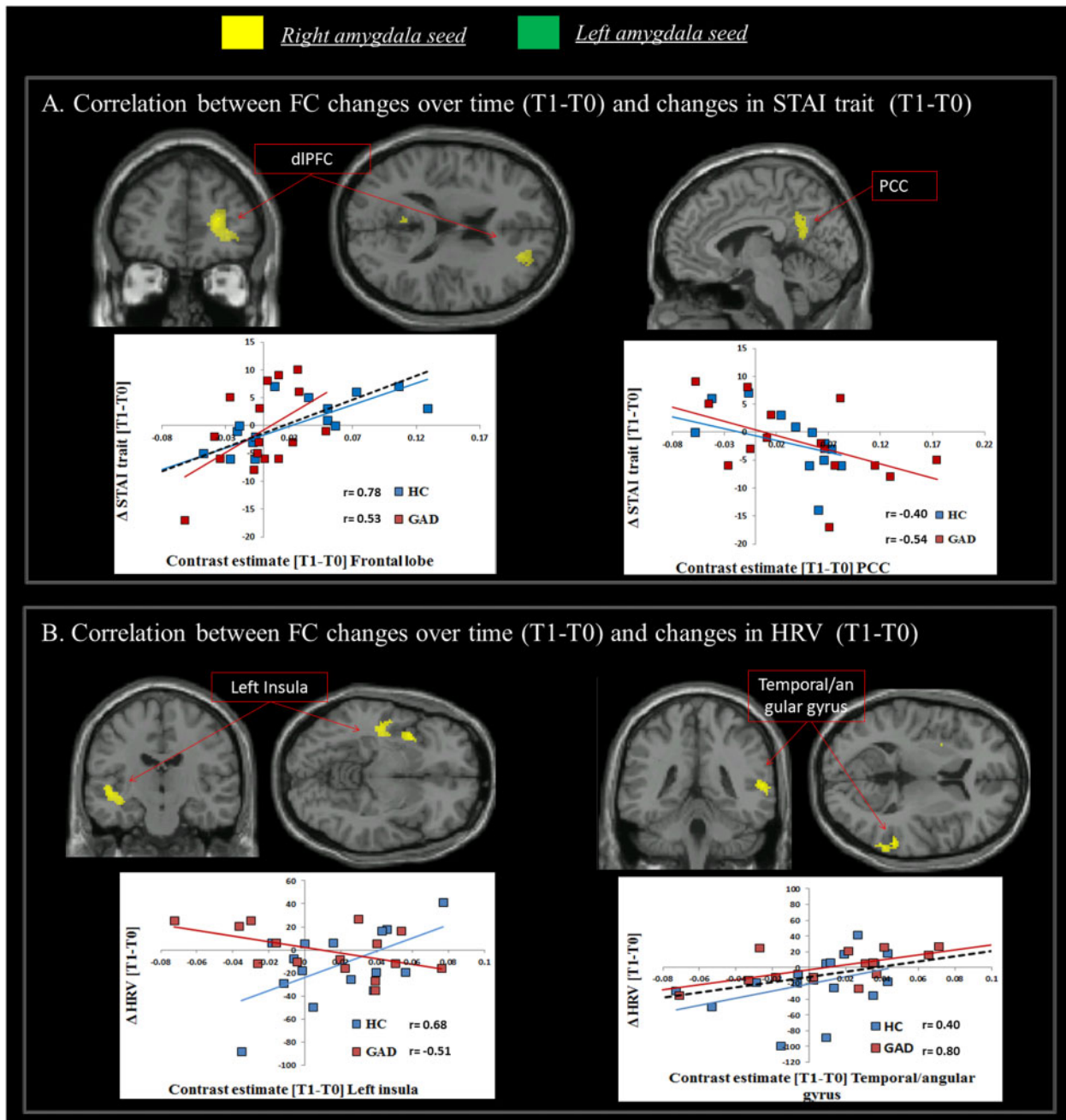
Fig. 2. Changes in functional connectivity from time 0 to time 1 reflect changes in GAD symptomatology (anxiety and autonomic dysfunction). A significant correlation was observed between: **(A)** ΔSTAI [time 1–time 0] and the shift in functional connectivity [time 1–time 0] between right amygdala and dlPFC (positive correlation) and the PCC (negative correlation); **(B)** ΔHRV [time 1–time 0] and the shift in functional connectivity [time 1–time 0] between right amygdala and left insula (significant interaction indicating a positive correlation in the group of HC and a negative correlation in GAD) and right temporal/angular gyrus area (positive correlation across both groups). *Note.* The correlational plots are for illustrative purposes only. The dashed black line indicates the regression line across both groups, coloured (blue and red) lines indicate the regression line for each group, separately, and are represented for illustrative purposes only (i.e. to elucidate the influence of each group individual slope on the overall regression).

Discussion

We combined rsfMRI with cognitive and physiological assessment at two time points, to quantify longitudinally alterations in amygdala functional connectivity with the rest of the brain in GAD. Autonomic and behavioural indices of emotion regulation capacities were acquired at time 0 before and after a perseverative cognition induction, and were tested for their prognostic value on longer-term changes in amygdala connectivity.

Moreover, we tested the hypothesis that changes derived subtracting amygdala functional connectivity at time 0 to that at time 1 correspond to changes in symptomatology (levels of anxiety, worry and autonomic dysregulation).

Our data indicate that the cognitive and autonomic response to an emotion regulation task has a prognostic value on the functional organization of emotion-relevant circuitry within the brain after 1 year. In both patients and controls, subjective increases in the amount of ruminative and worrisome thoughts

experienced after the worry induction predicted a decrease in functional connectivity between right amygdala and vmPFC and an increase in connectivity between left amygdala and the VTA up to 1 year later. Prefrontal cortex hypo-activation during emotion regulation is a recognized feature in GAD (Ball *et al.*, 2013) and earlier rsfMRI studies highlight a perturbation of amygdala-prefrontal functional connectivity underlying the core features of GAD (Hilbert *et al.*, 2014). A decrease in functional connectivity between amygdala and PFC is in fact reported in adults (Etkin *et al.*, 2009) and adolescents with GAD (Monk *et al.*, 2008; Roy *et al.*, 2013). Here, we extend these observations by demonstrating that a heightened reaction to a perseverative cognition induction has a prognostic value in predicting a longitudinal alteration within the amygdala-PFC network, highlighting a potential target for early intervention. Importantly, subregions of PFC are extremely active at rest and contribute to the so-called default mode network (DMN), a system directly implicated in mind-wandering and related self-generated thoughts. Plausibly, whenever mind wandering takes the form of perseverative cognition, prefrontal inhibition is tuned down, permitting an increase in physiological activity (Thayer and Lane, 2009). Given the pervasiveness of worry in GAD, it is suggested that such chronic and persistent physiological activation (Ottaviani *et al.*, 2016) may ultimately lead to somatic health problems (Perseverative Cognition Hypothesis; Brosschot *et al.*, 2006) including the increased cardiovascular risk that characterizes this clinical population (Larsen and Christenfeld, 2009). Therefore, the identification of biobehavioural markers longitudinally associated with alterations within this network is important not only to predict the psychological evolution of anxiety symptoms, but also prevent its negative physical consequences impacting the somatic health of GAD patients.

An increase in worrisome and ruminative thoughts after the induction at time 0 also predicted augmented functional connectivity between left amygdala and the VTA over time. The VTA has distributed connections across forebrain regions involved in emotional and cognitive processes, including hippocampus (Lisman and Grace, 2005), nucleus accumbens, amygdala and PFC (Roeper, 2013). Importantly, the VTA projections release of dopamine, a neurmodulator that shapes both appetitive motivational behaviours and acts potently on mechanisms underlying states of fear and anxiety. Dopaminergic neurotransmission influences each phase of memory for affective information, i.e. formation, expression, retrieval and extinction (Pezze and Feldon, 2004). In mice, the conditioning to a cue that predicts an aversive situation is impaired if the excitatory responses of dopamine neurons are altered, resulting in the development a generalized anxiety-like phenotype. In contrast, dopamine also facilitates contingency awareness during threat-learning, which is critical for the prevention of generalized anxiety (Zweifel *et al.*, 2011). In humans, increased functional connectivity between the VTA and vmPFC is linked to adaptive emotion regulation (Mulej Bratec *et al.*, 2015). Within our own data from people with and without GAD, we observed the longitudinal strengthening of connectivity between VTA and amygdala in participants who showed the most pathological response to the induction, pointing to a maladaptive neurobiological restructuring linked to impaired emotion regulation.

Generalized anxiety is not only characterized by repetitive worry but also by impaired autonomic regulation (Chalmers *et al.*, 2014) and these two core symptoms are likely to be strictly intercorrelated. Indeed, chronic reductions in resting-state HRV are associated with worry (Thayer *et al.*, 1996). Consistently, we previously reported a greater drop in HRV after perseverative cognition induction at time 0 in GAD patients compared to HC, that was mediated by lower baseline connectivity between bilateral amygdala and PFC/cingulum in GAD (Etkin *et al.*, 2009; Makovac *et al.*, 2016). In this study, we extended these observations by demonstrating that participants with least physiological capacity for emotional regulation (i.e. greatest drop in HRV after the induction at time 0) went on to manifest a functional decoupling of right amygdala with the thalamus over ensuing months. During states of high HRV (increased parasympathetic tone), the thalamus is more strongly coupled to amygdala (along with brainstem, putamen, ACC and dorsolateral prefrontal cortex; Chang *et al.*, 2013). Our data show that such "adaptive" communication between amygdala and thalamus might decrease over time in individuals who exhibit a dysfunctional physiological threat response (exaggerated parasympathetic withdrawal) during perseverative cognition. It is noteworthy that a GABAergic pathway from the amygdala to thalamus projects directly onto orbital cortex, forming a tight tripartite system that shapes the formation of emotions, and tempers the impact of direct amygdala-orbital projections to influence the neuronal representations through which emotions bias affective reasoning (Timbie and Barbas, 2015). Correspondingly, HRV represents a potential biomarker of progressive functional alterations between structures involved in both autonomic responses and anxiety symptoms in GAD. Recent studies already suggest that HRV can be efficient in predicting the risk of developing post-traumatic stress disorder (Shah and Vaccarino, 2015).

Our demonstration that baseline measures of emotional regulation capacity determine, over time, changes in amygdala-centred organization of functional networks across brain carries more importance as such changes are linked to anxiety symptomatology. Across groups, individuals who reported the greatest increase in levels of trait (i.e. *how they generally feel*) anxiety and worry up to 1 year later, also showed the deepest decrease in functional connectivity between right amygdala and PCC (a major DMN 'hub'). The functional connection between the PCC and the amygdala is recognized (Stein *et al.*, 2007; Roy *et al.*, 2009), where intrinsic activity within PCC is altered in anxiety disorders (Sylvester *et al.*, 2012; Andreescu *et al.*, 2011) and linked to self-referential thoughts and worry in anxiety (Paulesu *et al.*, 2010).

Contrary to our initial expectation, increases in trait anxiety over the year were associated with augmented amygdala connectivity with dlPFC. However, this result may reflect the differential contributions of the medial and lateral prefrontal cortices in fear and anxiety. In rodents, lesions of the mPFC may decrease anxiety, while lesions of the lateral PFC substantially increase freezing in fear-conditioning paradigms (Lacroix *et al.*, 2000). Similarly, we observed that more worrisome thoughts after the induction predicted a reduction in amygdala functional connectivity with vmPFC over time, while enhanced connectivity with lateral PFC was associated with increased trait anxiety after 1 year. There are earlier observations of this dissociation in humans. A positive increase in functional connectivity of amygdala to middle frontal gyrus, and a decrease in amygdala-vmPFC coupling is observed in high trait anxiety individuals during anticipatory anxiety (Vytal *et al.*, 2014). Moreover, following an effective mindfulness-based therapy, improvements in anxiety scores negatively correlate with changes in functional connectivity of right amygdala to mPFC (Hölzel *et al.*, 2013). Taken together, our current and previous findings add to

growing evidence for a dissociable function of lateral and ventromedial PFC coupling with amygdala in anxiety.

The changes in amygdala functional connectivity over time also resulted correlated with changes in resting HRV from time 0 to time 1 with the opposite pattern in GAD and controls. In the case of GAD, a stronger decrease in HRV over time was associated with increased connectivity between right amygdala and left insula whereas the opposite pattern (diminished connectivity) emerged in controls. An increased connectivity between amygdala and insula is already described in GAD, both at rest (Roy *et al.*, 2009) and when engaged in a task (McClure *et al.*, 2007). One influential account of role of the insula in anxiety disorders highlights the region's contribution to autonomic control (Paulus and Stein, 2006). This region integrates the information about the salience of environmental stimuli with the effects that these stimuli have on the body state (Critchley *et al.*, 2002; Paulus and Stein, 2006; Terasawa *et al.*, 2013; Craig, 2011) and this is assisted by bidirectional connections with the amygdala (Nieuwenhuys, 2012). The insula is also involved in the regulation of autonomic responses, consistent with a functional organization that integrates cardiovascular response patterns in the service of emotional behaviours. These processes might be disrupted in anxious individuals, making it difficult for them to differentiate normal fluctuations in interoceptive stimuli from potentially aversive body signals, resulting in an increased and persisting worry (Paulesu *et al.*, 2010). Thus, insula activation is observed in response to worry in a population suffering from depression and anxiety (Servaas *et al.*, 2014), in paediatric anxiety disorders (Hamm *et al.*, 2014), and in adults with GAD (Hoehn-Saric *et al.*, 2004). Worry induction in elderly GAD participants evoked stronger connectivity between insula and orbitofrontal cortex that diminished during reappraisal (Andreescu *et al.*, 2015). Stronger amygdala-insula functional connectivity is also reported in urban youth exposed to trauma (Thomason *et al.*, 2015) and adults with PTSD (Rabinak *et al.*, 2011), highlighting reproducible involvement of amygdala–insular interaction for anxiety disorders.

Lastly, changes in HRV over time were positively correlated with the functional connectivity between right amygdala and areas implicated in memory retrieval and attention, including right middle temporal gyrus and angular gyrus. More specifically, HRV reductions from time 0 to time 1 were associated with similar decreases in resting-state connectivity in regions underlying the perceptual, attentional and working memory deficits associated with trait anxiety (Modi *et al.*, 2015).

One limitation of this study is the relatively small sample size, due to difficulties in asking GAD patients to receive two MRI scans in a year. The prevalence of women in the sample reflects the unequal gender distribution in the GAD and, despite our best attempts at matching patients and controls, it may have biased the results. Another important limitation is that our measures of functional connectivity do not allow determining the direction of causal relationships between brain regions. Limitations notwithstanding, our data provides important new insight into neural mechanisms through which emotional regulation and autonomic dysfunction interact in GAD, and broadens this knowledge by clarifying the prognostic value of measures of emotion regulation on longitudinal functional connectivity alterations. Overall, the present study further validates the use of rsfMRI in the evaluation and monitoring of psychiatric disorders (Woodward and Cascio, 2015). If the robustness of our results will be confirmed by future longitudinal research, differences in connectivity may represent neurofunctional markers of emotion regulation capacities and anxiety symptoms progression.

Supplementary data

Supplementary data are available at *SCAN* online.

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