Abbreviated Title: Mindfulness and Amygdala Resting State Connectivity

Mindfulness Meditation Training Alters Stress-Related Amygdala Resting State Functional Connectivity: A Randomized Controlled Trial

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Abstract

Recent studies indicate that mindfulness meditation training interventions reduce stress and improve stress-related health outcomes, but the neural pathways for these effects are unknown. The present research evaluates whether mindfulness meditation training alters resting state functional connectivity of the amygdala, a region known to coordinate stress processing and physiological stress responses. We show in an initial discovery study that higher perceived stress over the past month is associated with greater bilateral amygdala-subgenual anterior cingulate cortex (sgACC) resting state functional connectivity (rsFC) in a sample of community adults (N=130). A follow-up, single-blind randomized controlled trial (RCT) shows that a 3-day intensive mindfulness meditation training intervention (relative to a well-matched 3-day relaxation training intervention without a mindfulness component) reduced right amygdala-sgACC rsFC in a sample of stressed unemployed community adults (N=35). Although stress may increase amygdala-subgenual anterior cingulate cortex rsFC, brief training in mindfulness meditation could reverse these effects. This work provides an initial indication that mindfulness meditation training promotes functional neuroplastic changes, suggesting an amygdala-sgACC pathway for stress reduction effects.

Keywords: Amygdala, Cingulate, Health, Mindfulness, Stress

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There has been significant recent interest in identifying interventions and pathways for stress resilience in at-risk individuals (Feder et al. 2009; Bonanno et al. 2011). Recent studies suggest that mindfulness meditation interventions, which train the capacity to be more open and aware of present-moment experience, may increase stress resilience (Ludwig and Kabat-Zinn 2008; Creswell 2014). For example, RCTs show that mindfulness meditation training interventions reduce reactivity to acute stressors (Nyklíček et al. 2013; Creswell et al. 2014) and improve health outcomes in stress-related disorders and diseases (e.g., anxiety, depression, PTSD, HIV-infection: Teasdale et al. 2000; Creswell et al. 2009; Hölzel et al. 2013; King et al. 2013). Moreover, cumulative evidence has linked individual differences in stress reactivity and the experience of stress to a broad range of physiological health outcomes (Everson-Rose and Lewis 2005; Cohen et al. 2007; Gianaros and Sheu 2009; Miller et al. 2009; McEwen and Gianaros 2010). Although there has been significant progress in understanding the neurobiology of mindfulness meditation training effects (Tang et al. 2015), there is still relatively little work directly relating the neural mechanisms of mindfulness training and stress resilience.

Resting state functional connectivity (rsFC) methods provide one approach for interrogating how mindfulness alters neural dynamics among stress-responsive brain regions. RsFC metrics of inter-regional dynamics specifically afford the advantage of being task-independent, providing reliable estimates of neural circuit functionality corresponding to structural topography (Fox and Raichle 2007; Greicius 2008). Here, we tested the extent to which mindfulness alters stress-related amygdala rsFC for three reasons: (1) The amygdala is a cell complex that is centrally involved in processing psychological stressors and coordinating

physiological stress responses (LeDoux 1994; Arnsten 2009). (2) Recent studies show that greater reported mindfulness is associated with reduced amygdala volumes and task-based amygdala activation (Goldin and Gross 2010; Hölzel et al. 2010; Way et al. 2010; Taren et al. 2013; Taylor et al. 2011; Desbordes et al. 2012). (3) Anatomical studies in animal models indicate robust amygdala connectivity to other brain regions considered to be integral for processing stressors and orchestrating stress reactions (e.g. anterior cingulate cortex [ACC] and medial prefrontal cortex, hypothalamus, periaqueductal gray, and pontine/medullary autonomic control regions) (Ulrich-Lai and Herman 2009; McEwen and Gianaros 2010). Moreover, recent human studies have shown that amygdala functional connectivity with the ACC is associated with greater stressor-evoked physiological reactivity (Gianaros et al. 2008) and amygdala-ACC functional connectivity is enhanced after acute stress exposures (van Marle et al. 2010; Veer et al. 2011).

Accordingly, to test whether mindfulness reduces stress-related amygdala-ACC rsFC, we conducted two studies: (1) an initial discovery study evaluated whether self-reported perceived stress was associated with greater amygdala-ACC rsFC in a large sample of community volunteers (N=130). Although some human studies implicate increased stress-related amygdala rsFC with spatially adjacent areas of the ACC (e.g., Gianaros et al. 2008), the discovery study tested for associations between perceived stress (and mindfulness) with amygdala rsFC across the whole brain. (2) We then conducted a randomized controlled trial (RCT) of mindfulness meditation training (compared to a matched relaxation training intervention without a mindfulness component) in a high-stress unemployed community sample to test whether mindfulness training prospectively reduces stress-related amygdala-ACC rsFC.

Methods

Discovery Study

Participants, Procedure, and Analysis

133 healthy adults were recruited from the community by mass mailings to residents of Allegheny County, PA (3 participants were removed due to poor co-registration during preprocessing, resulting in a final sample of N=130). This discovery study was drawn from a larger ongoing NIH-funded parent study focused on understanding the neurobiological, psychosocial, and behavioral correlates of health among community adults (the Pittsburgh Imaging Project). Demographic characteristics of this study sample are provided in Table 1. All participants gave written informed consent as part of protocols approved by the Institutional Review Boards of the University of Pittsburgh and Carnegie Mellon University. Inclusion criteria included no history of (1) cardiovascular disease (including treatment for or diagnoses of hypertension, stroke, myocardial infarction, congestive heart failure, and atrial or ventricular arrhythmias); (2) prior neurosurgery or neurological disorder; (3) current treatment for or self-reported psychiatric disorder; (4) typical consumption of greater than 15 alcoholic beverages per week; (5) daily use of corticosteroid inhaler; (6) current use of psychotropic, lipid lowering, or any cardiovascular medication, including any medication to control blood pressure; (7) metal implants or exposure; (8) colorblindness; and (9) claustrophobia. All participants were right-handed, as assessed by the Edinburgh Handedness Inventory (Oldfield 1971). Women were excluded if pregnant (verified by urine test). Participants completed a psychosocial survey battery, which included the Perceived Stress Scale (PSS). The PSS is a 10-item questionnaire measuring the frequency of stressful feelings and thoughts within the last month (0=Never, 4=Very Often). Four of the items are reverse-scored and an overall composite measure is computed, with higher values indicating a greater perceived stress (Cohen et al. 1983).

All participants completed a separate neuroimaging session, which included a 5-minute resting state functional connectivity scan where participants were asked to rest quietly with their eyes open. Images were acquired on a 3 Tesla Trio TIM whole-body scanner (Siemens, Erlangen, Germany), equipped with a 12-channel phased-array head coil. Three-dimensional magnetization prepared rapid gradient echo (MPRAGE) high-resolution T1-weighted neuroanatomical images were acquired for each subject over 7 minutes 17 seconds by these parameters: field of view = 256×208 mm, matrix size = 256×208 mm, time to repetition = 2100 ms, time-to-inversion = 1100 ms, time to echo = 3.29 ms, and flip angle = 8° (192 slices, 1mm thick, no gap). For each subject, a single 300 second run of resting state BOLD data was acquired with a repetition time of 2 seconds. Preprocessing of images was conducted in SPM8 (Welcome Department of Cognitive Neurology, London, UK; run on MATLAB, MathWorks, Inc., Natick, MA, USA) and rsFC analysis was conducted using the CONN toolbox (Whitfield-Gabrieli and Nieto-Castanon 2012). BOLD images for each participant were realigned to the first image of the series, corrected for distortion due to movement, and spatial normalized to the Montreal Neurological Institute 152 template. Normalization was conducted using the structural gray matter image segmented from the T1-weighted MPRAGE image; the structural gray matter image was co-registered with the mean realigned BOLD image, and registered to the MNI template by non-linear affine transformation. The normalized BOLD images were smoothed with a 6-mm full-width at half-maximum isotropic Gaussian kernel for statistical analysis. In the CONN toolbox, structural MPRAGE images were segmented to define gray matter, white matter, and cerebrospinal fluid areas. A time series was extracted from each region of interest using functional BOLD fMRI resting state data. Additional preprocessing was carried out in the CONN toolbox to account for further temporal confounding factors, including BOLD signal

from the subject-specific white matter and CSF masks, motion parameters (six dimensions), and the effect of rest (an average across the session) to account for global signal. A covariate for each subject's head motion was entered at the first level. A band-pass filter of 0.008-0.09 Hz was used. The CONN toolbox estimates orthogonal time series using principal component analysis of the BOLD signal in each noise ROI.

At the single subject level, functional connectivity was measured by calculating the average BOLD time series across all voxels in each seed region and calculating a bivariate correlation between each seed region of interest and every other voxel. A hemodynamic response function was used to down weight the initial scans within each resting state block to minimize potential ramping effects. Left and right amygdala seeds were anatomically defined using the Wake Forest University (WFU) pickatlas (Maldjian et al. 2003) for generating whole-brain rsFC maps in CONN, which were then related to PSS scores as a covariate of interest in separate group-level GLM analyses. Whole-brain analyses relating PSS to left and right amygdala rsFC were conducted using a discovery threshold of uncorrected $p<.001$ and $k>30$.

Randomized Controlled Trial of Mindfulness Meditation Training

Participants

Thirty-five stressed unemployed job-seeking community adults (who indicated moderate to high levels of perceived job-seeking stress over the past month, scoring >9 on an adapted 4-item PSS for job-seeking stress; $\alpha = 0.55$) participated in a single-blind RCT of 3-day intensive mindfulness meditation or relaxation training intervention (see Table 2 for participant characteristics). Participants were recruited via newspaper advertisements and through employment agencies in Pittsburgh, PA. Participants were English-speaking, had no pre-existing

health conditions, were willing and available to participate in all study assessments, and were

willing to be randomly assigned to one of two study conditions. Callers who met these

qualifications were invited to come to Carnegie Mellon University for an in-person screening

interview and baseline assessment. A more in-depth screening interview followed, including

assessments of basic cognitive ability, right- or left-handedness and internal metal content (for

fMRI eligibility), employment background (to probe for unemployment-related stress), medical

history, and health behavior. Subjects taking psychotropic medications were excluded. Figure 1

depicts the flow of participants through the RCT. This study was approved by the Carnegie

Mellon University Internal Review Board and all participants provided informed consent.

Procedure

We conducted this RCT between December 2010 and October 2011. Beginning four

weeks before the 3-day training intervention, participants completed a baseline neuroimaging

session. All participants began with a 5-minute resting state scan (where they passively viewed a

fixation cross), followed by three functional tasks in counterbalanced order (Multi-Source

Interference Task, Affect Labeling, and a Personalized Stress Task), and a 7-minute perfusion

MRI scan with a guided awareness of breathing task (the results of these functional tasks are not

reported here). After neuroimaging, participants were invited to a nearby residential retreat

center where they were randomized to either a 3-day intensive mindfulness meditation training

(N=19) or matched 3-day relaxation residential retreat intervention (N=16). Only the participant,

project manager, treatment program staff members, and the treatment program instructor were

aware of the participant's study condition. All other study personnel responsible for collecting

study assessments remained blinded. Participants returned for a neuroimaging assessment within

two weeks of completing the 3-day intervention and completed an identical scanning procedure

as at baseline, including the same 5-minute resting state scan. At both neuroimaging sessions, participants were instructed to passively view a fixation cross during the resting state scan period and not to sleep or engage in any meditation or relaxation practices (which was verbally confirmed in all participants at the conclusion of the neuroimaging session). 97% of randomized participants were retained at the post-intervention neuroimaging assessment (3% study attrition, see Figure 1). As part of the larger study, participants completed a comprehensive battery of psychosocial measures and provided a blood draw at baseline and at 4-month follow-up; the present report focuses on testing how mindfulness meditation training changes rsFC patterns using the 5-minute resting state BOLD scan at baseline and in the two weeks following the 3-day intensive training period (post-intervention). In order to measure cumulative hypothalamic-pituitary-adrenal (HPA) axis activation over the 4-month follow-up period, participants were invited to provide a hair sample at the 4-month follow-up appointment (Dettenborn et al. 2010; Stalder and Kirschbaum 2012).

Interventions

We adapted the standardized and manualized 8-week Mindfulness-Based Stress Reduction (MBSR) program (which includes a day-long retreat) (Kabat-Zinn 1982, 1990) into a condensed 3-day residential retreat format, entitled Health Enhancement through Mindfulness (HEM). Delivery of the HEM program in a structured residential retreat format improves compliance with training, reduces treatment attrition, and greater experimental control is afforded by offering a parallel matched relaxation training retreat (in a separate wing of the retreat center). The HEM instructor was a doctoral level psychologist with 7 years of MBSR teaching experience. Subjects were not informed that the mindfulness intervention was called HEM, so as to avoid any non-specific demand characteristics. Briefly, the HEM program

consists of mindfulness training through body scan awareness exercises, sitting and walking meditations, mindful eating, and mindful movement (gentle hatha yoga postures). After each formal meditation period, participants engaged in discussion of their observations about themselves and the practices. The instructor modeled and encouraged attitudes to foster mindfulness such as letting go of judgment and expectations, cultivating self-care, patience, and friendly curiosity toward present moment experience. On the third day, formal meditation practices were extended to discussions about how participants could use mindful awareness for their unemployment and job-seeking stress.

We developed a structurally matched Health Enhancement through Relaxation (HER) program that included similar behavioral training activities (e.g., walking, stretching, and didactics) as HEM, but all trainings emphasized participation in these activities in a restful way rather than a mindful way. The HER program instructor was a licensed social worker with over 2 decades of clinical experience in stress management. The use of a structurally-matched active comparison group was designed to control for non-mindfulness specific factors such as positive treatment expectancies, group support, teacher attention, physical activity, and mental engagement. An hour-by-hour outline of interventions is provided in Supplementary Materials.

Image Acquisition

Structural and functional images were acquired on a Siemens Verio 3T scanner using a 32-channel head coil. High-resolution T1-weighted gradient-echo images were acquired at the start of the scanning session, with a slice orientation of AC-PC aligned, temporal lobes up (TR=1800ms, TE=2.22ms, flip angle= 9º, matrix size= 256x256, number of slices= 256, FOV= (205mm, 0.8mm thick slices), GRAPPA accel. factor PE= 2, voxel size= 0.8x0.8x0.8mm). Four functional echo-planar imaging runs were acquired, including a 300 second resting state scan

(TR=2000ms, TE=30ms, flip angle=79º, matrix size=64x64, number of slices=36, FOV= 205mm, 3.2mm thick slices EPI with rate 2 GRAPPA, voxel size=3.2mmx3.2mmx3.2mm).

Image Preprocessing

Functional BOLD data were processed using SPM8 (Welcome Department of Cognitive Neurology, London, UK; implemented by MATLAB, MathWorks, Inc., Natick, MA, USA). First, the data were realigned to the mean image of the first run and then smoothed with a 4mm FWHM Gaussian kernel to be in the preferred format for the motion correction program, ArtRepair. Data were then submitted to motion correction using the ArtRepair utility (P. Mazaika et al. 2007; Mazaika et al. 2009), an interpolation-based motion correction utility program. Motion correction in ArtRepair follows a two-step process. In the first step, an algorithm is applied to each run of data to suppress interpolation errors due to large motion. The algorithm applies a larger correction to edge-wise voxels than to central voxels, since the effects of motion on BOLD signal are most pronounced in these areas. In the second step, TRs with large amounts of fast motion or large global signal variation are flagged for repair. A default motion threshold of 1mm was used, so that TRs with motion greater than 1mm were flagged for repair. Repair of the data was done through linear interpolation, so that volumes flagged for repair were filled in with the average signal value from the two nearest unrepaired TRs. To quantify head motion that could appreciably impact artifactual signal in our rsFC analyses, we had a hypothesis- and condition-blind coder count total fast head motion TRs for each subject at each resting state scan time point (quantified as a TR-TR change of .25mm or .25degrees rotation in any plane). A mixed linear model analysis indicated there were no head motion differences between groups (condition main effect p=.97) and no differential fast head motion change over time (condition*time interaction p=.98). After motion correction the functional data

was normalized to the standard Montreal Neuroimaging Template (MNI) T1 template using indirect normalization, in which the functionals are first coregistered to the MPRAGE, and then the MPRAGE is normalized to the T1 template. Finally, the images were smoothed a second time with a 7mm FWHM kernel, resulting in an overall FWHM smoothing of 8mm (P. Mazaika et al. 2007).

Connectivity and Data Analyses

Left and right amygdala seeded resting state BOLD fMRI images were generated in the CONN toolbox (Whitfield-Gabrieli and Nieto-Castanon 2012) (using the same procedures as the discovery study), which were then applied in a group-level flexible factorial analysis in SPM8 with two factors specified, time (pre- and post-intervention) and group (HEM vs HER groups). We generated a time-by-group spreading (or ordinal) interaction contrast that tested for baseline to post-intervention decreases in rsFC in the HEM program (relative to the HER program, in which we did not expect to see this rsFC decrease) using contrast weights: [1(pre,HEM), 1(pre,HER), -3(post, HEM), 1(post,HER)]. As our primary interest was in testing the hypothesis that the mindfulness training program group would show connectivity change from baseline to post-treatment, relative to no change in the relaxation control training group, this approach is superior to approaches that compare the two treatment groups at post-treatment only (such as using post only contrast weights 1, -1), as they neglect baseline values and fail to evaluate change over time. The discovery study indicated stress-related bilateral amygdala-sgACC rsFC, thus we pursued amygdala rsFC analyses with a bilateral anatomical (AAL atlas) ACC ROI (defined by the WFU Pickatlas (Maldjian et al. 2003)) in the mindfulness meditation training RCT. Cluster-level correction for multiple comparisons was obtained using a Monte Carlo simulation implemented by AlphaSim (National Institute of Mental Health, Bethesda, MD).

AlphaSim was implemented with a bilateral ACC mask generated from the AAL atlas using an 8mm smoothing kernel and 10000 iterations. Significant clusters ($p< 0.05$, corrected) were defined as those involving $k >27$ contiguous voxels, each at $p< .005$.

Measuring Stress in the Mindfulness Meditation Training RCT: Hair Sampling

In order to evaluate whether changes in amygdala-ACC rsFC prospectively predict stress-related biomarkers after mindfulness meditation training, we conducted an exploratory analysis testing whether pre-post intervention changes in rsFC were associated with hair-derived cumulative measures of HPA-axis activation during the 4-month follow-up period (Stalder and Kirschbaum 2012). Hair samples have been used to measure cumulative HPA-axis activation, with approximately 1cm of hair length corresponding to one month of HPA-axis activity. Recent studies indicate higher HPA-axis activation among the unemployed relative to employed adults in hair samples (Dettenborn et al. 2010). Hair was acquired and assayed using standard procedures described by Kirschbaum, Dettenborn and colleagues (2009; 2010). Briefly, about 40 hairs were cut with scissors as close to the back of the scalp as possible from a posterior vertex position. The follicle end of each hair sample was labeled and clipped to a piece of aluminum foil and then sent to a specialized laboratory in Dresden, Germany for assay. Each sample was washed, dried, and spun for steroid extraction (see Dettenborn et al. 2010). Cortisone and cortisol determination was then assessed using a commercially available immunoassay (with chemiluminescence detection, CLIA-IBL-Hamburg, Germany). The lab reports intra-assay and inter-assay coefficients of variance as below 8%.

Data on demographic and lifestyle factors that consistently affect hair glucocorticoid levels is limited, but previous studies suggest that age, gender, and exercise volume (as vigorous exercise is associated with HPA axis activation) may be associated with hair cortisol (Raul et al.

2004; Russell et al. 2012; Skoluda et al. 2012; Stalder and Kirschbaum 2012). Thus, participant age, gender, and frequency of vigorous exercise were included as covariates in hair analyses in order to reduce noise attributable to these potential confounding factors. We analyzed both hair cortisone and cortisol; both are glucocorticoids released by the adrenal glands, with similar physiologic functions. Although cortisol is the more active form; previous research reports that hair cortisone may be higher than hair cortisol (as opposed to in plasma, where cortisol concentrations are higher than cortisone) due to increased activity of the enzyme responsible for converting cortisol to cortisone in the hair bulb (Tiganescu et al. 2011; Russell et al. 2012)(Tiganescu et al., 2011). We first conducted a one-way ANCOVA comparing the mindfulness to relaxation groups on hair-derived measures of cortisone and cortisol at the 4-month follow-up appointment. Parameter estimates were then extracted in SPM8 using cluster analysis centered on the peak voxel in subgenual ACC at baseline and post-training. Exploratory analyses then tested whether greater pre-post (change score) training-related changes in amygdala-ACC rsFC co-varied with lower levels of these hair-derived markers of cumulative HPA-axis activation at 4-month follow-up. Specifically, associations between hair cortisone, cortisol and post-pre training change in functional connectivity across groups were examined using bivariate Pearson correlation analyses. N=4 participants were unable or unwilling to provide hair samples at the 4-month follow-up time point, and were excluded from these analyses. Statistical analyses were carried out in SPSS with a significance threshold of $p<0.05$.

Results

Discovery Study Results

A whole-brain analysis indicated a positive association between perceptions of stress over the previous month and greater amygdala coupling with the sgACC. Specifically, higher

levels of stress were associated with increased rsFC between right amygdala and subgenual ACC (sgACC) (MNI: 0,16,-13, k=120 voxels), the only region of significant correlation with the right amygdala in a whole-brain analysis (p<0.001, uncorrected) (Figure 2a, Table 3). Although smaller in spatial extent, greater self-reported stress was also associated with increased left amygdala-sgACC (MNI: 1,22,-4, k=24 voxels) rsFC (p<0.001, uncorrected) (Figure 2b). Higher levels of self-reported perceived stress were also associated with greater left amygdala rsFC with perigenual right anterior cingulate cortex, left parahippocampal gyrus, left insula, and right superior temporal cortex in the discovery study (Table 4).

Mindfulness Meditation Training RCT Results

Building on the discovery study findings showing that stress was associated with greater amygdala-sgACC rsFC, it was predicted that mindfulness meditation training (relative to a well-matched relaxation training program without a mindfulness component), would decrease right amygdala-sgACC rsFC in stressed, unemployed community adults. Consistent with this prediction, a flexible factorial random effects analysis showed that a 3-day intensive mindfulness meditation training program reduced right amygdala-sgACC (MNI: 0,18, -12) rsFC, compared to relaxation training without a mindfulness component (time × treatment interaction p<0.05, k=28, corrected for multiple comparisons) (Table 3, Figure 3). As shown in Figure 3, we observed positive right amygdala-sgACC rsFC pre-intervention in this high stress unemployed sample, which is consistent with the discovery study findings suggesting that stress is associated with elevated amygdala-sgACC rsFC (there were no significant difference in right amygdala-sgACC connectivity between the HEM and HER groups at baseline, p=0.877). But notably this was a spreading interaction driven by mindfulness meditation training reductions in right amygdala rsFC at post-treatment (Figure 3). A parallel flexible factorial random effects analysis also tested

for training related changes in left amygdala-ACC rsFC, but there was no significant differential decreases in left amygdala-ACC rsFC pre-post training (no significant time × treatment interaction). Although the primary intent was to test for stress-related amygdala-ACC rsFC in an ACC ROI analysis, exploratory uncorrected whole-brain results are provided in the Supplementary Materials.

Change in Amygdala rsFC Co-Vary with Biomarkers of Stress

An exploratory analysis evaluated whether hair-derived markers of cumulative HPA-axis were associated with brain activation during the 4-month follow-up period. There were no statistically significant differences between the mindfulness meditation and relaxation groups in cortisone (one-way ANCOVA $F(1,25)= 0.08$, $p=0.779$; mindfulness group $M=54.99$, $SE=43.63$; relaxation group $M=72.12$, $SE=40.75$)) or cortisol (one-way ANCOVA $F(1,25)= 0.783$, $p=0.385$; mindfulness group $M=52.80$, $SE=12.31$; relaxation group $M=37.72$, $SE=11.50$) at 4-month follow-up. However, pre-post intervention decreases in amygdala-sgACC rsFC were associated with less cumulative HPA-axis activation in hair across all subjects. Specifically, training-related decreases for right amygdala-sgACC rsFC were inversely associated with hair cortisone ($r(24)= -0.39$, $p=0.049$) and showed a trending association with hair cortisol ($r(24)= -0.31$, $p=0.102$).

Despite recommendations to continue home practice after the 3-day retreat with customized compact discs, the high stress participants in the HEM and HER programs did not complete much formal guided practice in the 4-month follow-up period. HEM group participants reported using their home practice CD an average of 1.24 times per week (SD = 1.28) over the last month (at 4-month follow-up), while HER group participants reported using their home practice CD an average of 0.382 times per week (SD = 0.86) over the past month ($t_{31} =2.272$, p = 0.03).

Discussion

We provide an initial indication that alterations in amygdala-ACC rsFC track with perceived stress and can be altered by a mindfulness meditation intervention. Specifically, self-reported perceived stress was found to be associated with greater amygdala-sgACC rsFC. Moreover, mindfulness meditation training decreased amygdala-sgACC functional coupling relative to a well-matched comparison relaxation treatment without a mindfulness component. These findings agree with a neural circuitry-based account of previous studies suggesting that mindfulness alters amygdala structure and function (Creswell et al. 2007; Goldin and Gross 2010; Hölzel et al. 2010; Way et al. 2010; Taren et al. 2013), identifying a candidate amygdala-sgACC pathway that may link mindfulness training with reduced stress and stress-related health outcomes (Ludwig and Kabat-Zinn 2008; Creswell 2014). While brain regions other than the amygdala are involved in stress reactivity, our approach was to first identify a candidate stress processing region that is central for gating stress responding (i.e., the amygdala) (Arnsten 2009; Ulrich-Lai and Herman 2009; McEwen and Gianaros 2010) and test how trained mindfulness alters stress-related rsFC.

The present discovery study indicates a positive association between perceived stress and amygdala–sgACC coupling. This finding is in accord with several studies, which link the right amygdala and ACC to stress. Specifically, the amygdala has well-known anatomical connectivity with regions of ACC (Carmichael and Price 1996; Ongür et al. 1998; Freedman et al. 2000; Johansen-Berg et al. 2008), including sgACC (Freedman et al. 2000; Johansen-Berg et al. 2008)—and neurobiological accounts highlight the importance of amygdala and ACC/medial PFC networks in driving central fight-or-flight stress response cascades via activation of the HPA and sympathetic-adrenal-medullary axes (Arnsten 2009). ACC regions spatially similar to

regions identified in the present study have been implicated in stress responding; stressor-evoked physiological reactivity has been positively associated with right amygdala-perigenual ACC connectivity (Gianaros et al. 2008). One important question for the present work is whether mindfulness training related changes in amygdala-sgACC rsFC could be prospectively driving changes in stress. We provide some initial suggestive evidence that pre-post training related decreases in right amygdala-sgACC are associated with lower cumulative markers of HPA-axis activation over the 4-month follow-up period in the mindfulness meditation RCT study. However, this exploratory analysis should be viewed with some caution (and tested in new studies) given the lack of any robust training differences between the mindfulness meditation and relaxation conditions in these cumulative HPA-axis activation biomarkers, and the small subsample who provided hair samples at 4-month follow-up in the RCT study (N=30).

One limitation of the present research is that there were no stress-related disease outcomes in the RCT study with high stress community adults, and thus no way to evaluate whether mindfulness training changes in amygdala rsFC might drive potential improvements in stress-related disease. Nonetheless, this work offers a novel candidate pathway for explaining how mindfulness interventions benefit stress sensitive psychiatric populations. Previous studies have implicated disrupted rsFC in stress-related psychiatric disorders (e.g., PTSD, generalized anxiety disorder, major depressive disorder) (Mayberg 2003; Bluhm et al. 2009; Etkin A et al. 2009; Lanius et al. 2010), and recent RCTs indicate robust effects of mindfulness training on improving psychiatric outcomes in these same patient groups (Ma and Teasdale 2004; Bhatnagar et al. 2013; Hoge et al. 2013; King et al. 2013; Zeidan et al. 2013). Moreover, higher levels of self-reported perceived stress have been linked to poorer mental and physical health outcomes (Keller et al. 2012). The present research offers a testable new prediction for future studies;

namely, mindfulness interventions may reduce amygdala-sgACC rsFC, which serves as a neurobiological mechanism for improvements in emotion regulation, stress reactivity, and improved stress-related health and disease outcomes.

There has been a recent interest in how mindfulness meditation alters structural and functional activity in the ACC. For example, integrative mind-body meditation training has been shown to increase network efficiency and connectivity of the ACC (Xue et al. 2011) and recent work has shown that this form of meditation training increases white matter integrity in the corona radiata – the major tract projecting through ACC (Tang et al. 2010). Notably, sgACC is thought to be an important hub in networks for negative affect and mood disorders (Mayberg 2003; Seminowicz et al. 2004) and anatomical studies have shown direct connections between sgACC and the amygdala, hypothalamus, nucleus accumbens, and orbitofrontal cortex (whereas pregenual ACC is more strongly connected to medial prefrontal and mid-cingulate cortex, sgACC, and subcortical regions)(Chiba et al. 2001). In combination, these studies and our work here suggest potential dissociable effects of mindfulness meditation training on spatially distinct regions of ACC; such as mindfulness meditation training increasing rsFC on more dorsal and pregenual ACC tracts associated with enhanced self-regulation (Tang et al. 2010; cf. Hasenkamp and Barsalou 2012) and pain modulation (Zeidan et al. 2011), while also decreasing stress-related functional connectivity of the sgACC with the amygdala. Together, this work suggests the possibility that mindfulness meditation training fosters dorsal/pregenual ACC connectivity for attention monitoring while decoupling stress-related sgACC connectivity for stress resilience.

An important limitation of the present seed-based rsFC analytic approach is that it precludes inferences about the directionality of amygdala-sgACC connectivity; in the future, effective connectivity analyses (e.g., with dynamic causal modeling) offer opportunities to test

causal interactions between these stress-sensitive nodes. Moreover, we utilized 5 minute resting state scans; longer scans periods as well as cardiorespiratory data can be used to reduce measurement error in future studies (Birn et al. 2006, 2013). Our aim in this study was to reveal stable stress-related rsFC changes by using the PSS as a measure of stress symptoms over the past month (Liston et al. 2009), however, the scanner environment itself can be stress-inducing, and the lack of a state-stress scanner measure in this study does not allow us to fully exclude potential state-stress effects. An additional limitation is the lack of inclusion of a usual care group, which would provide an additional level of comparison to the HER and HEM intervention groups, allowing examination of the effects of attending a 3-day relaxation retreat and the potential stress-buffering gained from this (above and beyond no intervention). Given prohibitive cost and subject burden concerns, hair samples were not collected at time 1, only at 4-month follow-up; thus, our analyses are necessarily limited to relating change in amygdala-ACC resting state functional connectivity to cortisone and cortisol levels at follow-up. Finally, this work more broadly suggests a new amygdala rsFC pathway for stress resilience; future work should evaluate whether similar changes in amygdala rsFC can be achieved with other clinically impactful psychological interventions (e.g., cognitive behavioral therapy) or anxiolytic pharmacological treatments in at-risk stressed populations.

Conclusions

The present findings significantly advance our understanding of resting state functional connectivity in stress and mindfulness training interventions, and implicate decoupling of the amygdala and sgACC as a potential neurobiological mechanism underlying mindfulness-based stress reduction effects.

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Table I. *Discovery Study Subject Characteristics (n = 130)*

Variable	Mean	St. Dev.
Age	40.15	6.14
Gender	71 male, 59 female	-
Household Income	$39,199	$17,713
Years of School	17.31	3.21
PSS-10: Perceived Stress	1.33 (Range 0.1 – 3.2)	0.61

Notes: PSS = Perceived Stress Scale

Table II. *Baseline Characteristics of Randomized Controlled Trial Participants.*

Characteristic	Mindfulness Group	Relaxation Group	Difference Statistic
Age [mean years (SD)]	37.94	41.00	$t(33)= -.48, p= 0.64$
	(10.96)	(9.55)	
Gender			$\chi^2(1)=.24, p= 0.63$
Male	11	9	
Female	7	8	
Ethnicity			$\chi^2(3)= 4.36, p= 0.23$
Caucasian	10	13	
African American	6	2	
Asian American	1	0	
Latino(a)	0	0	
Native American	0	0	
Other	0	1	
Years Unemployed	8.17	10.58	$t(33)= -.43, p= 0.67$
	(12.48)	(20.31)	
Education			$\chi^2(8)= 8.43, p= 0.39$
No high school degree	1	0	
GED	1	0	
High school degree	1	2	
Technical training	3	2	
Some college	4	3	
Associate degree	2	0	
Bachelor's degree	2	7	
Master's degree	3	3	
MD/PhD/JD/PharmD	1	0	

Notes: Standard deviation values are provided in parentheses. Mindfulness group refers to the 3-Day Health Enhancement thru Mindfulness (HEM) intervention. Relaxation group refers to the 3-Day Health Enhancement thru Relaxation (HER) intervention.

Table III. *Subgenual ACC clusters identified in amygdala rsFC analyses.*

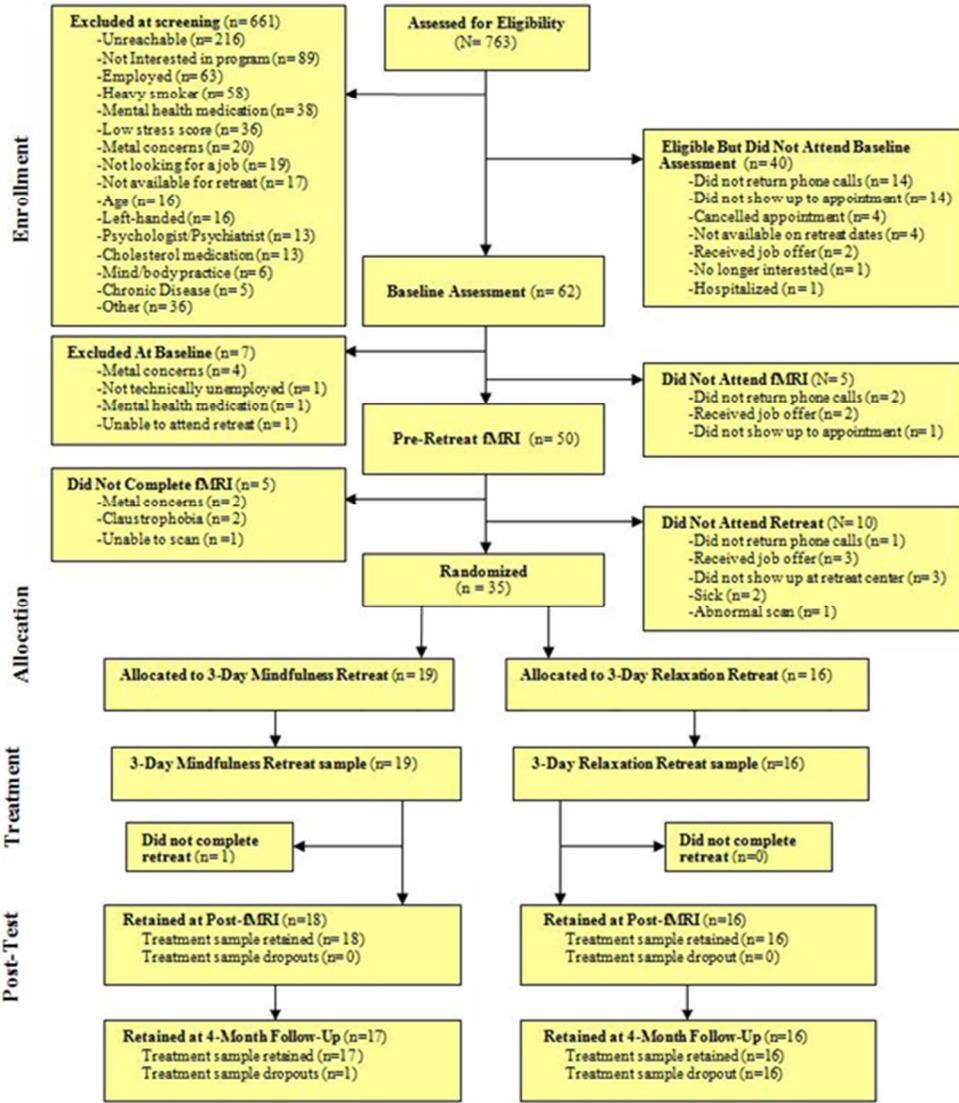| Analysis	Subgenual ACC MNI Peak Coordinates	k	p	T	Z
Study 1, PSS, Right Amygdala	(0, 15.5, -13)	120	<0.001, uncorrected	3.71	3.61
Study 1, PSS, Left Amygdala	(1, 21.5, -4)	24	<0.001, uncorrected	3.35	3.28
Study 2, Spreading Interaction HEM < HER at Post-Intervention relative to Baseline	(0, 18, -12)	28	<0.05, corrected	2.93	2.82

Table IV. Discovery Study. PSS Left Amygdala Seed Connectivity, Whole-Brain Analysis

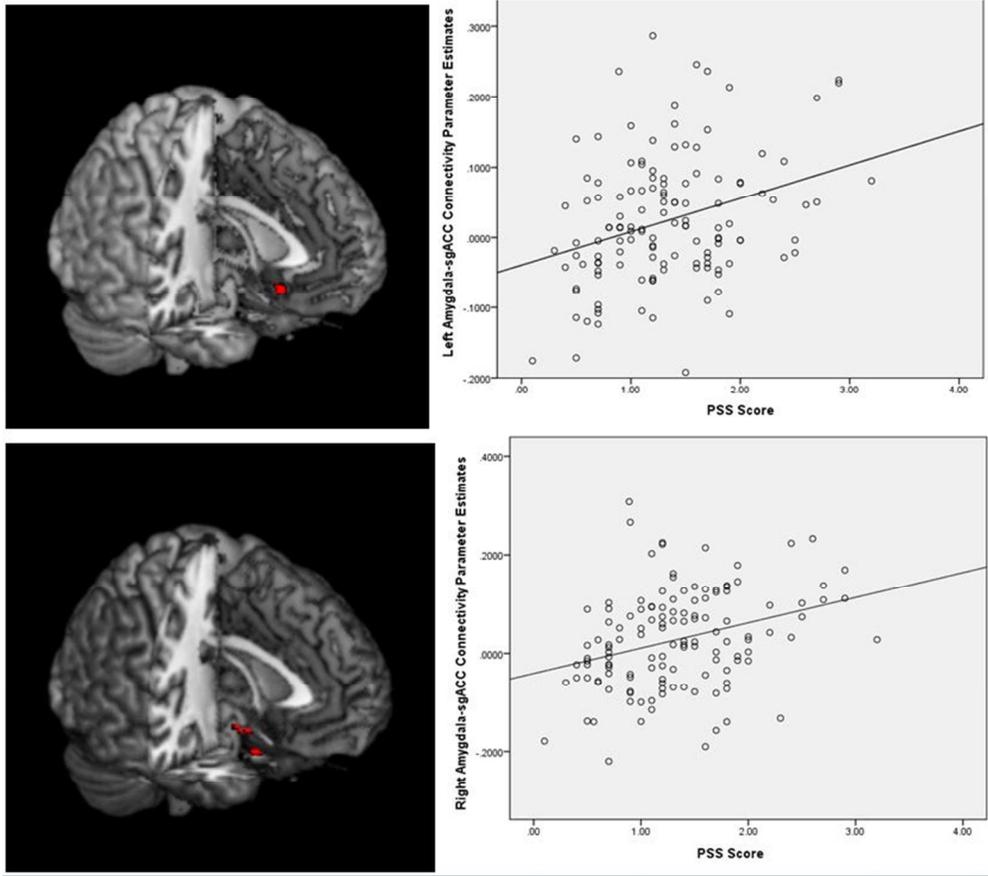| Increased Coupling associated with higher PSS score, $p < 0.001$, $k > 30$	Region (aal)	Peak MNI coordinate	Cluster Size	T	Z
	Left Parahippocampal Gyrus	-13 -11 -22	152	3.69	3.59
	Right Superior Temporal	56 -7 3	80	3.77	3.66
	Left Insula	-39 -13 11	112	3.92	3.80
	Right Perigenual Anterior Cingulum	11 30 14	236	4.48	4.31

 Figure 1. CONSORT flowchart of participants retained at each stage of the Mindfulness Meditation Training RCT.

Figure 2. Discovery Study: **(a, top left panel)** Greater self-reported perceived stress on the PSS (item average) is associated with greater resting state functional connectivity between right amygdala and subgenual ACC (p < 0.001). **(a, top right panel)** Scatterplot between perceived stress and right amygdala-sgACC rsFC parameter estimates (n = 130, R = -0.15). **(b, bottom left panel)** Greater self-reported perceived stress on the PSS (item average) is associated with greater resting state functional connectivity between left amygdala and ACC (p < 0.001). **(b, bottom right panel)** Scatterplot between perceived stress and left amygdala-sgACC rsFC parameter estimates (n = 130, R = -0.101).
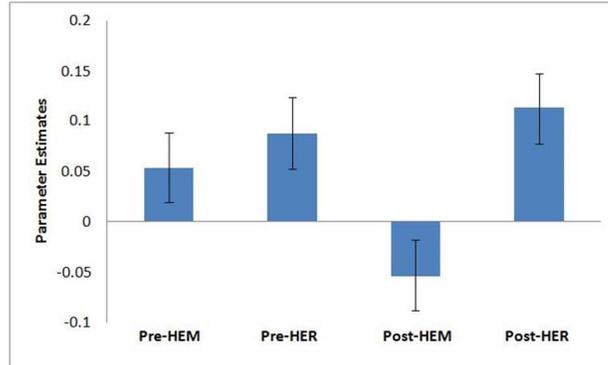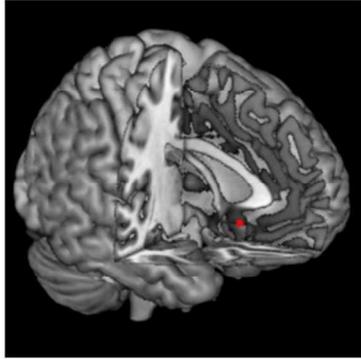
Figure 3. Mindfulness Meditation Training RCT. The left panel depicts the region of sgACC that showed decreased resting state functional connectivity with right amygdala from before to after mindfulness meditation training (HEM) relative to relaxation training (HER) (p<0.05, corrected for multiple comparisons). The right panel depicts the mean percent signal change for subgenual ACC cluster for the mindfulness (HEM) and relaxation (HER) training groups at each of the two time points (pre-intervention and post-intervention). Error bars depict +/- 1 standard error. Parameter estimates were extracted in SPM8 and plotted in a random effects mixed model conducted in SPSS.

CONSORT flowchart of participants retained at each stage of the Mindfulness Meditation Training RCT.
132x149mm (120 x 120 DPI)

Discovery Study: (a, top left panel) Greater self-reported perceived stress on the PSS (item average) is associated with greater resting state functional connectivity between right amygdala and subgenual ACC (p < 0.001). (a, top right panel) Scatterplot between perceived stress and right amygdala-sgACC rsFC parameter estimates (n = 130, R = -0.15). (b, bottom left panel) Greater self-reported perceived stress on the PSS (item average) is associated with greater resting state functional connectivity between left amygdala and ACC (p < 0.001). (b, bottom right panel) Scatterplot between perceived stress and left amygdala-sgACC rsFC parameter estimates (n = 130, R = -0.101).
177x156mm (120 x 120 DPI)

Mindfulness Meditation Training RCT. The left panel depicts the region of sgACC that showed decreased resting state functional connectivity with right amygdala from before to after mindfulness meditation training (HEM) relative to relaxation training (HER) ($p<0.05$, corrected for multiple comparisons). The right panel depicts the mean percent signal change for subgenual ACC cluster for the mindfulness (HEM) and relaxation (HER) training groups at each of the two time points (pre-intervention and post-intervention). Error bars depict +/- 1 standard error. Parameter estimates were extracted in SPM8 and plotted in a random effects mixed model conducted in SPSS.
228x85mm (120 x 120 DPI)