



Published in final edited form as:

Psychophysiology. 2017 May ; 54(5): 652–662. doi:10.1111/psyp.12829.

Association between Neural Reactivity and Startle Reactivity to Uncertain Threat in Two Independent Samples

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Abstract

Prior studies indicate that anxiety disorders are associated with heightened sensitivity to uncertain threat (U-threat). Individual differences in reactivity to U-threat have been measured in the laboratory with two methodologies - startle eyeblink potentiation and functional magnetic resonance imaging (fMRI). While startle and fMRI are purported to relate to each other, very little research exists on whether individual differences in one measure are associated with individual differences in another and thus, whether startle and fMRI capture shared mechanisms. Therefore, the current study was designed to investigate if and where in the brain measures of startle potentiation and fMRI blood-oxygen-level dependent (BOLD) signal correlate during response to U-threat across two independent samples. Participants in both studies completed two threat anticipation tasks – once during collection of startle potentiation and once during fMRI. In Study 1 ($n = 43$), the startle and fMRI tasks both used electric shock as the threat. As an extension, in Study 2 ($n = 38$), the startle task used electric shock but the fMRI task used aversive images. Despite these methodological differences, greater startle potentiation to U-threat was associated with greater dorsal anterior cingulate (dACC), caudate, and orbitofrontal cortex (OFC) reactivity to U-threat in both samples. The findings suggest that startle and fMRI measures of responding to U-threat overlap, and points towards an integrated brain-behavior profile of aberrant U-threat responding.

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All authors declare no conflicts of interest.

Keywords

unpredictable threat; anxiety; startle potentiation; fMRI

Introduction

Exaggerated responding to the anticipation of uncertain aversive events is a core feature of several anxiety disorders. For example, social anxiety disorder (SAD) and panic disorder (PD) are characterized by exaggerated anticipatory anxiety to the possibility of negative social evaluation and uncertain onset of panic attacks, respectively (APA, 2013). Moreover, elevated self-reported intolerance of uncertainty has been evidenced in SAD, PD, posttraumatic stress disorder (PTSD), and generalized anxiety disorder (GAD) (see Carleton, 2016a). Heightened reactivity to the anticipation of uncertain threat (U-threat) may therefore contribute to the pathogenesis of multiple internalizing disorders and has been proposed as a lower-order, fundamental individual difference factor underlying anxiety and neuroticism (Carleton, 2016b; Hong & Cheung, 2015). Reactivity to U-threat therefore reflects a potential transdiagnostic treatment target (Grupe & Nitschke; Barlow, 2000; Grillon et al., 2002) and identifying biobehavioral correlates of heightened reactivity to U-threat is critical in order to develop valid assays for treatment of this aberrant affective response tendency.

Electromyography (EMG) of the eyeblink startle reflex is one of the primary methods that are used to assess reactivity to U-threat. The eyeblink startle reflex is a rapid contraction of the orbicularis oculi muscle around the eye to a brief, unexpected, intense stimulus (e.g., short burst of white noise), and is reliably potentiated during aversive motivational states (e.g., threat) and attenuated during appetitive motivational states (e.g., pleasure) (Bradley, Cuthbert, & Lang, 1999; Lang, 1995). Relative to healthy controls, heightened potentiation of the startle response during the anticipation of U-threat has been evidenced in PD, SAD, specific phobia, and PTSD (Gorka et al., in press; Grillon et al., 2008, 2009; Shankman et al., 2013; Lieberman et al., in press). Of note is that all but one of these studies (Shankman et al., 2013) found that psychopathology was only related to exaggerated U-threat responding, and not predictable threat (P-threat) responding, suggesting specificity to U-threat. The distinction between U-threat and P-threat is also supported by animal (Davis, 1998; Davis et al., 2010) and pharmacological challenge (Grillon et al., 2006) studies, and the fact that startle potentiation to U-threat, but not P-threat, is associated with familial risk for anxiety disorders (Nelson et al., 2013). Taken together, heightened startle potentiation to U-threat is a potential psychophysiological indicator of anxiety-based psychopathology (Gorka et al., in press).

Studies have also used functional magnetic resonance imaging (fMRI) to identify the neural correlates of heightened reactivity to U-threat. Converging evidence suggests that there is a specific frontolimbic circuit, referred to as the anticipatory anxiety network (AAN), which is engaged during the processing of U-threat and may contribute to aberrant responding in anxiety psychopathology (Bach & Dolan, 2012; Grupe & Nitschke, 2013). The AAN consists of affect-generating limbic regions, such as the amygdala, anterior insula (aINS),

and bed nucleus of the stria terminalis (BNST), which have bidirectional connections with affect-modulating prefrontal regions, such as the prefrontal cortex, orbitofrontal cortex, and dorsal anterior cingulate cortex (dACC; Aupperle, Melrose, Stein, & Paulus, 2012; Grupe & Nitschke, 2013; Shankman et al., 2014; Walker & Davis, 1997). Several regions within the AAN have been implicated in anxiety psychopathology. In particular, hyperactivity of the aINS has been evidenced in various anxiety disorders (e.g., PD, SAD, GAD) during the anticipation of U-threat (Gorka et al., 2014; Simmons et al., 2011). The aINS has also been associated with interoceptive awareness during the anticipation of aversive events (Craig et al., 2009; Critchley et al., 2004; Khalsa et al., 2009), which is noteworthy given that heightened interoceptive awareness is involved in the pathogenesis of PD and SAD (Stevens et al., 2011; Ehlers & Bruer, 1992).

Hyperactivity of the dACC has also been observed in anxiety disorders during the anticipation of U-threat (Lieberman et al., in press; Straube, Mentzel & Miltner, 2007). Akin to the aINS, the dACC has been associated with interoceptive awareness and the generation and modulation of affective and physiological responding (Etkin, Egner & Kalisch, 2011; Milad & Rauch, 2007). Although the amygdala is another region involved in responding to threatening stimuli (Carleton et al., 2016b, Öhman, 2005; Thayer et al., 2012), few studies have found that amygdala reactivity differentiates anxiety disorder patients from controls during U-threat (e.g., Straube et al., 2007).

Taken together, anxiety disorders are associated with heightened startle potentiation to U-threat and hyperactivity of the aINS and dACC. These behavioral and neural responses to U-threat may therefore represent a transdiagnostic, anxiety disorder brain-behavior profile. However, the fMRI and startle literatures have been conducted in parallel and although it is often assumed that neural and startle reactivity to U-threat are related, very little research exists on whether individual differences in startle and fMRI response to U-threat converge. In other words, it is currently unclear whether individuals who display heightened startle potentiation to U-threat also exhibit hyperactivity of the aINS and dACC during the anticipation of U-threat, as expected. The question of convergence is further complicated by the fact that startle potentiation is often thought to be mediated by the amygdala, not the aINS or dACC (Davis et al., 2010; Lang & Davis, 2006), though the amygdala has dense projects to many AAN nodes including the aINS and dACC (Ochsner, Silvers, & Buhle, 2012).

The issue of multimethod convergence is particularly relevant to the National Institute on Mental Health's (NIMH) Research Domain Criteria (RDoC) Initiative. The RDoC Initiative seeks to reconceptualize psychopathology using a research framework based on transdiagnostic, multilayered, dimensional constructs that reflect core mechanisms of psychopathology (Sanislow et al., 2010; Cuthbert & Kozak, 2013). U-threat responding is a key construct in the RDoC framework and the RDoC Initiative implies that there is convergence across multiple units of analysis of a given construct (e.g., genes, circuits, physiology, behavior) (see Shankman & Gorka, 2015 for further discussion). Thus, startle indicators of reactivity to U-threat *should* converge with fMRI indicators of reactivity to U-threat. In order to establish this relation, it is necessary that studies assess whether individual

difference in startle potentiation during U-threat meaningfully predict fMRI blood oxygenation level-dependent (BOLD) signal during U-threat.

There is only one study addressing the literature gap by linking panic disorder symptoms with neural and startle responses to U-threat. In Lieberman et al. (2016), our lab demonstrated that panic symptoms were positively associated with startle potentiation to U-threat and dACC activation to U-threat. Moreover, within this sample, startle and dACC activation to U-threat was significantly correlated in a post hoc region-of-interest analysis. These findings suggest that startle potentiation to U-threat correlates with dACC reactivity to U-threat but because the Lieberman et al. analysis was post hoc, no other regions of the AAN were examined and it is presently unknown whether startle potentiation to U-threat relates to reactivity within any other AAN regions such as the aINS or amygdala.

Other studies have used startle potentiation as an index of aversive responding within fMRI paradigms, but have not looked at the correlations between the two measures (Eippert et al., 2007; van Well et al., 2012). Some studies have also collected simultaneous startle potentiation and fMRI to assess their associations yet none have examined this question during U-threat explicitly. For example, across several startle tasks, Neuner and colleagues (2010) found that non affect modulated startle (i.e., baseline startle) was correlated with activity in the somatosensory cortices, insula, thalamus, temporal pole, middle cingulate cortex, and cerebellum. Meanwhile, during an affective picture viewing task in healthy adults, Anders et al. (2004) found that greater startle changes to both positive and negative stimuli were associated with greater amygdala activity. These studies highlight that individual differences in startle potentiation relate to individual differences in specific neural structures; however, there is a need to directly examine the correspondence between startle and fMRI during the anticipation of U-threat given its relevance to psychopathology.

The current study was designed to examine whether individual differences in startle potentiation to U-threat were associated with neural reactivity to U-threat across two samples. In both studies, EMG startle responding to U-threat was assessed during modified versions of a well-validated threat-of-shock paradigm. In Study 1, fMRI BOLD response was assessed during an analogous threat-of-shock paradigm. In Study 2, however, fMRI BOLD response was assessed during the anticipation of uncertain aversive images, allowing us to examine whether startle and fMRI reactivity to U-threat converge even if the aversive stimulus differs between the two tasks. Study 2 therefore addresses whether there is an association between brain and behavioral (i.e., startle) responses to U-threat that generalizes across types of threat/stimulus. Based on the existing literature, we hypothesized that greater startle potentiation to U-threat would be associated with greater dACC, aINS, and amygdala activation to U-threat.

Method

Participants

Participants for Studies 1 and 2 were recruited from the community as a part of larger investigations on affective and physiological abnormalities related to internalizing disorders. Participants were recruited via advertisements posted in the Chicago community, local

psychiatric clinics, and nearby college campuses. A variety of advertisements were used to target different populations in an effort to enroll a diverse, internalizing disorder patient sample. Examining the impact of DSM diagnoses on the association between startle and fMRI measures of reactivity to U-threat is not an aim of the current study. However, exaggerated reactivity to U-threat has been observed across internalizing diagnoses (Carleton 2016b, Gorka et al., in press) and having individuals with a range of internalizing symptoms provides variability in individual differences to U-threat reactivity (Grillon et al., 2013; Shankman et al., 2013) and thus, variability in our startle and fMRI measures. Variability in responding is necessary to accurately detect statistical relationships making the current sample well-suited for the present aims.

Both studies took place at the University of Illinois-Chicago and were approved by the university Institutional Review Board. All participants provided written informed consent after review of the respective study protocols. Participants in each study completed a set of laboratory tasks and battery of questionnaires. All participants received cash as payment for participation.

Study 1—In line with the aims of the larger study, participants were included if they either (1) were seeking and would merit treatment with pharmacotherapy (selective serotonin reuptake inhibitors/SSRIs) or cognitive behavioral therapy (CBT) for their anxiety or depressive symptoms (i.e., patients); or (2) had no lifetime history of psychopathology (i.e., healthy controls). Participants were required to be between the ages of 18 and 65 years. Exclusion criteria included an inability to provide consent and read and write in English, a major active medical or neurological problem that could impact psychophysiological and brain function, lifetime history of mania or psychosis, any contraindication to receiving SSRIs, being already engaged in any form of psychiatric treatment, history of traumatic brain injury, left handedness, and being pregnant. All data used in the current study was assessed prior to treatment (i.e., baseline assessment) and none of the participants were taking psychoactive medications or engaged in psychotherapy at study entry. A total of 48 individuals met inclusionary criteria; however, 5 were excluded due to missing/poor quality raw startle data ($n = 4$), or poor quality fMRI data (i.e., excessive motion; $n = 1$). The final sample included 43 individuals, the demographics for which are listed in Table 1.

Study 2—As part of the aims for Shankman et al. (2013), participants in Study 2 were in one of three groups: (1) current PD with comorbid MDD ($n = 13$), (2) current MDD-only ($n = 9$), or (3) no lifetime history of any psychopathology ($n = 19$). Current and lifetime diagnoses were made using the Structured Clinical Interview for *DSM-IV* (SCID; First et al., 1996). For the larger study, individuals were excluded if they had a lifetime diagnosis of a psychotic disorder, bipolar disorder, or dementia, were unable to read or write in English, had a history of head trauma with loss of consciousness, or were left handed. A total of 39 individuals met inclusionary criteria; however, 1 was excluded due to missing fMRI data. The final sample included 38 individuals, the demographics for which are also listed in Table 1.

Procedure

For both studies, modified versions of the NPU-threat tasks (Schmitz & Grillon, 2012) were used to assess neural and startle reactivity to U-threat during fMRI and startle data collection, respectively. The startle and fMRI tasks were completed on separate days. Participants in both studies always completed the startle task first due to study constraints.

Prior to each shock task, a shock work-up procedure was completed in which participants received increasing levels of shock intensity until they reached a level that they described as “highly annoying but not painful.” Ideographic shock levels were used to ensure equality in perceived shock aversiveness (Rollman and Harris, 1987; max shock = 5mA). For Study 1 and 2, the startle task shock electrodes were placed on participants’ left wrist. For Study 1, the fMRI shock electrodes were placed on participants’ left foot in order to minimize movement and potential scan artifacts. After shock electrode placement, to prevent early exaggerated startle responding, participants completed a 2.5 min baseline habituation task during which 9 acoustic startle probes were presented.

Startle Threat Tasks and Data Collection—The startle tasks included three within-subjects conditions - no shock (N), predictable shock (P), and unpredictable shock (U). Text at the bottom of the computer monitor informed participants of the current threat condition and each condition lasted for 90 s. In Study 1, the cue was a 6 s countdown, displayed five times within each condition, and in Study 2 the cue was an 8 s geometric shape (blue circle for N, red square for P, and green star for U; not counterbalanced), presented four times within each condition. Interstimulus intervals (ISIs; i.e., times between cues) ranged from 7 to 17 s during which only the text describing the condition was on the screen.

During N, no shocks were delivered. During P, participants received a shock at the end of each cue. During U, shocks were administered at random (i.e., any time). Of note, shocks were not administered during every U_{Cue} and U_{ISI} as to ensure that the U condition was fully unpredictable. In Study 1, participants received 20 shocks (10 each during P and U) and 48 startle probes (16 each during N, P, and U). In Study 2, participants received 12 shocks (6 during P and 6 during U) and 72 startle probes (24 during N, 24 during P, and 24 during U).

In Study 1, EMG startle data was acquired and presented using BioSemi Active Two system (BioSemi, Amsterdam, The Netherlands) and Presentation (Albany, CA), respectively. In Study 2, EMG startle data were acquired and presented using Neuroscan 4.4 (Compumedics, Charlotte, NC) and PSYLAB (Contact Precision Instruments, London, UK), respectively. For both studies, acoustic startle probes were 40 ms duration, 103 dB bursts of white noise with near instantaneous rise time presented binaurally through headphones, and electric shocks lasted 400 ms.

Startle responses were recorded from two 4 mm Ag/AgCl electrodes placed over the orbicularis oculi muscle below the left eye in Study 1 and right eye in Study 2. The ground electrode was located at the frontal pole (Fpz) of an electroencephalography cap that participants were wearing as part of the larger studies. One startle electrode was placed 1 cm below the pupil and the other was placed 1 cm lateral of that electrode. Data for Study 1

were collected using a bandpass filter of DC-500 Hz at a sampling rate of 2000 Hz, and for Study 2 using a bandpass filter of DC-200 Hz at a sampling rate of 1000 Hz.

fMRI Threat Task and Data Collection

Study 1: The fMRI threat task was designed to be analogous to the startle threat task. There were three within-subjects conditions – safe, predictable, and unpredictable (see Figure 1). Rather than text at the bottom of the screen indicating the condition, three discrete images of different rooms were used to signal each condition (counterbalanced across participants). Within each room image, a lamp, which turned on and off, was the cue. In N, participants never received a shock. In P, participants received a shock when the lamp was turned on. In U, participants were shocked at random (though not during every trial). Each condition lasted 6 s, during which the lamp was turned on once, for a duration of 2 s. The task consisted of 90 trials, with 30 trials in each condition. Light onset occurred at either 0 s (for 1/3rd of trials), 2 s (for 1/3rd of trials), or 4 s (for 1/3rd of trials) across all conditions. A fixation cross was presented in between trials, ranging from 1 to 8 s ($M = 4.3$ s).

Functional MRI data was collected using a 3T GE magnetic resonance scanner at the University of Illinois at Chicago Medical Center. Functional images were acquired using a gradient-echo echo-planar images (2s TR, 25ms TE, 82° flip, 64×64 matrix, 200 mm FOV, 3mm slice thickness, 0mm gap, with 40 axial slices).

Study 2: The fMRI threat sensitivity task was modified to test the convergence between reactivity to electric shock and negative aversive images (i.e., two different forms of threat). Participants viewed a series of count-ups (CU; e.g., 1-2-3-4) that ended with the presentation of a negative (Neg) or neutral (Neut) image selected from the International Affective Picture System (IAPS; Lang et al., 2008; see Figure 1). The timing of the presentation of each image was either predictable (P; i.e., it was known when the image would appear) or unpredictable (U; i.e., it was unknown when the image would appear). Therefore, the task included two within-subjects factors: timing (P vs. U) and picture valence (Neg vs. Neut). For each trial, text initially appeared at the bottom of the screen for 2 s indicating the condition including image valence and P or U timing (i.e., P-Neut, P-Neg, U-Neut, or U-Neg). Next, the CU was presented for 4 to 11 s and at the end of the CU the image appeared for 1–5 s. In the P condition, text at the bottom of the screen told participants when the CU would end and the valence of the image that would appear (e.g., “Neutral image at 5”). In the U condition, text at the bottom of the screen indicated the valence of the image but did not specify when the image would appear (e.g., “Negative image at anytime”). For each condition, trials were presented during 42 s blocks during which the CU was presented four times. Each condition block (P-Neut, P-Neg, U-Neut, U-Neg) was presented four times, counterbalanced across two runs. In-between blocks, a fixation cross was presented for 10 s.

After completing the task, participants were shown each IAPS image again in a random order and provided valence and arousal ratings on a 1 to 9 scale (valence: very unpleasant to very pleasant; arousal: not at all arousing to very arousing). Mean valence and arousal ratings, as well as score ranges, for the current sample are presented in Table 1. A list of the individual IAPS images used in the task and the normative valence and arousal ratings are

included in Supplemental Methods. It is necessary to highlight that some participants rated the negative and neutral stimuli as highly positive and consequently, the range of scores for negative and neutral images does not match the range of scores from the normative IAPS data. However, the mean valence and arousal ratings are comparable across the datasets suggesting that on average, the current sample responded as intended.

Functional MRI was performed on a 3.0 Tesla GE MR 750 scanner (General Electric Healthcare; Waukesha, WI) using an 8-channel phased-array radio frequency head coil. A standard T2-sensitive gradient-echo echoplanar imaging (EPI) sequence was used (2s TR; 22.2ms TE; 90° flip; 64×64 matrix; 22cm FOV; 44 axial slices; 3.44 × 3.44 × 3.0 mm voxels; 336 volumes).

Data Processing and Analyses—Startle blinks were scored according to published guidelines (Blumenthal et al., 2005), and in an identical manner across the two studies. Data processing included applying a 28 Hz high-pass filter, rectifying, and then smoothing using a 40 Hz low-pass filter. Blink response was defined as the peak amplitude of EMG activity within the 20–150 ms period following startle probe onset relative to baseline. Each peak was identified by software but examined by hand to ensure acceptability. Blinks were scored as non responses if EMG activity during the 20–150 ms post stimulus timeframe did not produce a blink peak that was visually differentiated from baseline activity. Blinks were scored as missing if the baseline period was contaminated with noise, movement artifact, or if a spontaneous or voluntary blink began before minimal onset latency and thus interfered with the startle probe-elicited blink response. Blink amplitude values were used in all analyses. Consistent with prior studies (e.g., Gorka et al., 2013, 2016, in press), we created startle potentiation scores for the P- and U-threat conditions to account for baseline individual differences in startle amplitude. Specifically, for P-threat, we subtracted startle amplitude during N_{Cue} from P_{Cue} . For U-threat, we subtracted average startle amplitude during N_{Cue} and N_{ISI} from average startle amplitude during U_{CD} and U_{ISI} because both phases of the U conditions (and N conditions) had the same meaning during the task. Startle potentiation variables were normally distributed within each sample (all skew values < 1.2).

Like the startle data, fMRI data across the two studies were processed using identical steps implemented in Statistical Parametric Mapping software (SPM8, Wellcome Department of Imaging NeuroScience, London, UK). Images were spatially realigned to correct for head motion, warped to standardized Montreal Neurological Institute (MNI) space using the participants' mean functional image, resampled to 2 mm³ voxels, and smoothed with an 8 mm³ kernel to minimize noise and residual differences in gyral anatomy. The general linear model was applied to the time series, convolved with the canonical hemodynamic response function and with a 128 s high-pass filter. Condition effects were modeled with event-related regressors representing the occurrence of each anticipation phase (variable durations). Effects were estimated at each voxel and for each participant.

The two fMRI tasks differed in their design but both included anticipation phases for temporally uncertain threat. Therefore, to be consistent across the two studies, individual contrast maps (statistical parametric maps) for U-threat anticipation > fixation were created for each subject. It is necessary to highlight that anticipation phases of both fMRI tasks are

most analogous to the startle potentiation scores as they were collected during the anticipation of shock (Study 1) or pictures (Study 2), not during the actual receipt of shock/picture. For Study 1, at the first level, U-threat anticipation, P-threat anticipation, and no-threat anticipation were modeled separately. For Study 2, at the first level, U-threat anticipation, P-threat anticipation, U-neutral anticipation, and P-neutral anticipation were modeled separately. Parameter estimates were included as regressors in all first level models to account for motion.

To test our hypotheses, for both studies, contrast maps for U-threat anticipation > fixation and P-threat anticipation > fixation were entered into second-level, one-sample *t*-tests in SPM. This approach allowed for an examination of the main effects of the task, independent of startle potentiation. Next, individual U-threat startle potentiation values were entered as a regressor-of-interest for each U-threat model (Study 1 and Study 2). We considered activations that survived $p < 0.001$ (uncorrected), with a cluster extent threshold of greater than 20 contiguous voxels (volume > 160mm³), as significant. This threshold has been used by others (Banks et al., 2007; Labuschagne et al., 2010) but is considered lenient and we intentionally chosen to focus on replication rather than adopt conservative statistical threshold cutoffs.

We next extracted BOLD signal responses (i.e., parameter estimates, β weights [arbitrary units]) from 10mm (radius) spheres surrounding significant peak activations across models. Then, to test the relative specificity of the findings to U-threat, we explored whether the associations between U-threat startle and peak BOLD signal responses remained significant when controlling for startle potentiation to P-threat using partial correlation analyses. We also tested whether any of the U-threat findings were also observed during P-threat.

Results

Sample Descriptives

Descriptive information regarding participants is presented in Table 1. Mean startle amplitude for all task conditions is presented in Figure 2. The two samples were comparable in age, sex, and race/ethnicity. Despite the fact that both studies were designed to recruit individuals with a range of internalizing symptoms, there were important clinical differences between samples. For example, Study 1 included a greater proportion of individuals with current anxiety disorders whereas Study 2 included a greater proportion of individuals with depression. Study 2 also included a greater proportion of individuals with lifetime alcohol and substance use disorders relative to Study 1. Lastly, the two samples differed on use of psychotropic medication such that no individual in Study 1 was taking medication at the time of evaluation but a small percentage of individuals in Study 2 (10.5%) were taking medications.

Main Effects of fMRI Threat Tasks

Neural activation elicited by U- and P-threat for both tasks is presented in Table 2. Despite the fact that the two tasks used different forms of threat, activation patterns were remarkably consistent. For example, individuals exhibited robust occipital lobe, insula and hippocampus

activation during U-threat in both tasks. As for differences, individuals in Study 1, but not Study 2, notably demonstrated right amygdala activation during U-threat.

Whole Brain Association between Startle and fMRI Measures

Significant correlations between U-threat startle and U-threat neural activation for Study 1 and Study 2 are presented in Table 3. In Study 1, greater startle potentiation to U-threat was associated with greater dACC, right caudate, and right orbitofrontal cortex (OFC) activation (Figure 3). Importantly, these correlations were replicated in Study 2 such that greater startle potentiation to U-threat was associated with greater dACC, bilateral caudate, and mid OFC activation (Figure 3). In addition to the consistency of location, in both studies, the magnitude of the correlations between startle potentiation and neural activation were consistent (r s ranging from 0.33 to 0.46; see Figure 3).

Post Hoc Analyses

In order to test whether the above U-threat findings were better accounted for by broad threat responding we conducted a series of follow up analyses. First, we conducted partial correlations between startle to U-threat and neural activation during U-threat while controlling for startle to P-threat. For Study 1, results indicated that the U-threat startle and dACC ($r = 0.31$), right caudate ($r = 0.32$) and right OFC ($r = 0.43$) correlations remained significant. For Study 2, the U-threat startle and dACC ($r = 0.36$) and bilateral caudate ($r = 0.34$) remained significant; however, the U-threat startle and OFC correlation became non significant ($r = 0.18$) suggesting that the OFC finding was not specific to U-threat (i.e., independent of the effects of general threat responding). Second, we confirmed that startle to P-threat was not correlated with dACC, OFC, or caudate activation during U-threat (all p s > 0.15) or P-threat (all p s > 0.18).

Given that both samples included many individuals with DSM internalizing disorders, we also explored whether the associations between startle and neural responding during U-threat differed as a function of current MDD and/or anxiety disorder diagnoses using a series of hierarchical linear regression analyses. Two diagnostic variables were created – one reflecting current MDD (yes/no) and the other reflecting any current anxiety disorder (yes/no; PD, SAD, GAD, PTSD, or specific phobia). For each model, startle potentiation to U-threat, MDD status and anxiety disorder status were entered in Step 1. The two-way interactions between startle and depression, and startle and anxiety, were entered in Step 2 and the three-way interaction was entered in Step 3. Neural activation (dACC, OFC or caudate) was specified as the dependent variable. Across all models there were no two-way or three-way interactions indicating that the present startle-brain correlations did not differ between those with and without internalizing disorders (all p s > 0.55).

Discussion

Separate studies have shown that individuals with anxiety disorders display heightened startle potentiation (e.g., Shankman et al., 2013) and hyperactivity of the aINS and dACC during the anticipation of U-threat (e.g., Simmons et al., 2011); however, it has been unclear whether measures of startle potentiation and fMRI BOLD signal during U-threat are

correlated and thus reflect similar individual differences. To address this important gap in the literature, the current study investigated if and where in the brain startle potentiation and fMRI BOLD signal response were correlated during U-threat anticipation tasks in two independent samples. Results revealed that across all subjects, regardless of DSM diagnoses, individuals with greater startle potentiation to U-threat exhibited greater dACC, caudate and OFC activation to U-threat. These results were notably replicated in two samples using varying tasks, different forms of threat, and participants with differing types of psychopathology. The consistency of the findings is striking and strongly supports that startle and fMRI during U-threat capture overlapping individual differences.

Consistent with our hypotheses, startle and dACC reactivity to U-threat were positively correlated, which fits with prior studies and extant theory. As previously noted, various anxiety disorders have been associated with heightened startle potentiation to U-threat (Grillon et al., 2008, 2009; Lieberman et al., in press), and in separate studies, hyperactivity of the dACC during the anticipation of U-threat (Straube, Mentzel & Miltner, 2007); indirectly suggesting that individual differences in startle and dACC activity are related. In more direct support, our lab has recently shown that within individuals with a range of panic symptoms, startle potentiation to U-threat was positively associated with dACC reactivity to U-threat (Lieberman et al., in press). The current study corroborates this prior finding and extends the work by demonstrating the consistency of startle-dACC correlations across samples and varying threat tasks.

The dACC is a particularly interesting site of convergence given that there are several putative roles that the dACC may play in exaggerated responding to U-threat. First, the dACC has been implicated in the psychophysiological expression of aversive responding to threatening stimuli (Milad & Quirk, 2012; Hartley et al., 2011; Milad et al., 2007) and is speculated to play a modulatory role in startle reactivity and/or other autonomic defensive responses (e.g., skin conductance and heart rate) to U-threat. That is, individuals with greater dACC reactivity to aversive stimuli also appear to exhibit heightened defensive responding and relatedly, exaggerated anticipatory anxiety to U-threat. In addition to anxiety expression, the dACC has been implicated in threat appraisal, or the evaluation of the degree of danger associated with a stimulus or situation (Etkin, Egner & Kalisch, 2011; Maier et al., 2012). Greater dACC reactivity could therefore reflect exaggerated appraisal of U-threat, which may prime the defensive motivational system and potentiate the startle reflex.

Although not originally hypothesized as a site of overlap, current findings suggest that OFC reactivity to U-threat is also positively correlated with startle. The OFC is part of the AAN (Grupe & Nitschke, 2013) and implicated in responding to U-threat. OFC hyperactivity is also linked to several forms of psychopathology including obsessive-compulsive disorder (OCD) (Chamberlain et al., 2008; Evans, Lewis & Iobst, 2004; Milad & Rauch, 2007), which is characterized by high intolerance of uncertainty and maladaptive defensive responding (Chamberlain et al., 2007; Evans, Lewis & Iobst, 2004; Tolin et al., 2003). The broader literature indicates that the OFC contributes to the regulation of emotional responding to aversive stimuli and appraisals of threat salience (Holtz et al., 2012; Milad & Rauch, 2007), similar to the dACC. Interestingly, though, whereas the dACC finding was

relatively specific to U-threat, results suggest that OFC reactivity may have been more reflective of individual differences in general threat responding.

In addition to the dACC and OFC, results indicate that caudate and startle reactivity to U-threat are also related. The caudate is often activated in tasks involving conscious threat appraisals (see Mechias et al., 2010 for a review) and is posited to mediate emotional arousal (Colibazzi et al., 2010). Relatedly, there have been a few studies noting increased caudate activity during threat anticipation (e.g., Choi et al., 2012) and one study which found that caudate activation was positively correlated with heart-rate reactivity to social threat (Wager et al., 2009). Therefore, although not frequently considered part of the AAN or anxiety-related networks, the caudate has a clear role in threat responding and reactivity. In addition, the caudate is known to regulate the feedback loop between the OFC and thalamus (Hansen et al., 2002; Milad & Rauch, 2007) and may therefore be related to OFC reactivity to threat. Indeed, like the OFC, caudate hyperactivity is commonly implicated in the pathophysiology of OCD and it has been speculated that exaggerated caudate reactivity is related to maladaptive thinking styles during aversiveness (Milad & Rauch, 2012; Tolin et al., 2003). Together, this literature implies that the dACC, OFC and caudate are all regions implicated in threat responding and based on the present results, may be particularly important for reactivity to U-threat.

Although the current findings fit with the existing literature, we did not find in either study that startle reactivity to U-threat was correlated with amygdala or aINS reactivity, as we initially hypothesized. We did, however, find that that across all participants both threat tasks robustly elicited the aINS, and in Study 1 (the threat-of-shock task) participants also displayed right amygdala activation. These findings suggest that the aINS and amygdala are indeed engaged by U-threat but that activation in these regions does not necessarily correlate with the magnitude of startle potentiation to U-threat. The aINS and amygdala null findings have potentially important implications as to date much of the anxiety disorder literature has been focused on individual differences in aINS and amygdala reactivity (e.g., Gorka et al., 2014; Klumpp, Angstadt & Phan, 2012; Stein et al., 2007). The current findings suggest that although these are two important threat-related regions, we may also consider further investigating the dACC, OFC and caudate as potential anxiety disorder treatment targets, especially for those who display an exaggerated psychophysiological reactivity to U-threat.

The convergence of fMRI and startle during U-threat is important from a methodological standpoint but also has broader implications. First, the study supports one of the basic premises of the NIMH RDoC Initiative which is that two ‘units of analysis’ (i.e., the columns in the RDoC matrix) within the same construct/domain (i.e., potential threat) are related. This relation was not moderated by internalizing psychopathology suggesting that psychophysiology relates to neural functioning similarly across individuals. Second, given that heightened startle potentiation to U-threat, and hyperactivity of the dACC, caudate and OFC have each been implicated in various anxiety disorders, the pattern of responding identified in the present study may point towards a biobehavioral profile of aberrant affective responding that contributes to the pathogenesis of anxiety psychopathology. Future studies should continue to explore this brain-behavior profile as a transdiagnostic, anxiety prevention and/or intervention target.

The present study had many strengths but also some important limitations. First, fMRI and startle data was not collected simultaneously. Doing so may have revealed greater overlap between startle and neural reactivity to U-threat and this is an important avenue for future work. Second, both studies recruited individuals with and without psychopathology. Although this approach allowed for the necessary variability in psychophysiological responding, it is unclear whether the current findings would generalize to other populations (e.g., primary externalizing disorder patients). Third, both study sample sizes were relatively small and although the present results were replicated in a second study, the analyses were likely underpowered to detect associations with regions that did not have robust signals. Lastly, a subset of participants in Study 2 reported that the negative (and neutral) images presented during the fMRI task were positive in valence. It is unclear whether this reflects an error in self-report or if some participants actually appraised the negative stimuli as pleasant. Although the findings replicate across the two studies, it is possible that the Study 2 fMRI task did not robustly elicit aversive responding across all individuals and it is therefore necessary that future studies attempt to corroborate the current findings.

In sum, the current study suggests that two separate measures of sensitivity to U-threat, across two units of analysis, correlate such that individuals with greater startle potentiation exhibit greater dACC, caudate, and OFC activation. This study is one of the first in a series of investigations that are needed to fully understand the extent to which different psychophysiological methodologies converge in assessing core constructs as the field moves towards a more dimensional, multi-measure conceptualization of normal and abnormal affective processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institute of Mental Health (R01MH101497 [PI: Phan] and R21MH080689 [PI: Shankman]) and the Brain and Behavior Research Foundation (PI: Shankman). The funding sources had no role in the present manuscript.

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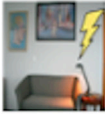







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A)

	Cue	ISI	Cue	ISI
Condition				
No-Shock (N)				
Predictable Shock (P)				
Unpredictable Shock (U)				

B)



Condition					
Predictable (P) Negative Image	1 Negative Image at 5	2 Negative Image at 5	3 Negative Image at 5	4 Negative Image at 5	
Unpredictable (U) Negative Image	1 Negative Image Anytime	2 Negative Image Anytime	3 Negative Image Anytime		1 Negative Image Anytime

Fig 1.
Illustration of the fMRI tasks administered in Study 1 (A) and Study 2 (B).

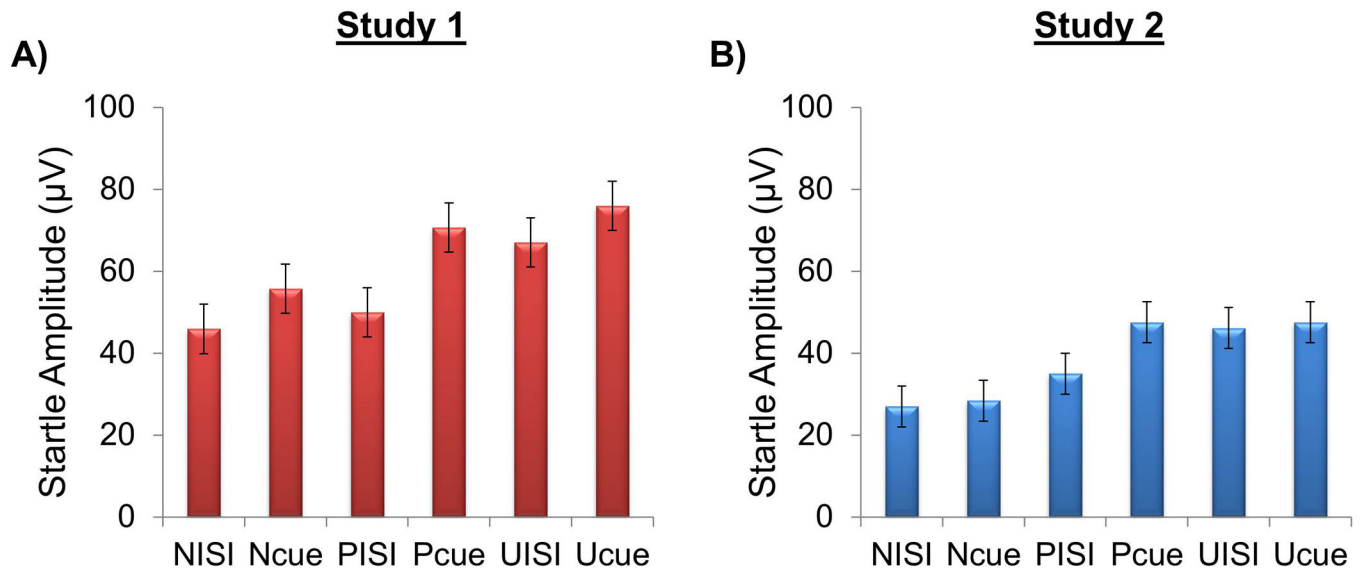
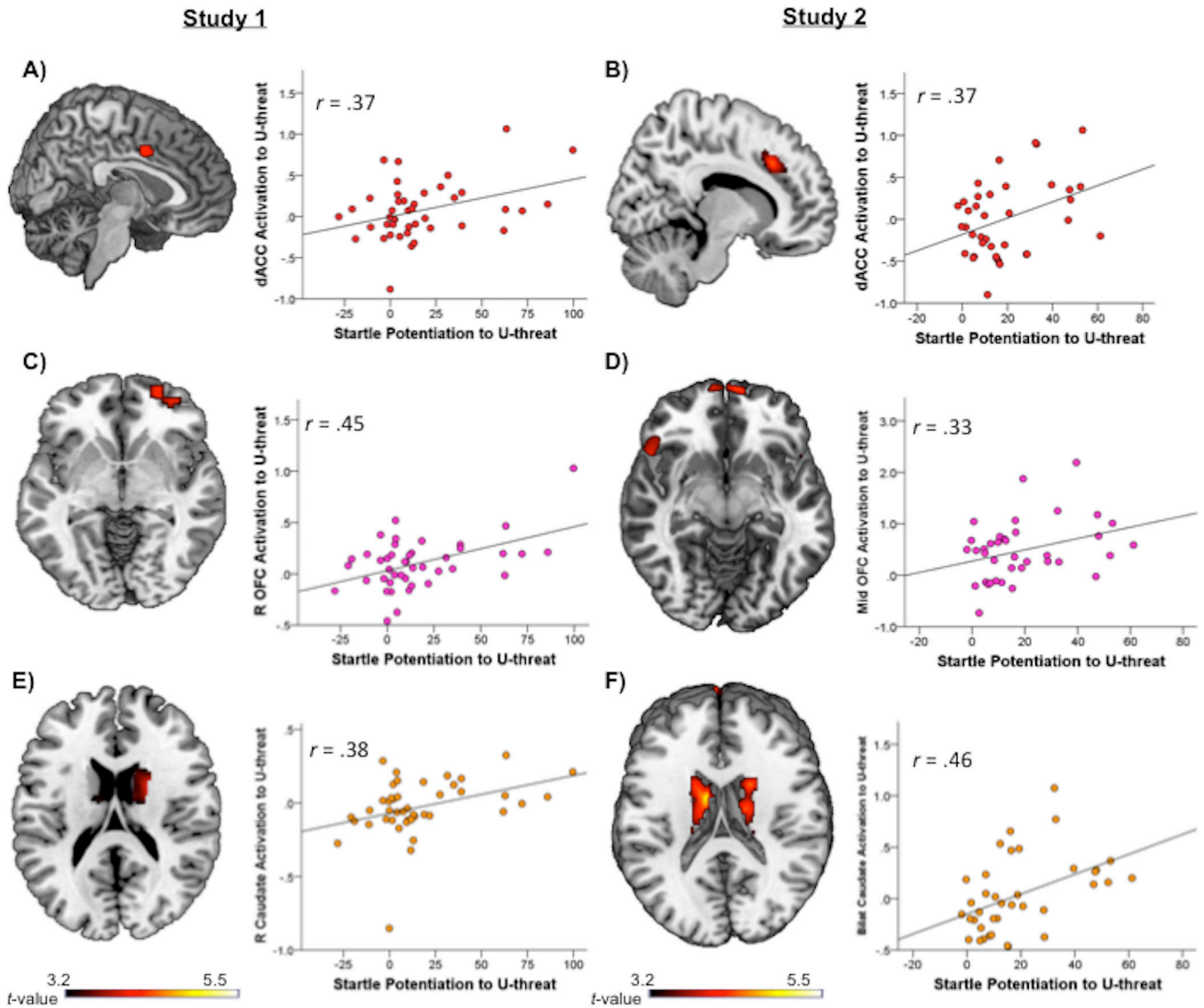


Fig 2. Mean startle amplitude for each of the startle task conditions in Study 1 (A) and Study 2 (B). N = no-shock; P = predictable shock; U = unpredictable shock; Cue = numeric countdown in Study 1 and geometric shape in Study 2; ISI = interstimulus interval. Bars reflect standard error.

**Fig. 3.**

Associations between startle potentiation to U-threat and neural activation to U-threat. All brains display voxel-wise statistical t -maps on canonical brains reflecting significant correlations at $p < 0.001$, uncorrected, and a cluster extent threshold greater than 20 contiguous voxels. Scatter plots reflect the relation between extracted BOLD parameter estimates and startle potentiation to U-threat. A) and B) depict significant startle and dACC correlations for Study 1 and Study 2. C) and D) depict significant startle and OFC correlations for Study 1 and Study 2. E) and F) depict significant startle and caudate correlations for Study 1 and Study 2. U-threat = unpredictable threat; dACC = dorsal anterior cingulate cortex; OFC = orbitofrontal cortex.

Table 1

Participant demographics and clinical characteristics

	Sample 1 (<i>n</i> =43)	Sample 2 (<i>n</i> =38)
Demographics	Mean (SD) or %	Mean (SD) or %
Age (years)	25.2 (7.6) _a	31.2 (11.9) _a
Sex (% female)	68.2% _a	70.7% _a
Race (% Caucasian)	61.9% _a	52.5% _a
Clinical Variables		
Current Major Depressive Disorder	36.6% _a	53.7% _b
Current Anxiety Disorder	58.1% _a	31.7% _b
Current Comorbid Anxiety Disorders	27.9% _a	0.0% _b
Current Comorbid Depression and Anxiety	44.2% _a	31.6% _a
Lifetime Major Depressive Disorder	51.2% _a	53.7% _a
Lifetime Anxiety Disorder	62.8% _a	31.7% _b
Lifetime Alcohol Use Disorder	9.3% _a	26.3% _b
Lifetime Substance Use Disorder	11.6% _a	31.6% _b
Taking Psychiatric Medication	0.0% _a	10.5% _b
Startle Variables		
Startle Potentiation to U-Threat	12.9 (22.1) _a	18.7 (19.4) _a
Startle Potentiation to P-Threat	16.4 (28.0) _a	19.0 (17.3) _a
IAPS fMRI Image Ratings		
Arousal (Mean; Range)		
U-threat Negative Images	-	4.9 (1.9); 1.0 – 8.9
U-threat Neutral Images	-	2.7 (1.7); 1.0 – 7.4
P-threat Negative Images	-	4.7 (1.9); 1.0 – 8.9
P-threat Neutral Images	-	2.8 (1.6); 1.0 – 7.2
Valence (Mean; Range)		
U-threat Negative Images	-	3.3 (1.4); 1.2 – 8.5
U-threat Neutral Images	-	5.8 (0.9); 4.0 – 8.1
P-threat Negative Images	-	3.3 (1.4); 1.0 – 8.4
P-threat Neutral Images	-	5.7 (0.9); 4.1 – 7.7

Note. Means (and standard deviations) or percentages with different subscripts across rows were significantly different in pairwise comparisons ($p < .05$, chi-square test for categorical variables and Tukey's honestly significant difference test for continuous variables). IAPS = International Affective Picture System.

Table 2

Main effect of the threat task, whole-brain corrected, for Study 1 and Study 2.

Region	MNI Coordinates			Volume (mm ³)	Z-score
	X	Y	Z		
<u>U-Threat > Fixation</u>					
Study 1					
Occipital Lobe	-12	-104	6	144640	7.56
L Orbitofrontal Cortex	-40	48	-8	7776	4.88
R Orbitofrontal Cortex	46	46	-6	3344	4.84
R Middle Frontal Gyrus	52	30	36	3816	4.08
L Hippocampus	-22	-26	-8	728	3.77
R Hippocampus	22	-26	-6	664	3.43
R Insula	52	12	0	4272	3.22
L Middle Frontal Gyrus	-50	4	50	208	3.10
R Inferior Parietal Lobe	58	-44	50	232	3.10
R Amygdala	34	-2	-16	848	3.09
<u>Study 2</u>					
Occipital Lobe	-12	-96	-10	143392	5.82
L Hippocampus	-24	-28	-4	6048	5.82
R Hippocampus	24	-28	-2	9304	5.77
R Orbitofrontal Cortex	46	34	-16	329680	5.16
L Orbitofrontal Cortex	-48	36	-16	319280	5.05
R Superior Frontal Gyrus	12	42	54	2160	3.50
R Insula	40	24	-2	800	3.49
L Superior Frontal Gyrus	-10	60	28	1560	3.45
R Inferior Temporal Lobe	54	6	-44	208	3.34
<u>P-Threat > Fixation</u>					
Study 1					
Occipital Lobe	28	-94	20	127064	6.16
L Middle Frontal Gyrus	-48	36	28	9776	4.50

Region	MNI Coordinates			Volume (mm ³)	Z-score
	X	Y	Z		
L Insula	-46	14	2	9624	3.88
R Insula	40	24	0	4360	3.75
L Hippocampus	-20	-30	-6	1344	3.73
L Superior Frontal Gyrus	-12	6	72	800	3.49
R Hippocampus	22	-26	-4	832	3.44
R Middle Frontal Gyrus	38	54	24	1240	3.25
Study 2					
R Insula	36	22	-18	28720	4.78
R Thalamus	6	-10	18	85232	4.23
R Superior Frontal Gyrus	6	30	54	3744	4.03
R Occipital Lobe	40	-68	-8	3488	3.48
L Insula	-28	20	-20	184	3.41
L Orbitofrontal Cortex	-46	36	-18	320	3.35
R Superior Frontal Gyrus	18	62	14	384	3.20
R Inferior Parietal Lobe	54	-52	50	800	3.20
R Superior Temporal Gyrus	58	-48	20	312	3.18

Note: Reporting of all significant clusters at $p < 0.001$, uncorrected, with a cluster extent threshold of k (number of contiguous voxels) > 20 . All associations are positive. L = Left; R = Right; MNI = Montreal Neurologic Institute.

Table 3
Neural regions during U-threat that significantly correlated with startle potentiation to U-threat

Region	MINI Coordinates			Volume (mm ³)	Z-score
	X	Y	Z		
Study 1: Positive Correlations					
L Occipital Lobe	-34	-90	24	170	3.94
R Middle Temporal Gyrus	36	-64	16	158	3.80
R Occipital Lobe	26	-80	20	126	3.68
<i>R Orbitofrontal Cortex</i>	32	48	-14	72	3.58
L Precentral Gyrus	-40	-14	30	108	3.57
R Precentral Gyrus	40	-10	30	63	3.49
R Precuneus	18	-60	56	136	3.47
R Superior Frontal Gyrus	20	66	2	57	3.46
<i>Dorsal Anterior Cingulate Cortex</i>	2	4	40	66	3.37
<i>R Caudate</i>	4	16	-4	22	3.25
Study 1: Negative Correlations					
No significant clusters					
Study 2: Positive Correlations					
L Inferior Parietal Lobe	-34	-38	46	917	4.71
L Middle Frontal Gyrus	-28	44	26	2196	4.63
L Fusiform Gyrus	-36	-16	-28	41	4.25
R Postcentral Gyrus	56	-30	40	466	4.11
R Fusiform Gyrus	42	-12	-22	306	4.09
R Superior Parietal Lobe	30	-54	64	265	3.98
L Superior Parietal Lobe	-28	-64	64	418	3.92
<i>R Caudate</i>	18	-6	26	121	3.86
R Superior Frontal Gyrus	30	50	20	89	3.86
<i>L Caudate</i>	-10	-2	16	98	3.72
Inferior Frontal Gyrus	52	16	-4	114	3.66
Supp. Motor Area	0	10	60	78	3.62

Region	MINI Coordinates			Volume (mm ³)	Z-score
	X	Y	Z		
<i>Orbitofrontal Cortex</i>	-8	64	-10	283	3.60
R Middle Frontal Gyrus	48	10	44	111	3.46
R Precuneus	6	-64	40	59	3.35
<i>Dorsal Anterior Cingulate Cortex</i>	-6	20	40	110	3.34
L Medial Frontal Gyrus	-14	36	32	29	3.33
<u>Study 2: Negative Correlations</u>					
No significant clusters					

Note: Reporting of all significant clusters at $p < 0.001$, uncorrected, with a cluster extent threshold of k (number of contiguous voxels) > 20 . L = Left; R = Right; MINI = Montreal Neurologic Institute.