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Anticipatory Threat Responding: Associations With Anxiety, Development, and Brain Structure

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ABSTRACT

BACKGROUND: While translational theories link neurodevelopmental changes in threat learning to pathological anxiety, findings from studies in patients inconsistently support these theories. This inconsistency may reflect difficulties in studying large patient samples with wide age ranges using consistent methods. A dearth of imaging data in patients further limits translational advances. We address these gaps through a psychophysiology and structural brain imaging study in a large sample of patients across the lifespan.

METHODS: A total of 351 participants (8–50 years of age; 209 female subjects; 195 healthy participants and 156 medication-free, treatment-seeking patients with anxiety) completed a differential threat conditioning and extinction paradigm that has been validated in pediatric and adult populations. Skin conductance response indexed psychophysiological response to conditioned (CS+, CS−) and unconditioned threat stimuli. Structural magnetic resonance imaging data were available for 250 participants. Analyses tested anxiety and age associations with psychophysiological response in addition to associations between psychophysiology and brain structure.

RESULTS: Regardless of age, patients and healthy comparison subjects demonstrated comparable differential threat conditioning and extinction. The magnitude of skin conductance response to both conditioned stimulus types differentiated patients from comparison subjects and covaried with dorsal prefrontal cortical thickness; structure–response associations were moderated by anxiety and age in several regions. Unconditioned responding was unrelated to anxiety and brain structure.

CONCLUSIONS: Rather than impaired threat learning, pathological anxiety involves heightened skin conductance response to potential but not immediately present threats; this anxiety-related potentiation of anticipatory responding also relates to variation in brain structure. These findings inform theoretical considerations by highlighting anticipatory response to potential threat in anxiety.

Keywords: Anticipation, Anxiety, Conditioning, Development, Extinction, Threat

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Translational theories link neurodevelopmental changes in threat learning to pathological anxiety (1–5). However, findings in patients inconsistently support these theories (3,6,7). Disconnect between theory and data may reflect difficulties in recruiting large patient samples, inconsistent methods across studies, and failure to examine wide age ranges within studies. Furthermore, few studies in patients relate physiology to brain measures, limiting translational advances across developmental stages. Here, we address these gaps by integrating psychophysiology with structural brain imaging to study threat learning in individuals with anxiety and healthy individuals spanning childhood, adolescence, and adulthood (n = 351; 8–50 years of age).

Threat learning encompasses conditioning and extinction. Conditioning is a highly conserved process through which a neutral stimulus becomes associated with a threat, such that subsequent encounters with the stimulus elicit anticipatory responding to the danger that might follow; extinction reflects the attenuation of conditioned threat responding when the stimulus no longer predicts the occurrence of threat (2,8–11). A core feature of pathological anxiety is an exaggerated fear of anticipated threats (12,13), and contemporary theories attribute anxiety to aberrant threat learning, which is conceptualized as rapid or exaggerated conditioning or impaired extinction (1,3,4,14). Other data suggest that developmental changes in threat learning contribute to the emergence of anxiety disorders in late childhood and early adolescence (2,5,15–18). Research guided by these theories aims to inform anxiety treatment (1,2,5,19–23).

While studies in nonhuman animals and healthy humans provide support for these theories (2,19,24,25), meta-analyses of studies comparing threat learning between healthy participants and participants with anxiety in both pediatric (6) and adult (3,7) samples yield mixed findings. Thus, all meta-analyses find evidence of perturbed threat learning but differ in the specific affected processes they

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identify. Such inconsistency generates a need for more data on associations among pathological anxiety, age, and threat learning (6,25,26). The current report addresses this need.

Paradigms model threat learning by pairing a neutral conditioned stimulus (CS) with an unconditioned threat stimulus (UCS) (11,27); with learning, the CS comes to elicit a conditioned response in anticipation of danger (9,13,14,28,29). Threat learning may therefore rely on both conditioned and unconditioned threat responding (30). While ample research focuses on anxiety-related differences in conditioned responding (3,6,7,13,31), fewer studies address aspects of unconditioned threat responding (13,31), particularly as such responding changes with development (25,32). Thus, comparing conditioned and unconditioned threat response among pediatric and adult anxiety patients and healthy volunteers addresses important gaps.

Finally, we extended insight from neuroanatomical data. Substantial research, particularly in healthy participants, links conditioned threat responding to structure and function in the prefrontal cortex (PFC), amygdala, and hippocampus (2,8,33–40). Moreover, other data suggest that age moderates the neural architecture of threat learning (2,25,41). However, no previous studies have related individual differences in brain structure to psychophysiological threat response measures that relate most strongly to anxiety disorders across age. We first identified such skin conductance measures and then identified their structural correlates. Finally, we evaluated the moderation of these associations between skin conductance response and brain structure by age and anxiety diagnosis.

To achieve these goals, we studied a large sample of children, adolescents, and adults with anxiety and similarly aged subjects without anxiety (n = 351). All participants completed a differential (i.e., involving both threat and safety learning) conditioning and extinction paradigm that had been previously validated in pediatric and adult populations (32,42); a subset (n = 250) completed structural imaging. Analyses proceeded in 3 stages, testing specific hypotheses arising from prior research. First, we tested anxiety and age effects on skin conductance response (SCR) indices of conditioning and extinction. Based on prior findings (3,6,7), we hypothesized that there are comparable differential conditioning and extinction effects in patients and comparison subjects but enhanced anxiety-related responding to both conditioned threat and safety cues during the task, in both youths and adults. Second, we examined response to unconditioned threat; given no prior reports of anxiety-related differences in UCS responding and the prominence of anticipatory fears in anxiety, we hypothesized that anxiety effects manifest more strongly in response to conditioned than to unconditioned threats. Finally, we examined correlations between brain structure (cortical thickness and gray matter volume [GMV]) and conditioned responding, and their moderation by anxiety and age. Given prior research on structure−SCR associations (19,36–38,40,43), we hypothesized that effects emerge in prefrontal regions as well as the amygdala and hippocampus. Primary hypothesis tests in all 3 areas considered SCR, given data on reliability (44), the ease with which SCR responses to the CS and UCS can be compared, and the availability of prior data on brain structure correlates of SCR. Secondary analyses examined anxiety and age effects on startle-probe–related electromyography (EMG) and self-reported fear.

METHODS AND MATERIALS

Participants

A total of 387 individuals underwent conditioning and extinction; analyses included n = 351 (Table 1 and Supplement), with 195 healthy participants (108 female subjects; 8–46 years of age) and 156 participants with anxiety (101 female subjects; 8–50 years of age) who did not differ in age, sex, or IQ, with all p values $>$.08. All participants were studied at the National Institute of Mental Health. Written informed consent was acquired from adult participants and from parents of youth participants, and written assent was acquired from youth participants for an institutional review board–approved protocol. Previously reported psychophysiology data for 162 participants (72 with anxiety, 90 healthy) (32,44) were combined with unpublished data for 189 participants to generate the sample (n = 351).

Anxiety Diagnosis. Psychiatric status was determined using structured interviews by trained clinicians. Pediatric patients met criteria for generalized anxiety, social anxiety, and/or separation anxiety disorder as the primary diagnosis and the presenting complaint for treatment. Adult patients were additionally eligible for panic disorder. Healthy participants were diagnosis free. See the Supplement.

Auxiliary analyses used standard anxiety symptom questionnaires. Youths and their parents completed the Screen for Child Anxiety Related Emotional Disorders (45), and adults completed the trait subscale of the State-Trait Anxiety Inventory (46). Data were combined by z scoring (see the Supplement).

Table 1. Sample Demographic and Clinical Characteristics[a]

Characteristic	Healthy Subjects, n = 195	Subjects With Anxiety, n = 156
Age, Years, Mean (SD)	21.46 (9.14)	19.78 (9.99)
IQ, WASI, Mean (SD)	114.30 (11.55)	114.31 (13.33)
Female, n (%)	108 (55.4)	101 (64.7)
Diagnosis, n (%)		
Generalized anxiety disorder	–	121 (77.6)
Social anxiety disorder	–	94 (60.3)
Separation anxiety disorder	–	29 (18.6)
Specific phobia	–	28 (17.9)
Panic disorder	–	11 (7.1)
Attention-deficit/hyperactivity disorder	–	5 (3.2)
Major depressive disorder	–	4 (2.5)
Selective mutism	–	2 (1.3)
Oppositional defiant disorder	–	1 (0.6)

WASI, Wechsler Abbreviated Scale of Intelligence.
[a]Total n = 351.

Threat Conditioning and Extinction Task

We used an uninstructed, differential threat learning task that had been previously shown to produce conditioning with acceptable dropout rates in youths and adults (18,26,32,42,44,47,48). In the task (Figure 1), photographs of 2 women displaying neutral expressions (49) served as CS+ and CS−. The UCS (presented at CS+ offset) was a 1-second presentation of the CS+ woman displaying fear co-occurring with a 95-dB female scream for all participants. The task involved 3 phases. During preconditioning, each CS appeared 4 times. During conditioning, each CS appeared 10 times; the CS+ was followed by the UCS with an 80% reinforcement schedule. During extinction, CSs each appeared 8 times. See the Supplement for additional details.

Psychophysiology. SCR was determined by the square-root-transformed difference in base-to-peak amplitude within 5 seconds after stimulus onset, in line with previous studies (32,35,44,47,50). Additionally, startle probes were delivered 5 to 6 seconds after stimulus onset, and response was measured using eye-blink startle EMG. Primary analyses used SCR; the Supplement provides EMG methods, results, and discussion on combining psychophysiology measures.

Subjective Fear Ratings. Before and following conditioning, and following extinction, participants rated their fear of the CSs using a 10-point Likert scale (1 = no fear, 10 = extreme fear) (32,47). These ratings complemented psychophysiological responses to the CSs.

Brain Imaging

Magnetic resonance images (1 mm^3) were collected for 250 of the participants (71%; 145 healthy [82 female subjects, mean age = 21.3 years]; 105 with anxiety [71 female subjects, mean age = 19.0 years]) in a separate visit. Data were processed using FreeSurfer (http://surfer.nmr.mgh.harvard.edu/). Analyses tested associations between structural imaging measures and psychophysiological indices and the moderation of these associations by anxiety and age using permutation tests (51). We considered whole-brain cortical thickness, using the threshold-free cluster enhancement statistic (52), and subcortical GMV. Magnetic resonance imaging data from 115 participants appear in previous reports that use different analyses (41,53). Analyses applied familywise error rate correction. See the Supplement for additional details.

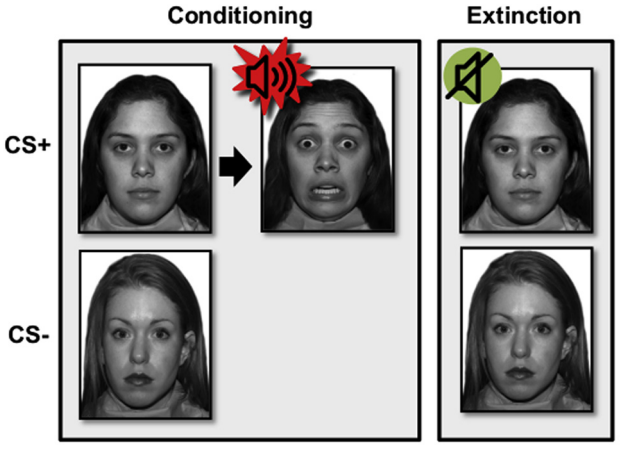

Figure 1. Schematic representation of the threat conditioning and extinction paradigm. During conditioning, one face (CS+) was repeatedly paired with a fearful face coterminating with a scream (unconditioned stimulus [UCS]); the other face (CS−) was never paired with the UCS. During extinction, both faces were presented in the absence of the UCS.

Data Analysis

First, we examined anxiety and age effects on differential threat conditioning and extinction through omnibus anxiety × age × phase × CS interactions on SCR to conditioned cues; trial-by-trial analyses complemented analyses on averaged SCR (11). In auxiliary analyses, EMG and self-reported data were analyzed in a similar manner. Second, effects on unconditioned responding were tested through the anxiety × age interaction on SCR to the UCS. Finally, we examined relationships between brain structure and SCR responses, with the primary analysis using the SCR measure that best differentiated healthy comparison subjects from patients, and moderation of structure−response relations by anxiety and age. To do so, we regressed SCR on cortical thickness and GMV measures. All analyses used general linear models, whereby anxiety status (with anxiety or healthy) was a between-subjects factor; age was a continuous covariate. Effect sizes are reported as η_p^2. All tests were 2-sided, and significance was set at $\alpha = .05$.

RESULTS

Response to Conditioned Cues

Skin Conductance Response. Averaged psychophysiological responding to conditioned cues by task phase is summarized in Figure 2A. Repeated-measures analysis of covariance testing the anxiety × age × phase × CS effect on averaged SCR yielded a significant phase × CS interaction, $F_{2,696} = 21.62$, $p < .001$, $\eta_p^2 = .06$, with follow-up paired-samples t tests indicating greater response to CS+ relative to CS− during conditioning, $t_{350} = 8.12$, $p < .001$, but not during preconditioning or extinction, p values $> .16$. This pattern indicates successful conditioning followed by extinction.

We also observed a main effect of anxiety on SCR, $F_{1,348} = 10.46$, $p = .001$, $\eta_p^2 = .03$, whereby patients exhibited greater mean response to the conditioned cues across the task relative to that of healthy control subjects. Further group comparisons indicated that patients generated stronger responses relative to that of control subjects to both CS− and CS+ in each task phase, and all p values were $< .032$.

Additionally, we noted a main effect of age, $F_{1,348} = 83.90$, $p < .001$, $\eta_p^2 = .19$, indicating decreasing response with increasing age. This effect was qualified by an age × phase × CS interaction, $F_{2,696} = 7.77$, $p < .001$, $\eta_p^2 = .02$. Follow-up analyses yielded a significant age × CS interaction during conditioning, $F_{1,348} = 27.91$, $p < .001$, $\eta_p^2 = .07$, indicating decreased differential conditioning with age but no age differences during preconditioning or extinction, p values $> .86$.

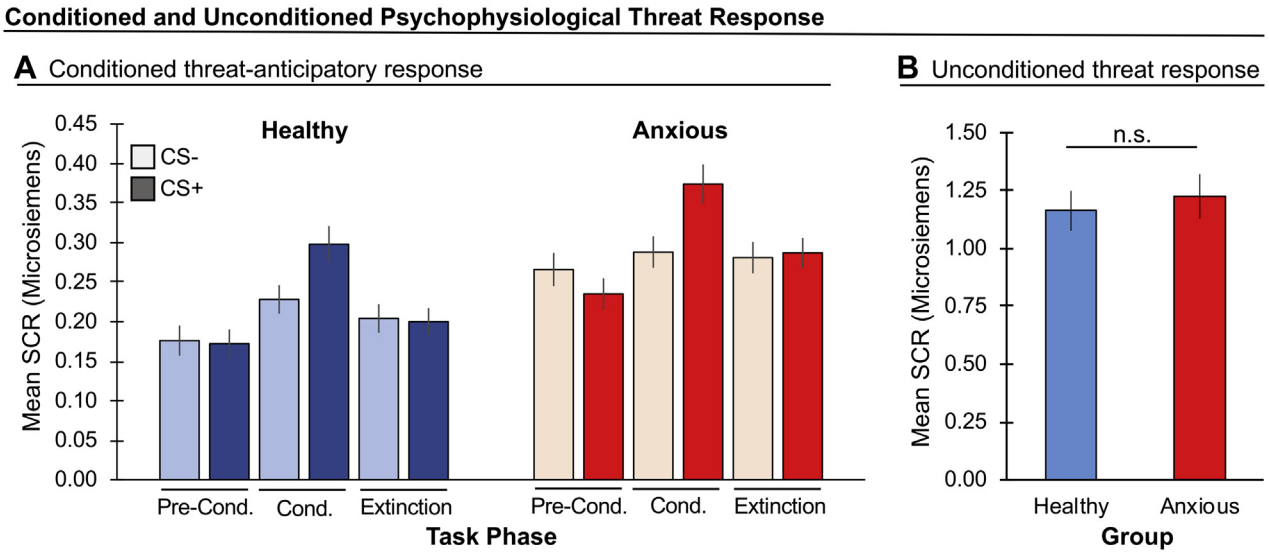

Figure 2. Conditioned and unconditioned psychophysiological threat response. **(A)** Conditioned skin conductance responses (SCRs) by stimulus type (CS−, CS+) averaged across each phase of the task (preconditioning, conditioning, extinction), by anxiety group (healthy or with anxiety). **(B)** Averaged SCR to the unconditioned stimulus by anxiety group (healthy or with anxiety). SCR data were square-root-transformed microsiemens. Error bars represent 1 standard error of the mean. Cond., Conditioning; n.s., not significant; Pre-Cond., preconditioning.

No significant anxiety interaction effects emerged, either across the task or separately during conditioning or extinction, indicating no difference in differential threat learning processes. Additional trial-by-trial SCR analyses are reported in the Supplement, indicating similar findings. A complementary dimensional analysis of anxiety-symptom severity indicated a significant positive association between symptoms and averaged SCR to the conditioned cues, $r = .14$, $p = .010$.

EMG analyses appear in the Supplement. These indicate two notable nonsignificant effects: phase × CS interaction ($p = .061$, CS+ > CS− only during conditioning and extinction) and anxiety main effect ($p = .090$, anxiety > healthy).

Self-reported Fear. Analyses of subjective fear responses are reported in the Supplement. Subjective fear paralleled SCR, demonstrating increased fear of CS+ relative to that of CS− following conditioning that was diminished following extinction. Moreover, fear of CS+ following conditioning correlated positively with the magnitude of SCR to CS+, supporting convergence of subjective and psychophysiological measures. Finally, as with SCR, we noted a main effect of anxiety on fear reports, indicating greater fear of conditioned cues throughout the task but no anxiety interaction effects.

Brain Structure Correlates. Since anxiety group differences emerged in SCR responding across CSs and task phases, averaged SCR across CSs and phases was used to index conditioned responding. Analyses tested the main effect of cortical thickness, as well as moderation by anxiety and age using the 3-way thickness × anxiety × age interaction, in predicting the magnitude of conditioned response. These analyses indicated a significant association between cortical thickness and conditioned response (controlling for age and anxiety) in a left-hemisphere cluster extending from the dorsomedial to dorsolateral PFC (Figure 3A, Table 2), whereby less thickness predicted greater conditioned response. Another cluster in the left retrosplenial cortex demonstrated a positive association between thickness and conditioned response. Furthermore, anxiety moderated the association between cortical thickness and conditioned responding in the bilateral ventral occipital cortex (Figure 3B) such that patients exhibited a more positive thickness–SCR association in this region. Finally, age moderated the thickness–response associations in several clusters (Figure 3C), including the bilateral posterior insula and temporal occipital cortex, right midcingulate cortex, and left middle-frontal gyrus. Analysis of GMV revealed age moderation in the bilateral hippocampus. The effect of age was consistent across all regions, such that among younger participants, thicker cortex or greater GMV was positively associated with conditioned responding, but with age this association became negative.

In summary, analyses of psychophysiological responses indicate comparable threat conditioning and extinction between patients and healthy control subjects. Compared with control subjects, patients demonstrated increased conditioned SCR responding to both CS− and CS+. The magnitude of this responding was inversely related to dorsal PFC thickness; anxiety and age moderation effects emerged in other cortical regions as well as bilateral hippocampus.

Response to UCS

Skin Conductance Response. Analysis of SCR to the UCS indicated comparable response to the unconditioned threat stimulus in the patient and healthy groups, $F_{1,348} = 0.24$, $p = .62$, $\eta_p^2 < .01$ (Figure 2B). We noted a significant main effect of age, $F_{1,348} = 34.29$, $p < .001$, $\eta_p^2 = .02$, indicating

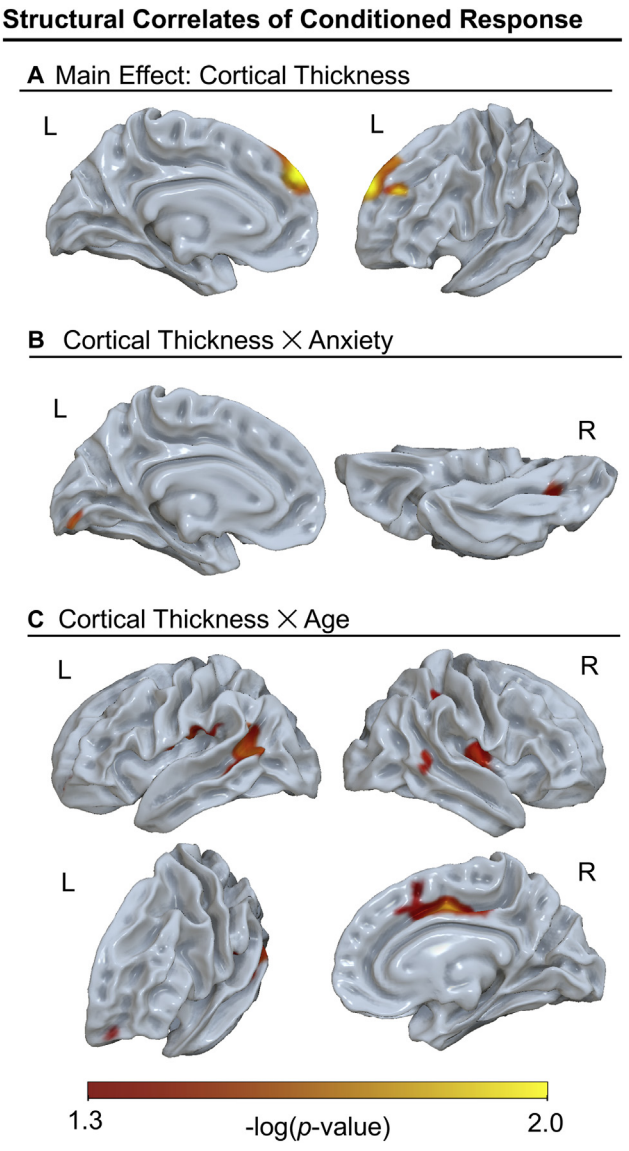

Figure 3. Brain structure correlates of psychophysiological response to conditioned cues. Result of analysis predicting individual averaged skin conductance responses to conditioned cues (CS−, CS+) across the task by cortical thickness, anxiety status (healthy or with anxiety), and age (in years). **(A)** Association between cortical thickness and conditioned response, controlling for anxiety status and age. **(B)** Moderation of association between cortical thickness and conditioned response by anxiety status. **(C)** Moderation of association between cortical thickness and conditioned response by age. Each surface's color reflects −log(*p* value) of the threshold-free cluster enhancement statistic; brighter colors represent stronger effects (threshold: $p_{FWE} < .05$). FWE, familywise error; L, left; R, right.

decreasing response with increasing age. Additional trial-by-trial analyses indicated diminishing unconditioned response across trials and with age (Supplement; Supplemental Figure S1B), but no anxiety effects. Of note, these analyses indicate that absence of anxiety effects on unconditioned responding is not due to ceiling effects.

An auxiliary analysis directly comparing averaged SCR to the conditioned cues with averaged SCR to the UCS within a single model yielded a significant stimulus × anxiety interaction, $F_{1,348} = 7.86$, $p = .005$, $\eta_p^2 = .02$, further indicating that the anxiety effect on response was specific to increased conditioned but not unconditioned responding. Dimensional analysis indicated that symptom severity and averaged SCR to the UCS were not correlated, $r_{319} = .03$, $p = .65$.

Self-reported Fear. Full statistics are provided in the Supplement. Subjective fear of the conditioned cues did not depend on the magnitude of unconditioned response to threat.

Brain Structure Correlates. Analyses of cortical thickness and GMV indicated no significant association, either direct or moderated by anxiety or age, between variation in brain structure measures and magnitude of unconditioned response.

In summary, the magnitude of unconditioned threat responses diminished with age. However, unlike anticipatory responses to the conditioned cues, response to the unconditioned stimulus did not differ as a function of anxiety, did not relate to conditioned subjective fear, and did not relate to variation in brain structure.

DISCUSSION

This study examined the associations that anxiety exhibits with conditioned and unconditioned threat responding across age. Three key findings emerged. First, as hypothesized, across age, patients with anxiety and healthy comparison subjects demonstrated comparable differential threat conditioning and extinction. Second, despite intact threat learning, the magnitude of conditioned SCR responding was greater to both CS− and CS+ in patients relative to that in healthy comparison subjects. The magnitude of such responding also covaried with subjective fear of the conditioned cues and brain structure in several hypothesized regions. Third, the magnitude of unconditioned psychophysiological responding did not relate to anxiety status, subjective fear of conditioned cues, or variation in brain structure. Together, these findings suggest that differential threat learning remains intact in pathological anxiety. Instead, anxiety involves heightened SCR to both CS+ and CS− but not UCS; the magnitude of such diagnosis-related SCRs also correlates with variation in brain structure.

This study is the largest single report comparing threat conditioning and extinction between patients with anxiety and healthy comparison subjects across development. The findings of comparable differential threat conditioning and extinction in patients and comparison subjects is consistent with prior meta-analyses (3,6,7). Thus, findings do not unequivocally support theories that relate anxiety to aberrant threat conditioning or extinction (3,4). Moreover, the use of a single, established paradigm informs theories on development and anxiety (2,6,25). Importantly, age did not moderate anxiety effects on these processes.

Instead, our findings highlight greater responding to both conditioned cues as differentiating participants with anxiety from healthy participants. This finding is consistent with previous findings generated in separate studies among youths

Table 2. Location, Peak Significance Level[a], and Size[b] of Clusters Showing Significant Associations Between Cortical Thickness or Gray Matter Volume and Magnitude of Conditioned Psychophysiological Response

Effect	Location	Peak *p* Value[c], FWE-Corrected	Cluster Size, No. of Vertices
Cortical Thickness	L dmPFC–dlPFC	.009	115
	L retrosplenial cortex	.021	27
Cortical Thickness × Anxiety	L visual association cortex	.029	16
	R visual association cortex	.039	15
Cortical Thickness × Age	R midcingulate cortex	.019	173
	L temporo-occipital cortex	.025	156
	L posterior insula	.025	146
	R posterior insula	.035	92
	R temporo-occipital cortex	.042	40
	R parieto-occipital cortex	.046	22
	L visual association cortex	.046	9
	L ventral medial frontal gyrus	.047	8
Gray Matter Volume × Age	R hippocampus	.017	–
	L hippocampus	.033	–

dlPFC, dorsolateral prefrontal cortex; dmPFC, dorsomedial prefrontal cortex; FWE, familywise error; L, left; R, right.
[a]$p_{FWE} < .05$.
[b]Number of vertices.
[c]For gray matter volume, *p* values refer to the entire structure. For cortical thickness, *p* values refer to the threshold-free cluster enhancement statistic.

and adults demonstrating anxiety-related enhancement of responding to CS−, CS+, or both (3,6,7). Greater response to CS− has been hypothesized to reflect anxiety-related aberrations in safety learning (54–56); greater response to CS+ has been hypothesized to reflect enhanced threat learning or reduced fear extinction (3,4,57). Both patterns emphasize some form of perturbed learning, and neither hypothesis is fully supported by our data. This finding calls for alternative explanations of the observed patterns.

A notable finding from the current study stems from comparing responses to the unconditioned stimulus. This finding indicates that enhanced SCRs in patients do not occur in response to the UCS, suggesting that there is perturbed anticipatory responding as opposed to acute-threat responding. This distinction could arise from differences in the function of conditioned and unconditioned responses to threat. Unconditioned threat stimuli signal unambiguous, immediate danger; as such, they elicit reflexive defensive responses that require minimal computation to execute (9,29). In contrast, conditioned stimuli predict only the potential for, as opposed to immediate occurrence of, danger; such prediction may be influenced by multiple processes that jointly estimate the probability, magnitude, or proximity of danger and accordingly influence adaptive defensive responding (55,58,59). Excessive conditioned responding may reflect perturbations in any of these processes, each involving biased threat estimates in response to any stimulus that predicts danger.

The uninstructed and probabilistic nature of the conditioning schedule used here may have led participants with anxiety relative to healthy participants to view both CS+ and CS− as conveying relatively high levels of danger, leading to greater anticipatory responses to both cues in patients (55,58). Such an effect was also observed prior to the presentation of the first UCS, whereby patients demonstrated greater responding to the initial face presentations during preconditioning (Supplement). As participants were aware of the aversive nature of the paradigm, patients may have shown increased anticipatory responding to the first stimuli presented in the task; this group difference diminished during preconditioning as the stimuli were continually nonreinforced.

Several brain structure correlates of anticipatory threat response were identified. Analyses specifically examined correlations with SCR measures that differentiated patients from healthy comparison subjects, to inform understanding of clinical psychophysiological correlates. Less left dorsomedial PFC and left dorsolateral PFC thickness was associated with greater anticipatory psychophysiological responding. Considerable functional imaging literature implicates these regions, particularly left-sided ones, in threat learning (33,34). Furthermore, other work suggests that altered function or structure in these regions contributes to maladaptive anticipation (55,60–62) and emotion regulation (63,64) processes. Our results bridge these findings, offering the possibility that dorsomedial PFC and dorsolateral PFC support effective regulation of anticipatory responses to potential danger. Indeed, preliminary findings from lesioned patients also support this possibility (65).

A positive association emerged between cortical thickness in left retrosplenial cortex and conditioned anticipatory response. The retrosplenial cortex is one of several regions implicated in threat conditioning (33), and it is suggested that it mediates the encoding of episodic or contextual memory of CS–UCS associations (66,67). Our results extend these findings by showing that structural variation in this region relates directly to the expression of conditioned psychophysiological responses indicative of diagnostic differences in physiology.

Additionally, the association between thickness in bilateral clusters in ventral visual association areas and anticipatory response varied with anxiety status. Prior research links structure and function in the occipital cortex to anxiety

disorder clinical features and treatment response (41,68–71). Reciprocal connections between the amygdala and visual cortex may account for such findings, as these connections are thought to facilitate the processing of biologically relevant stimuli in the context of threat conditioning (72–76). Our findings add to this literature, potentially linking patients' increased psychophysiological responding to visual threat stimuli to perturbations in cortical regions mediating visual processing. Additional research using conditioned stimuli of other modalities is needed to explore the specificity of this effect.

Age-dependent associations emerged between several structures and individual differences in conditioned responding. The consistent pattern of age moderation suggests that a group of regions may constitute a network supporting threat anticipation processes in ways that change with development. Broadly, other work finds these regions to show relatively protracted maturation with age (77,78). Some data suggest that the midcingulate cortex, particularly its anterior extent, acts as a key hub in networks mediating threat conditioning, modulation of negative affect, and anticipation (33,55,79). Consistent with these prior findings, our data may suggest that the midcingulate cortex supports anticipatory responding to threat. Similarly, prior functional and structural imaging work relates posterior insula to threat conditioning (33,37); this region has also been linked to the integration of interoceptive information (80). Given such prior work, our data also implicate the posterior insula in conditioned anticipatory preparation for harm. Finally, associations between GMV and anticipatory responding were also observed in bilateral hippocampus, a structure implicated in threat learning processes, potentially via context representation (33,39,81). As thinning in these regions is associated with greater anticipatory response, it is possible that some of their functions are regulatory, involving integration of somatic, affective, and contextual information. Nevertheless, it is important to emphasize that brain structure might not directly map onto function (82); thus, inferences on functional roles for these regions are limited. Longitudinal studies focusing on both the structure and function of these regions are necessary to more completely understand age moderation of associations among brain structure, function, and conditioned response to threat.

Of note, our findings suggest that neither aberrant differential conditioning nor extinction exhibits strong, direct associations with pathological anxiety across development. Nevertheless, it remains possible that more-nuanced anxiety differences in differential learning of anticipatory threat responses exist. One possibility is that analytical challenges in capturing dynamic learning processes mask such subtle differences. Methods that directly model associative learning processes (14,83,84) may be more powerful in identifying such differences. Alternatively, it has been suggested that other effects that derive from threat learning, such as tests of extinction recall or generalization of learned threat, may better capture anxiety deficits (19,26,56,85,86). Such effects may also reflect the elicitation of anticipatory responses (e.g., to generalized stimuli) and could prove valuable avenues for research linking anxiety, anticipation, and response to learned threat.

Exaggerated fear of potential danger is a core feature in the presentation of anxiety symptoms. Here, we identify a potential psychophysiological correlate of this maladaptive anticipatory fear response. Importantly, the magnitude of the anticipatory response differentiated between patients and healthy comparison subjects but also correlated with reported fear of the conditioned cues, thereby linking psychophysiological and subjective fear responses to potential threat. As such, this paradigm provides an experimental setting primed for uncovering the nature of associations among anticipatory psychophysiological responding, subjective fear, and anxiety symptoms. Follow-up studies could use repeated assessments of conditioned fear alongside anxiety ratings embedded in the threat learning paradigm to examine how anticipatory psychophysiological responses and subjective fear might interact to contribute to the experience of anxiety symptoms (10,26,87–89).

Along these lines, identifying a psychophysiological correlate of a pathological process in anxiety could potentially inform treatment development (5,90). For example, increased anticipatory psychophysiological response could serve as a specific target for interventions, such as particular forms of cognitive behavioral therapy and biofeedback techniques, that aim to directly reduce physiological arousal. Future research could explore whether neuroscience-guided interventions, such as brain stimulation methods and neurofeedback (91,92), could potentially downregulate neural processes mediating anticipatory responses or upregulate regulatory processes. Given the absence of anxiety differences in response to the unconditioned acute-threat stimulus, psychotherapy and cognitive behavioral therapy might focus on addressing anticipation-focused cognition and somatic responses. Additional research could further explore whether the magnitude of anticipatory psychophysiological response could serve as a biomarker for anxiety treatment outcome.

Several limitations should be acknowledged. First, this was a cross-sectional study, limiting the extent of inference about causality; a longitudinal design would allow stronger inferences about developmental and causal processes (93). Second, this study was not designed to directly link individual differences in threat learning and treatment outcome, thus limiting the scope of therapeutically relevant inference. Third, establishing baseline (11) for SCR to UCS is inherently challenging because of potential anticipation effects once associations have been learned. Here, CS+ and UCS events were separated by an adequate duration, as recommended (11); nevertheless, future research should consider this issue. Fourth, since brain structure variably maps onto function (82), inference on the functional role of identified brain regions is limited. Fifth, we measured both SCR and EMG; the use of multiple psychophysiological indicator variables could interfere with their indexing of the target processes (94). Sixth, we used a paradigm that is well suited for developmental research but uses a preset volume level for all participants; this limits comparison with prior studies in adults in which UCS aversiveness was set individually.

Several strengths mitigate these limitations and address general shortcomings in threat learning research (11,27). First, the large sample size increases precision in the estimates of associations (95). Second, participants were carefully assessed and free of medications known to impact threat learning and psychophysiology (11). Third, a wide age range

generates inferences on age differences with reasonable statistical power. Finally, task and setting were identical for all participants, reducing measurement confounds and noise.

In summary, the current study examined associations among conditioned and unconditioned responses to threat, anxiety, and age. Our findings highlight anticipatory threat responding as differentiating between patients and healthy control subjects and identify brain structure correlates of this response. These findings may bear implications for our conceptualization of anxiety and its treatment and study.

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ARTICLE INFORMATION

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Anticipatory Threat Responding: Associations with Anxiety, Development, and Brain Structure

Supplemental Information

Supplemental Methods

Participants

Prior to participation in the study, patients' psychiatric symptoms and commitment to seeking treatment were assessed on three separate occasions, via (1) telephone screen with a psychiatric nurse, (2) in-person, standardized diagnostic assessment (see below) with a trained clinician, and (3) independent assessment and confirmation of diagnosis by a senior psychiatrist. All patients agreed to enter treatment for their anxiety disorder; as such, the patient data reported reflect populations of youth and adults with anxiety in need of treatment.

Individuals were included if they were medication-free, physically healthy, and had an IQ>70, based on the Vocabulary and Matrix Reasoning subscales of the Wechsler Abbreviated Scale of Intelligence (1). A primary diagnosis of major depressive disorder, bipolar disorder, obsessive compulsive disorder, disruptive mood dysregulation disorder, or posttraumatic stress disorder was exclusionary. Patients with anxiety were permitted to have comorbid additional anxiety disorders or attention-deficit/hyperactivity disorder if presenting as a secondary, minor problem, relative to the primary diagnosis (see Table 1 in main text). Healthy participants were diagnosis-free. Exclusion criteria for both groups included current psychotropic medications, inclusion of family relatives in the study, physical health problems, or contraindications for neuroimaging.

Data from 32 participants were excluded due to aborting the task (22 anxious, eight healthy) or technical problems (one anxious, one healthy). Data from 4 additional participants (2 anxious, 2 healthy) were excluded from analyses because they inquired and were then informed of the CS contingencies prior to the conditioning phase (2).

Anxiety diagnosis

Diagnosis of an anxiety disorder was determined by trained clinicians using the Kiddie Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (3) for youths (age <18 years) and the Structured Clinical Interview for DSM-IV-TR Axis I Disorders (4) for adults (age ≥18 years). All clinicians were trained on an initial series of recorded interviews and then were regularly monitored through a review of interview tapes and reassessments of patients.

Anxiety symptoms

Some analyses were repeated using anxiety symptom severity scores instead of a categorical anxiety status variable to examine dimensional associations with other factors. Youth completed the Screen for Child Anxiety Related Emotional Disorders (SCARED) while adults completed the State-Trait Anxiety Inventory (STAI), both standard, age-appropriate measures of trait anxiety symptoms. These data were collected within 3 months of the task, and were available for 325 of the participants, as detailed below.

Screen for Child Anxiety Related Emotional Disorders (SCARED). The SCARED is a child- and parent-report measure comprising 41 items assessing recent anxiety symptoms (past 3 months) rated on a 3-point Likert scale(5,6) Item scores are summed to a total score (range: 0-82).

The SCARED possesses strong psychometric properties (5,6). To reduce informant discrepancies (7), child- and parent-report scores were averaged. Data for 177 youths (93 anxious, 84 healthy) were available. Anxious (M=31.7, SD=11.4) and healthy (M=6.8, SD=4.4) youth significantly differed in total SCARED scores, $t(146)=14.2$, $p<0.001$.

State-Trait Anxiety Inventory (STAI). The trait subscale of the STAI (8) was used, consisting of 20 items relating to general anxious moods answered on a 4-point scale. Item scores are summed to a total score (range: 20-80). The STAI possesses strong psychometric properties (9). Data for 148 adults (52 anxious, 96 healthy) were available. Anxious (M=50.0, SD=12.0) and healthy (M=28.4, SD=6.5) adults significantly differed in total STAI scores, $t(175)=18.7$, $p<0.001$.

Combined symptom severity score. Given that youth and adult participants completed different anxiety measures, we Z-transformed each of the two measures within the sample who completed it (i.e., SCARED scores were Z-transformed across the entire youth sample; STAI scores were Z-transformed across the entire adult sample). These Z-scores were then combined across samples and used in dimensional analyses of anxiety.

Threat conditioning and extinction task

A schematic representation of the threat conditioning and extinction task is provided in Fig 1. The task consisted of a pre-conditioning phase, a conditioning phase, and an extinction phase. Each conditioned cue (CS+ and CS-) was presented for 7s during conditioning, and 8s during pre-conditioning and extinction. The unconditioned stimulus (UCS) was a 1s presentation of the actress designated as CS+ displaying fear and co-occurring with a 1-second 95dB female scream delivered via headphones and presented at CS+ offset. Participants were instructed that they could learn to predict when the UCS would occur, but they were not explicitly informed of the

contingency. Throughout all phases, presentation order of the CSs and an inter-trial interval (a gray screen presented for 8-21s, averaging 15s) was pseudo-randomized (two different orders counterbalanced across participants). The conditioning and extinction phases were separated by a 5-to-10-minute break during which the participant rested and reported fear of each stimulus. All task phases were completed in the same experimental room. The task was programmed and administered (including psychophysiological data collection) using PsyLab psychophysiological recording system (PsyLab SAM System, Contact Precision Instruments, London). Of note, as in prior administration of this task, the volume level for the scream sound was not calibrated individually as is typically done in studies that use stimuli such as electric stimulation as an unconditioned stimulus. Thus, the current study used an identical UCS across subjects whereas many prior studies used distinct, individually-tailored UCS. Future studies using the paradigm in the current study may wish to incorporate such calibration in order to reduce inter-subject variability in perceived aversiveness (for example, due to individual differences in auditory sensitivity). However, this approach is complicated by habituation to the UCS; employing a pre-conditioning calibration procedure could significantly attenuate the aversiveness of the UCS and reduce the capacity for the procedure to induced conditioned fear.

Of note, while the face stimuli in the task may be considered “social”, this task is not considered an observational conditioning task. This is because all participants personally experience the aversive unconditioned stimulus, as opposed to merely observing another person’s response to experienced aversive stimuli (10).

In light of prior research, participants were not removed from analyses if they exhibited relatively low psychophysiological responses during the task (11). Five participants (3 anxious, 2 healthy) were considered as non-responders (did not show any skin conductance response [SCR]

to any UCS or any CS presentation during conditioning); results did not change when these participants were excluded, and as such we retained them in analyses.

Skin conductance was recorded at 1,000Hz from two Ag/AgCl electrodes from the medial phalanx of the middle and ring non-dominant-hand fingers. In addition to skin conductance, eye-blink reflex startle electromyography (EMG) and electrocardiography (ECG) were recorded. ECG data were not analyzed. EMG task data for 306 participants were available for analysis; data were not available for the remaining subjects due to technical issues. Analyses in the main manuscript focus on SCR as opposed to startle response. This decision reflected three factors, which led us to expect SCR to best support tests of hypotheses arising from three study aims. First, in our threat learning work, we find stronger reliability for SCR than EMG measures (12); since reliability impacts a study's ability to detect between-group differences, this led to a prioritization of the SCR data. Second, we also were particularly interested in contrasting the response to conditioned and unconditioned stimuli. For SCR, this is a simple issue, since SCR is reliably evoked in direct response to the stimuli presented in the paradigm (CS or UCS). In contrast, startle response requires a secondary stimulus to assess potentiation; thus, while SCR is directly evoked by a primary stimulus, startle potentiation to the primary stimulus is assessed by evoking startle with a secondary, air-puff probe stimulus. This secondary, air-puff probe stimulus cannot be delivered with sufficient proximity to briefly-presented stimuli, such as the unconditioned stimulus in the current study. Thus, unlike SCR which is quantified in a similar manner to the CS and UCS, startle potentiation to the UCS could not be quantified in the same manner as startle potentiation to the CS. Third, considerable prior research links SCR in the context of threat learning to brain structure (e.g., 13,14,15); no such research uses startle response. As a result, we could place our SCR results in a broader context of other, similar studies.

For startle response, startle probes (i.e., 40ms, 4-10 psi of compressed air delivered to the forehead) were presented during the CS trials (5-6 seconds post-stimulus onset; i.e., outside the SCR window) and during the inter-trial interval (ITI). At the start of the task, six startle probes in the absence of any stimuli were presented to habituate response to the probes and were not included in analyses. Recording and pre-processing follow our previous work (12,16). Startle response was measured using two 6mm tin cup EMG electrodes filled with standard electrolyte solution placed under the participant's left eye. A ground electrode was attached to the participant's left forearm. EMG data were recorded using a sampling rate of 1000Hz and filtered using an amplifier bandwidth of 30-500Hz. EMG data were rectified and smoothed using moving averages with 20ms windows. The EMG response to the startle probe during each CS+, CS-, and ITI was calculated as the difference between the peak EMG response (within 150ms following the startle probe) and the baseline activity (50ms prior to the startle probe). In a similar fashion as SCR, averaged startle response by CS type was calculated separately for each task phase. Averaged response for CS- and for CS+ was divided by the average response during the ITI for that phase to diminish inter-subject variability.

It should be noted that using multiple psychophysiological measures, and particularly SCR and EMG, requires some consideration. On the one hand, divergent findings suggest that these measures may reflect different processes associated with threat learning. Thus, including assessments of multiple measures provides a more comprehensive physiological assessment (17). On the other hand, evidence suggests that the presence of startle probes interferes with threat conditioning as measured by SCR (18). While our findings indicate strong conditioning effects as well as comparable baseline response to startle probes across anxiety groups (see Results), future research should nevertheless consider potential interacting effects in task design.

Analysis. Extant evidence is not consistent in terms of identifying developmental periods that are particularly sensitive for threat learning processes (19). Since it is not known *a-priori* when specific developmental effects emerge, we used age as a continuous measure so that effects could be detected anywhere along the age continuum, as opposed to using multiple categorization schemes and thus multiple tests. Analyses on SCR and EMG data were conducted using standard Repeated-Measures ANOVA commands in SPSS 25 (Armonk, NY: IBM Corp.).

Imaging data processing and analysis

All participants underwent MRI scanning acquired on a 3-Tesla MR750 GE scanner with a 32-channel head coil at the National Institute of Mental Health Functional Magnetic Resonance Imaging Core Facility. Participants completed a high-resolution, T1-weighted magnetization-prepared rapid-conditioning gradient-echo scan (MPRAGE) with the following parameters: sagittal conditioning; 176 slices; 256x256 matrix; 1mm^3 isotropic voxels; flip angle = 7°; repetition time (TR) = 7.7ms, echo time (TE) = 3.42ms. Imaging was conducted within 90 days of the task.

Image Processing. To facilitate reproducibility, a summary of the analysis pipeline, including commands used and references to documentation for custom functions, is detailed at the end of this document. Scans were analyzed with FreeSurfer (version 5.3.0, http://surfer.nmr.mgh.harvard.edu). Surface-based analysis followed the procedures in Fischl and Dale (20) and Dale et al. (21). T1-weighted images were corrected for magnetic field inhomogeneities, affine-registered to the Talairach-Tournoux atlas (22), and then skull-stripped. White matter (WM) voxels were identified based on their locations, their intensities, and the intensities of neighboring voxels, and grouped into a mass of connected voxels using a six-neighbor connectivity scheme. A mesh of triangular faces was the constructed using two triangles

per exposed voxel face. The mesh was next smoothed based on local intensity in the original images using trilinear interpolation (23); a second smoothing iteration was then applied, resulting in a realistic representation of the interface between grey and white matter. The external cortical surface was produced by identifying a point where tissue contrast is maximal, maintaining constraints on smoothness and possibility of self-intersection (20).

The subcortical volume-based analysis stream is designed to automatically preprocess MRI volumes and label subcortical tissue classes (24,25). First, images were affine-registered to MNI305 space. Next, initial volumetric labeling was conducted and variation in intensity due to the B1 bias field was corrected. Finally, a high-dimensional nonlinear volumetric alignment to the MNI305 atlas was performed, and structures were labeled. These included left and right amygdala, hippocampus, midbrain, thalamus, caudate, putamen, pallidum, and nucleus accumbens. The permutations tests corrected for the number of structures tested (see below).

Bias-corrected images from FreeSurfer were segmented into gray matter, white matter, and cerebrospinal fluid using the FAST module of FSL. The outputs of FAST are images in which the value at each voxel corresponds to the proportion of the volume of the voxel that is occupied by each of these tissue classes (26). We tested for effects on gray matter volume (GMV).

Of note, our sample features a wide age range (8-50 years), and the brain develops with age; this could have potentially impacted the accuracy of alignment and registration into common space. However, these processes rely on gray and white matter tissue contrasts, and while such contrasts show substantial developmental trajectories until the age of 2 years, they thereafter remain stable through adulthood (27). In addition, FreeSurfer uses surface-based algorithms which have been shown to produce superior registration to volume-based algorithms in data from children as young as 4 years old (28). Finally, we visually inspected the FreeSurfer outputs.

Analyses. Statistical significance was assessed using permutation tests. Family-wise error (FWE) rate correction controlled for multiple testing across surfaces (cortical thickness) or structures (GMV) and the number of effects tested, and all reported *p*-values reflect corrected values. All statistical tests were performed using PALM (Permutation Analysis of Linear Models; 29), based on 1000 permutations, followed by an approximation to the tail of the permutation distribution of the maximum statistic using a generalized Pareto distribution(30). For each morphometry measure, analyses of volumes included global whole-brain estimates of the measure as nuisance, as recommended in prior research (31). Thus, for subcortical GMV, we controlled for total intracranial volume; for cortical thickness, we controlled for global average thickness. Sex was also used as a nuisance variable. For the cortical analysis, only the surface vertices that represent actual cortex were included, masking out a sub-callosal region of each hemisphere that is included in the surfaces only to ensure the topology of a sphere. Results were visualized using the tool Surf Ice (https://www.nitrc.org/projects/surfice/).

Supplemental Results

Response to conditioned cues

SCR. To further delineate patterns of conditioned responding, we examined Anxiety×Age×CS×Trial interactions on SCR separately in each task phase. While our focus was on conditioning and extinction processes, we first examined differences in initial habituation in responding to the novel stimuli in the task as reflected in responses during the pre-conditioning, habituation phase. A repeated-measures ANCOVA on pre-conditioning SCR revealed a significant Anxiety×Trial interaction indicating differential habituation by group. Follow-up group comparisons, controlling for age, revealed greater responding in patients relative to healthy controls during the first two presentations of the (yet-to-be conditioned) CS- and CS+, $F(1,347)$s≥5.67, ps≤0.018, $\eta_p^2 s$≥0.02; however, by the last two presentations, response levels were not significantly differ between the groups, $F(1,347)$s≤2.32, ps≥0.13, $\eta_p^2 s$≤0.01. Thus, the increased average response among patients during this phase was driven primarily by initial increased responding to the novel stimuli; however, the conditioning phase started with comparable levels of psychophysiological responding.

Trial-by-trial SCR data by CS type and anxiety group for the conditioning and extinction phases are presented in Fig. S1A. For the conditioning phase, we noted a main effect of Trial (increasing response across trials), $F(9,3132)=15.36$, $p<0.001$, $\eta_p^2=0.04$, and a main effect of CS (greater response to CS+ relative to CS-), as reported above. In addition, we noted a main effect of Age (decreasing response with age), $F(1,348)=72.54$, $p<0.001$, $\eta_p^2=0.17$, and a main effect of Anxiety (anxious > healthy), $F(1,348)=5.36$, $p=0.021$, $\eta_p^2=0.02$. These effects were qualified by several significant two-way interactions: a CS×Trial interaction, $F(9,3132)=18.14$, $p<0.001$,

η_p^2=0.05, a CS×Age interaction, F(1,348)=27.91, p<0.001, η_p^2=0.07, and a Trial×Age interaction, F(9,3132)=4.24, p<0.001, η_p^2=0.01. These effects were further qualified by a CS×Trial×Age interaction, F(9,3132)=5.30, p<0.001, η_p^2=0.02. No other significant effects were observed for the conditioning phase.

For the extinction phase, we observed a main effect of Trial (decreasing response across trials), F(7,2436)=13.10, p<0.001, η_p^2=0.04, a main effect of Age (decreasing response with age), F(1,348)=70.63, p<0.001, η_p^2=0.17, and a main effect of Anxiety (anxious > healthy), F(1,348)=10.69, p=0.001, η_p^2=0.03. These effects were qualified by a significant Trial×Age interaction effect, F(7,2429)=4.59, p<0.001, η_p^2=0.01. No other significant effects were noted, including no Anxiety interaction effects.

We also tested the Anxiety×Age×Trial interaction on SCR separately in CS+ and CS- trials. For the conditioning phase, analysis of CS+ resulted in significant effects of Trial, F(9,3132)=21.20, p<0.001, η_p^2=0.06, Age, F(1,348)=78.00, p<0.001, η_p^2=0.18, Anxiety, F(1,348)=5.14, p=0.024, η_p^2=0.02, and Trial×Age, F(9,3132)=6.59, p<0.001, η_p^2=0.02. Analysis of CS- resulted in similar findings, with significant effects of Trial, F(9,3132)=11.54, p<0.001, η_p^2=0.03, Age, F(1,348)=54.10, p<0.001, η_p^2=0.14, Anxiety, F(1,348)=4.68, p=0.031, η_p^2=0.01, and Trial×Age, F(9,3132)=2.65, p=0.005, η_p^2=0.01. For the extinction phase, analyses of CS+ and CS- data yielded similar results. For CS+ trials, we noted significant effects of Trial, F(7,2436)=7.21, p<0.001, η_p^2=0.02, Age, F(1,348)=64.72, p<0.001, η_p^2=0.16, Anxiety, F(1,348)=11.19, p=0.001, η_p^2=0.03, and Trial×Age, F(7,2436)=2.69, p=0.009, η_p^2=0.01. For CS- trials, we observed significant effects of Trial, F(7,2436)=7.98, p<0.001, η_p^2=0.02, Age,

$F(1,348)=60.13$, $p<0.001$, $\eta_p^2=0.15$, Anxiety, $F(1,348)=7.89$, $p=0.005$, $\eta_p^2=0.02$, and Trial×Age, $F(7,2436)=2.13$, $p=0.037$, $\eta_p^2=0.01$. No other effects were noted.

Startle response. In a parallel manner to the primary SCR analyses, we examined differences in startle response during the presentation of the CS- and CS+ by testing the Anxiety×Age×Phase×CS interaction on averaged startle response. Startle response data by anxiety, task phase, and CS are presented in Fig. S2. This analysis resulted in a trend-level non-significant Phase×CS interaction effect, $F(2,606)=2.81$, $p=0.061$, $\eta_p^2=0.01$, with follow-up analyses indicating greater response to CS+ than to CS- during conditioning, $F(1,303)=4.60$, $p=0.033$, $\eta_p^2=0.02$, and during extinction, $F(1,303)=19.03$, $p<0.001$, $\eta_p^2=0.06$, but not during pre-conditioning, $F(1,303)=0.28$, $p=0.60$, $\eta_p^2<0.01$. In addition, we noted a trend-level non-significant main effect of Anxiety, $F(1,303)=2.89$, $p=0.090$, $\eta_p^2=0.01$, with greater startle response across the task among anxious relative to healthy participants. Of note, anxious and healthy participants did not differ in startle response during pre-conditioning, $F(1,303)=0.94$, $p=0.33$, $\eta_p^2<0.01$.

In light of prior findings reporting that the delivery of startle probes interfered with threat learning as assessed with SCR (18), we repeated the primary SCR analyses testing the Anxiety×Age×Phase×CS effect and included the averaged startle response during CSs presentation as a covariate. The results of this analysis remain unchanged when startle response was included.

In addition, we repeated the structural imaging analyses testing for associations with SCR, using the mean startle response across the task as the dependent measures. Unlike the SCR analyses, these analyses did not reveal any brain regions significantly associated with startle response.

Self-reported fear. We analyzed self-reported fear of the conditioned cues, collected before conditioning, following conditioning, and following extinction, using a repeated-measures ANCOVA testing the Anxiety×Age×Phase×CS interaction on self-report data (values 0-10). Self-report data by task phase, CS, and anxiety group are presented in Fig. S3. We noted a significant main effect of CS (CS+>CS-), $F(1,347)=38.23$, $p<0.001$, $\eta_p^2=0.10$, and a main effect of Phase, $F(2,694)=12.64$, $p<0.001$, $\eta_p^2=0.04$. These effects were qualified by a significant Phase×CS interaction, $F(2,694)=23.27$, $p<0.001$, $\eta_p^2=0.06$, with follow-up analyses indicating comparable fear of the cues before conditioning, $p=0.99$, and increased fear of the CS+ relative to CS- after conditioning, $F(1,347)=58.38$, $p<0.001$, $\eta_p^2=0.14$, which was maintained following extinction(32), $F(1,347)=17.40$, $p<0.001$, $\eta_p^2=0.05$.

Finally, we observed a significant main effect of Anxiety, with anxious participants reporting increased fear of both conditioned cues relative to healthy controls, $F(1,347)=6.83$, $p=0.009$, $\eta_p^2=0.02$. No other effects were observed.

In addition, to examine associations between psychophysiological responses and conditioned subjective fear to the threat stimulus, we calculated correlations between anticipatory SCR to the conditioned stimuli and subjective fear reports of these stimuli. Averaged SCR to the conditioned stimuli across the task correlated positively with post-conditioning subjective fear of the CS+, $r(350)=0.13$, $p=0.015$, but not with fear of the CS-, $r(350)=0.07$, $p=0.18$. SCR specifically to the CS+ during conditioning also correlated with post-conditioning subjective fear of the CS+, $r(350)=0.18$, $p=0.001$, but not with fear of the CS-, $r(350)=0.06$, $p=0.29$. Thus, the magnitude of anticipatory psychophysiological response was associated with the magnitude of conditioned subjective fear.

Response to the unconditioned stimulus

SCR. In addition to analyses on averaged psychophysiological response to the UCS, we examined trial-by-trial response to the UCS during the conditioning phase (Fig. S1B). A repeated-measures ANCOVA of SCR to the UCS in the eight reinforced trials revealed a significant main effect of Trial, $F(7,2422)=45.33$, $p<0.001$, $\eta_p^2=0.12$, indicating decreasing response to the UCS across the phase. In addition, we noted a main effect of Age (decreasing response with age), $F(1,348)=32.99$, $p<0.001$, $\eta_p^2=0.09$. These effects were qualified by a significant Trial×Age interaction effect, $F(7,2422)=8.06$, $p<0.001$, $\eta_p^2=0.02$, indicating stronger decrease in younger participants. No main or interaction effect of Anxiety was noted, ps>0.59, indicating that, in contrast to response to the conditioned cues, anxious and healthy individuals did not differ in their psychophysiological response to the aversive UCS. Moreover, the range of psychophysiological response to the UCS across UCS presentations, as well as the comparable response between groups across presentations, indicate that the absence of group difference in averaged response to the UCS is not due to a ceiling effect on psychophysiological response.

In addition, averaged SCR to the conditioned cues and to the UCS correlated positively, $r(350)=0.62$, $p<0.001$. This indicates that the magnitude of conditioned response is commensurate with the magnitude of unconditioned response; it further does not provide evidence for conditioned diminution of the unconditioned response (33).

Self-reported fear. We analyzed associations between the magnitude of unconditioned threat response and the magnitude of subjective fear of the conditioned stimuli. Averaged SCR to the UCS did not significantly correlate with post-conditioning subjective fear of either conditioned stimulus, $r(350)$s≤0.09, ps≥0.11. Thus, subjective fear of conditioned cues did not depend on the magnitude of unconditioned threat response.

Additional information

See Fig. S4 for age distributions in the study sample.

Figures

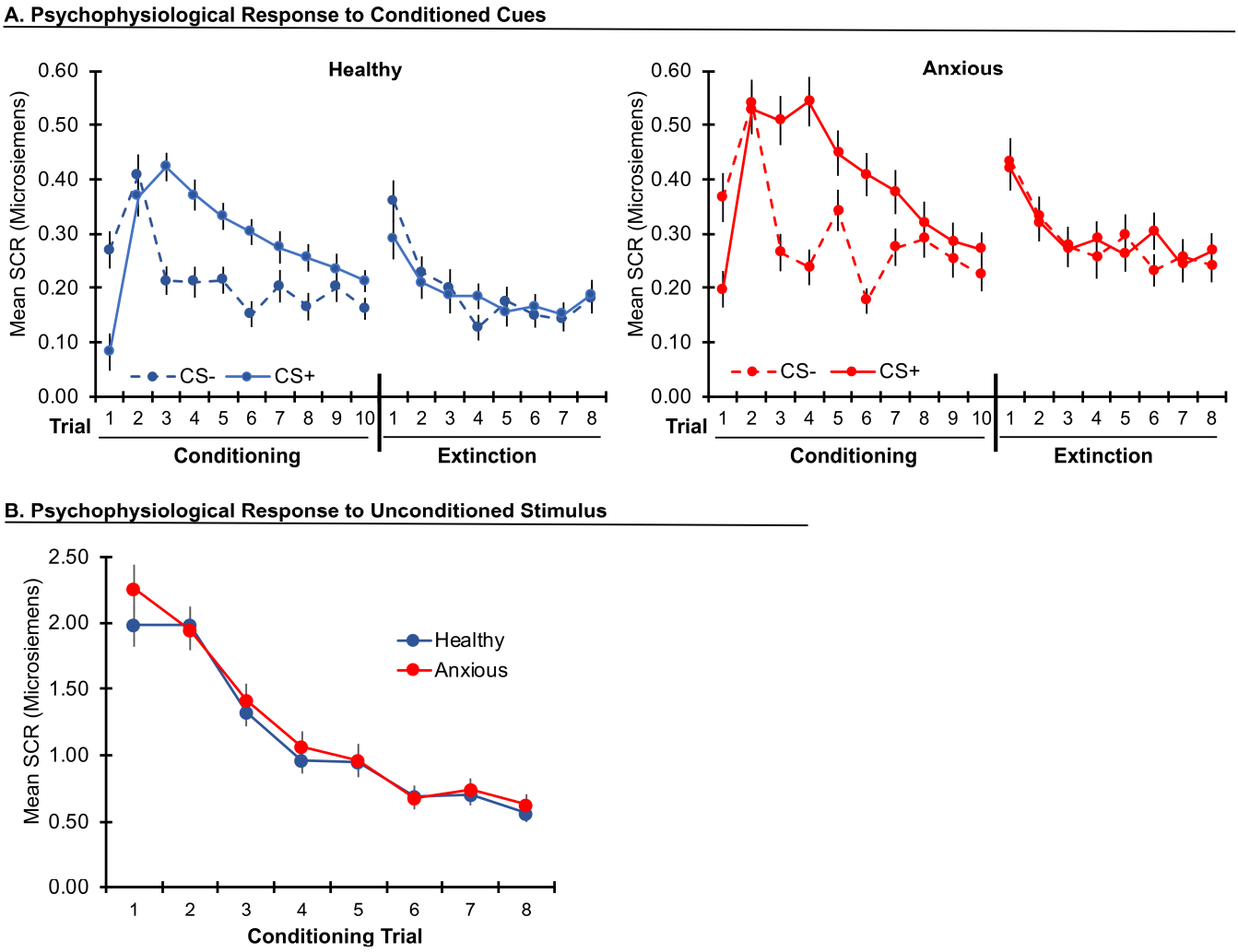

Figure S1. **Trial-by-trial psychophysiological responses**.

Skin conductance responses (SCR) to A) the conditioned cues (CS-, CS+) during the conditioning and extinction phases, and to B) the unconditioned stimulus (UCS) during the conditioning phase, averaged by anxiety group (healthy, anxious).

Note: SCR data were square-root-transformed microsiemens units. Bars represent one standard error of the mean.

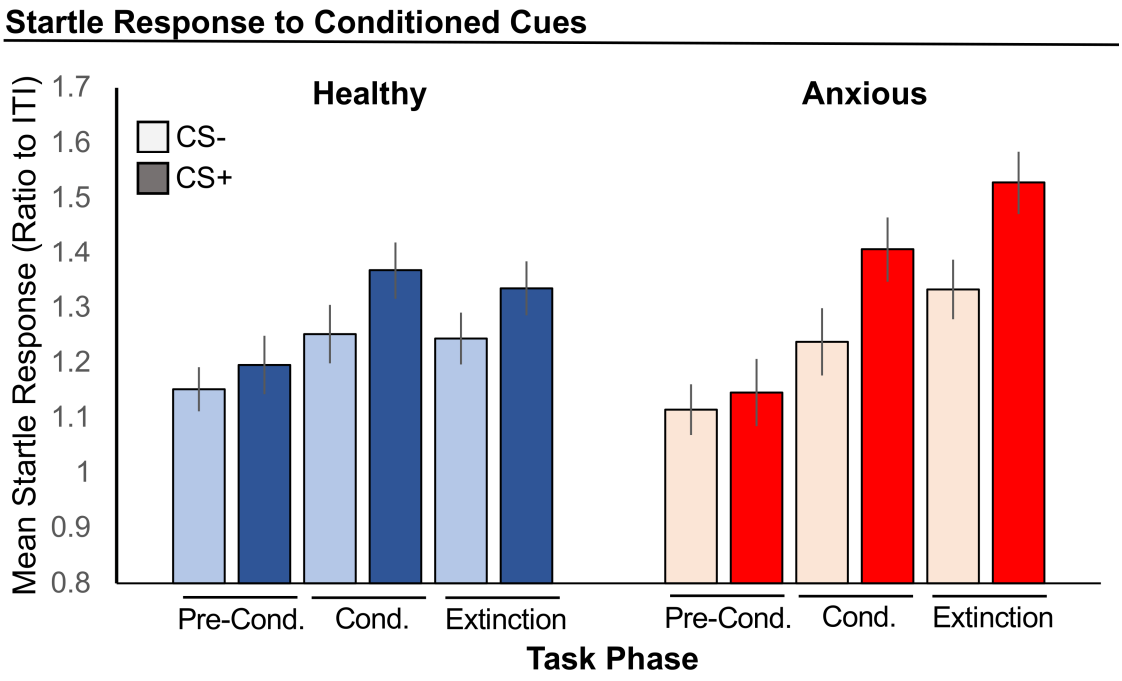

Figure S2. Startle response to conditioned cues.

Startle response to the conditioned cues (CS-, CS+) averaged by task phase (pre-conditioning, conditioning, extinction) and by anxiety group (healthy, anxious). Scores reflect averaged response for each trial type divided by averaged response during ITI.

Note: Bars represent one standard error of the mean. Pre-cond = Pre-conditioning, ITI = inter-trial interval.

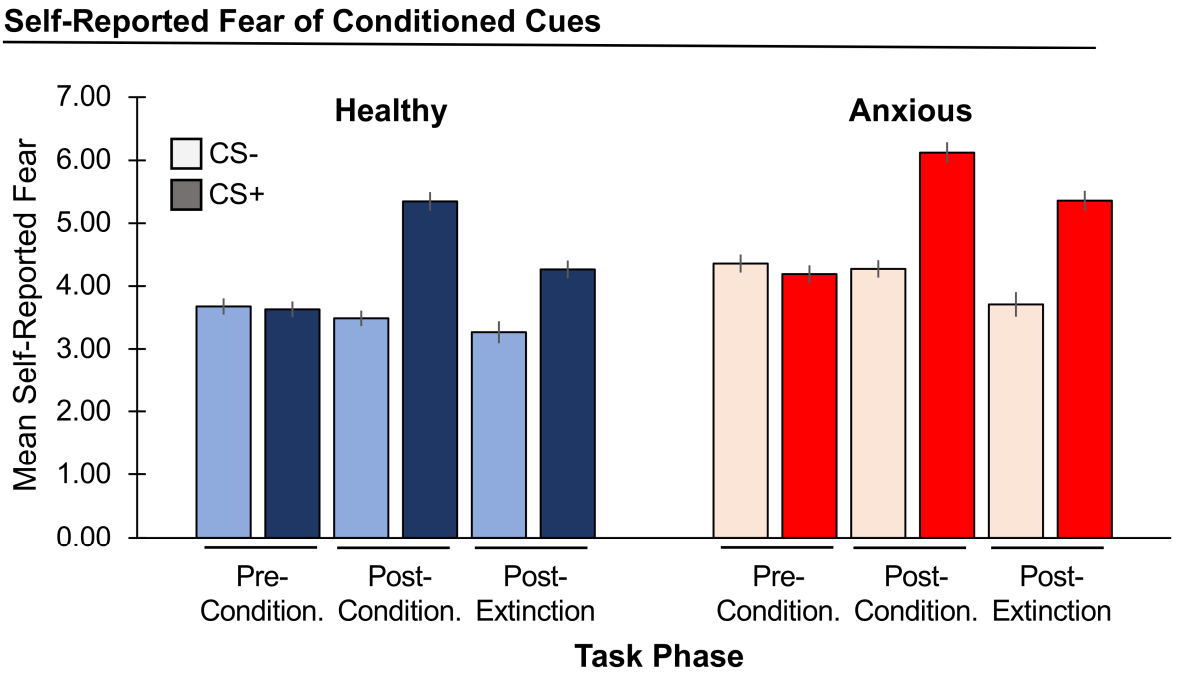

Figure S3. Self-reported fear of conditioned cues.

Self-reported fear to the conditioned cues (CS-, CS+) collected before conditioning, following conditioning, and following extinction, averaged by anxiety group (healthy, anxious).

Note: Bars represent one standard error of the mean. Pre-cond = Pre-conditioning.

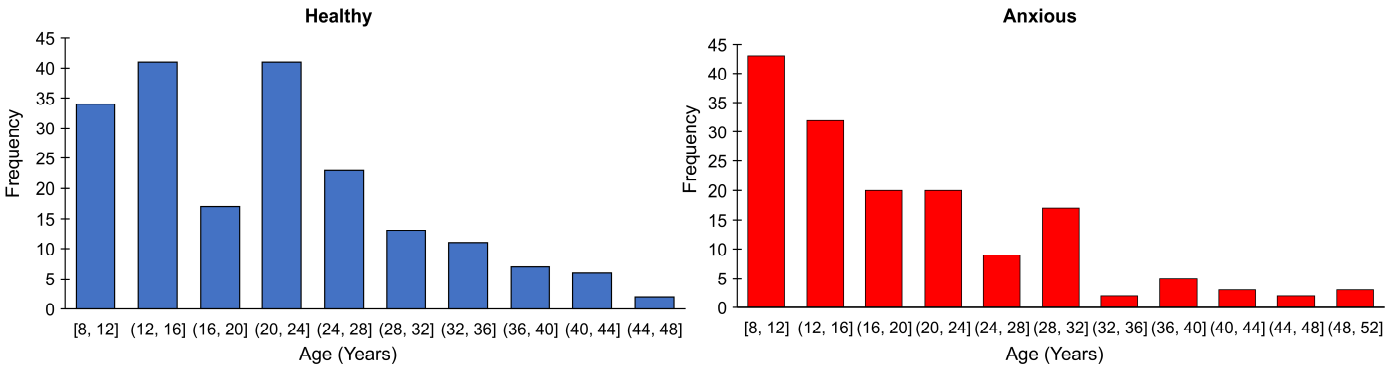

Figure S4. Age distribution in the healthy and anxious sub-samples.

The histograms depict the distribution of age among the healthy (left) and anxious (right) participants.

Analysis Pipeline for Imaging Data

1. Run FreeSurfer for each participant to align, register, and segment the brain images:

   recon-all -subjid subj -i output/subj.nii.gz -all

2. Removal of poor surface reconstructions after visual inspection using procedure described in: https://brainder.org/2011/09/10/quickly-inspect-freesurfer-cortical-surfaces/

3. Run mris_preproc to smooth and put all participants in the same common grid for between-subject comparisons:

   e.g.: mris_preproc subj --hemi rh --meas thickness –out rh.thickness.mrispreproc.mgh --nocleanup --fwhm-src 20 --target fsaverage5

4. Merge hemispheres to allow multiple testing correction across both hemispheres (bh):

   palm_hemimerge lh* rh* (documentation: https://github.com/andersonwinkler/PALM/blob/master/palm_hemimerge.m)

5. Extract volumes of subcortical structures from the outputs of FreeSurfer:

   asegstats2table -s subj

6. Compute the amount of gray matter within each subcortical structure, using asegpve, which in turn uses FSL FAST for segmentation into GM/WM/CSF:

   asegpve -s subj (documentation: https://github.com/andersonwinkler/toolbox/blob/master/bin/asegpve)

7. Prepare design matrices and contrast files for PALM analysis. While this could have been accomplished manually, a MATLAB script was created:

```
% Load variables data: ID, age, diagnosis, sex, DV
D = strcsvread('variables_data.csv'),',');

% Get the DV data (e.g, SCR), save
SCR = cell2mat(D(2:end,strcmp(D(1,:),'scr')));
csvwrite(fullfile(palmdir,'scr.csv'),SCR);
```

```
% Get age and center
age = cell2mat(D(2:end,strcmp(D(1,:),'age')));
age = bsxfun(@minus,age,mean(age));

% Get the diagnostic group and center
dx  = D(2:end,strcmp(D(1,:),' diagnosis'));
idx = strcmp(dx,'healthy');  dx(idx) = {1};
idx = strcmp(dx,'anxious'); dx(idx) = {-1};
dx = cell2mat(dx);
dx = bsxfun(@minus,dx,mean(dx));

% Get the sex and center
sex  = D(2:end,strcmp(D(1,:),'sex'));
idx = strcmp(sex,'Male');   sex(idx) = {1};
idx = strcmp(sex,'Female'); sex(idx) = {0};
sex = cell2mat(sex);
sex = bsxfun(@minus,sex,mean(sex));

% Create an intercept and a dummy
I = ones(size(age));
dummy = I*9999;

% Load the global variables (global thickness, ICV)
G = strcsvread('globals.csv'),'\t');
Globals = cell(2,1);
Globals{1} = cell2mat(G(2:end,strcmp(G(1,:),'bh.MeanThickness')));
Globals{2} = cell2mat(G(2:end,strcmp(G(1,:),'EstimatedTotalIntraCranialVol'))./1e6;
Global_names = {'globalthickness','globalicv'};

% Subcortical imaging data:
img = load(‘gmv_data.csv');
csvwrite(‘gmv.csv', img);
csvwrite(‘gmv.age.csv', img.*age);
csvwrite(‘gmv.dx.csv', img.*dx);
csvwrite(‘gmv.age.dx.csv', img.*age.*dx);
mask = ones(1,size(img,2));
csvwrite('mask.csv'),mask);

% Cortical imaging data:
x = palm_miscread('bh.thickness.mrispreproc.mgz');
img = permute(x.data,[4 1 2 3]);
x.data = permute(img,[2 3 4 1]); x.filename = 'bh.thickness'; palm_miscwrite(x,false);
x.data = permute(img.*age,[2 3 4 1]); x.filename = 'bh.thickness.age';
palm_miscwrite(x,false);
```

```
x.data = permute(img.*dx,[2 3 4 1]); x.filename = 'bh.thickness.dx';
palm_miscwrite(x,false);
x.data = permute(img.*age.*dx,[2 3 4 1]); x.filename = 'bh.thickness.age.dx';
palm_miscwrite(x,false);

% Compute the average area per vertex, to be used for spatial statistics
x = palm_miscread('bh.area.mgz');
x.data = mean(x.data,4);
x.filename = 'bh.avg_area_per_vertex';
palm_miscwrite(x,false);

% Create the designs; dummies are replaced by img*age, for example, to predict DV
M = [I       ...  % intercept    (1)
   dummy    ...  % imaging data (2)
   age      ...  % age          (3)
   dx       ...  % dx (group)   (4)
   sex      ...  % sex          (5)
   age.*dx ...  % age*dx       (6)
   dummy    ...  % img*age      (7)
   dummy    ...  % img*dx       (8)
   dummy];      % img*age*dx   (9)

for g = 1:numel(Globals)
csvwrite('design_incl_global_%s.csv',Global_names{g})),horzcat(M,Globals{g}))
end

% Create the contrast files
C = [...
   0 0 0 0 0 0 0 0 +1;
   0 0 0 0 0 0 0 0 -1;
   0 0 0 0 0 0 0 +1 0;
   0 0 0 0 0 0 0 -1 0;
   0 0 0 0 0 0 +1 0 0;
   0 0 0 0 0 0 -1 0 0;
   0 +1 0 0 0 0 0 0 0;
   0 -1 0 0 0 0 0 0 0];
C = horzcat(C,zeros(size(C,1),1));
csvwrite('contrasts_incl_global.csv',C);
```

8. Run PALM (documentation: https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/PALM):

```
PALM -i predicted_var.csv -d design_incl_global_globalthickness.csv -t
contrasts_incl_global.csv -evperdat bh.thickness.mgz 2 1 -evperdat
bh.thickness.age.mgz 7 1 -evperdat bh.thickness.dx.mgz 8 1 -evperdat
bh.thickness.age.dx.mgz 9 1 -m bh.FS.ic5.aparc.mask.dpv -s bh.white.srf
```

bh.avg_area_per_vertex.mgz -T -designperinput -logp -nouncorrected -approx tail -n 1000

9. Split output files in merged hemispheres format into left and right hemispheres:

   palm_hemisplit bh* (documentation:

   https://github.com/andersonwinkler/PALM/blob/master/palm_hemisplit.m)

10. Figures were generated by hand using Surf Ice: https://www.nitrc.org/projects/surfice/

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