

Structural and functional bases of inhibited temperament

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Children born with an inhibited temperament are at heightened risk for developing anxiety, depression and substance use. Inhibited temperament is believed to have a biological basis; however, little is known about the structural brain basis of this vulnerability trait. Structural MRI scans were obtained from 84 (44 inhibited, 40 uninhibited) young adults. Given previous findings of amygdala hyperactivity in inhibited individuals, groups were compared on three measures of amygdala structure. To identify novel substrates of inhibited temperament, a whole brain analysis was performed. Functional activation and connectivity were examined across both groups. Inhibited adults had larger amygdala and caudate volume and larger volume predicted greater activation to neutral faces. In addition, larger amygdala volume predicted greater connectivity with subcortical and higher order visual structures. Larger caudate volume predicted greater connectivity with the basal ganglia, and less connectivity with primary visual and auditory cortex. We propose that larger volume in these salience detection regions may result in increased activation and enhanced connectivity in response to social stimuli. Given the strong link between inhibited temperament and risk for psychiatric illness, novel therapeutics that target these brain regions and related neural circuits have the potential to reduce rates of illness in vulnerable individuals.

Keywords: behavioral inhibition; shy; salience; anxiety

INTRODUCTION

Individuals with an inhibited temperament exhibit profound behavioral differences that are evident early in childhood (Calkins *et al.*, 1996; Kagan *et al.*, 1998) and persist across development (Fox *et al.*, 2001; Kagan *et al.*, 2007). Inhibited children are quiet, reticent, and avoid novel situations and people. Importantly, these behavioral differences predict long-term psychiatric outcomes. For example, very inhibited children are at increased risk for anxiety (Hirshfeld *et al.*, 1992; Schwartz *et al.*, 1999; Chronis-Tuscano *et al.*, 2009; Essex *et al.*, 2010; Clauss and Blackford, 2012), depression (Caspi *et al.*, 1996; Beesdo *et al.*, 2007) and substance use (Williams *et al.*, 2010; Lahat *et al.*, 2012). Neuroscientifically based preventions promise to reduce the burden of psychiatric illness in these vulnerable children; however, the neurobiological basis of inhibited temperament remains poorly understood.

Inhibited temperament is evolutionarily conserved and is observed across species, including rhesus monkeys (Kalin and Shelton, 1989; Fox *et al.*, 2008; Oler *et al.*, 2010; Shackman *et al.*, 2013) and other mammals (for an extensive review, see Gosling and John, 1999). Although

the trait exists across a continuum, ranging from inhibited to uninhibited, a considerable number of individuals are either very inhibited or very uninhibited. These extreme groups may be maintained by natural selection, preserving genes for extreme responses to novelty. Both very inhibited and very uninhibited individuals have an evolutionary advantage, with relative strengths in different contexts (Biro and Post, 2008). For example, in contexts of predator threat, inhibited individuals are more likely to stay close to shelter and survive, whereas uninhibited individuals are more likely to venture out and be attacked by a predator. In contexts of famine, the pattern is opposite: uninhibited individuals are more likely to explore and find food, whereas inhibited individuals are more likely to stay close to shelter and starve. In humans, inhibited and uninhibited temperament can be reliably and validly measured (Cheek and Buss, 1981; Garcia Coll *et al.*, 1984; Jones *et al.*, 1986), and extreme groups are typically defined as the top and bottom 15% of the continuum (Kagan *et al.*, 1998).

Although the behavioral correlates of inhibited temperament have been well characterized, the origin of these differences remains poorly understood. Inhibited temperament likely has a neurobiological origin and is associated with physiological differences—such as a high and stable heart rate, increased salivary cortisol and greater asymmetry in frontal electroencephalography (EEG) (Kagan *et al.*, 1998; Fox *et al.*, 2005a). Furthermore, both inhibited humans and non-human primates consistently show altered amygdala function (Pérez-Edgar *et al.*, 2007; Beaton *et al.*, 2008; Fox *et al.*, 2008; Oler *et al.*, 2010; Blackford *et al.*, 2011; Schwartz *et al.*, 2012; Shackman *et al.*, 2013), which may reflect differences in gene expression within the amygdala (Fox *et al.*, 2012). Given prior work that demonstrates that brain structure is associated with brain function (Kalin *et al.*, 2004; Machado *et al.*, 2008; Bliss-Moreau *et al.*, 2010), we propose that these differences in behavior and brain function may be related to variation in brain structure. One prior study examined cortical thickness and found that inhibited temperament was associated with thicker ventromedial prefrontal cortex and thinner lateral orbitofrontal cortex

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(Schwartz *et al.*, 2010). To our knowledge, no prior studies have explored the link between inhibited temperament and amygdala volume. We hypothesize that inhibited temperament is associated with larger amygdala volume.

MATERIALS AND METHODS

Participants

Consistent with both the existing animal and human literature, we used an extreme discordant phenotype approach (Nebert, 2000) and compared inhibited ($n=40$; 21 female) and uninhibited ($n=44$; 26 female) young adults. To focus on a stable trait and ensure that valid groups were identified, the trait was assessed retrospectively (childhood) and currently using the Retrospective Self-Report of Inhibition (RSRI) and the Adult Self-Report of Inhibition (ASRI) (Reznick *et al.*, 1992), respectively. Both questionnaires have excellent reliability (Cronbach's $\alpha=0.79$ for the RSRI and $\alpha=0.78$ for the ASRI), demonstrate convergent validity (Reznick *et al.*, 1992; Rohrbacher *et al.*, 2008) and minimize self-report bias by focusing on reports of concrete behaviors in specific situations instead of subjective feelings.

Young adults were selected from a larger, ongoing study ($n=114$ at the time of this analysis). Inhibited and uninhibited young adults were recruited using a systematic recruitment strategy designed to target members of extreme groups who identified as being 'especially shy or outgoing as a child'. Subjects were screened for extreme inhibited or uninhibited temperament on both the RSRI and the ASRI [standard top/bottom 15% guideline used in most human studies, based on normative data (Reznick *et al.*, 1992)]; therefore, all subjects had stable inhibited temperament. For the larger study, 53% of subjects who were screened met criteria for extreme temperament. Subject characteristics are reported in Table 1. For this report, we selected inhibited and uninhibited adults who had completed a structural MRI scan. To minimize environmental effects on brain structure, adults were excluded for significant drug use (defined by substance abuse or dependence within the past 6 months), current psychotropic medication use, major medical illness, or history of brain trauma. To minimize disorder-related effects on brain volume, adults were excluded if they had a psychiatric disorder; however, because anxiety is common in inhibited adults, anxious subjects in the inhibited group were not excluded. Although inhibited temperament is also associated

with risk for depression and substance abuse, incidence is lower and likely develops secondary to anxiety (Beesdo *et al.*, 2007). Psychiatric diagnoses were established by trained interviewers using the Structured Clinical Interview for DSM-IV and 10 inhibited adults met criteria for an anxiety disorder (4 social anxiety disorder, 1 generalized anxiety disorder, 2 anxiety not otherwise specified, 1 specific phobia, and 2 comorbid social anxiety disorder and generalized anxiety disorder). Inhibited individuals with an anxiety diagnosis had significantly higher scores on both the RSRI ($M=3.66$, $s.d.=0.46$ vs $M=3.2$, $s.d.=0.48$; $P=0.01$) and ASRI ($M=3.49$, $s.d.=0.57$ vs $M=3.1$, $s.d.=0.39$; $P=0.02$) relative to inhibited individuals without an anxiety diagnosis.

The study was approved by the Vanderbilt University Institutional Review Board and we obtained written informed consent after providing subjects with a complete study description.

Measures

Gray matter volume

High resolution anatomic images were collected using a 3 Tesla Philips scanner (Philips Healthcare, Inc., Best, The Netherlands). T1-weighted images were obtained in the sagittal direction, with a three-dimensional turbo field echo, 1 mm^3 voxel-size, 256 mm field of view, 170 slices, 1 mm slice thickness, 0 mm gap, 8 ms TR and 3.7 ms TE.

Three complimentary methods were used to assess amygdala structure: manual segmentation, surface mapping and voxel-based morphometry (VBM). Manual segmentation was used to identify the size and shape of each subject's amygdalae. The amygdala is well-suited for manual segmentation and surface mapping because of its small structure, clear boundaries and well-established segmentation methods (Pruessner *et al.*, 2000); the low reliability of automated segmentation methods makes manual segmentation the gold standard (Nugent *et al.*, 2013), but see Hanson *et al.* (2012). The amygdala was manually traced by two blinded raters (A.L.S. and R.M.V., trained by an expert rater J.U.B.) using 3DSlicer (version 3.4; www.slicer.org), a software package that provides simultaneous visualization in all orientations. Amygdalae were traced according to standard protocols (Honeycutt *et al.*, 1998; Pruessner *et al.*, 2000) with reference to an anatomical atlas (Mai *et al.*, 2008). Consistent with standards for manual segmentation (Pruessner *et al.*, 2000; Bergouignan *et al.*, 2009) and previous studies (Dedovic *et al.*, 2010; van der Plas *et al.*, 2010), images were normalized to a standard T1 Montreal Neurological Institute (MNI) template using standard normalization in SPM5 (affine registration, followed by non-linear deformations) prior to manual tracing. The amygdala was initially traced from the superior to the inferior border in the axial view, with the coronal and the sagittal views used for confirmation. The posterior boundary of the amygdala was defined using the alveus of the hippocampus or the inferior horn of the lateral ventricle. The lateral border was defined as the vertical border tangential to the most medial adjacent white matter. The medial border was defined in slices superior to the uncus, as the border with the ambient cistern, and in slices inferior to the uncus, as the white matter separating the amygdala from the entorhinal cortex. In superior slices, the anterior border of the amygdala was defined in the axial view as the border with the subarachnoid space. In the inferior slices, the anterior border was defined in the coronal view as the slice just posterior to the slice containing the anterior commissure. The superior border was defined in the coronal view, by drawing a horizontal line between the superolateral part of the optic tract and the inferior portion of the circular sulcus of the insula. Amygdala volume was estimated for each of the subjects in 3-D Slicer. Reliability was estimated using intra-class correlations (Shrout and Fleiss, 1979); the intra-rater correlation and the inter-rater correlation were both 0.81.

Table 1 Subject characteristics by group

	Inhibited temperament ($n=40$)		Uninhibited temperament ($n=44$)		P-value
	Mean	s.d.	Mean	s.d.	
Inhibited temperament					
Child	3.26	0.50	1.55	0.47	<0.001
Adult	3.19	0.48	1.78	0.43	<0.001
Intracranial volume	1.71	0.16	1.71	0.18	ns
Age (years)	23.4	4.0	22.8	3.7	ns
	Percent	<i>N</i>	Percent	<i>N</i>	P-value
Gender (% female)	52.5	21	59.1	26	ns
Handedness (% right)	80.0	32	90.9	40	ns
Ethnicity					ns
% Caucasian	70.0	28	70.5	31	
% African American	12.5	5	20.5	9	
% Asian	17.5	7	4.5	2	
% Other	0.0	0	4.5	2	

ns, not significant.

Surface models were estimated using weighted spherical harmonic representation methods (Chung *et al.*, 2008). Briefly, amygdala surface was extracted from the manual segmentation in the form of a triangular mesh, and then inflated onto a sphere. A weighted spherical harmonic representation was estimated for each subject's left and right amygdala. The weighted spherical harmonic representation was projected onto a sphere with 2562 vertices and 5120 faces, and the surface spheres were averaged across all subjects to create an average for the left amygdala and right amygdala. Individual differences in amygdala shape were estimated as the deviation of each subject's surface from the average surface. Surface models were estimated using Matlab.

Because comparisons of overall amygdala volume can only identify gross structural differences, it is critical to also test for regional differences, especially for brain structures composed of multiple functionally heterogeneous subnuclei, such as the amygdala. Regional differences in gray matter volume were assessed using VBM—an automated technique for comparing gray matter volume in each voxel of the brain (Ashburner and Friston, 2000). With VBM, individual brains are normalized into a common space while retaining the original gray matter volume. For example, for a brain region that is compressed to fit the standard space, gray matter volume will be >1 in the compressed voxels; for brain regions that are expanded, gray matter volume will be <1 in the expanded voxels. VBM analysis was performed using SPM8 (Statistical Parametric Mapping, Wellcome Department of Imaging Neuroscience, London, UK) implemented in Matlab.

Following an established VBM protocol (Bergouignan *et al.*, 2009), preprocessing steps included checking for artifacts and anatomic abnormalities, manually realigning the anterior and posterior commissures to the same horizontal line, and setting the image origin at the anterior commissure. Preprocessed images were segmented into gray matter, white matter and cerebrospinal fluid using the a priori templates included in SPM8 and standard SPM8 segmentation. As traditional normalization is less effective for medial temporal lobe structures (Yassa and Stark, 2009), such as the amygdala, we used an improved method—diffeomorphic anatomical registration through exponentiated lie algebra (DARTEL) (Ashburner, 2007). This method is more exact for normalizing medial temporal lobe structures than other automated normalization programs (Bergouignan *et al.*, 2009; Yassa and Stark, 2009). Using DARTEL, segmented images were skull-stripped and averaged to create a template of gray and white matter for all subjects. This average template was used to register and derive Jacobian-modulated warped-tissue class images of gray and white matter for each subject and transform the images into a standard space (MNI T1 template). Finally, the images were smoothed with an isotropic Gaussian kernel of 6 mm at FWHM. The resulting images provided a measure of gray matter volume accounting for shape differences for each 1 mm^3 voxel across the entire brain. To adjust for any remaining differences in brain size, total intracranial volume (sum of gray matter, white matter and cerebrospinal fluid volumes) was also computed for use as a covariate. Total intracranial volume did not differ significantly between groups (Table 1).

Functional activation and connectivity

Functional MRIs (fMRIs) were acquired on a subset of 26 subjects (14 inhibited, 12 uninhibited) who had participated in a study of face processing (Blackford *et al.*, 2011). In the fMRI task, novel and familiar neutral faces were presented using a slow event-related design. Twelve novel and 12 familiar faces were presented once per run across four runs, for a total presentation of 48 novel face presentations and 48 familiar face presentations (of the six familiarized faces). No novel faces were repeated across runs. Faces were familiarized by showing them eight times per face across the course of a familiarization block,

prior to the present task. Face stimuli were selected from two standard sets of emotional expressions (Lundqvist *et al.*, 1998; Gur *et al.*, 2001). Brain activation was measured during the presentation of neutral faces, relative to baseline. Data from this fMRI study have been previously published and detailed methods are available (Blackford *et al.*, 2011). Importantly, this subsample was representative of the larger sample of subjects; the groups did not differ on any demographic or other study variables (all P s > 0.13).

To determine if gray matter volume (as measured by VBM) was significantly correlated with functional activation in brain regions with significant volume differences, a voxel-to-voxel correlation analysis was performed. Using the Biological Parametric Mapping toolbox (Casanova *et al.*, 2007) implemented in SPM8, voxel-level correlations were estimated between the fMRI images (all faces $>$ baseline) and gray matter volume images (from the VBM analysis). Correlation analyses were performed across all individuals, because the structure–function relationship was expected to be similar for both groups.

To identify the impact of gray matter volume differences on neuro-circuit function, a functional connectivity analysis was performed using the Generalized Psychophysiological Interaction Toolbox (McLaren *et al.*, 2012). For this analysis, we used 'seeds' based on the regions where group differences in gray matter volume were identified using VBM. We created a map of network connectivity for each seed region (FWE-corrected $\alpha = 0.05$). To determine the relationship between volume and connectivity, we correlated gray matter volume (using VBM, extracted from the region with significant group differences) and functional connectivity between each seed region and all other voxels in the connectivity map.

Statistical analyses

Structural differences

Group differences in amygdala volume were tested using an analysis of covariance (ANCOVA) with age, gender, race and handedness included as covariates. Statistical analyses ($\alpha = 0.05$) were performed using SAS (SAS Institute, Cary, NC). Group differences in amygdala shape were tested using a general linear model, implemented in SurfStat (<http://www.math.mcgill.ca/keith/surfstat/>), with age, gender, race and handedness included as covariates. Group differences were represented by a t -value at each dimension (vertex) of the average amygdala. Uncorrected P -values were estimated using SurfStat and P -values corrected for multiple comparisons were estimated using a false-discovery rate method implemented in Matlab (mafdr function; version 7.5.0; MathWorks, Natick, MA). The specific location of group differences was identified by comparing significant regions with a detailed anatomic atlas of the amygdala (Mai *et al.*, 2008). Group differences in gray matter volume (VBM) within the amygdala were tested using an ANCOVA with age, gender, race and handedness included as covariates. Analyses were restricted to an amygdala region of interest defined by manual tracing on an average study-specific brain. A combination of voxel P -values and cluster-based thresholds was used to control for Type I error due to multiple comparisons (simulations computed using AlphaSim). For the amygdala VBM analysis, family-wise error correction at $\alpha = 0.05$ was provided by a voxel $P = 0.05$ and cluster size of 282 voxels (left amygdala) or 275 voxels (right amygdala). For the whole brain analysis, the ANCOVA included age, gender, race, handedness and intracranial volume as covariates. Family-wise error correction at $\alpha = 0.05$ was provided by a voxel-level $P < 0.001$ and a cluster size of 295 voxels.

Functional differences

Correlations between gray matter volume and functional activation were computed for the left and right amygdalae, and for other regions

identified as having significantly different gray matter volumes across the brain. A one-sample *t*-test in SPM was used to test for significant correlations. To control for multiple comparisons, AlphaSim was used to determine cluster sizes at voxel-level $P < 0.05$ and $\alpha = 0.05$.

To determine the functional impact of greater gray matter volume on functional connectivity, a regression analysis was performed with gray matter volume in the amygdala and any other regions identified as having significantly different gray matter volumes across the brain as the predictor variables. Analyses were performed within the overall functional connectivity map for each region to constrain the analyses to relevant regions (overall map constrained with voxel-level $P < 0.05$, cluster-corrected at $\alpha = 0.05$). For correlation analyses, family-wise error was controlled at $\alpha = 0.05$ with a voxel-level $P < 0.05$ and a cluster threshold determined by AlphaSim.

Diagnosis effects

To determine the effects of subjects with an anxiety disorder on the results, we compared inhibited subjects with an anxiety diagnosis to inhibited subjects without a diagnosis. Because the inhibited subjects with anxiety disorders also had higher temperament scores, we performed an additional analysis controlling for temperament scores.

RESULTS

Amygdala analyses

Due to the known role of the amygdala in responding to novel stimuli (Wright et al., 2003; Blackford et al., 2010) and mediating fear and avoidance behaviors (Davis, 1992), we first examined differences in amygdala volume. Using manual segmentation to measure overall amygdala volume, we found that inhibited adults had significantly larger volume in the right amygdala (Figure 1B; 8% larger; $P = 0.05$), with a similar trend for the left amygdala (6% larger; $P = 0.13$).

To examine differences in amygdala shape, we used surface modeling methods (Chung et al., 2008). Relative to uninhibited adults, inhibited adults had regions of increased convexity (Figure 1C; right amygdala peak difference: corrected $P = 0.02$, uncorrected $P < 0.0001$; left amygdala peak difference: corrected $P = 0.055$, uncorrected $P = 0.0003$) located primarily in the basolateral and lateral subnuclei (Mai et al., 2008), regions known to play a crucial role in both fear conditioning and anxiety (Davis, 1992; Phelps and LeDoux, 2005; Etkin and Wager, 2007).

Next, we tested for regional differences in amygdala gray matter volume using VBM. Inhibited adults, relative to uninhibited adults, had regions of greater gray matter volume in both the left and right amygdalae (Figure 1C; $P < 0.05$ FWE corrected at the cluster level). The regions of greater volume were mainly in the basal and lateral subnuclei, consistent with the amygdala shape analysis. Together, these findings provide converging evidence that inhibited adults have overall larger amygdalae with specific volume increases in the basal and lateral amygdala subnuclei.

To determine the functional impact of greater amygdala volume in these regions, we tested for structure–function relationships in a subsample with fMRI data. Left amygdala gray matter volume was positively correlated with left amygdala activation to faces ($P < 0.05$ FWE, $k > 10$; Figure 2A), suggesting that the functional impact of greater amygdala volume is increased activation to biologically salient stimuli.

To determine the impact of amygdala activation on neurocircuit function, we tested whether amygdala volume predicted differences in amygdala functional connectivity across the brain. Larger volume in the left amygdala was correlated with greater connectivity between the left amygdala and the right temporal lobe (including the parahippocampal gyrus, hippocampus and amygdala), right putamen and right insula (all $P < 0.05$ FWE, $k > 99$; Figure 2B and Table 2).

Greater volume in the right amygdala was correlated with greater connectivity between the right amygdala and the right temporal lobe (including the parahippocampal gyrus, amygdala and hippocampus), bilateral medial visual cortex (including the lingual gyrus, parahippocampal gyrus and fusiform gyrus), right insula and bilateral cerebellum (all $P < 0.05$ FWE, $k > 94$; Figure 2C and Table 2). There were no regions where larger amygdala volume was associated with less functional connectivity. The regions of enhanced connectivity are consistent with neural networks activated during face perception (Haxby et al., 2000) and social interactions (Blakemore, 2008).

Whole brain analyses

Next, we hypothesized that other brain regions, in addition to the amygdala, might show structural differences in inhibited adults. To identify novel structural substrates of inhibited temperament, we tested for group differences in gray matter volume across the whole brain using VBM. Inhibited adults had significant larger volume in a region of the left caudate than the uninhibited adults ($P < 0.05$ FWE corrected at the cluster level; Figure 3A).

To assess the functional impact of caudate volume differences, we tested for a correlation between caudate gray matter volume and activation to faces. Gray matter volume was positively correlated with activation to faces ($P < 0.05$ FWE, $k > 23$; Figure 3B). Caudate volume differences also predicted differences in functional connectivity. Individuals with larger caudate volumes had greater connectivity between the left caudate and regions which respond to social stimuli, including the right caudate, putamen and insula. Larger caudate volume was also associated with reduced connectivity between the left caudate and the cerebellum and lateral occipital complex ($P < 0.05$ FWE, $k > 94$; Figure 3C and Table 3).

Diagnosis effects

As a *post hoc* analysis, we examined the impact of anxiety diagnosis on our results. Specifically, we compared inhibited individuals with an anxiety diagnosis to those without on each of the structural findings. Inhibited subjects with a diagnosis had a region of significantly larger left amygdala volume (VBM; $P = 0.02$) relative to inhibited individuals without a diagnosis, and a trend toward larger regional volume in the right amygdala (VBM; $P = 0.06$). The groups were similar on right amygdala volume (manual; $P = 0.38$) and left caudate volume (VBM; $P = 0.29$). Because inhibited individuals with an anxiety diagnosis also had more extreme inhibited temperament scores which could have contributed to volume differences, we performed an additional analysis covarying for temperament score. The inhibited group with a diagnosis had a trend toward a larger left amygdala volume (VBM; $P = 0.07$). The groups were not significantly different for right amygdala volume (VBM; $P = 0.27$, manual $P = 0.30$) or left caudate volume (VBM; $P = 0.50$).

DISCUSSION

Inhibited adults had structural brain differences in two key regions that distinguished them from uninhibited adults, providing compelling evidence for a structural basis to this foundational temperament trait. Using three complimentary methods, we demonstrated that inhibited adults have significantly larger amygdalae and critically, these anatomical differences had functional consequences for both activation and connectivity. We also identified a novel brain region that distinguished inhibited adults—the caudate. Together these findings provide converging evidence for a structural and functional correlates of inhibited temperament.

Inhibited adults had overall amygdalae volumes that were 8% larger in the left hemisphere and 6% larger in the right hemisphere. These

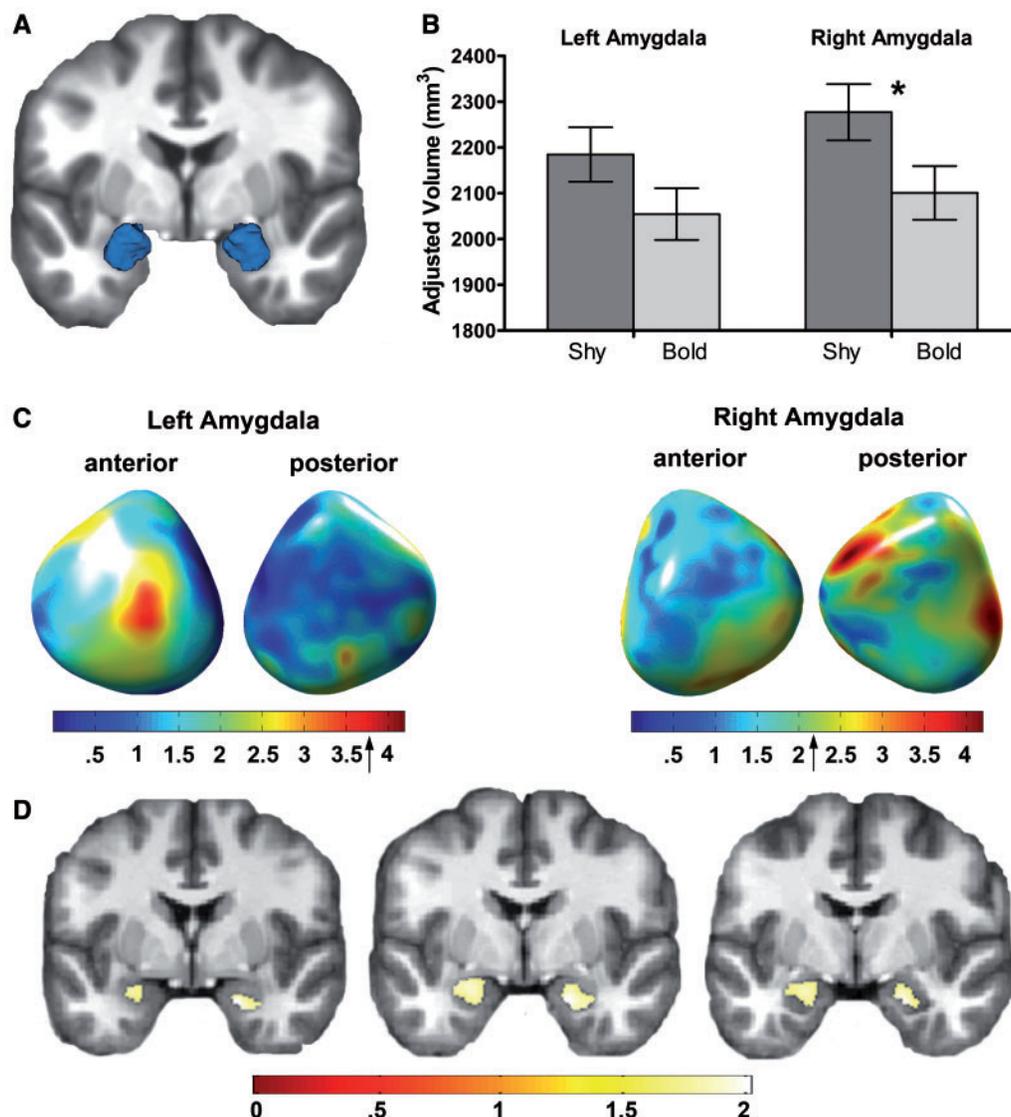


Fig. 1 Inhibited adults have larger amygdalae. (A) Average manually traced amygdala displayed in 3-D. (B) Overall amygdala volume was greater in the inhibited compared with the uninhibited group in the right amygdala (inhibited: $2277 \pm 62 \text{ mm}^3$; uninhibited: $2101 \pm 59 \text{ mm}^3$; $P = 0.045$). (C) In the inhibited group, shape analysis reveals areas of greater convexity in the left and right amygdala. The peak areas of convexity are shown in red. Group differences in surface amygdala shape are displayed on an average amygdala surface. (D) Inhibited adults had regions of significantly larger gray matter volume in both the left and right amygdalae ($P < 0.05$ FWE corrected at the cluster level). For the left amygdala, the significant cluster was 442 voxels (35% of 1252 voxels; peak voxel: $x = -21, y = -4, z = -16$). For the right amygdala, the significant cluster was 402 voxels (33% of 1217 voxels; peak voxel: $x = 18, y = -3, z = -22$). Areas of significant between-group differences are illustrated on multiple coronal slices of an average brain.

differences are consistent with volume differences reported for individuals with severe behavioral alterations; for example, the well-documented hippocampal atrophy in schizophrenia represents a 4% reduction in volume (Nelson *et al.*, 1998). The structural differences identified using VBM and surface mapping converged on the basal and lateral subnuclei of the amygdala. These regions have widespread efferent projections from sensory cortices and afferent projections to prefrontal cortical regions that modulate the amygdala (Stefanacci and Amaral, 2000). Interestingly, increases in basolateral amygdala volume produce increases in anxious behavior in rodents (Vyas *et al.*, 2002; Mitra and Sapolsky, 2008). In humans, alterations in amygdala structure and function are associated with anxiety, depression and substance abuse (De Bellis *et al.*, 2000; Etkin and Wager, 2007; Shin and Liberzon, 2010; Price and Drevets, 2012). In support of this view, the inhibited subjects with an anxiety diagnosis in this sample had larger amygdala volumes and more extreme inhibited

temperament. Based on these findings, we propose that increased amygdala volume in inhibited individuals may produce the characteristic shy and cautious behavior, increased amygdala activation to faces, and increased risk for psychiatric illness.

Given that most theories of inhibited temperament have focused on brain regions mediating fear and anxiety, larger caudate volume in inhibited adults is a novel and intriguing finding. The caudate is a diverse brain region involved in many functions including motor learning and habit formation (Jueptner *et al.*, 1997; Kesner and Gilbert, 2006), cognition (Grahn *et al.*, 2008) and reward (Knutson *et al.*, 2000; Choi *et al.*, 2014). One possible explanation is that increased caudate volume and activity are associated with the caudate's role in reward processing. Consistent with this view, increased caudate activation has been reported in inhibited and anxious adolescents during anticipation of reward (Guyer *et al.*, 2006, 2012) and loss of reward (Helfinstein *et al.*, 2011). However, we propose an alternative

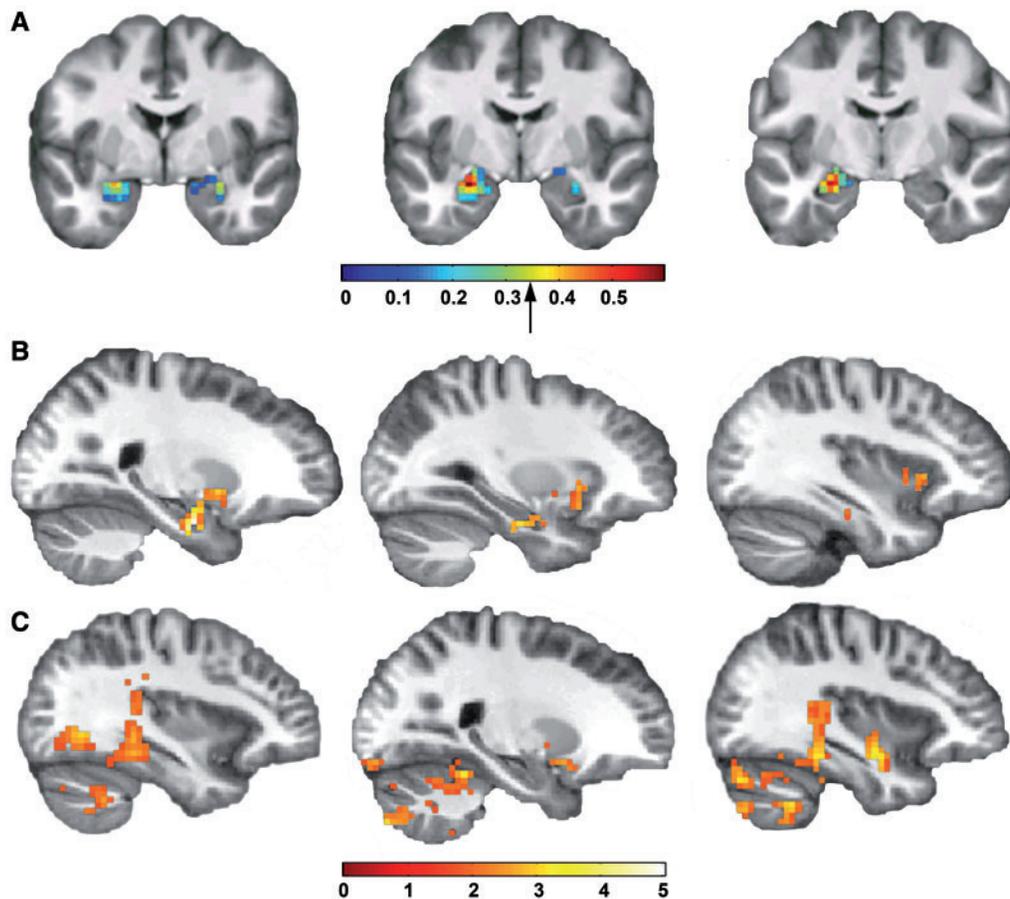


Fig. 2 Larger amygdala volume predicted greater activation and connectivity. (A) Gray matter volume was correlated with activation to faces in the left amygdala. Correlations are shown on three coronal slices of an average structural brain. The correlations are thresholded at $r = 0.01$ for illustration; the arrow on the color bar indicates $P = 0.05$, corrected ($r = 0.33$). (B) Greater gray matter volume in the left amygdala was associated with greater connectivity between the left amygdala and multiple other brain regions including the right temporal lobe, right putamen and right insula ($P < 0.05$ FWE corrected at the cluster level). Functional connectivity is illustrated across multiple sagittal slices of an average brain. (C) Greater gray matter volume in the right amygdala was associated with greater connectivity between the right amygdala and multiple other brain regions including the right temporal lobe, bilateral medial visual cortex, right insula and bilateral cerebellum ($P < 0.05$ FWE corrected at the cluster level). Functional connectivity is illustrated across multiple sagittal slices of an average brain.

Table 2 Brain regions that show significant positive correlations between amygdala gray matter volume and functional connectivity

Cluster (volume mm ³)	Gyrus/region
Connectivity with the left amygdala	
Temporo-basal gangliar (3699)	
Temporal	R hippocampus, R parahippocampal gyrus, R amygdala
Basal ganglia	R putamen
Connectivity with the right amygdala	
Temporo-occipito-cerebellar (65 610)	
Temporal	B hippocampus, B parahippocampal gyrus, B superior temporal gyrus, B inferior temporal gyrus, B transverse temporal gyrus
Occipital	B lingual gyrus, B fusiform gyrus, B middle occipital gyrus, B inferior occipital gyrus
Cerebellum	B dentate, B vermis
Temporo-basal gangliar-insular (2592)	
Temporal	R parahippocampal gyrus, R amygdala, R hippocampus
Basal ganglia	R putamen
Insula	R insula

B, bilateral; L, left hemisphere; R, right hemisphere.

explanation. An emerging perspective is that the caudate is sensitive to saliency and is only engaged under conditions where response choice matters (Tricomi *et al.*, 2004; Zink *et al.*, 2004), consistent with previous findings in other regions of the basal ganglia (Cooper and Knutson, 2008; Bar-Haim *et al.*, 2009; Zaehle *et al.*, 2013). In this study, larger caudate volume predicted greater caudate activation during face processing, consistent with the idea that faces are more salient for inhibited individuals. Therefore, although the amygdala has been studied most extensively in inhibited temperament, due to its role in fear and threat evaluation, the current findings highlight the need to broaden our ideas about the inhibited brain. Instead of focusing on neural systems governed by heightened threat detection, fear and avoidance, we propose that the inhibited brain is governed by increased sensitivity to salient stimuli, including aversive, novel, rewarding and social stimuli. This sensitivity is likely manifest in multiple ways including heightened reactivity to salient stimuli (Schwartz *et al.*, 2003) and broader windows of what stimuli elicit brain responses (Blackford *et al.*, 2011). Interestingly, a recent study found that adults with high dispositional mindfulness—a trait associated with low stress reactivity—have smaller amygdala and caudate volumes (Taren *et al.*, 2013), providing a possible link between enhanced volume, salience detection and stress reactivity.

These findings raise interesting questions. For example, ‘What are the origins of these brain differences?’ Inhibited temperament has

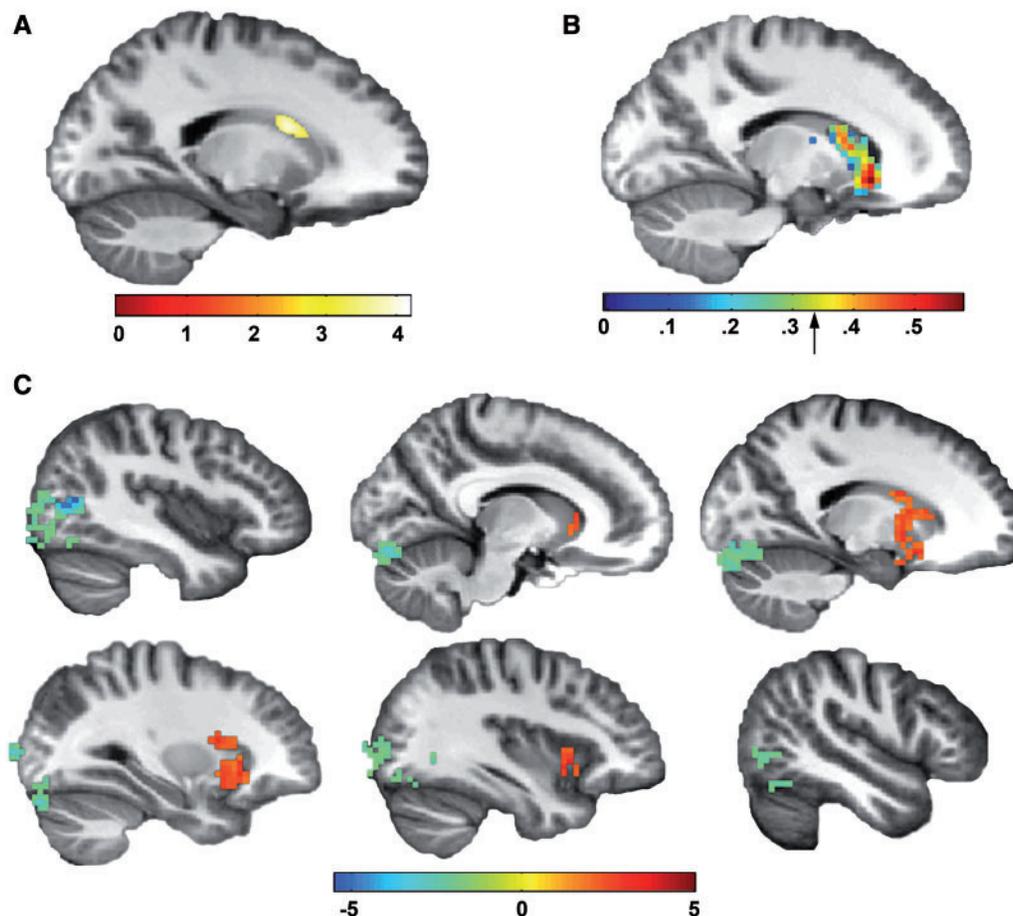


Fig. 3 Greater caudate volume and function in inhibited adults. (A) Inhibited adults had greater gray matter volume in the left caudate (496 voxels; $P < 0.05$ FWE corrected at the cluster level; peak voxel: $x = -19, y = 6, z = 17$). The region of group difference is illustrated on a sagittal slice of an average brain. (B) Gray matter volume was positively correlated with functional activation to faces in the left caudate. The correlations are thresholded at 0.01 for illustration; the arrow on the color bar marking $P < 0.05$ FWE corrected at the cluster level ($r = 0.33$). (C) Larger gray matter volume in the left caudate was associated with greater functional connectivity between the left caudate and right caudate, putamen, and insula ($P < 0.05$ FWE corrected at the cluster level) and with reduced functional connectivity between the left caudate and lateral occipital complex and cerebellum. Functional connectivity is illustrated across multiple sagittal slices of an average brain.

Table 3 Brain regions that show significant correlations between left caudate gray matter volume and functional connectivity

Cluster (volume mm ³)	Gyrus/region
<i>Positive correlation</i>	
Basal gangliar-insular (5238)	
Basal ganglia	R putamen, R caudate, R globus pallidus
Insula	R insula
<i>Negative correlation</i>	
Temporo-occipital (6075)	
Temporal	L middle temporal gyrus, L inferior temporal gyrus
Occipital	L middle occipital gyrus, L inferior occipital gyrus
Insular (3294)	L insula
Temporo-occipito-cerebellar (7182)	
Temporal	R inferior temporal gyrus, R middle temporal gyrus
Occipital	R middle occipital gyrus, R inferior occipital gyrus
Cerebellum	R declive, R posterior lobe

B, bilateral; L, left hemisphere; R, right hemisphere.

genetic origins—it is conserved across evolution, is heritable (Plomin and Daniels, 1986; Emde *et al.*, 1992; Robinson *et al.*, 1992; Eley *et al.*, 2003), and linked to specific genes (Arbelle *et al.*, 2003; Fox *et al.*, 2005b; Smoller *et al.*, 2005). Although nature is clearly

important, we propose that nurture is equally important. Key to this proposition is the fact that not all inhibited children become inhibited adults (Essex *et al.*, 2010; Clauss and Blackford, 2012). Developmental research has highlighted the contributions of parental environment (Williams *et al.*, 2009; Kiel and Buss, 2011; Marakovitz *et al.*, 2011; Tang *et al.*, 2012) and family stress (Volbrecht and Goldsmith, 2010) to the developmental trajectories of inhibited children. As with many other biological processes, we expect that nature and nurture are intertwined and have reciprocal influences on inhibited temperament across development. Many factors interact between nature and nurture, including intra-uterine environment (Buss *et al.*, 2012), epigenetic influences (McEwen, 2012; Monk *et al.*, 2012) and shaping of the environment by parents (Tang *et al.*, 2012). In this study, the observed structural differences may reflect both innate differences and the effect of being inhibited over many years. Much remains to be discovered about how genetic and environmental influences shape brain development in inhibited children.

Another question is ‘What is the mechanism by which structure may impact function?’ Studies in rodents and humans provide compelling evidence that brain structure and function can be altered by both learning (Markham and Greenough, 2004; Zatorre *et al.*, 2012) and drugs (Mitra and Sapolsky, 2008). Larger volume and greater functional activation may reflect underlying differences in gray matter—including axon sprouting, dendritic branching and synaptogenesis,

neurogenesis, glial changes, angiogenesis—or white matter, for example, changes in myelin formation, myelin remodeling or fiber organization (for a review, see Zatorre et al., 2012). In an attempt to link rodent studies with human MRI findings, a recent study found that structural MRI changes in rodents due to learning reflect changes in presynaptic terminal morphology. Although we cannot draw conclusions about the specific mechanism, future advances in technology may improve the ability to examine cellular changes *in vivo* and to link these changes to altered brain function and behavior in humans.

A limitation of the study is that the fMRI data were only available on a subsample of subjects. Although the sample size was similar to other functional studies and the subjects were representative of the larger sample, the functional findings should be considered preliminary. Also, the correlational nature of the study prevents us from drawing strong conclusions about the direction of the effects. We propose that these differences may be the underlying neurobiological basis of inhibited temperament; however, the differences may instead reflect the consequence of years of being inhibited. Animal models will be critical for teasing apart the directionality of this relationship. For example, a non-human primate model of inhibited temperament (anxious temperament) has been well-established (Fox et al., 2008; Oler et al., 2010; Shackman et al., 2013) and has the potential to inform the question of whether larger amygdala and caudate volume is a cause or a consequence of inhibited temperament.

In conclusion, this study provides evidence that inhibited temperament is associated with structural brain differences and that these differences have functional consequences for face processing. Given that inhibited individuals are at high risk for adverse long-term outcomes, these structural differences may also inform our understanding of why inhibited individuals have increased rates of developing psychiatric disorders. Inhibited individuals are more sensitive to the environment and more likely to avoid novel stimuli, which may result in heightened anxiety, and subsequently, depression and substance use. We propose that larger amygdala and caudate volume may mediate this enhanced environmental sensitivity. Studies aimed at identifying the cellular and molecular basis of larger amygdala and caudate volume may provide novel and specific targets for treatment. For example, studies in rodents have identified that increases in amygdala dendritic arborization are associated with anxious behavior (Vyas et al., 2002). Novel therapeutics that target dendritic branching or increase inhibitory projections to the amygdala may reduce inhibited or anxious behavior in humans. In addition, prevention strategies that focus on enriching the environments of high-risk children hold promise (Fox et al., 2013; Rapee, 2013).

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