

5-HTTLPR polymorphism impacts human cingulate-amygdala interactions: a genetic susceptibility mechanism for depression

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Carriers of the short allele of a functional 5' promoter polymorphism of the serotonin transporter gene have increased anxiety-related temperamental traits, increased amygdala reactivity and elevated risk of depression. Here, we used multimodal neuroimaging in a large sample of healthy human subjects to elucidate neural mechanisms underlying this complex genetic association. Morphometrical analyses showed reduced gray matter volume in short-allele carriers in limbic regions critical for processing of negative emotion, particularly perigenual cingulate and amygdala. Functional analysis of those regions during perceptual processing of fearful stimuli demonstrated tight coupling as a feedback circuit implicated in the extinction of negative affect. Short-allele carriers showed relative uncoupling of this circuit. Furthermore, the magnitude of coupling inversely predicted almost 30% of variation in temperamental anxiety. These genotype-related alterations in anatomy and function of an amygdala-cingulate feedback circuit critical for emotion regulation implicate a developmental, systems-level mechanism underlying normal emotional reactivity and genetic susceptibility for depression.

Depression is among the four leading causes of disability and disease burden throughout the world and is associated with serious medical conditions and mortality across the lifespan^{1,2}. The importance of serotonergic neurotransmission for the pathogenesis of depression is suggested clinically by the efficacy of serotonin re-uptake inhibitors (SSRIs), the first-line treatment of depression and most related anxiety disorders¹ and by induction of depression by tryptophan depletion in susceptible individuals². Post-mortem and *in vivo* studies of the serotonin transporter (5-HTT) and receptors support a role for this neurotransmitter system in depression and anxiety disorders¹. Furthermore, serotonin (5-HT) appears to be critical for the development of emotional circuitry in the brain, and even transient alterations in 5-HT homeostasis during early development modify neural connections implicated in mood disorders and cause permanent elevations in anxiety-related behaviors during adulthood³⁻⁵. Importantly, 5-HT has broad developmental effects, promoting differentiation not only of serotonergic but also of glutamatergic neurons, which transiently express 5-HTT in limbic regions such as cingulate cortex³.

Since heritability of depression approaches 70% (ref. 2), and anxious temperament is related to risk for depression², there has been intense interest in candidate genes related to serotonergic function and these phenotypes. A variable number of tandem repeats in the 5' promoter region (5-HTTLPR) of the human serotonin transporter gene (*SLC6A4*) has been shown both *in vitro* and *in vivo* to influence

transcriptional activity and subsequent availability of the 5-HTT^{6,7}. Specifically, the 5-HTTLPR short allele (*s*) has reduced transcriptional efficiency compared with the long allele (*l*), and individuals carrying the *s* allele tend to have increased anxiety related temperamental traits^{8,9}, which are related to increased risk for depression¹⁰. It has further been shown that *s* carrier status elevates the risk of depression in the context of environmental adversity¹¹ (a unique gene-environment interaction that has been independently replicated^{12,13}), and *s* alleles adversely affect outcome of SSRI treatment¹⁴. Thus, there is converging evidence that 5-HTTLPR genotype is related to the biology and risk for depression and anxiety-related temperamental traits. Previously, using functional magnetic resonance imaging (fMRI) we found that healthy, non-depressed *s* allele carriers show an exaggerated amygdala response to threatening visual stimuli compared with individuals with the *ll* genotype, suggesting a possible link between variation in the gene and a basic brain mechanism involved in processing of negative emotion^{15,16}. This finding has been independently replicated¹⁷. However, it remained unclear how this neurobiological association relates to clinical endpoints, as variation in amygdala response did not account for individual differences in behavioral measures of emotional reactivity^{15,16}.

Here, we used a multimodal neuroimaging strategy to identify mechanisms on the level of neural systems contributing to behavioral and, potentially, clinical effects associated with 5-HTTLPR. Because even simple emotionally-charged stimuli (for example, masked fearful

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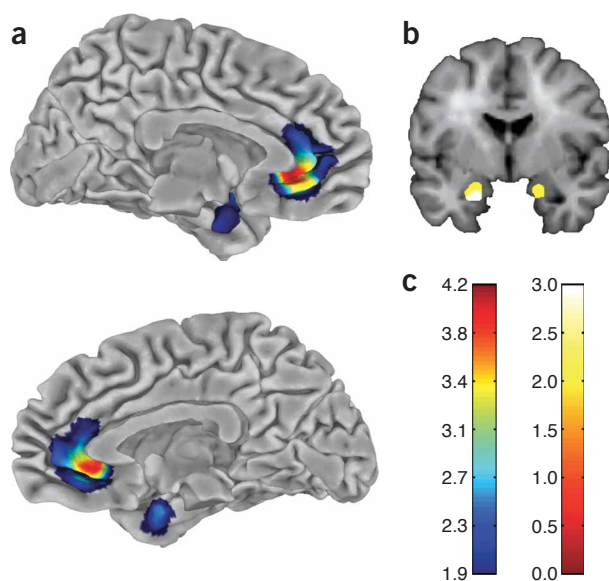


Figure 1 Thresholded ($P < 0.05$) statistical maps restricted to the limbic cortex and amygdala illustrating gray matter volume reductions of *s* allele carriers in comparison to *ll* genotype ($n = 114$). **(a)** Surface projections display significant volume reductions of bilateral perigenual anterior cingulate cortex and medial amygdala. Note that peak differences within the whole brain are found within the subgenual cingulate, a region implicated in the biology of depression and anxiety. **(b)** A coronal section through the amygdala displays significant bilateral volume reductions. **(c)** Color scales used for surface (left) and coronal (right) views represent *t*-scores.

eye-whites) are processed in the human brain by distributed interactive networks¹⁸, we hypothesized that the phenotypic expression of the 5-HTTLPR genotype would involve the structure and function of neural circuitries involved in emotion processing. We used voxel-based morphometry (VBM), a neuroanatomical MRI technique, to test for genetic association with the morphology of limbic circuitry,

consistent with neurodevelopmental studies of animals with altered 5-HT function^{3–5}. We then explored the functional relevance of the observed structural manifestations with an fMRI strategy, focusing on interactions within distributed mood circuitry important for affect generation and regulation, to test the hypothesis that 5-HTTLPR genotype affects development and patterns of neural activation within this circuitry. Assuming that neural mechanisms of disease susceptibility exist in clinically healthy individuals inheriting risk alleles, we restricted our study to a large sample of healthy Caucasian subjects without any lifetime psychiatric diagnosis or treatment, allowing us to exclude disease-related heterogeneity and environmental confounders.

We studied the effect of a functional variation in the serotonin transporter gene on limbic circuitry implicated in mood disorders. We found that *s* carriers had reduced gray matter volume in perigenual cingulate and amygdala. During processing of fearful stimuli, these same regions showed strong functional interactions. In *s* carriers this circuit was relatively uncoupled, and the magnitude of cingulate-amygdala interaction was a strong predictor of variation in temperamental anxiety, indicating genotype-related alterations in anatomy and function of a limbic feedback circuit critical for negative emotion.

RESULTS

Morphometry

First, we performed a structural imaging study using ‘optimized’ VBM^{19,20}, a sophisticated and automated method designed to measure gray matter volume changes with sufficient sensitivity to detect genotype effects of single nucleotide polymorphisms²¹. In comparison to *ll* genotype subjects, *s* allele carriers showed significantly reduced volume of the perigenual anterior cingulate cortex (pACC) and amygdala (**Fig. 1**, **Table 1**). Structural volume changes were more pronounced in the pACC than in amygdala (**Fig. 2a**), and only pACC and right amygdala remained significant ($P < 0.05$) after correction for multiple testing (however, the appropriateness of correcting for multiple tests with respect to the amygdala is debatable, considering prior data showing 5-HTTLPR effects on amygdala function^{15,16}). It is noteworthy that the rostral subgenual portion of the anterior cingulate cortex (rACC), a structure implicated in depression²², was the punctum maximum of observed gray matter volume reductions in *s* allele carriers within the whole brain (**Supplementary Figure 1** online).

Covariance of gray matter structures

Given the prior anatomical evidence of interconnection between amygdala and pACC^{23,24}, we determined the degree to which amygdala volume was related to volume of the pACC across all subjects by calculating measures of ‘structural covariance’ based on VBM data²⁵: using the general linear model, we estimated across the brain the degree to which regional (amygdala) volume covaried with that of a target region (pACC), putatively reflecting an aspect of neuronal ‘wiring’ with that region²⁵. We found significant positive covariation of amygdala and pACC volume, again with a maximum in the rACC and another local maximum in the more caudal supragenual portion of the anterior cingulate cortex (cACC; **Fig. 2b**, **Table 1**).

Table 1 Cluster maxima of morphometric analyses ($n = 114$)

Region	Subregion	<i>t</i>	<i>z</i>	<i>P</i> ^a	<i>x</i> ^b	<i>y</i>	<i>z</i>
Morphometry							
<i>ll</i> genotype > <i>s</i> carrier							
Subgenual anterior cingulate cortex ^c	BA 24	4.01	3.86	<0.001*	–3	33	–2
Supragenual anterior cingulate cortex	BA 24	2.87	2.81	0.002	0	30	4
Supragenual anterior cingulate cortex	BA 32	2.20	2.17	0.015	0	35	13
Left amygdala	—	2.39	2.35	0.009	–31	3	–15
Right amygdala	—	2.35	2.31	0.010*	18	–2	–22
Structural covariance							
Main effect							
Subgenual anterior cingulate cortex	BA 32	3.16	3.08	0.001*	3	36	–9
Supragenual anterior cingulate cortex	BA 32	3.30	3.22	0.001*	3	36	16
<i>ll</i> genotype > <i>s</i> carrier							
Subgenual anterior cingulate cortex	BA 25	3.11	3.04	0.001*	–1	18	–10
Supragenual anterior cingulate cortex	BA 32	2.28	2.25	0.010	3	33	22

^aUncorrected *P*-values. ^bCoordinates have been transformed from MNI space to that of Talairach and Tournoux; * $P < 0.05$ after correction for multiple comparisons based on a ROI of the limbic cortex or amygdala; ^cRegion with the maximal volume difference within the entire brain (post-hoc analysis); statistics have been thresholded with a *t*-score value corresponding to uncorrected $P = 0.05$.

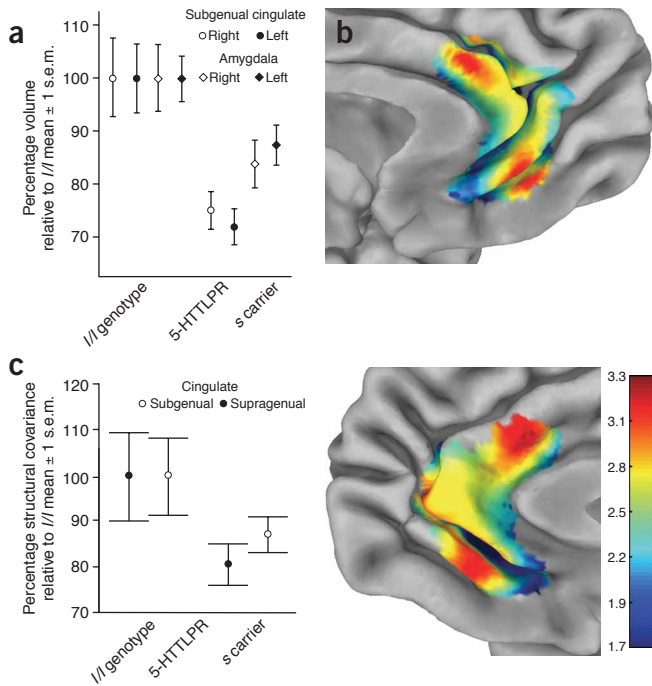


Figure 2 Structural data illustrating peak volume changes and results of structural covariance analyses ($n = 114$). Plots represent extracted peak results normalized to volume measures relative to the // genotype group mean. (a) Subgenual anterior cingulate cortex volume is markedly reduced (by >25%) in *s* allele carriers in comparison to // individuals. (b) Statistical map of structural covariance analysis displays different degrees of positive correlation between bilateral amygdala volume and perigenual cingulate volumes, with two local peaks located supra- and subgenually. (c) Plot displays reduced structural covariance in *s* carriers in comparison to // genotype individuals, particularly in the subgenual anterior cingulate cortex.

Relationship between regional volume and BOLD signal

Since our earlier reports of increased amygdala activation in response to threatening stimuli included individuals in this VBM analysis^{15,16}, we tested whether those functional results might be confounded by these structural changes (Supplementary Table 1). We did not observe a significant correlation between amygdala or pACC volume and fMRI blood oxygen level-dependent (BOLD) activation, suggesting that genotype differences in this functional parameter of amygdala response are not driven by local structural alterations.

Functional connectivity

The structural evidence that amygdala and pACC volumes were correlated suggests that they represent a functional circuitry modulated by genetic variation of the serotonergic system. We thus measured functional connectivity between these regions using fMRI to acquire BOLD signal during the perceptual processing of fearful and threatening facial expressions¹⁵. 'Functional connectivity' is a measure of correlated activity, derived from BOLD fMRI data, between a reference (amygdala) and target region (pACC) used widely in the imaging community as a simple and robust characterization of aspects of functional integration^{25–27}. Converging lines of evidence suggest that this measure reflects anatomically and functionally relevant coupling within neuronal circuitries²⁵; however, a finding of 'functional connection' should not be interpreted as proving the presence of structural or causal connections, as this measure is correlative in nature. We found in the entire dataset that amygdala and pACC were significantly

'functionally connected' (Fig. 3a, Table 2). Notably, amygdala connectivity distinguished two functionally divergent regions within pACC: rACC, which was positively correlated with the amygdala, and cACC, which was negatively correlated with it (Fig. 3a). These distinct zones of functional connectivity within the cingulate cortex (rACC, cACC) also showed strong positive connectivity with each other, suggesting that they might form a feedback loop with amygdala (Supplementary Table 2).

Functional connectivity and structural covariance

These various findings suggest that disruption of amygdala-pACC feedback circuitry could underlie the earlier observation of increased amygdala activity in *s* carriers during the processing of fearful stimuli (using the same procedure as here)^{15,16}. Therefore, we analyzed the effect of genotype on functional coupling between amygdala, rACC and cACC. Short allele carriers showed a highly significant reduction of amygdala-pACC connectivity in comparison to // genotype individuals (Fig. 3b, Table 2), particularly prominently in rACC (Fig. 4a). Within the cingulate, rACC-cACC functional connectivity did not differ by genotype (Supplementary Table 2). A similar finding was present in structural covariance, where *s* carriers showed significantly lower structural covariance between amygdala and rACC than did // individuals (Fig. 2c, Table 1).

Temperament correlates

As an important external validation, we reasoned that if functional uncoupling of this mood circuit underlies reported associations of 5-HTTLPR with emotional phenotypes, functional connectivity between amygdala and rACC should predict normal variation in temperamental trait measures related to anxiety and depression that also have been related to 5-HTTLPR^{8,9}. Therefore, we performed a correlation analysis based on temperament ratings evaluated by the

Figure 3 Statistical functional connectivity maps between bilateral amygdala and perigenual anterior cingulate cortex representing degree of functional coupling between these structures ($n = 94$). (a) Subgenual cortical regions in left hemisphere (top) and right hemisphere (bottom) correlate positively with amygdala activity during the perception of threatening faces, whereas supragenual regions correlate negatively (color bar represents *t*-scores). (b) 5-HTTLPR *s* allele carriers show significantly less functional coupling between amygdala and perigenual anterior cingulate cortex than // individuals, particularly in the subgenual region (color bar represents absolute *t*-scores).

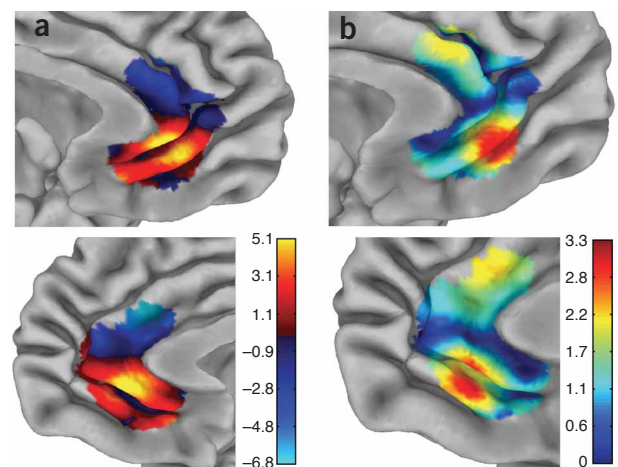


Table 2 Cluster maxima of functional connectivity analyses between bilateral amygdala and perigenual cingulate cortex ($n = 94$).

Region	Subregion	t	z	P^a	x^b	y	z
Main effect							
Subgenual anterior cingulate cortex	BA 32	5.57	5.16	<0.001*	0	37	-2
Supragenual anterior cingulate cortex	BA 32	-10.27	Inf	<0.001*	0	34	26
// genotype > s carrier							
Subgenual anterior cingulate cortex	BA 32	3.27	3.18	0.001*	4	40	-6
Supragenual anterior cingulate cortex	BA 32	-2.54	2.49	0.006*	-4	38	17

^aUncorrected P -values. ^bCoordinates have been transformed from MNI space to that of Talairach and Tournoux; * $P < 0.05$ after correction for multiple comparisons based on ROIs derived from our structural analysis (perigenual cingulate). Inf, infinite. Statistics have been thresholded with a t -score value corresponding to uncorrected $P = 0.05$.

Tridimensional Personality Questionnaire (TPQ)²⁸, choosing the total harm avoidance subscale, a well-validated heritable measure linked to anxiety and risk for depression that has been weakly related to 5-HTTLPR status²⁹. Almost 30% of the variance in harm avoidance scores was predicted by our measure of amygdala-rACC functional connectivity. In contrast, local functional or structural measures of single regions were of no predictive value (Fig. 4b, Supplementary Table 3), consistent with earlier results^{15,16}.

Analysis of amygdala–medial prefrontal cortex relationship

A recent fMRI study¹⁷ reported that 5-HTTLPR genotype affected functional coupling of the amygdala with ventromedial prefrontal cortex (Brodmann area (BA) 10) in a sample of 29 normal male subjects analyzed with the same statistical approach employed herein. In contrast to our data in pACC, this study¹⁷ showed increased coupling of both amygdala and a region of ventral medial prefrontal cortex (BA 10) in *s* allele carriers compared with // genotype. Cortical regions analyzed in this and our study were largely non-overlapping (Supplementary Figure 2); our analysis was based on a region of interest defined *a priori* by a genotype effect on brain structure and contained most of the ACC. In contrast, these authors¹⁷ created a spherical search region in medial prefrontal cortex, which did not cover major parts of the ACC and specifically not the region where we report

maximal effects for genotype (rACC) (Supplementary Figure 2). To compare these results further, we constructed in our dataset the same search region as reported¹⁷ and performed a new functional connectivity analysis based on this region and the amygdala. We found a strong main effect on functional connectivity with amygdala and BA10 in the same direction observed previously¹⁷ (Supplementary Table 4). With respect to genotype we found a weak effect of increased functional connectivity in *s* carriers in comparison to // genotype (Supplementary Figure 3) that became more pronounced if only males were studied (Supplementary Table 4). This suggests that our functional connectivity data are consistent with the main effect of functional connectivity as recently reported¹⁷ and are, with respect to genotype, at least qualitatively consistent across these studies.

DISCUSSION

Our multimodal imaging approach has identified an effect of 5-HTTLPR genotype on structure, function and interconnections of a neural circuit encompassing both amygdala and regions of the rACC and cACC. These structures have been prominently implicated in studies of depression and negative emotion^{22,30,31}. Moreover, the measure of amygdala-rACC connectivity impacted by this polymorphism predicted a notable degree of variation in a measure of anxious temperament that also has been linked to risk for depression¹². Convergent evidence from neuroimaging and neuropathological studies suggests a key regulatory role for these regions in negative emotion processing, particularly the rACC (BA 32/25/24) but also the cACC (BA 32; ref. 23). Reduced activity of rACC is found in depression²² and induced sadness³¹ and can be reversed by various antidepressant therapies, including SSRIs^{32,33}, sleep deprivation³⁴ and even cingulotomy³⁵. Notably, the region showing the greatest effect of 5-HTTLPR genotype (pACC) is within the phylogenetically older archicortical portion of the cingulate cortex³⁶, a region that displays the highest density of 5-HTT terminals within the human cortex³⁷ and that is a target zone of dense projections from the amygdala²⁴.

The functional connectivity analysis demonstrates that amygdala and pACC are significantly ‘functionally connected.’ It delineates two distinct subregions that agree well with cytoarchitectonic and functional subdivisions (rACC, pACC) of this region as discussed above. This pattern of functional connectivity derived from fMRI data is markedly analogous to anatomical studies in primate brain showing massive amygdala projections to rACC and efferent projections from cACC back to the amygdala²⁴. Convergent evidence strongly suggests that these interactions represent a functional feedback circuitry that regulates amygdala processing of environmental adversity: stimulation of perilimbic prefrontal cortex inhibits amygdala function³⁸, medial prefrontal cortex neurons also exert an inhibitory influence on the amygdala³⁹ and lesions of this region markedly impair fear extinction⁴⁰.

Given the evidence that rACC modulates amygdala activity by inhibition^{39,41}, our finding of reduced amygdala connectivity in *s* carriers to rACC provides a potential mechanistic account for the observed increase in amygdala activity in *s* carriers because reduced coupling would translate into altered feedback regulation of amygdala activity^{15,16} (Supplementary Figure 4). It would be of interest to extend our observations on functional connectivity using ‘effective connectivity’ methods that allow inferences about directionality in the interactions observed here.

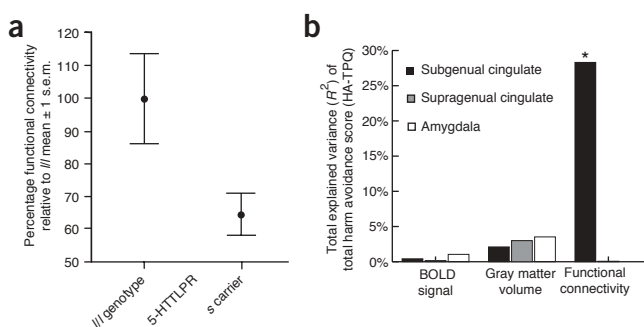


Figure 4 Functional connectivity between the subgenual cingulate and amygdala is dissociated by genotype and significantly explains harm avoidance scores, whereas other functional or structural measures do not. (a) Short allele carriers show significantly reduced functional connectivity compared to // genotype ($n = 94$). Plot represents extracted peak results normalized to the mean absolute functional connectivity, relative to the // genotype group. (b) Only functional connectivity between subgenual cingulate and amygdala explains (Bonferroni corrected, $P < 0.05$) harm avoidance scores, in contrast to other structural and functional measures ($n = 26$ subjects with both functional and structural data). Amygdala was used as reference for connectivity measures. A small vertical line indicates values close to zero. Error bars, s.e.m.

Taken together, our data show that 5-HTTLPR genotype affects the structure and putative wiring of a core region within the limbic system thought to be crucial for depression and anxiety-related temperamental traits. Since our morphometric analysis was age- and gender-independent and based on data from healthy Caucasian individuals without any history of psychiatric illness or treatment, we conclude that 5-HTTLPR affects brain development of these core regions and the functionality of related brain circuitries. Our finding that 5-HTTLPR impacts cortical structure is consistent with longstanding evidence that 5-HT plays an important role in cortical development, shaping neuronal circuitries by regulating synaptic plasticity and neuronal activity patterns of serotonergic and non-serotonergic neurons³. Serotonergic neurons are among the first neurons to be generated, and even non-serotonergic neurons may transiently express 5-HTT within cingulate cortex in animals³. This pattern of expression in non-serotonergic neurons within a specific temporal window has been hypothesized to underlie the formation and fine-tuning of specific connectivity patterns³, possibly through regulation of synaptogenesis and growth cone motility⁴². Reduced relative connectivity in *s* allele carriers may be further compounded by the ability of 5-HT to alter differentiation of glutamatergic neurons, the major projection neurons for cortico-cortical interactions³.

Our data further demonstrate that 5-HTTLPR specifically affects a functional interaction between amygdala and rACC, an effect that predicted a large degree of variance in normal subjects in a temperamental trait related to neuroticism and associated with greater risk for depression. These findings echo those from studies suggesting a key role for rACC in neural interactions corresponding to affective personality styles, which are risk factors for depression⁴³. As noted, a substantial body of preclinical and clinical work shows the functional relevance of this circuitry in inhibitory modulation of the amygdala by the rACC, with a specific role in fear extinction and depression. The amygdala is densely directly connected to the rACC as mentioned above. In contrast, amygdala connections to ventromedial prefrontal cortex (BA10), which may also participate in regulating amygdala activity¹⁷, if they exist at all^{44,45}, are sparse⁴⁶. Thus, the previously reported finding¹⁷ of increased functional connectivity in *s* allele carriers in this region (supported in our own data set) is likely based on indirect anatomical interconnections. It is, therefore, tempting to speculate that ventromedial BA10 may function as an indirect regulatory area, and the observation of increased 'functional connectivity' in *s* allele carriers may then represent a compensatory mechanism for alterations in the primary regulatory loop involving the cingulate delineated here.

Two differences may have contributed to the effect in BA10 being stronger in the previous study¹⁷ than in our own data. First, differences in task design, which there¹⁷ required explicit processing of emotion-laden complex visual scenes, resulting in a more pronounced top-down regulation of limbic structures³⁹ expected to engage more upstream modulatory prefrontal regions. Second, the previous study¹⁷ examined only males, and both our reanalysis and a recent report on emotional processing of negatively perceived verbal attributes indicate a possible gender effect in BA10 (ref. 47). We therefore propose that decreased coupling in amygdala-rACC feedback circuitry leads to a relatively dysregulated amygdala response, and that prior observations of amygdala hyperactivity and increased anxiety-related traits in *s* allele carriers are based on changes in this circuit and probably lead to compensatory overactivity in the ventromedial prefrontal cortex.

Our findings suggest a causal mechanism linking developmental alterations in 5-HT-dependent neuronal pathways to impaired interactions in a regulatory network mediating emotional reactivity. The

effects of 5-HTTLPR genotype converge on the rACC in our structural and functional connectivity analyses, which, coupled with evidence of uniquely dense serotonergic innervation of this region, argues that genetically driven variation in 5-HT signaling shapes the connectivity of amygdala with this region. These alterations are manifested in anxiety-related temperamental traits, possibly reflecting inadequate regulation and integration of amygdala-mediated arousal, leading to an increased vulnerability for persistent negative affect and eventually depression in the context of accumulating environmental adversity. While investigations of localized structural and functional abnormalities have provided insights about depression, our data underscore the importance of studying genetic mechanisms of complex brain disorders at the level of dynamically interacting neural systems. We suggest that such relationships better capture the functional consequences of neurodevelopmental processes altering circuitry function implicated in human temperament and psychiatric disorders.

METHODS

Assessment of subject data. *Demographics.* Subjects were culled from a larger population after careful screening⁴⁸ to ensure they were free of any lifetime history of psychiatric or neurological illness, psychiatric treatment or drug or alcohol abuse (**Supplementary Table 5**). Only Caucasians of European ancestry were studied to avoid stratification artifacts. All available scans of subjects meeting these criteria were used. Subjects gave written informed consent and participated in the study according to the guidelines of the National Institute of Mental Health Institutional Review Board. Structural MRIs from 114 subjects were used and customized templates were created from a larger sample of 214 healthy volunteers²¹. Functional MRIs of 94 subjects were studied. Twenty-six (28%) subjects from the functional analyses were also part of our morphometric analyses.

Mood and personality assessment. The harm avoidance subset of the TPQ was administered, as it has been noted as a putative index of heritable behavior traits reportedly related to anxiety, amygdala function and 5-HTTLPR status. Harm avoidance scores were available for 109 subjects included in the morphometric study and 79 subjects included in the functional study.

Genotyping. Genotyping was performed as described previously¹⁵. In addition, our sample was genotyped with a panel of 100 unlinked SNP loci to survey for occult genetic stratification between 5-HTTLPR genotype groups and showed no significant differences in frequency at any of these SNPs, including several that have been associated with variation in brain function (for example, *COMT*, *BDNF*, *GRM3*, *GAD1*, *APOe4*; available upon request).

Imaging. *Functional task.* During fMRI scanning, subjects completed a simple perceptual task previously described to robustly engage the amygdala^{15,16}. During two blocks of an emotion task, subjects viewed a trio of faces, selecting one of the two faces (bottom) that was identical to the target face (top). Per block, six images were presented sequentially for 5 s, three of each gender and target affect (angry or afraid) derived from a standard set of pictures of facial affect. Emotion tasks alternated with three blocks of a sensorimotor control task where faces were replaced with simple geometric shapes.

Structural image processing. Three-dimensional structural MRI scans were acquired on a 1.5-T GE scanner using a T1-weighted SPGR sequence (TR/TE/NEX 24/5/1, flip angle 45°, matrix size 256 × 256, FOV 24 × 24 cm) with 124 sagittal slices (0.94 × 0.94 × 1.5 mm resolution) and pre-processed as previously described²¹ followed by an optimized VBM protocol using customized templates^{19,20}. Resulting gray matter images were smoothed with a 12-mm Gaussian kernel prior to statistics. Analysis was performed on Linux workstation (RedHat) using MATLAB 6.52SP2 (MathWorks) within the General Linear Model⁴⁹ in SPM2 (<http://www.fil.ion.ucl.ac.uk/spm>). The specification of a design matrix identical to the one used in this study was described in detail elsewhere²¹. Briefly, effects of 5-HTTLPR on gray matter volume were examined by using an analysis of covariance model including the following covariates of no interest: total gray matter volume, orthogonalized first- and second-order polynomials of age, and gender. A hypothesis-driven regions of interest (ROI) approach was used to investigate structural alterations induced by genotype within the limbic cortex, including bilateral amygdala, hippocam-

pal formation, and cingulate cortex. Gray-matter volume changes were assessed statistically using one-tailed *t*-contrasts after small-volume correction for the limbic cortex or amygdala. False discovery rate estimations were used to correct for multiple comparisons, and a probability of 0.05 was considered to be significant. In addition, the *t*-statistic map representing decreased gray matter volumes of *s* carriers in comparison to *l/l* genotype was used to define the search region (ROI) for subsequent functional connectivity analyses by creating a binary mask including voxels showing a significant reduction at $P < 0.001$ (uncorrected). Anatomical amygdala ROIs were based on Talairach labels using the WFU Pick atlas (<http://www.fmri.wfubmc.edu>) software.

Functional image processing. BOLD fMRI was performed on a GE Signa 3-T scanner using gradient echo EPI (24 axial slices, 4 mm thickness, 1 mm gap, TR/TE = 2000/28 ms, FOV = 24 cm, matrix = 64 × 64). Images were processed as described previously^{15,16} using SPM99 (<http://www.fil.ion.ucl.ac.uk/spm>). Briefly, images were realigned to the middle image of the scan run, spatially normalized into a standard stereotactic space (MNI template) using an affine and nonlinear (4 × 5 × 4 basis-functions) transformation, smoothed with a 8-mm FWHM Gaussian filter and ratio normalized to the whole-brain global mean. A statistical image for the contrast of the emotion task versus the sensorimotor control was obtained for each subject and analyzed in a second-level random effects model (ANOVA and one-tailed *t*-test) to identify significant ($P < 0.05$, corrected for multiple comparisons) activations within and between genotype groups.

Functional connectivity analyses. Our methods to measure 'functional connectivity' have been described previously^{26,50}. This measure examines the covariation across the brain with the activation in a region (volume) of interest. We used anatomical masks derived from VBM analyses to define these regions. After mean and drift correction of the time series, median activity within this region of interest was calculated (we prefer median as a robust estimator that coincides with the mean under the assumption of normality) for each scan. These values were then correlated across the brain with all voxel time series, resulting in a map which contained, in each voxel, the correlation coefficient of the time series in this voxel with that of the reference regions. These maps, one per subject, were then analyzed in a random-effects model in SPM. To identify regions significantly positively or negatively connected with the target region, a one-sample *t*-test was used. To compare connectivity with the target region across genotypes, a two-sample *t*-test was used. In each case, statistical parametric maps reflected one-tailed significance (as is standard in the employed software package, SPM) and were produced using appropriate *t*-contrasts. Using the Gaussian random fields approach implemented in SPM, correction for multiple comparisons was applied with inference restricted to appropriate regions of interest as mentioned above.

Structural covariance analysis. 'Structural covariance' uses a similar measure of coupling as functional connectivity, only this time not between functional data (fMRI time series), but using voxel-wise gray matter volume maps derived from VBM (see above). Voxels identified as significant in this approach will have regional volume that is significantly positively or negatively correlated with the target area across subjects. Summed volume in an anatomically defined region of interest in standardized space was computed by adding the local volume in all voxels comprising the ROI and used as a covariate of interest in a random-effects general linear model in SPM that also included nuisance covariates as described above for VBM. Statistical inference proceeded exactly as described for VBM and functional connectivity by using appropriate contrasts to derive SPMs for the significance of the volume covariate (to identify regions positively or negatively covarying in volume with the target region) or difference in correlation between the volume covariate split between genotype groups (to identify regions differing in their dependence on target area volume by genotype), again achieving multiple comparison correction with the Gaussian random fields approach and restricting inference to appropriate regions of interest as detailed in the manuscript.

Creation of surface maps. SPM images files containing *t*-statistics have been converted into AFNI format and warped from MNI into Talairach space using AFNI software (<http://afni.nimh.nih.gov/afni>). C. Holmes' pial brain surface in Talairach space has been used as projection target, whereas its volume representation in Talairach space served as grid parent (<http://afni.nimh.nih.gov/afni/suma>). Only voxels with a maximal distance of 4 mm along the segment of the cortical gray matter band were considered for mapping onto the

surface. A mapping function was chosen which maps only maximal statistical values along the gray matter band segment in the brain volume onto the surface. For surface maps displaying positive and negative statistics, maximal absolute values have been mapped at the surface. Statistical maximal projection maps were displayed within above-mentioned ROIs derived from VBM on C. Holmes' pial brain surface with SUMA, choosing Matlab's standard color bar where appropriate. A customized color bar has been generated for results of the connectivity analysis irrespective of genotype in order to visualize positive and negative 'correlations' in different subdivisions of the anterior cingulate.

Creation of plots. Plots represent extracted relative volumes, functional connectivity or structural covariance β coefficients of SPMs at cluster peak locations as reported in tables. Extracted values have been normalized to mean values relative to the *l/l* genotype group using SPSS 11.0 (SPSS) for Mac OS X (Apple Computers) resulting in percentage changes relative to *l/l* genotype. Functional connectivity or structural covariance data have been transformed to absolute measures before normalization in order to display connectivity/covariance data on a positive scale.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Nemeroff, C.B. & Owens, M.J. Treatment of mood disorders. *Nat. Neurosci.* **5** (suppl.), 1068–1070 (2002).
- Wong, M.L. & Licinio, J. Research and treatment approaches to depression. *Nat. Rev. Neurosci.* **2**, 343–351 (2001).
- Gaspar, P., Cases, O. & Maroteaux, L. The developmental role of serotonin: news from mouse molecular genetics. *Nat. Rev. Neurosci.* **4**, 1002–1012 (2003).
- Gross, C. & Hen, R. The developmental origins of anxiety. *Nat. Rev. Neurosci.* **5**, 545–552 (2004).
- Ansorge, M.S., Zhou, M., Lira, A., Hen, R. & Gingrich, J.A. Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science* **306**, 879–881 (2004).
- Lesch, K.P. *et al.* Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* **274**, 1527–1531 (1996).
- Murphy, D.L., Lerner, A., Rudnick, G. & Lesch, K.P. Serotonin transporter: gene, genetic disorders, and pharmacogenetics. *Mol. Interv.* **4**, 109–123 (2004).
- Schinka, J.A., Busch, R.M. & Robichaux-Keene, N. A meta-analysis of the association between the serotonin transporter gene polymorphism (5-HTTLPR) and trait anxiety. *Mol. Psychiatry* **9**, 197–202 (2004).
- Sen, S., Burmeister, M. & Ghosh, D. Meta-analysis of the association between a serotonin transporter promoter polymorphism (5-HTTLPR) and anxiety-related personality traits. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **127**, 85–89 (2004).
- Lotrich, F.E. & Pollock, B.G. Meta-analysis of serotonin transporter polymorphisms and affective disorders. *Psychiatr. Genet.* **14**, 121–129 (2004).
- Caspi, A. *et al.* Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* **301**, 386–389 (2003).
- Kendler, K.S., Kuhn, J. & Prescott, C.A. The interrelationship of neuroticism, sex, and stressful life events in the prediction of episodes of major depression. *Am. J. Psychiatry* **161**, 631–636 (2004).
- Eley, T.C. *et al.* Gene-environment interaction analysis of serotonin system markers with adolescent depression. *Mol. Psychiatry* **9**, 908–915 (2004).
- Murphy, G.M. Jr., Hollander, S.B., Rodrigues, H.E., Kremer, C. & Schatzberg, A.F. Effects of the serotonin transporter gene promoter polymorphism on mirtazapine and paroxetine efficacy and adverse events in geriatric major depression. *Arch. Gen. Psychiatry* **61**, 1163–1169 (2004).
- Hariri, A.R. *et al.* Serotonin transporter genetic variation and the response of the human amygdala. *Science* **297**, 400–403 (2002).
- Hariri, A.R. *et al.* A susceptibility gene for affective disorders and the response of the human amygdala. *Arch. Gen. Psychiatry* **62**, 146–152 (2005).
- Heinz, A. *et al.* Amygdala-prefrontal coupling depends on a genetic variation of the serotonin transporter. *Nat. Neurosci.* **8**, 20–21 (2005).
- Whalen, P.J. *et al.* Human amygdala responsivity to masked fearful eye whites. *Science* **306**, 2061 (2004).
- Good, C.D. *et al.* A voxel-based morphometric study of ageing in 465 normal adult human brains. *Neuroimage* **14**, 21–36 (2001).



20. Ashburner, J. & Friston, K.J. Voxel-based morphometry—the methods. *Neuroimage* **11**, 805–821 (2000).
21. Pezawas, L. *et al.* The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology. *J. Neurosci.* **24**, 10099–10102 (2004).
22. Drevets, W.C. *et al.* Subgenual prefrontal cortex abnormalities in mood disorders. *Nature* **386**, 824–827 (1997).
23. Phillips, M.L., Drevets, W.C., Rauch, S.L. & Lane, R. Neurobiology of emotion perception II: Implications for major psychiatric disorders. *Biol. Psychiatry* **54**, 515–528 (2003).
24. Paus, T. Primate anterior cingulate cortex: where motor control, drive and cognition interface. *Nat. Rev. Neurosci.* **2**, 417–424 (2001).
25. Ramnani, N., Behrens, T.E., Penny, W. & Matthews, P.M. New approaches for exploring anatomical and functional connectivity in the human brain. *Biol. Psychiatry* **56**, 613–619 (2004).
26. Meyer-Lindenberg, A. *et al.* Evidence for abnormal cortical functional connectivity during working memory in schizophrenia. *Am. J. Psychiatry* **158**, 1809–1817 (2001).
27. Friston, K.J., Frith, C.D., Liddle, P.F. & Frackowiak, R.S. Functional connectivity: the principal-component analysis of large (PET) data sets. *J. Cereb. Blood Flow Metab.* **13**, 5–14 (1993).
28. Cloninger, C.R., Svrakic, D.M. & Przybeck, T.R. A psychobiological model of temperament and character. *Arch. Gen. Psychiatry* **50**, 975–990 (1993).
29. Reif, A. & Lesch, K.P. Toward a molecular architecture of personality. *Behav. Brain Res.* **139**, 1–20 (2003).
30. Drevets, W.C. *et al.* Glucose metabolism in the amygdala in depression: relationship to diagnostic subtype and plasma cortisol levels. *Pharmacol. Biochem. Behav.* **71**, 431–447 (2002).
31. Mayberg, H.S. *et al.* Reciprocal limbic-cortical function and negative mood: converging PET findings in depression and normal sadness. *Am. J. Psychiatry* **156**, 675–682 (1999).
32. Drevets, W.C., Bogers, W. & Raichle, M.E. Functional anatomical correlates of antidepressant drug treatment assessed using PET measures of regional glucose metabolism. *Eur. Neuropsychopharmacol.* **12**, 527–544 (2002).
33. Mayberg, H.S. *et al.* Regional metabolic effects of fluoxetine in major depression: serial changes and relationship to clinical response. *Biol. Psychiatry* **48**, 830–843 (2000).
34. Wu, J.C., Buchsbaum, M. & Bunney, W.E., Jr. Clinical neurochemical implications of sleep deprivation's effects on the anterior cingulate of depressed responders. *Neuropsychopharmacology* **25**, S74–S78 (2001).
35. Dougherty, D.D. *et al.* Cerebral metabolic correlates as potential predictors of response to anterior cingulotomy for treatment of major depression. *J. Neurosurg.* **99**, 1010–1017 (2003).
36. Mega, M.S., Cummings, J.L., Salloway, S. & Malloy, P. The limbic system: an anatomic, phylogenetic, and clinical perspective. *J. Neuropsychiatry Clin. Neurosci.* **9**, 315–330 (1997).
37. Varnas, K., Halldin, C. & Hall, H. Autoradiographic distribution of serotonin transporters and receptor subtypes in human brain. *Hum. Brain Mapp.* **22**, 246–260 (2004).
38. Stefanacci, L. & Amaral, D.G. Some observations on cortical inputs to the macaque monkey amygdala: an anterograde tracing study. *J. Comp. Neurol.* **451**, 301–323 (2002).
39. Maren, S. & Quirk, G.J. Neuronal signalling of fear memory. *Nat. Rev. Neurosci.* **5**, 844–852 (2004).
40. Sotres-Bayon, F., Bush, D.E. & LeDoux, J.E. Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction. *Learn. Mem.* **11**, 525–535 (2004).
41. Rosenkranz, J.A., Moore, H. & Grace, A.A. The prefrontal cortex regulates lateral amygdala neuronal plasticity and responses to previously conditioned stimuli. *J. Neurosci.* **23**, 11054–11064 (2003).
42. Haydon, P.G., McCobb, D.P. & Kater, S.B. Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. *Science* **226**, 561–564 (1984).
43. Keightley, M.L. *et al.* Personality influences limbic-cortical interactions during sad mood induction. *Neuroimage* **20**, 2031–2039 (2003).
44. Carmichael, S.T. & Price, J.L. Limbic connections of the orbital and medial prefrontal cortex in macaque monkeys. *J. Comp. Neurol.* **363**, 615–641 (1995).
45. Ghashghaie, H.T. & Barbas, H. Pathways for emotion: interactions of prefrontal and anterior temporal pathways in the amygdala of the rhesus monkey. *Neuroscience* **115**, 1261–1279 (2002).
46. Amaral, D.G. & Price, J.L. Amygdalo-cortical projections in the monkey (*Macaca fascicularis*). *J. Comp. Neurol.* **230**, 465–496 (1984).
47. Shirao, N., Okamoto, Y., Mantani, T., Okamoto, Y. & Yamawaki, S. Gender differences in brain activity generated by unpleasant word stimuli concerning body image: an fMRI study. *Br. J. Psychiatry* **186**, 48–53 (2005).
48. Egan, M.F. *et al.* Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. *Proc. Natl. Acad. Sci. USA* **98**, 6917–6922 (2001).
49. Friston, K.J. *et al.* Statistical parametric maps in functional imaging: a general linear approach. *Hum. Brain Mapp.* **2**, 189–210 (1994).
50. Meyer-Lindenberg, A. *et al.* Neural basis of genetically determined visuospatial construction deficit in Williams syndrome. *Neuron* **43**, 623–631 (2004).

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