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ORIGINAL RESEARCH ARTICLE

A regulatory variant of the human tryptophan hydroxylase-2 gene biases amygdala reactivity

SM Brown[1], E Peet[2], SB Manuck[2], DE Williamson[1], RE Dahl[1], RE Ferrell[3] and AR Hariri[1]

[1]*Department of Psychiatry, University of Pittsburgh, Pittsburgh, PA, USA;* [2]*Department of Psychology, University of Pittsburgh, Pittsburgh, PA, USA;* [3]*Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA, USA*

Recent studies have indicated that a newly identified second isoform of the tryptophan hydroxylase gene (*TPH2*) is preferentially involved in the rate-limiting synthesis of neuronal serotonin. Genetic variation in the human TPH2 gene (*hTPH2*) has been associated with altered *in vitro* enzyme activity as well as increased risk for mood disorders. Here, we provide the first *in vivo* evidence that a relatively frequent regulatory variant (G(−844)T) of *hTPH2* biases the reactivity of the amygdala, a neural structure critical in the generation and regulation of emotional behaviors.
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Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the synthesis of serotonin (5–HT), a key modulator of mood and affect. Thus, the identification of genetic variation contributing to functional changes in TPH enzymatic activity is of great interest in determining the biological pathways underpinning individual differences in emotional behaviors and risk for psychiatric disorders, including depression, anxiety, and suicidality. Recent molecular and cellular studies have revealed the existence of a second TPH isoform, tryptophan hydroxylase-2 (*TPH2*), exclusively expressed in the murine brain.[1]

Functional assays demonstrate that *TPH2*—and not the original isoform, referred to now as *TPH1*—is responsible for regulating TPH expression and 5-HT synthesis in the murine central nervous system.[2] In contrast, both *TPH1* and *TPH2* are expressed in the human brain[3] and genetic variation in both isoforms have been associated with alterations in mood and 5-HT function.[4,5] Furthermore, a relatively rare single nucleotide polymorphism in human *TPH2* (*hTPH2*) associated with depression has been shown to alter TPH enzymatic efficiency *in vitro*.[6] However, the functional consequence of genetic variation in *hTPH2* on the neural circuitry of mood and emotional behaviors is unknown.

In the current study, we used functional magnetic resonance imaging (fMRI) to examine the effects of a single nucleotide polymorphism (G(−844)T) in the upstream regulatory region of *hTPH2* on the reactivity of the amygdala, a central structure in the mediation of behavioral and physiologic arousal associated with emotional behaviors. Although the molecular and cellular effects of the G(−844)T polymorphism are unknown, we focused on this regulatory variant for several reasons. First, unlike other *hTPH2* variants with demonstrated impact on enzymatic activity,[6] the G(−844)T has a relatively high minor allele frequency (T allele = 38%) and thus, has the potential to contribute more broadly to brain function and risk for mood disorders. Second, this variant is located within 1 kb (844 bp upstream) of the transcription initiation site of *hTPH2* and is likely a constituent of the proximal promoter of the gene. Regulatory variants in general often impact gene expression[7] and several specific promoter polymorphisms in other 5-HT subsystem genes have demonstrated effects on brain function and emotional behaviors.[8–10] For example, a frequent variant in the promoter region of the human serotonin transporter gene (5-HTTLPR) impacts gene expression, transporter availability and 5-HT reuptake,[10] as well as the response of the amygdala to environmental threat.[11–15] Finally, queries of transcriptional regulatory databases (http://www.genomatix.de) reveal evidence of transcription factor recognition sequence homology surrounding the −844 promoter variant and indicate that the specific G to T allele substitution potentially modifies the binding of several transcription factors including octamer-binding factor 6, special AT-rich sequence-binding protein 1 as well as homeodomain proteins MSX-1 and MSX-2.[16]

Materials and methods

Subjects

A total of 31 healthy adult volunteers participated after providing informed consent according to the guidelines of the University of Pittsburgh Institutional Review Board. All subjects were recruited from

Correspondence: Dr AR Hariri, PhD, Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA. E-mail: haririar@upmc.edu
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a larger parent study of genetic and behavioral risk factors for cardiovascular disease. Subjects were generally healthy and exclusion criteria included (1) medical diagnoses of cancer, stroke, diabetes requiring pharmacological treatment, chronic kidney, or liver disease, and a lifetime history of psychotic symptoms; (2) use of psychotropic, glucocorticoid, or hypolipidemic medication; (3) conditions affecting cerebral blood flow and metabolism (eg hypertension); and (4) diagnosed DSM-IV Axis I disorders.

Candidate genotyping
DNA was isolated and amplified from blood samples obtained from all subjects. The *hTPH2* G(−844)T promoter single nucleotide polymorphism (dbSNP accession number rs4570625) was genotyped using fluorescence polarization.[17] The 5-HTTLPR was genotyped using PCR amplification.[18] To determine if there were any differences in nongenotype confounds between the genotype groups, one-way ANOVA and χ^2 tests were used.

Amygdala reactivity paradigm
The experimental fMRI paradigm consisted of four blocks of a face-processing task interleaved with five blocks of a sensorimotor control task. Subject performance (accuracy and reaction time) was monitored during all scans. During the face-processing task, subjects viewed a trio of faces (expressing either anger or fear) and selected one of two faces (bottom) identical to a target face (top). Each face processing block consisted of six images, three of each gender and target affect (angry or fearful) all derived from a standard set of pictures of facial affect.[19] During face-processing blocks, each image was presented for 4 s with a variable interstimulus interval (2–6 s). During the sensorimotor control task, subjects viewed a trio of simple geometric shapes (circles, vertical and horizontal ellipses) and selected one of two shapes (bottom) identical to a target shape (top). Each sensorimotor control block consisted of six different images presented sequentially for 4 s. As we were not interested in neural networks associated with face- or affect-specific processing *per se*, but rather in eliciting a maximal amygdala response across all subjects that we could then interrogate for *hTPH2* effects, we chose not to use neutral faces as control stimuli because neutral faces can be subjectively experienced as affectively laden or ambiguous and thus engage the amygdala.[20,21]

Blood oxygenation-level dependent fMRI acquisition parameters
Each subject was scanned using a Siemens 3 T Allegra scanner. Blood oxygenation level-dependent (BOLD) functional images were acquired with a gradient echo EPI sequence and covered 34 axial slices (3 mm thick) beginning at the cerebral vertex and encompassing the entire cerebrum and the majority of the cerebellum (TR/TE = 2000/25 ms, FOV = 20 cm, matrix = 64×64). All scanning parameters were selected to optimize the quality of the BOLD signal while maintaining a sufficient number of slices to acquire whole-brain data. Before the collection of fMRI data for each subject we acquired a reference EPI scan that we visually inspected for artifacts (eg ghosting) as well as good signal across the entire volume of acquisition, including the medial temporal lobes. The fMRI data from all 31 subjects included in this study were cleared of such problems.

Image processing and analysis
Whole-brain image analysis was completed using the general linear model of SPM2 (http://www.fil.ion.ucl.ac.uk/spm). Images for each subject were realigned to the first volume in the time series to correct for head motion, spatially normalized into a standard stereotactic space (Montreal Neurological Institute template) using a 12-parameter affine model and smoothed to minimize noise and residual difference in gyral anatomy with a Gaussian filter, set at 6 mm full-width at half-maximum. Voxel-wise signal intensities were ratio normalized to the whole-brain global mean.

Predetermined condition effects at each voxel were calculated using a *t*-statistic, producing a statistical image for the contrast of the face-processing task *vs* the sensorimotor control for each subject. These individual contrast images were then used in second-level random effects models that account for both scan-to-scan and subject-to-subject variability to determine task-specific regional responses at the group-level with one-sample *t*-tests (main effects of task) and analysis of variance (direct comparisons of genotype groups). As a result of our *a priori* interest in the differential response of the amygdala and our use of a rigorous statistical model, a statistical threshold of $P < 0.05$, corrected for multiple comparisons across the volume of the amygdala, was used to identify significant responses for all comparisons. Genotype effects were explored in amygdala clusters exhibiting a main effect of task.

Results

Sample demographics
The allele and genotype frequencies from the total cohort of 31 subjects were in Hardy–Weinberg equilibrium. From this initial cohort, two genotype groups were established based on the *hTPH2* G(−844)T promoter variant: G/G homozygotes ($n = 11$) and T allele carriers (G/T: $n = 9$, T/T: $n = 2$). To control for nongenotype confounds, the two *hTPH2* genotype groups were matched for age ($F(1,20) = 0.116$; $P = 0.737$), sex (eight females and three males per group) and race (eight Caucasians and three African–Americans (all females) per group). As all of our subjects were cleared of medical or psychiatric disease or treatment, the two genotype groups did not differ on these parameters either. Furthermore, the two groups did not differ in 5-HTTLPR genotype status ($\chi^2 = 0.004$, $P = 0.95$),

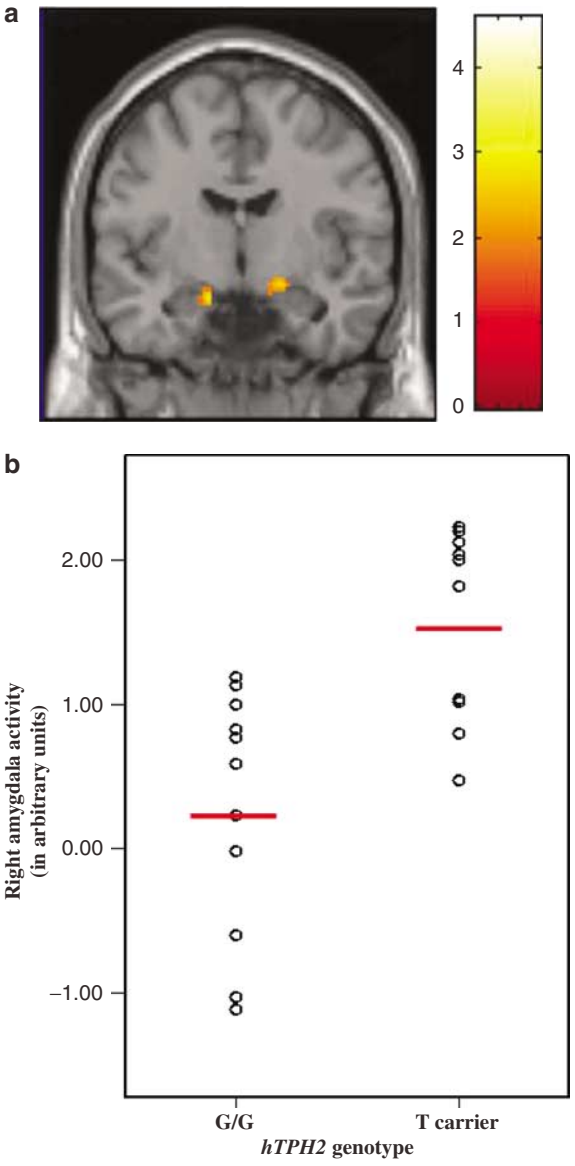

Figure 1 (a) Statistical parametric map illustrating greater bilateral dorsal amygdala activity in *hTPH2* T allele carriers in comparison with G/G homozygotes ($P<0.05$, corrected). Differential amygdala activity (T allele carriers >G/G homozygotes) is shown overlaid onto an averaged structural MRI. (b) Mean right amygdala activity across all 50 suprathreshold amygdala voxels from (a) for each subject (except the female T allele carrier outlier) grouped by *hTPH2* genotype with genotype group means designated by red lines ($F(1,20)=12.517$; $P=0.002$).

another 5-HT subsystem regulatory variant that may obscure *hTPH2* effects. Accuracy ($F(1,20)=0.093$; $P=0.76$) and reaction time ($F(1,20)=0.909$; $P=0.35$) on the face processing task did not differ between genotype groups.

hTPH2 effects on amygdala reactivity

Consistent with prior studies using this amygdala reactivity paradigm,[13,22–24] analysis of the fMRI data revealed significant bilateral amygdala activation in all subjects. Direct genotype group comparisons revealed greater activity in the bilateral dorsal amygdala of *hTPH2* T allele carriers in comparison with G allele homozygotes (Figure 1, Table 1). This pattern was still evident after excluding a single apparent outlier (female T allele carrier), whose amygdala activity was greater than three standardized residuals from the group mean. Importantly, these *hTPH2* effects on brain function were independent of 5-HTTLPR genotype status, as the distribution of 5-HTTLPR variants was not different between the *hTPH2* genotype groups. Notably, these *hTPH2* effects were replicated in the overall sample of 31 subjects (20 G/G homozygotes and 11 T allele carriers) when not explicitly controlling for the potential confounding effects of age, sex, and race.

Discussion

This is the first demonstration of the *in vivo* significance of *hTPH2* on regional brain activity relevant to the generation and regulation of emotional behavior. Specifically, *hTPH2* T allele carriers exhibited relatively greater activity in the amygdala than G allele homozygotes. This genetically driven bias on the functional reactivity of the amygdala suggests that the G(−844)T promoter variant impacts the expression of TPH and subsequent synthesis and availability of 5-HT. Increases in synaptic 5-HT concentrations within limbic circuits (eg amygdala and medial prefrontal cortex) have been associated with heightened stress responsivity and anxiety.[25–28]

Previous studies of another 5-HT subsystem gene variant, the 5-HTTLPR, suggest that increased 5-HT availability associated with reduced reuptake results in relatively heightened amygdala reactivity. Thus, the *hTPH2* T allele may be associated with greater promoter activity and TPH2 expression resulting in increased 5-HT synthesis. Notably, the right dorsal amygdala cluster identified in our current study exhibits significant overlap with that previously associated with the 5-HTTLPR short allele.[13,14] Thus,

Table 1 fMRI results

Main effect of genotype: T allele carriers >G allele homozygotes	*x*	*y*	*z*	*cluster size*	*F-value*	*P-value*
Right amygdala	14	−1	−10	50	12.517	0.002
Left amygdala	−14	−9	−15	37	7.162	0.015

both 5-HT subsystem variants appear to bias the response of the dorsal amygdala, where the major output nucleus (central) mediating behavioral and physiologic arousal is located in primates. Moreover, this dorsal region of the amygdala exhibits the highest density of serotonergic fibers and thus is likely affected by variation in 5-HT availability.[29,30]

While this interpretation is consistent with the *in silico* evidence for functionality of this polymorphism[16] additional *in vitro* and *in vivo* studies to directly examine the molecular and cellular effects of the *hTPH2* G(−844)T promoter variant on gene expression and activity are warranted. Such studies would also shed light on the likelihood that the functional association observed between the G(−844)T variant and amygdala reactivity reflect effects of additional variants that may be in linkage disequilibrium with the G(-844)T. Furthermore, because of our limited sample size of T allele homozygotes ($n = 2$), we were unable to explore allele dosage effects and additional studies with larger samples are needed to identify the nature of these effects (ie, dominant or codominant).

Our current report provides further insight into the biological significance of *hTPH2* in the human central nervous system and provides a critical next step in our understanding of the importance of this newly identified second tryptophan hydroxylase isoform for human brain function. In addition, this report marks an important advance in the application of functional neuroimaging to the study of genes, brain and behavior. In contrast to previous studies of genetic effects on brain function, where the molecular and cellular effects of the candidate variants had been previously demonstrated (eg 5-HTTLPR,[13] MAO-A,[31] COMT[32] and BDNF[33]), our fMRI data provide the first evidence that a single nucleotide polymorphism in the regulatory region of the human TPH2 gene likely affects biological function. In this way, the initial identification of a systems level effect of a specific polymorphism provides impetus for the subsequent characterization of its functional effects at the molecular and cellular level. Thus, this study highlights the potential reciprocal nature by which functional imaging and molecular genetics approaches can be mutually informative in advancing our understanding of the biological mechanisms of behavior.

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