Serotonin Transporter Genetic Variation and the Response of the Human Amygdala

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by activating the translation of ribosomal components, fly cells become abnormally small (*14*, *45*). Pathways that control critical cell size at Start in budding yeast may provide further insight into mechanisms that couple growth and division in higher organisms.

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Materials and Methods
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Serotonin Transporter Genetic Variation and the Response of the Human Amygdala

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A functional polymorphism in the promoter region of the human serotonin transporter gene (*SLC6A4*) has been associated with several dimensions of neuroticism and psychopathology, especially anxiety traits, but the predictive value of this genotype against these complex behaviors has been inconsistent. Serotonin [5-hydroxytryptamine, (5-HT)] function influences normal fear as well as pathological anxiety, behaviors critically dependent on the amygdala in animal models and in clinical studies. We now report that individuals with one or two copies of the short allele of the serotonin transporter (5-HTT) promoter polymorphism, which has been associated with reduced 5-HTT expression and function and increased fear and anxiety-related behaviors, exhibit greater amygdala neuronal activity, as assessed by BOLD functional magnetic resonance imaging, in response to fearful stimuli compared with individuals homozygous for the long allele. These results demonstrate genetically driven variation in the response of brain regions underlying human emotional behavior and suggest that differential excitability of the amygdala to emotional stimuli may contribute to the increased fear and anxiety typically associated with the short *SLC6A4* allele.

The elucidation of underlying biological mechanisms that contribute to individual differences in both normal and abnormal behavior remains a crucial and largely unmet challenge. Advances in both molecular genetics and noninvasive functional neuroimaging, however, have begun to address this issue, particularly in regards to affective behaviors, such as fear and anxiety, which exhibit considerable individual variability (*1*). In humans, two common alleles, the short (s) and long (l), in a variable repeat sequence of the promoter region of the serotonin transporter gene (5-HTTLPR) have been differentially associated with anxiety-related behavioral traits in healthy subjects (*2*, *3*). Likewise, both positron emission tomography and functional magnetic resonance imaging (fMRI) studies have revealed links between the physiological responses of brain regions, such as the prefrontal cortex and amygdala, and individual differences in affect and temperament (*4*, *5*).

At the physiological level, the serotonin transporter promoter polymorphism alters both *SLC6A4* transcription and level of serotonin transporter function. Cultured human lymphoblast cell lines homozygous for the l allele have higher concentrations of 5-HTT mRNA and express nearly twofold greater 5-HT reuptake compared with cells possessing either one or two copies of the s allele, which may act dominantly (*2*). Nearly identical differences in 5-HTT binding levels in human brain were detected between individuals with the l/l versus l/s or s/s genotypes,

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using in vivo SPECT imaging (*6*) and postmortem ligand binding (*7*). At the behavioral level, individuals carrying the s allele are slightly more likely to display abnormal levels of anxiety (*2*, *8*–*10*), acquire conditioned fear responses (*11*), and develop affective illness (*3*) compared with those homozygous for the l allele. These behavioral and personality differences most likely reflect relative differences in 5-HTT expression and subsequent levels of synaptic 5-HT, a potent modulator of emotional behavior (*12*). Not surprisingly, however, the relation between *SLC6A4* genotype and subjective measures of emotion and personality has been weak and inconsistent (*8*, *13*–*16*), likely reflecting the vagueness and subjectivity of the behavioral measurements, but also raising concern that the relation may be spurious (*17*).

Although the potential influence of a genetic variation in 5-HTT function on human anxiety and fear behavior has been corroborated in studies of 5-HTT knockout mice (*18*), the underlying neurobiological correlates of this functional relation are unknown. Recent human (*11*) and animal studies (*19*) have revealed abnormal fear conditioning, a phenomenon dependent on the amygdala (*20*), to be associated with 5-HTT function, suggesting that this structure may be critical in mediating the effects of 5-HT on emotional behavior. Because the physiological response of the amygdala during the processing of fearful stimuli may be more objectively measurable than the subjective experience of emotionality, a functional polymorphism in *SLC6A4* may have a more obvious impact at the level of amygdala biology than at the level of individual responses to questionnaires or ratings of emotional symptoms.

We used fMRI to explore the relation between this functional polymorphism and the response of the amygdala to fearful stimuli in two independent cohorts of healthy volunteers (*21*). We hypothesized that individuals with either one or two copies of the s allele, who presumably have relatively lower 5-HTT function and expression and relatively higher levels of synaptic 5-HT and who have been associated in earlier studies with more anxiety and fearfulness, would exhibit a greater amygdala response than those homozygous for the l allele, who presumably have lower levels of synaptic 5-HT and have been reported to be less anxious and fearful.

In each cohort, subjects were divided into two equal groups on the basis of their 5-HTTLPR genotype (*22*): individuals with either one or two copies of the s allele (s group) and individuals homozygous for the l allele (l group). The two groups were matched for age, gender, and IQ in each cohort (*23*). During fMRI, all subjects completed a blocked paradigm in which a sensorimotor control task was interleaved with an emotion task that required subjects to match the affect (angry or afraid) of one of two faces to that of a simultaneously presented target face (Fig. 1). Such perceptual processing of facial expressions has been shown to effectively and consistently engage the amygdala (*24*, *25*).

Analysis of the imaging data (*22*) revealed a similar and significant bilateral BOLD (blood oxygen level–dependent) response in the amygdala during the emotion task in each cohort. In addition, there were significant responses in the bilateral posterior fusiform gyri, inferior parietal lobules, and frontal eye fields—a network of brain regions implicated in face processing (*26*). Direct comparisons revealed that the response of the right amygdala was significantly greater in the s carrier group compared with the l homozygous group in each cohort (Figs. 2 and 3). The laterality of this amygdala difference is consistent with the proposed general role of right hemisphere brain regions in processing faces, as well as recent reports implicating a specific role for the right amygdala in processing angry and fearful facial expressions (*27*, *28*). The responses of regions within the distributed perceptual network, including the right posterior fusiform gyrus, were also greater in the s groups, possibly reflecting excitatory feedback from the amygdala to object-specific processing regions in an effort to improve recognition and refine behavioral responses (*20*, *29*, *30*). There were no significant effects of gender or a gender-by-genotype interaction for any of these brain responses. Furthermore, the 5-HTT allele–dependent effect on amygdala excitability did not reflect a nonspecific tendency of s allele carriers to overactivate a complex brain response, as a subsequent fMRI analysis of these same subjects, grouped again by 5-HTT genotype, performing a working memory task revealed no significant group differences in any brain regions involved with the task (*31*).

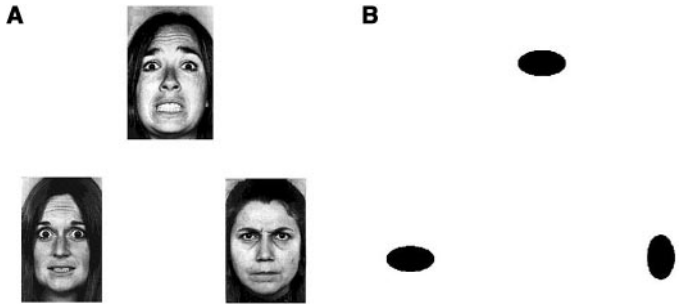

Fig. 1. fMRI paradigm. Two blocks of an emotion task were interleaved with three blocks of a sensorimotor control task. (**A**) During the emotion task, subjects viewed a trio of faces and selected one of two faces (bottom) that expressed the same emotion as the target face (top). The identity of all three faces was always different. Each emotion block consisted of six images, three of each gender and target affect (angry or afraid) all derived from a standard set of pictures of facial affect (*42*), presented sequentially for 5 s. (**B**) During the sensorimotor control, the subjects viewed a trio of simple geometric shapes (circles, vertical and horizontal ellipses) and selected one of two shapes (bottom) identical to the target shape (top). Each control block consisted of six different images presented sequentially for 5 s. Subject performance (accuracy and reaction time) was monitored during all scans.

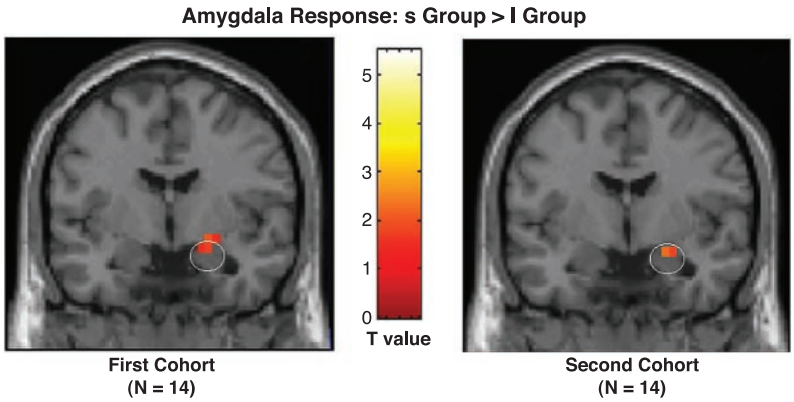

Fig. 2. Genotype-based parametric comparisons illustrating significantly greater activity in the right amygdala of the s group versus the l group in both the first and second cohort. BOLD fMRI responses in the right amygdala (white circle) are shown overlaid onto an averaged structural MRI in the coronal plane through the center of the amygdala. Talairach coordinates and voxel level statistics ($P < 0.05$, corrected) for the maximal voxel in the right amygdala for the first and second cohort are as follows: $x = 24$ mm, $y = -8$ mm, $z = -16$ mm; cluster size = 4 voxels; voxel level corrected P value = 0.021; T score = 2.89, and $x = 28$ mm, $y = -4$ mm, $z = -16$ mm; cluster size = 2 voxels; voxel level corrected P value = 0.047; T score = 2.03, respectively.

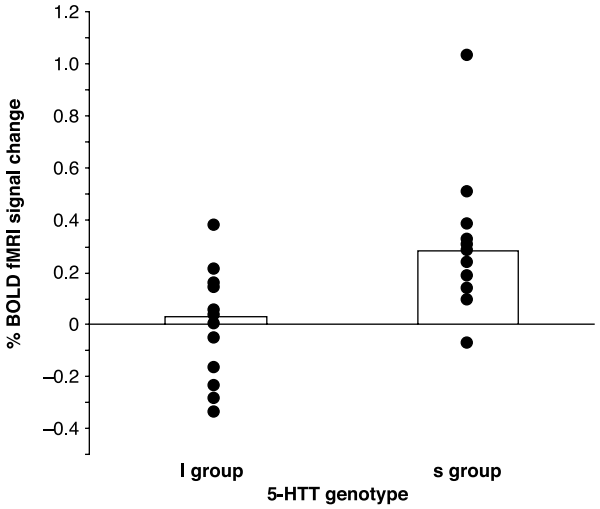

Fig. 3. Effect of 5-HTT genotype on right amygdala activity. Bar graphs represent the mean BOLD fMRI percent signal change in a region of interest (ROI) comprising the entire right amygdala in the s (n = 14) and l (n = 14) groups collapsed across both cohorts. Individual circles represent the activity for each subject in this ROI. Consistent with the statistical parametric maps (Fig. 2), which identified significant voxels within the right amygdala, analysis of variance for the entire amygdala ROI, including voxels that were not differentially activated according to statistical parametric mapping, still revealed significant group differences in the mean (±SEM) BOLD fMRI percent signal change [s group = 0.28 ± 0.08 and l group = 0.03 ± 0.05; $F(1,26) = 6.84$, $P = 0.01$].

The genotype groups did not differ in performance (accuracy and reaction time) on the emotion task (*32*), indicating that general attentional, perceptual, and cognitive phenomena did not contribute to the observed amygdala differences. The lack of a genotype effect on the brain response during the working memory task further supports the conclusion that the results are not driven by nonspecific factors. Moreover, there were no significant group differences in anxiety-like or fear-related traits, as indexed by the Tridimensional Personality Questionnaire (*33*). However, given the small effect (3 to 4%) of this 5-HTT polymorphism on behavior in previous studies (*2*), a lack of significant genotype differences in these personality traits is not surprising in view of the considerable individual variability in these measures and our relatively small sample size.

The heightened amygdala response of individuals possessing the s allele most likely reflects increased neuronal excitability leading to larger local field potentials and subsequent increases in the BOLD fMRI signal (*34*). Relatively increased amygdala neuronal excitability in s carriers may result from the relatively decreased 5-HTT expression and increased available synaptic 5-HT acting on excitatory 5-HT receptor subtypes (*35*). Such heightened amygdala activity might also reflect partial desensitization of inhibitory 5-HT_{1A} receptors following increased synaptic 5-HT (*36*). Furthermore, the differential response of the amygdala that we observed in adult subjects may be rooted in early postnatal developmental processes that are critical for establishing emotional behavior and are influenced by serotonergic neurotransmission (*37*).

Our results directly implicate a genetically determined link between 5-HTT function and the response of brain regions critical for emotion processing. Specifically, individuals carrying the less efficient s allele of the 5-HTT gene promoter exhibit an increased amygdala response to fearful stimuli compared with those homozygous for the l allele. Thus, the increased anxiety and fear associated with individuals possessing the s allele may reflect the hyperresponsiveness of their amygdala to relevant environmental stimuli. Such genetically driven variation in 5-HTT function and subsequent amygdala reactivity may also contribute to previously reported abnormalities of the serotonergic system in depression and suicidal behavior (*38*). The differences we describe at the neurobiological level were marked in a relatively small sample population in the absence of significant differences in behavioral measures of personality, underscoring the power of a direct assay of brain function (i.e., fMRI) to identify a phenotype related to a functional polymorphism in a gene likely important for human emotion. The application of such techniques appears to provide a unique opportunity to explore and evaluate the functional impact of brain-relevant genetic polymorphisms more rapidly and with greater sensitivity than existing behavioral assessments (*39*).

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21. Twenty-eight right-handed healthy subjects (20 females and 8 males) participated in the study according to the guidelines of the National Institute of Mental Health Institutional Review Board. All subjects were screened for and cleared of neurological, psychiatric, or substance-abuse problems and had no history of other medical problems or medical treatment relevant to cerebral metabolism and blood flow.
22. Materials and methods are available as supporting material on *Science* Online.
23. In both cohorts, the two genotype groups were matched for gender (first and second cohort: 5 females and 2 males in each group), age [first cohort: mean ± SEM, s group = 27.4 ± 5.6 years and l group = 32.1 ± 3.8 years; $F(1,12) = 3.35$, $P = 0.09$; second cohort: s group = 32.7 ± 4.9 years and l group = 32.7 ± 3.8 years; $F(1,12) = 0.12$, $P = 0.73$], and mean IQ [first cohort: mean ± SEM, s group = 106.9 ± 9.8 and l group = 110.3 ± 6.2; $F(1,12) = 0.61$, $P = 0.45$; second cohort: s group = 106.1 ± 4.8 and l group = 96.9 ± 3.9; $F(1,12) = 2.25$, $P = 0.16$]. In the first cohort, there were 12 Caucasians and 2 African Americans (both female l/l homozygotes), and in the second there were 13 Caucasians and 1 African American (female l/l homozygote).
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31. All subjects completed a version of the "*n*-back" working memory task known to engage the dorsolateral prefrontal cortex and a network of related cortical regions (*40*). In this task, subjects are required to remember the sequence of serially presented numbers in order to identify numbers that appeared "*n*-back" (i.e., 1-back, 2-back, 3-back). There were no significant group differences in performance (accuracy or reaction time) on this task. Analysis of the imaging data (*22*) revealed no significant differences in any brain regions, including the dorsolateral prefrontal cortex, between the s and l groups.
32. Analysis of variance of performance measures on the emotion task revealed no significant group differences in either cohort for mean accuracy [first cohort: % correct ± SEM, s group = 93.1 ± 7.0 and l group = 86.1 ± 4.5; $F(1,12) = 0.69$, $P = 0.43$; second cohort: s group = 96.4 ± 1.7 and l group = 98.8 ± 1.2; $F(1,12) = 1.33$, $P = 0.27$] or mean reaction time [first cohort: ms ± SEM, s group = 2274.1 ± 120.8 and l group = 2367.8 ± 108.7; $F(1,12) = 0.33$, $P = 0.58$; second cohort: s group = 1761.5 ± 132.6 and l group = 1941.3 ± 159.6; $F(1,12) = 0.75$, $P = 0.40$].
33. Analysis of variance of total scores from the Harm Avoidance subset of the Tridimensional Personality Questionnaire (*41*), designed to represent behaviors, such as fear and anxiety, influenced by serotonergic neurotransmission, revealed no significant group differences in either the first [mean ± SEM, s group = 8.7 ± 2.0 and l group = 9.9 ± 1.1; $F(1,12) = 0.24$, $P = 0.63$] or second [mean ± SEM, s group = 10.4 ± 1.6 and l group = 10.0 ± 2.3; $F(1,12) = 0.02$, $P = 0.88$] cohort. It should be

noted, however, that the Tridimensional Personality Questionnaire has not been as robust as other measures (i.e., NEO personality inventory) in identifying genotype effects on fear and anxiety (*3*) and that the use of more robust measures in the current study may have revealed group differences in these behaviors.

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Supporting Online Material
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Materials and Methods

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Enhanced CpG Mutability and Tumorigenesis in MBD4-Deficient Mice

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The mammalian protein MBD4 contains a methyl-CpG binding domain and can enzymatically remove thymine (T) or uracil (U) from a mismatched CpG site in vitro. These properties suggest that MBD4 might function in vivo to minimize the mutability of 5-methylcytosine by removing its deamination product from DNA. We tested this hypothesis by analyzing *Mbd4*$^{-/-}$ mice and found that the frequency of of C → T transitions at CpG sites was increased by a factor of three. On a cancer-susceptible *Apc*$^{Min/+}$ background, *Mbd4*$^{-/-}$ mice showed accelerated tumor formation with CpG → TpG mutations in the *Apc* gene. Thus MBD4 suppresses CpG mutability and tumorigenesis in vivo.

Deamination of 5-methylcytosine (m^5C) to T at CpG sites is probably the single most important cause of point mutations in humans, accounting for more than 20% of all base substitutions that give rise to genetic disease (*1*). Estimates based on the in vitro deamination rate of m^5C (*2*) suggest that approximately four m^5C residues deaminate per diploid genome per day in the germ line. It is likely that many of the resulting mismatches are repaired, but appropriate repair must take into account that the incorrect base at the T:G mismatches is invariably T, not G. Two mismatch-specific T glycosylases that accomplish this discrimination in vitro have been discovered. Both thymine DNA glycosylase [TDG; (*3*, *4*)] and MBD4 (*5*, *6*) can specifically remove T from a T-G mismatch within a CpG context without cleaving the DNA strand. This study concerns MBD4, which contains a COOH-terminal glycosylase domain (*7*, *8*) and an NH_2-terminal methyl-CpG binding domain [MBD; (*7*)]. The MBD binds to symmetrically methylated CpG sites but has a higher affinity in vitro for m^5CpG/TpG mismatches, which arise due to deamination at methyl-CpG (*5*). Neither TDG nor MBD4 has been tested for its effect on mutations in vivo.

To investigate whether MBD4 suppresses the mutability of methylated CpG sites in vivo we generated mice bearing a mutated *Mbd4* gene by means of targeted allele replacement in embryonic stem cells (*9*). The mutated allele contained a cassette within intron 1 containing a splice acceptor and polyadenylation signal. Mice homozygous for this allele were viable and fertile. Northern blot (*10*) and reverse transcriptase–polymerase chain reaction (RT-PCR) analyses confirmed that MBD4 expression was less than 0.1% of the wild-type level in tissues from homozygous-mutant mice (fig. S1). We refer to this allele as *Mbd4*$^{-}$ throughout this study.

To determine whether MBD4 deficiency leads to an increase in mutations at CpG sites, we crossed mice with the "Big Blue" reporter locus, comprising 40 head-to-tail copies of a recoverable lambda transgene (*11*), onto the *Mbd4*$^{-/-}$ background and measured in vivo mutation frequencies at the λ *cII* locus (*12*). Bisulfite sequencing (*9*) of the *cII* locus from both wild-type and *Mbd4*$^{-/-}$ mice showed that, on average, 95% of the CpG sites in the *cII* gene are methylated (Fig. 1A). The frequency of *cII* mutations in liver and spleen of 105-day-old mice and in spleens of 183-day-old mice was determined by plating packaged genomic DNA (*9*, *12*). The total mutant frequency in *Mbd4*$^{-/-}$ mice (6.8×10^{-5}) was significantly higher than in wild-type animals (3.2×10^{-5}) by a maximum likelihood ratio test [$P < 0.0001$; Fig. 1B; Table 1; (*9*)]. This difference was reduced, but remained statistically significant ($P < 0.05$), when a correction for the number of independent mutations was applied [Fig. 1B; (*9*)]. The true mutation frequency is likely to lie between the uncorrected and corrected values, as correction may underestimate the real number of independent mutational events.

A striking difference between wild-type and *Mbd4*$^{-/-}$ mice emerged when the spectrum of mutations was examined. The most abundant mutational change in both wild-type and mutant mice involved CG → TA transitions, but this category was more frequent in *Mbd4*$^{-/-}$ mice than in *Mbd4*$^{+/+}$

Table 1. Analysis of mutation frequencies at the *cII* locus of a bacteriophage λ transgene in *Mbd4*$^{-/-}$ and *Mbd4*$^{+/+}$ mice. Numbers of "mutants" were derived from raw counts of mutant plaques. Numbers of "mutations" were deduced by correcting mutant numbers for likely clonal expansion of a single mutant cell (*9*). "Mutant" and "mutation" frequencies reflect the uncorrected and corrected data, respectively.

Genotype (*n*)	Age (days)/ tissue	Plaques screened (*n*)	Mutants (*n*)	Mutant frequency ($\times 10^{-5}$)	Individual mutations (*n*)	Mutation frequency ($\times 10^{-5}$)
Mbd4$^{+/+}$ (3)	105/liver	656,165	15	2.29	15	2.29
Mbd4$^{-/-}$ (3)	105/liver	991,900	39	3.93	32	3.23
Mbd4$^{+/+}$ (4)	105/spleen	1,340,250	45	3.36	38	2.84
Mbd4$^{-/-}$ (4)	105/ spleen	1,201,833	88	7.32	39	3.25
Mbd4$^{+/+}$ (1)	183/ spleen	200,500	11	5.49	11	5.49
Mbd4$^{-/-}$ (1)	183/ spleen	227,433	38	16.71	27	11.87

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