

Genotype over diagnosis in amygdala responsiveness: affective processing in social anxiety disorder

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Background: Although the amygdala is thought to be a crucial brain region for negative affect, neuroimaging studies do not always show enhanced amygdala response to aversive stimuli in patients with anxiety disorders. Serotonin (5-HT)-related genotypes may contribute to interindividual variability in amygdala responsiveness. The short (s) allele of the 5-HT transporter linked polymorphic region (5-HTTLPR) and the T variant of the G-703T polymorphism in the tryptophan hydroxylase-2 (*TPH2*) gene have previously been associated with amygdala hyperresponsivity to negative faces in healthy controls. We investigated the influence of these polymorphisms on amygdala responsiveness to angry faces in patients with social anxiety disorder (SAD) compared with healthy controls. **Methods:** We used positron emission tomography with oxygen 15-labelled water to assess regional cerebral blood flow in 34 patients with SAD and 18 controls who viewed photographs of angry and neutral faces presented in counterbalanced order. We genotyped all participants with respect to the 5-HTTLPR and TPH2 polymorphisms. **Results:** Patients with SAD and controls had increased left amygdala activation in response to angry compared with neutral faces. Genotype but not diagnosis explained a significant portion of the variance in amygdala responsiveness, the response being more pronounced in carriers of s and/or T alleles. **Limitations:** Our analyses were limited owing to the small sample and the fact that we were unable to match participants on genotype before enrolment. In addition, other imaging techniques not used in our study may have revealed additional effects of emotional stimuli. **Conclusion:** Amygdala responsiveness to angry faces was more strongly related to serotonergic polymorphisms than to diagnosis of SAD. Emotion activation studies comparing amygdala excitability in patient and control groups could benefit from taking variation in 5-HT-related genes into account.

Contexte : Même si l'on considère l'amygdale comme une région du cerveau cruciale pour l'affect négatif, les épreuves de neuro-imagerie ne révèlent pas toujours un rehaussement de la réactivité amygdalienne aux stimuli aversifs chez les patients souffrant de troubles anxieux. Les génotypes liés à la sérotonine (5-HT) pourraient contribuer à la variabilité interindividuelle de la réponse amygdalienne. L'allèle (s) court du gène polymorphe 5-HTTLPR (*serotonin-transporter-linked polymorphic region*), un transporteur de la 5-HT, et la variante T du polymorphisme G-703T du gène *TPH2* (*tryptophan hydroxylase-2*) ont été associés antérieurement à une hyperréactivité amygdalienne aux visages exprimant une émotion négative chez des témoins en bonne santé. Nous avons voulu mesurer l'influence de ces polymorphismes sur la réactivité amygdalienne aux visages exprimant la colère chez des patients atteints de phobie sociale, comparativement à des témoins en bonne santé. **Méthodes :** Nous avons utilisé la tomographie par émission de positrons avec de l'eau radioactive marquée à l'oxygène 15 pour mesurer le débit sanguin cérébral régional chez 34 patients souffrant de phobie sociale et 18 témoins à qui l'on présentait des photographies de visages en colère ou neutres en séquence contrebalancées. Nous avons établi le génotype de tous les participants pour ce qui est des polymorphismes 5-HTTLPR et TPH2. **Résultats :** Les patients souffrant de phobie sociale et les témoins ont présenté une activation amygdalienne gauche accrue en réponse aux visages en colère, par rapport aux visages neutres. Il a été possible d'expliquer une portion significative de la variance de la réactivité amygdalienne par le génotype, mais

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J Psychiatry Neurosci 2009;34(1):30-40.

Submitted Jan. 22, 2008; Revised May 26, Jun. 3, 2008; Accepted Jun. 6, 2008

non par le diagnostic, la réponse ayant été plus prononcée chez les porteurs des allèles s ou T. **Limites** : Nos analyses ont été limitées en raison du petit échantillon et du fait que nous n'ayons pas pu assortir les participants selon leurs génotypes avant leur inscription à l'étude. De plus, d'autres techniques d'imagerie, non utilisées lors de notre étude, auraient pu révéler certains effets additionnels des stimuli émotionnels. **Conclusion** : La réactivité amygdalienne aux visages exprimant la colère a été plus intimement liée aux polymorphismes sérotoninergiques qu'au diagnostic de phobie sociale. Les études d'activation des émotions comparant l'excitabilité de l'amygdale chez des groupes de patients et de témoins pourraient utiliser avantageusement les variations des gènes liés à la 5-HT.

Introduction

The amygdala has long been associated with fear reactions and detection of danger signals.¹ Studies in animals and humans have confirmed that the amygdala is a critical structure in Pavlovian fear conditioning and that it responds rapidly to environmental threat cues.²⁻⁵ The amygdala is also believed to play a prominent role in anxiety disorders as these are characterized by exaggerated fear responses and increased vigilance to potential threat stimuli.⁶

Neuroimaging studies of symptom provocation (e.g., exposure to the feared stimuli) have yielded mixed findings regarding the expected amygdala hyperexcitability in anxiety populations. As reported in a recent meta-analysis,⁷ several neuroimaging trials have demonstrated exaggerated amygdala activation during anxious states in patients with social anxiety disorder (SAD), posttraumatic stress disorder (PTSD) and specific phobia. However, many studies of these disorders have been negative,⁸⁻¹⁴ and seemingly counter-intuitive findings such as higher amygdala activation to aversive stimuli in healthy controls than in patients with anxiety¹⁴⁻¹⁶ or higher amygdala activation to emotionally neutral than to anxiogenic tasks^{15,17} have also been reported.

One reason for the lack of consistency across studies may be that the amygdala does not remain activated during sustained emotional states after initial processing of threat-related stimuli. Consequently, anxiety-provoking tasks may be less powerful in evoking amygdala activity compared with affective information-processing tasks that require prolonged or intermittent attention to emotional stimuli but do not generate strong feelings. In line with this, numerous brain imaging studies of healthy volunteers have demonstrated increased activity of the amygdala during perception of emotional faces (e.g., presentation of pictures of fearful or angry facial expressions contrasted with neutral faces).^{4,18}

Patients with anxiety disorders may show exaggerated amygdala responses to negative faces compared with controls, presumably because their core fear systems are over-activated.⁷ Neuroimaging data support amygdala hyper-responsivity to negative faces in patients with PTSD¹⁹⁻²¹ but not in patients with specific phobia²² or obsessive-compulsive disorder.²³ However, the use of threat-signalling faces may be particularly ecologically valid in patients with SAD, because individuals with this disorder are anxious of being criticized or rejected by others²⁴ and are thus hypersensitive to facial expressions that may be interpreted as dislike or hostility. Compromised social information-processing, including recognition and recall biases for negative faces, has been found in this clinical population.²⁵ Functional magnetic resonance imaging (fMRI) studies have reported elevated amygdala re-

sponses to harsh compared with happy faces^{26,27} and to angry schematic or photographic faces compared with neutral expressions^{28,29} in patients with SAD compared with controls. One fMRI trial demonstrated enhanced amygdala activation not only to angry faces but also to happy facial expressions selectively in patients with SAD,³⁰ whereas 2 other studies failed to replicate this finding.^{27,31} Cooney and colleagues³¹ reported exaggerated right amygdala response (SAD > controls) to neutral faces relative to baseline, but the reverse pattern (SAD < controls) was noted for the left amygdala. Facial expressions of disgust did not evoke differential amygdala activation patterns in patients with SAD and controls.³²

Taken together, results from neuroimaging investigations show variability between and within the anxiety disorders regarding the amygdala response to emotionally relevant tasks. Allelic variation in serotonin (5-HT)-related genes could be an important contributing factor to this variability. The amygdala is densely innervated by serotonergic fibres,³³ and amygdala reactivity appears to be modulated by serotonergic synapses.³⁴ In line with this, a number of studies have demonstrated that carriers of the low expression short (s) allele of the promoter polymorphism of the 5-HT transporter gene (the polymorphic region linked to 5-HTT; 5-HTTLPR) show enhanced amygdala response to negative faces compared with those who are homozygous for the long (l) allele.³⁵⁻³⁷ The tryptophan hydroxylase-2 (*TPH2*) gene, which encodes the enzyme that catalyzes the rate-limiting step of 5-HT biosynthesis in the brain,³⁸ also seems to modulate amygdala activity. Two independent studies demonstrated that carriers of the T allele of the G-703T (rs4570625) single nucleotide polymorphism in the *TPH2* gene exhibited elevated amygdala responsiveness to negative (angry or fearful) faces compared with those who were homozygous for the G allele.^{39,40} Thus with regard to amygdala responsiveness, the s and T variants appear to be "high-response alleles," whereas the l and G variants (i.e., ll and/or GG homozygosity) are "low-response alleles" of the 5-HTTLPR and *TPH2* polymorphisms, respectively.

To our knowledge, no brain imaging study has taken genetic variation into account when comparing amygdala excitability in patients with anxiety disorders and controls. Thus the main objective of the present study was to compare patients with SAD and controls with respect to differences in amygdala activation in response to angry and neutral faces and to investigate the predictive power of 5-HT-related genotypes, diagnosis and behavioural measures of anxiety on amygdala responsiveness. In a previous imaging study of SAD,⁴¹ we noted elevated right amygdala activity in 5-HTTLPR s allele carriers relative to ll homozygotes during a stressful public speaking task compared with a private

speech control condition. We sought to replicate and extend this finding by using a face emotion processing probe of amygdala functioning, genotyping the individuals with regard to the TPH2 polymorphism G-703T in addition to the 5-HTTLPR and including a comparison group of healthy volunteers. As in our previous study,⁴¹ we used oxygen-15 positron emission tomography (PET) and a region-of-interest (ROI) approach focusing on regional cerebral blood flow in the amygdala. We expected higher regional cerebral blood flow response in the amygdala to angry compared with neutral faces. We expected this difference to be exaggerated in patients with SAD compared with controls. Within the SAD and control groups, we expected amygdala reactivity (angry > neutral) to be enhanced in carriers of the high-response variants of the 5-HTTLPR and TPH2 genotypes.

Methods

Participants and recruitment

We recruited participants through newspaper advertising. A clinical psychologist evaluated psychiatric status using the anxiety disorder section of the structured clinical diagnostic interview⁴² for the *Diagnostic and statistical manual for mental disorders*, fourth edition (DSM-IV).²⁴ In addition, a psychiatrist administered the Mini-International Neuropsychiatric Interview⁴³ to exclude other serious psychiatric disorders. We also performed medical examinations.

Social anxiety disorder was the main diagnosis for all patients, whereas we required all controls to be free from psychiatric illness. The main criteria for exclusion were treatment of social anxiety in the 6 months preceding the study, current serious or dominant psychiatric disorder other than SAD (e.g., psychosis, major depressive disorder, bipolar disorder), chronic use of prescribed medication, abuse of alcohol or narcotics, pregnancy, menopause, left handedness, previous PET examination and any somatic or neurologic disorder that could be expected to influence the outcome of the study.

The Uppsala University Medical Faculty Ethical Review Board and the Uppsala University Isotope Committee approved our study. We obtained written informed consent from all participants after the nature and consequences of the study had been explained.

Imaging assessments

We performed PET investigations at Uppsala Imanet, GE Healthcare (Sweden). We scanned participants using a 32-ring ECAT EXACT HR+ scanner (Siemens/CTI), which enables acquisition of 63 contiguous planes of data with a distance of 2.46 mm, resulting in a total axial field of view of 155 mm.

Participants fasted for 3 hours and refrained from tobacco, alcohol and caffeine for 12 hours before the investigation. We then positioned participants in the scanner with the head gently fixated, and we inserted a venous catheter for tracer injections. We performed a 10-minute transmission scan using 3 retractable ⁶⁸Ge rotating line sources. We then injected the ¹⁵O-water tracer, about 10 MBq/kg body weight,

intravenously. The emission scan started automatically in 3-dimensional mode when the bolus reached the brain (50 000 counts/s) and consisted of 3 frames lasting 30 seconds. The experimental face processing task (see next section) commenced immediately after tracer injection.

We reconstructed emission scans with a filter back projection using an 8-mm Hanning filter, resulting in a spatial resolution of about 5 mm in the field of view. We corrected data for photon attenuation, decay, scattered radiation and random coincidences. After reconstruction, we made a summation image of the 3 frames to obtain a better statistical reference for realignment and subsequent analyses.

Emotional face-processing task

During the ¹⁵O-water PET scans, participants viewed black and white photographs of angry and neutral faces of different individuals (6 men and 8 women), taken from the set of Ekman and Friesen.⁴⁴ During each scan, we presented 30 slides of 1 facial affect, either angry or neutral, 1 at a time on a computer screen. We presented slides of the other affect during the subsequent scan, performed about 20 minutes later. We counterbalanced the order of presentation of angry or neutral faces among participants. During each condition, we presented photographs of 15 men and 15 women. In accordance with previous PET studies,^{45,46} each presentation lasted 3 seconds, followed by a 2-second interval in which the screen was blank. To monitor adherence to the experimental task, we asked participants to click on a mouse button each time a new face was presented.

Behavioural measures

We obtained affective ratings for angry and neutral faces using the Spielberger State Anxiety Inventory;⁴⁷ after each scan, participants retrospectively rated how anxious they felt when they viewed the facial condition. Before PET assessments, we asked participants to complete the Liebowitz Social Anxiety Scale,⁴⁸ the Social Phobia Scale,⁴⁹ the Social Interaction Anxiety Scale⁴⁹ and the Social Phobia Screening Questionnaire.⁵⁰ In addition, we evaluated trait anxiety using the Spielberger Trait Anxiety Inventory.⁴⁷ We also asked participants to complete the Global Assessment of Functioning self-report.⁵¹

Genotyping

To avoid mass comparisons, we focused our analyses on 2 polymorphisms: the 5-HTTLPR of the 5-HT transporter gene *SLC6A4*, and the rs4570625 of the *TPH2* gene. We extracted genomic DNA from plasma samples using a Qiamp DNA extraction kit (QIAGEN). We achieved amplification by means of polymerase chain reaction on a Perkin Elmer 9700 (PerkinElmer) thermal cycler.

The primer sequences used for the 5-HTTLPR, reported by Gelernter and colleagues,⁵² were 5'-ATGCCAGCACCTAACCCCTAATGT-3' (forward primer) and 5'-GGACCGCAAGGTGGCGGGA-3' (reverse primer), resulting in a

419-bp-long polymerase chain reaction product for the 16-repeat-allele (l) and a 375-bp-long polymerase chain reaction product for the 14-repeat-allele (s). The 15 μ L reaction mixture contained 50 ng of genomic DNA, 1.5 mM of $MgCl_2$, 0.3 μ M of each primer, 300 μ M of dNTPs and 1 unit of HotstarTaq polymerase from QIAGEN. The temperature profile consisted of an initial denaturation at 95°C for 15 minutes, followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 66°C and 60 seconds at 72°C, followed by final incubation for 7 minutes at 72°C. We separated polymerase chain reaction products on a 3% agarose gel supplemented with ethidium bromide and they were visualized by ultraviolet transillumination.

The primer sequences used for the TPH2 G-703T (rs4570625) polymorphism were 5'-TGTGGCTAAATTGAACCCTTACCT-3' (forward primer) and 5'-TGTGCTCCCGAACACTAGATCTTA-3' (reverse primer). The 20 μ L reaction mixture contained 50 ng of genomic DNA, 1.5 mM of $MgCl_2$, 0.15 μ M of each primer, 200 μ M of dNTPs and 1 unit of HotstarTaq polymerase from QIAGEN. The temperature profile consisted of initial denaturation at 95°C for 15 minutes, followed by 45 cycles of 15 seconds at 95°C, 30 seconds at 62°C and 30 seconds at 72°C, followed by final incubation for 7 minutes at 72°C. We performed genotyping of the TPH2 polymorphism using a Pyrosequencer PSQ 96MA and the PSQ 96 SNP Reagent Kit (QIAGEN). To identify the polymorphism, we used 15 pmol of the sequencing primer 5'-GCTTTTCTGACTTGACAT-3'.

Statistical analysis

Behavioural and demographic measures

We evaluated group differences in affective ratings (Spielberger State Anxiety Inventory) associated with each facial condition using repeated-measurement analysis of variance (ANOVA). For the other behavioural measures, we evaluated group differences using unpaired *t* tests, whereas we analyzed dichotomous variables using a Fisher exact probability test. We set the significance level at $p < 0.05$.

Gene data

We dichotomized the 5-HTTLPR genotype such that participants carrying at least 1 short allele (s) were compared with those who were homozygous for the long allele (ll).^{35,41} With regard to the TPH2 gene, T allele carriers were compared with G allele (GG) homozygotes.^{39,40}

PET data

We realigned PET images to correct for different positions between scans, and we normalized them to the Montreal Neurological Institute's (MNI) stereotactic template ICBM152 using SPM2 (Wellcome Trust Centre for Neuroimaging). We then smoothed images using a 12-mm Gaussian kernel and scaled them to give all scans the same global signal. We evaluated PET data using within- and between-group comparisons defined in SPM2 with regional cerebral blood flow data fitted to the general linear model.⁵³ We evaluated between-group differences using group by condition interactions in the form of double subtractions (e.g., [SAD_{angry} - SAD_{neutral}] -

[control_{angry} - control_{neutral}]). Contrasts generated *t* maps, which were subsequently converted to *z* scores for interpretation. We describe brain locations as MNI coordinates (*xyz*).

In line with our a priori hypotheses, our analyses focused on the amygdala. We defined the amygdala volume by the WFU PickAtlas (ANSIR Laboratory, Wake Forest University School of Medicine), an automated method to generate ROI masks.⁵⁴ We also extracted mean voxel values from the amygdala volume for all participants. We evaluated effects on regional cerebral blood flow at the voxel level (1 voxel = 2 × 2 × 2 mm) by examining statistically significant changes corrected family-wise for multiple comparisons and with a minimum extension of 10 voxels.

Prediction of neural response

We used multiple regression analysis to assess the predictive power of genotype, diagnosis and behavioural measures on amygdala responsiveness (i.e., change in regional cerebral blood flow scores [angry v. neutral] extracted from the whole amygdala volume).⁵⁴

Besides the SPM2 evaluations, we performed statistical analyses using SPSS 12.1 (SPSS Inc.).

Results

Study population

Thirty-four medication-free patients with SAD and 18 healthy controls participated; descriptive characteristics are outlined in Table 1. We scanned 2 additional patients with SAD, but because they did not leave a blood sample for genotyping, we did not include their data. Twenty-five (73.5%) of the patients with SAD had received diagnoses of the generalized subtype. For the remaining 9 patients, we could not establish with confidence that their fears encompassed most social situations and, accordingly, we classified them as having the nongeneralized subtype.²⁴ Seven (20.5%) patients met the criteria for a comorbid anxiety diagnosis (specific phobia, $n = 5$; panic disorder, $n = 1$; agoraphobia, $n = 1$). The mean age and the number of men and women did not differ significantly between the SAD and control groups.

Behavioural measures

As expected, patients with SAD and controls scored significantly differently on all social anxiety scales and on the trait anxiety and Global Assessment of Functioning instruments (Table 1). Repeated-measurement ANOVA of the state anxiety ratings associated with each facial expression revealed significant main effects of diagnosis ($F_{1,50} = 18.69$, $p < 0.001$; SAD > control) and condition ($F_{1,50} = 5.68$, $p = 0.021$; angry > neutral). We observed no significant diagnosis by condition interaction ($F_{1,50} = 1.64$, $p = 0.21$).

Genotypes

The distribution of the 5-HTTLPR and TPH2 alleles did not differ significantly between the SAD and control groups (Table 1). Also, the portion of s and ll carriers was not

different across the T and GG allelic variants ($\chi^2_1 = 0.61$, $p = 0.77$). We found at least 1 high-response allele (s or T) in 65% of the patients and in 67% of the controls. Patients carrying the TPH2 T allele had higher trait anxiety scores compared with GG homozygous patients ($t_{32} = 2.67$, $p = 0.012$) and they tended to display higher scores on the Social Interaction Anxiety Scale ($t_{32} = 1.94$, $p = 0.062$). Apart from this, genotypes were not related to behavioural measures. Age, sex and SAD subtype distribution did not differ significantly among the genetic subgroups (s, ll, T, GG and their combinations).

Regional cerebral blood flow analyses

Amygdala response in patients versus controls

We noted a significantly enhanced regional cerebral blood flow response to angry compared with neutral faces in the left amygdala within the SAD ($xyz = -22 -4 -16$, $z = 3.00$, $p = 0.016$) and the control ($xyz = -26 2 -20$, $z = 2.75$, $p = 0.029$) groups. We noted trend effects in the right amygdala within the SAD group (angry > neutral, $xyz = 28 -2 -14$, $z = 2.29$, $p_{\text{uncorr}} = 0.011$) compared with controls (SAD > control, angry > neutral, $xyz = 30 0 -22$, $z = 1.87$, $p_{\text{uncorr}} = 0.031$). There were, however, no significant differences in amygdala responsiveness between patients and controls at corrected levels (Fig. 1).

Effects of 5-HT-related genes on amygdala reactivity

In patients with SAD, the left amygdala response (angry > neutral) was significantly higher in s carriers com-

pared with ll homozygotes, and in T carriers compared with GG homozygotes (Table 2, Fig. 2). In controls, we noted similar genetic influences in the left (T > GG, angry > neutral) and right (s > ll, angry > neutral) amygdala, albeit at uncorrected p levels only (Table 2, Fig. 2).

Gene-gene effects on amygdala reactivity

In patients carrying both high-response (s+T) alleles, left amygdala reactivity (angry > neutral) was significantly elevated compared with patients with only 1 high-response allele and with patients homozygous for both low-response (ll+GG) alleles (Table 2, Fig. 2). The 5-HTTLPR by TPH2 interaction plot indicated a synergistic effect between the 2 polymorphisms (Fig. 3).

Because only 3 controls carried both the s and T alleles, we did not consider a corresponding analysis of the 2 serotonergic genes in the control group to be meaningful. However, we noted a similar interaction effect in the right amygdala such that controls carrying at least 1 high-response allele had increased reactivity, whereas controls carrying both low-response (ll+GG) alleles had decreased reactivity (Table 2, Fig. 2).

Genetic subgroup comparisons among patients and controls

To test whether regional cerebral blood flow differences between patients with SAD and controls were present in certain genetic subgroups, we compared carriers of high- and low-response alleles across diagnostic entities.

We observed no significant differences in amygdala reactivity in comparisons of high-response patients versus high-response controls. At the uncorrected level, we noted higher reactivity (angry > neutral) in ll+GG patients compared with ll+GG controls in the left ($xyz = -20 -10 -10$, $z = 2.34$, $p_{\text{uncorr}} = 0.010$) and right ($xyz = 24 0 -26$, $z = 2.19$, $p_{\text{uncorr}} = 0.014$) amygdala. We noted higher reactivity (angry > neutral) in the left ($xyz = -22 -8 -22$, $z = 2.66$, $p = 0.035$) and right ($xyz = 30 0 -18$, $z = 2.88$, $p = 0.020$) amygdala in patients carrying the TPH2 T allele compared with GG homozygous controls. We also noted a significant difference in right amygdala reactivity ($xyz = 24 0 -26$, $z = 2.68$, $p = 0.033$) between patient s carriers and control ll carriers. Finally, patients carrying both high-response (s+T) alleles showed enhanced reactivity to

Table 1: Descriptive characteristics of study participants

Characteristic	Group; mean (SD)*		Statistics	
	SAD (n = 34)	Control (n = 18)	t_{50}	p value
Age (SD) [range], yr	37.59 (8.60) [20–50]	34.50 (9.45) [22–49]	1.19	0.24
Sex, female/male	20/14	9/9	—	0.57†
Behavioural measures				
LSAS	68.50 (20.64)	7.22 (5.46)	12.32	< 0.001
SPS	37.88 (13.17)	2.11 (2.54)	11.36	< 0.001
SIAS	51.32 (11.73)	7.50 (4.36)	15.25	< 0.001
SPSQ	31.09 (7.28)	4.61 (3.96)	14.31	< 0.001
GAF	73.44 (10.71)	93.06(10.16)	6.39	< 0.001
STAI-T	50.97 (9.40)	29.00 (6.80)	8.76	< 0.001
STAI-S angry	41.27 (11.04)	28.83 (7.54)	4.27	< 0.001
STAI-S neutral	38.50 (9.42)	28.00 (7.77)	4.05	< 0.001
Genotype, no.				
5-HTTLPR/s or ll	16/18	9/9	—	0.99†
TPH2/T or GG	13/21	6/12	—	0.77†
≥ 1 high/both low	22/12	12/6	—	0.99†
Both high (s+T)/1 high (s or T)	7/15	3/9	—	0.99†

5-HTTLPR = serotonin transporter promoter polymorphic region (s = short allele carriers, ll = homozygous for long allele); GAF = Global Assessment of Functioning self-report; high = high-response alleles (s, T); low = low-response alleles (ll, GG); LSAS = Liebowitz Social Anxiety Scale; SAD = social anxiety disorder; SD = standard deviation; SIAS = Social Interaction Anxiety Scale; SPS = Social Phobia Scale; SPSQ = Social Phobia Screening Questionnaire; STAI-S = Spielberger State Anxiety Scale; STAI-T = Spielberger Trait Anxiety Scale; TPH2 = tryptophan hydroxylase-2 polymorphism (T allele carriers or GG = homozygous for G allele).

*Unless otherwise indicated.

†Based on Fisher exact probability test.

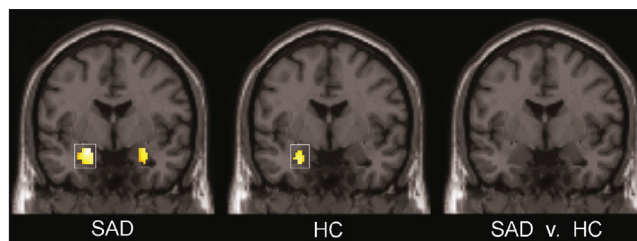


Fig. 1: Coronal views of enhanced amygdala activation to angry compared with neutral faces in patients with social anxiety disorder (left) and healthy controls (middle). The magnitude of amygdala activation did not differ significantly in patients compared with controls at corrected levels (right). The mask threshold corresponds to $z > 2$, and activations that remained significant after correction for multiple comparisons are displayed within squares.

angry faces in the left ($xyz = -20 -10 -12, z = 3.03, p = 0.014$) and right ($xyz = 26 2 -20, z = 3.01, p = 0.014$) amygdala relative to low-response (ll+GG) controls (Fig. 4).

We also noted enhanced left amygdala responsiveness (angry > neutral) in controls carrying at least 1 high-response allele compared with low-response (ll+GG) patients, although this finding was significant at the uncorrected level only ($xyz = -26 2 -18, z = 2.36, p_{\text{uncorr}} = 0.009$) (Fig. 4).

Within-group analyses of genetic subgroups

We observed significantly exaggerated reactivity in the left amygdala (angry > neutral, $p < 0.05$) only in carriers of the s, T and s+T alleles within the SAD group and in controls carrying at least 1 high-response allele. Coordinates, z scores and p values are available on request.

Predictors of the amygdala response

Multiple regression analysis showed that the TPH2 genotype (T/GG) was a significant predictor of reactivity in the left amygdala (angry > neutral), whereas diagnosis (SAD/control), 5-HTTLPR (s/ll) or state anxiety change scores did not predict amygdala responsiveness (Table 3). None of these variables was a significant predictor of reactivity in the right amygdala. Presence of both high-response alleles was also predictive of left amygdala responsiveness ($p = 0.016$) when replacing the individual genotypes in the regression model. Social anxiety scales, trait anxiety and GAF scores correlated highly with diagnosis ($r = 0.67-0.91, p < 0.001$) and were therefore not included in the analysis. When limiting the analysis to patients with SAD, TPH2 remained a significant predictor of regional cerebral blood flow changes in the left amygdala ($p = 0.020$). In addition, Liebowitz Social Anxiety

Scale values predicted left amygdala reactivity in patients with SAD ($p = 0.032$); higher values were associated with lower amygdala reactivity.

Amygdala activity during the neutral baseline

We evaluated group differences at baseline by calculating main effects of diagnosis and genotype on regional cerebral blood flow during the neutral face condition. Amygdala activity levels did not differ significantly between patients and controls. Correlations between baseline activity and reactivity measures did not exceed $r = 0.20$ (in each case $p > 0.16$).

Discussion

Patients with SAD and healthy volunteers exhibited higher activation in the left amygdala in response to angry compared with neutral facial expressions but, contrary to our initial expectation, we did not observe a significant differential amygdala response between the 2 groups. In line with our expectation, increased amygdala activation to angry faces within the SAD group was associated with 5-HT-related high-response alleles: the s allele of the 5-HT transporter promoter polymorphism (5-HTTLPR) and the T allele of the TPH2 polymorphism.^{35-37,39,40} Patients carrying either s or T alleles displayed greater amygdala reactivity (angry > neutral) compared with patients being homozygous for the corresponding low-response alleles (ll or GG), and amygdala reactivity was particularly elevated in patients with both high-response alleles. Genetic influences on amygdala responsiveness were statistically less robust in controls, although analyses confirmed that healthy individuals carrying a high-response allele (s or T) showed significantly elevated

Table 2: Amygdala reactivity (angry > neutral faces) in genetic subgroups of patients with social anxiety disorder and controls

Genotype; amygdala location	SAD group						Control group					
	Statistics			MNI coordinates			Statistics			MNI coordinates		
	No.	p value	z score	x	y	z	No.	p value	z score	x	y	z
5-HTTLPR/s > ll	16/18						9/9					
Left		0.013	3.04	-26	2	-20		> 0.10				
Right		> 0.10						0.014*	2.19	22	-8	-10
TPH2/T > GG	13/21						6/12					
Left		0.006	3.33	-24	-8	-22		0.030*	1.88	-16	-6	-14
Right		> 0.10						> 0.10				
At least 1 high/ both low	22/12						12/6					
Left		0.010*	2.33	-26	2	-20		0.016*	2.14	-20	-2	-14
Right		> 0.10						0.041	2.58	18	-6	-14
Both high/> 1 high	7/15											
Left		0.024	2.82	-22	-8	-22						
Right		> 0.10										
Both high/both low	7/12											
Left		0.025	2.80	-22	-4	-26						
Right		> 0.10										

5-HTTLPR = serotonin transporter promoter polymorphic region (s = short allele carriers, ll = homozygous for long allele); high = high-response alleles (s, T); low = low-response alleles (ll, GG); MNI = Montreal Neurological Institute; SAD = social anxiety disorder; TPH2 = tryptophan hydroxylase-2 polymorphism (T allele carriers or GG = homozygous for G allele).
*Uncorrected for multiple comparisons.

amygdala reactivity (angry > neutral) compared with the low-response (II+GG) subgroup.

We found a significant group difference in amygdala responsiveness between patients and controls only when we compared patients who had high-response alleles with controls who had low-response alleles. Intriguingly, we also found suggestive evidence for greater amygdala activation in response to angry faces among controls carrying high-response alleles compared with patients carrying low-response variants. Taken together, these results indicate that serotonin-related allelic variation, particularly in the *TPH2* gene, is important for amygdala responsiveness to emotionally salient stimuli and that the serotonergic poly-

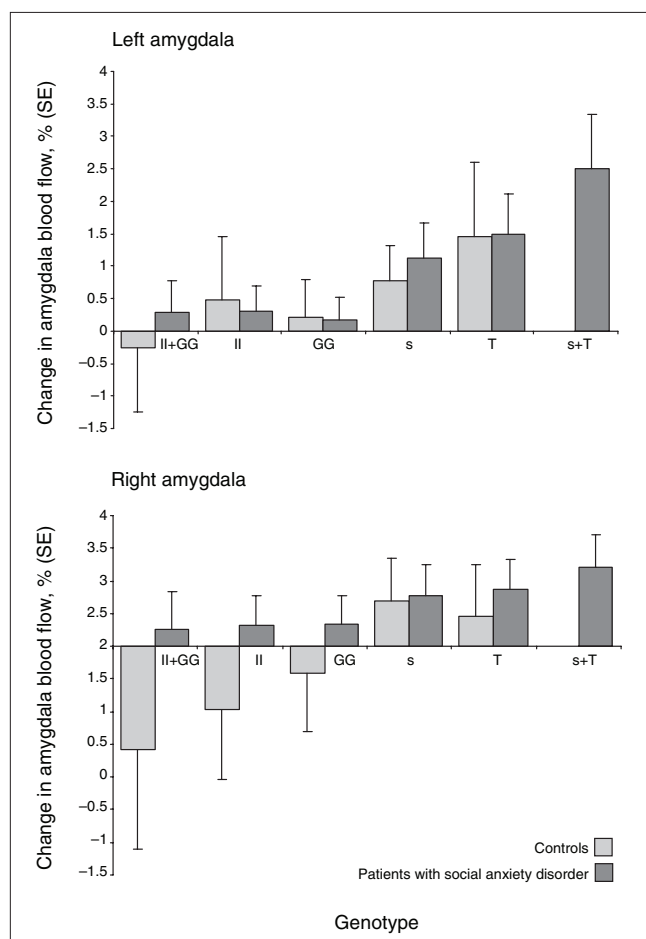


Fig. 2: Mean (and standard error of the mean) percent amygdala blood flow changes from the neutral to the angry face condition in genetic subgroups of patients with social anxiety disorder (SAD) and healthy controls. The 6 bars from the left show groups dominated by low-response alleles of the serotonin transporter promoter and tryptophan hydroxylase-2 polymorphisms (i.e., II and/or GG homozygous individuals). The II+GG bars represent individuals with low-response alleles only. The 5 bars from the right display groups dominated by high-response carriers (i.e., s and/or T carriers) having higher amygdala reactivity. Because only 3 controls were carriers of both high-response alleles, the s+T bar is only shown for patients with SAD.

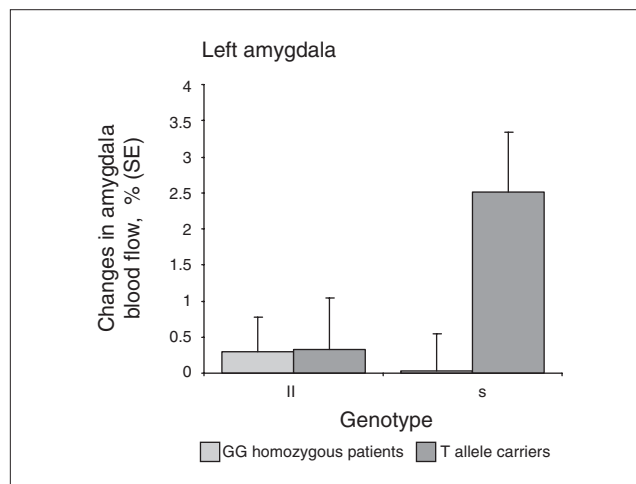


Fig. 3: Gene-gene effects on left amygdala responsiveness in patients with social anxiety disorder. Bars show mean (and standard error of the mean) percent amygdala blood flow changes from the neutral to the angry face condition. The markedly enhanced cerebral blood flow response in carriers of both high-response alleles (s+T) indicated synergistic effect between the serotonin transporter promoter and tryptophan hydroxylase-2 polymorphisms.

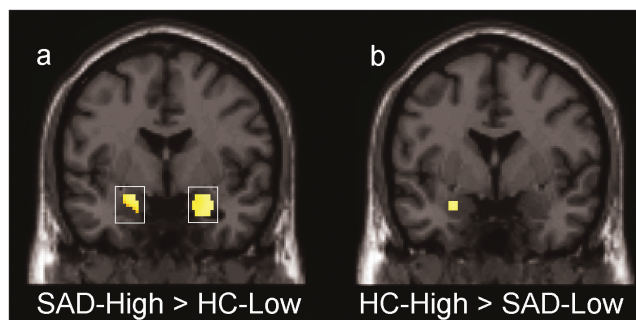


Fig. 4: Coronal views of increased amygdala responsiveness to angry compared with neutral faces in (a) patients with social anxiety disorder (SAD) carrying both high-response (s+T) alleles of the serotonin-transporter promoter and tryptophan hydroxylase-2 polymorphisms relative to controls carrying low-response (II+GG) variants and (b) controls carrying high-response (s or T) alleles relative to patients with SAD carrying low-response (II+GG) variants. The mask threshold corresponds to $z > 2$, and activations that remained significant after correction for multiple comparisons are displayed within squares.

Table 3: Coefficients for predictor variables of left amygdala reactivity (angry > neutral) entered into the multiple regression model for patients and controls (n = 52)

Variable	B	SE B	β	p value
Diagnosis (SAD/control)	0.04	0.60	0.01	0.95
5-HTTLPR (s/II)	0.55	0.56	0.14	0.33
TPH2 (T/GG)	1.26	0.58	0.30	0.035
STAI-S (Δ angry-neutral)	-0.01	0.06	-0.03	0.84

5-HTTLPR = serotonin transporter promoter polymorphic region (s = short allele carriers, II = homozygous for long allele); β = standardized regression coefficient; B = unstandardized regression coefficient; SAD = social anxiety disorder; SE = standard error; STAI-S = Spielberger State Anxiety Scale; TPH2 = tryptophan hydroxylase-2 polymorphism (T allele carriers or GG = homozygous for G allele).

morphisms are stronger predictors of amygdala reactivity than a diagnosis of SAD. This was confirmed by multiple regression analysis in which the TPH2 polymorphism alone and the presence of both high-response alleles emerged as significant predictors of left amygdala responsiveness. Diagnosis and behavioural measures, on the other hand, accounted for small and insignificant portions of variance in amygdala reactivity.

The absence of a robust diagnosis-related effect on amygdala reactivity was somewhat surprising considering the respectable number of studies that have reported such an effect.⁷ At a liberal statistical threshold, there was a difference in the expected direction (SAD > control) in the right amygdala, partly driven by decreased regional cerebral blood flow (angry < neutral) among controls, especially in carriers of low-response alleles. Although other imaging paradigms could be more sensitive to detect small group differences in neural responding, our data suggest that 5-HT-related polymorphisms explain much more of the variance in amygdala reactivity than a diagnosis of SAD. The robustness and generalizability of this finding could be explored in future studies of SAD and other disorders associated with amygdala dysfunction.

Our results are consistent with our previous PET study of patients with SAD in which we noted that the 5-HTTLPR polymorphism modulated amygdala activation during a stressful public speaking task,⁴¹ even though the lateralization patterns differed. The results also add to a growing corpus of imaging data demonstrating serotonergic genetic influence on the amygdala response to social-emotional stimuli.^{35-37,39,40} Gene-gene analyses in the current study suggested a synergistic effect between the 5-HTTLPR and TPH2 polymorphisms such that patients with SAD carrying both high-response alleles (s+T) showed markedly increased left amygdala response (angry > neutral) relative to subgroups of patients carrying only 1 high-response allele and relative to low-response (ll+GG) patients and controls. Other investigators have noted additive effects of the s and T alleles on neural responding to emotional stimuli.^{55,56}

In line with previous association studies failing to demonstrate a link between SAD and the 5-HT transporter or other monoamine-related genes,^{57,58} the allelic distribution in the current study did not differ between patients and controls, although the statistical power to find such a difference was low. It should be noted that one-third of the patients were homozygous for both low-response alleles and did not show elevated amygdala reactivity to angry faces, whereas two-thirds of the controls carried at least 1 high-response allele and did show exaggerated amygdala reactivity. Thus, it seems that 5-HT-related high-response alleles and amygdala hyperresponsivity are neither necessary nor sufficient to cause SAD to develop.

In contrast to our previous PET study,⁴¹ carriers of the s and ll alleles in either the patient or control group did not differ on any behavioural measure. In large sample studies of nonpsychiatric populations, the serotonin transporter gene has been associated with anxiety-related traits,⁵⁹⁻⁶² although there have been some inconsistent findings.⁶⁰ The only significant influence of genotype on behavioural measures in the

present study was a higher level of trait anxiety in patients carrying T alleles compared with GG homozygotes. Other investigators have also reported that the TPH2 polymorphism was associated with personality disorders and with traits characterized by emotional instability; however, the nature of the association was not always the same.^{63,64}

Neither trait nor state anxiety predicted amygdala response to angry faces in the present study, although some imaging data suggest that these affective components can modulate the amygdala response to emotionally salient stimuli.^{20,65,66} Moreover, we could not replicate the findings of 2 previous imaging studies demonstrating significant positive correlation between right amygdala activation to negative faces and severity of SAD as measured by the Liebowitz Social Anxiety Scale.^{27,29} In fact, we noted that lower values on the scale were predictive of elevated left amygdala response in the SAD group. Despite having significantly higher levels of trait and state anxiety, patients with SAD did not exhibit higher baseline amygdala activity during the neutral face condition compared with controls, and amygdala responsiveness (angry v. neutral) did not correlate with activity during the neutral baseline.

There are several methodologic factors that may affect assessments of amygdala response during emotional face processing. For example, the magnitude of the amygdala activation may be influenced by attention demands⁶⁷ and by different qualities of the facial stimuli such as valence of the emotional expression,^{3,4} emotional intensity level,⁶⁸ degree of novelty/familiarity,⁶⁹ gaze direction⁷⁰ and use of schematic or photographic stimuli.²⁸ In within-group designs, it remains to be tested how these putative amygdala-modulating variables interact with 5-HT-related genotypes. There are also issues with the selection of participants related to characteristics such as age,^{71,72} sex,^{73,74} personality and temperamental traits^{75,76} that may be of importance for group differences in amygdala responsiveness. Studies using between-group designs could benefit from taking genetic variation into account before attributing the source of differential amygdala response to the group factor of interest.

Limitations

Our study had some limitations. First, sample sizes were modest when comparing genetic subgroups. The small number of participants prevented us from properly evaluating gene-gene interactions in controls and from demonstrating possible group differences of small effect sizes. Because we were not able to match participants on genotype beforehand, we were restricted to a post-hoc analytic approach and unbalanced groups. Second, other imaging techniques or designs may uncover additional effects of emotional stimuli on the magnitude and time course of amygdala activation. For example, Campbell and colleagues⁷⁷ noted that the early and late temporal components of the amygdala fMRI response to negative faces differed among patients with SAD and controls. Third, although perception of emotional faces does not take place in a single brain region⁷⁸ the present study focused on the amygdala response only, a decision

motivated by the central role of the amygdala in emotional processing¹⁻⁵ and by the complexity generated by the large number of genetic subgroups and contrasts. It should also be noted that we based some analyses and plots on voxel values extracted from the whole amygdala volume; however, it is possible that only certain subregions of the amygdala are activated by face processing tasks.

Conclusion

Future imaging genetic studies of emotional processing could explore other serotonergic and nonserotonergic polymorphisms and their interactions. For example, amygdala reactivity appears to be modulated by polymorphisms in the 5-HT_{1A} receptor gene (*5-HT_{1A} 1019C/G*)⁷⁹ and the catechol-O-methyltransferase gene (*COMT Val158Met*).⁸⁰ Functional connectivity analyses could be used to evaluate how genes affect not only the amygdala response but also the dynamic interplay between relevant nodes in a larger affective processing network. For instance, the 5-HTTLPR has been demonstrated to influence an amygdala–anterior cingulate cortex feedback circuit putatively involved in the regulation of emotion.⁸¹ Future studies could also evaluate effects of genotype on treatment response and concomitant neurofunctional changes. Interestingly, Stein and colleagues⁸² noted that the 5-HTTLPR s allele was associated with poorer response to selective serotonin reuptake inhibitors in patients with SAD. Treatment may modulate amygdala activity differentially owing to genetic makeup.

In conclusion, we demonstrate that variation in 2 genes of major importance for central serotonergic function influence amygdala responsiveness during affective processing to a larger degree than a diagnosis of SAD. Further research is needed to determine the relevance of serotonergic genotypes and amygdala function in the etiology of anxiety disorders. Meanwhile, the present study underscores the importance of accounting for serotonergic polymorphisms when studying group differences in amygdala responsiveness.

Acknowledgements: The study was supported by the Swedish Research Council, the Swedish Brain Foundation and GlaxoSmithKline Co. We thank the staff at Uppsala Imanet and Quintiles for providing excellent research conditions.

Competing interests: None declared by Drs. Furmark, Appel, Orelund, Eriksson and Fredrikson, Mses. Henningsson, Pissioti and Faria and Messrs. Åhs and Linnman. Drs. Bani and Pich are employed by GlaxoSmithKline.

Contributors: Drs. Furmark, Appel, Bani, Merlo Pich, Eriksson and Fredrikson designed the study. Drs. Furmark, Appel, Eriksson and Fredrikson and Ms. Henningsson acquired and analyzed data and wrote the article. Messrs. Åhs and Linnman, Mses. Pissioti and Faria and Dr. Orelund acquired data. Mr. Linnman and Dr. Merlo Pich analyzed data. All authors reviewed the article and gave final permission for publication.

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Correction

A comparison of affected and unaffected relatives of patients with bipolar disorder using proton magnetic resonance spectroscopy

In the print version of the article by T. Hajek, D. Bernier, C. Slaney, L. Propper, M. Schmidt, N. Carrey, G. MacQueen, A. Duffy and M. Alda (*JPN* 2008;33[6]:531-40), the citations of references 22-36 in the text were incorrect. This affected only the print version; for correct citations, please refer to the online version, available at cma.ca/jpn.

We apologize for this error.