Genetic variations in the serotonergic system contribute to amygdala volume in humans

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The amygdala plays a critical role in emotion processing and psychiatric disorders associated with emotion dysfunction. Accumulating evidence suggests that amygdala structure is modulated by serotonin-related genes. However, there is a gap between the small contributions of single loci (less than 1%) and the reported 63–65% heritability of amygdala structure. To understand the “missing heritability,” we systematically explored the contribution of serotonin genes on amygdala structure at the gene set level. The present study of 417 healthy Chinese volunteers examined 129 representative polymorphisms in genes from multiple biological mechanisms in the regulation of serotonin neurotransmission. A system-level approach using multiple regression analyses identified that nine SNPs collectively accounted for approximately 8% of the variance in amygdala volume. Permutation analyses showed that the probability of obtaining these findings by chance was low (p = 0.043, permuted for 1000 times). Findings showed that serotonin genes contribute moderately to individual differences in amygdala volume in a healthy Chinese sample. These results indicate that the system-level approach can help us to understand the genetic basis of a complex trait such as amygdala structure.

Keywords: serotonin, gene, amygdala, brain structure, missing heritability

Introduction

The amygdala, an almond-shaped brain structure which resides in the medial temporal lobe of the brain (Whalen and Phelps, 2009), is key in emotion processing (Sergerie et al., 2008). Lesion studies suggest that the amygdala plays a central role in the perception of emotional stimuli (Campanella et al., 2014), and fMRI studies show that the amygdala activates in response to emotional stimuli...
Our 417 participants (mean age 20.4 years, SD = 0.9; 179 males and 238 females) were a subset of a larger study of 480 healthy Chinese college students (mean age = 19.9 years, SD = 0.9; 208 males and 272 females) from Beijing Normal University, Beijing, China (Li et al., 2011), for whom structural imaging data was available. All participants were Han Chinese and were free of neurological and psychiatric disorders. This study was approved by the IRB of the State Key Laboratory of Cognitive Neuroscience and Learning at Beijing Normal University, China. All experiments were performed in accordance with approved guidelines and regulations. Written informed consent was obtained from each participant.

**Gene Selection**

We selected genes using the serotonin pathway defined in the Kyoto Encyclopedia of Genes and Genomes database, a collection of pathway maps widely used in gene-set analysis. Genes in the following four serotonin subsystems were selected: (1) the serotonin synthesis subsystem, which converts hydroxylation (by TPH) to 5-HT: TPH1, TPH2; (2) the degradation subsystem, which directly breaks down released 5-HT at the synapse into inactive metabolites (MAOA, MAOB); (3) the transportation subsystem, which pumps serotonin from synaptic spaces into presynaptic neurons [SLC6A4 (also known as 5-HTT)] or integrates the membrane of intracellular vesicles of presynaptic neurons and transported monoamines into the synaptic vesicles [SLC18A1 (also known as VMAT1), SLC18A2 (also known as VMAT2)]; (4) the serotonin receptor subsystem (HTR1A, HTR1B, HTR1D, HTR1E, HTR2A, HTR2B, HTR2C, HTR3A, HTR3B, HTR3C, HTR3D, HTR3E, HTR4, HTR5A, HTR5B, HTR6, HTR7). Together, the selected genes represent all major genes involved in the four serotonin subsystems in humans (Chen et al., 2015). Several tag SNPs (tSNPs) defined by the HapMap project1 [Phase 3] (Frazer et al., 2007) were selected to sample the genetic diversity of these genes. Details of these genes and the selected loci (129 polymorphisms, including 127 SNPs and 2 VNTR polymorphisms) are shown in Supplementary Table S1.

**Genotyping Techniques**

Genotyping was conducted as previously described (Li et al., 2011). Briefly, 4 ml venous blood sample was collected from each subject, and then genomic DNA was extracted according to standard methods. SNPs were genotyped using the Illumina GoldenGate Genotyping protocol (see Illumina GoldenGate Assay Protocol for details2). In addition, two genetic markers (5-HTTLPR, MAOA VNTR) were ascertained by standard PCR procedures (Chen et al., 2015).

**Gene Data Preprocessing**

Quality control of the genetic data was carried out based on the larger sample of 480 participants. Two subjects met the criteria of over 10% null genotyping, and were thus excluded from subsequent analyses. Of the 60228 genotypes (126 SNPs by 478 subjects), 120 genotypes (0.2%) were excluded because of low GenCall (<0.25). If any SNP had fewer than 10 (2%) heterozygotes or minor homozygotes, these two genotype groups were combined. If the combined group still had fewer than 10 subjects, that SNP was excluded from further analysis.

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1www.hapmap.org
2www.southgene.com.cn
We found that five SNPs showed significant Hardy–Weinberg disequilibrium ($p < 0.01$) based on a $df$ of 1 (for SNPs located on X chromosome, only females were included in HWE calculation since males have only one X chromosome). However, these SNPs were retained because the HW disequilibrium here did not seem to result from genotyping error but rather reflected the characteristics of college students due to social selection (i.e., overrepresentation of alleles linked to school achievement and motivation; Chen et al., 2013). Because both tag SNPs and additional SNPs in regions detected in recent selection (Hawks et al., 2007) were selected in the current study, there was high LD, and whether they were included in the main analyses.

The statistical procedure was comprised of three major analyses. First, analysis of variance (ANOVA) was conducted for each of these loci to detect variants which met the inclusion criterion ($p < 0.05$, uncorrected, to control for Type II error). Second, these loci were then entered into a regression model to estimate their overall contribution to amygdala size. In the regression model, all loci with significant main effects based on the ANOVA results were included with a forward stepwise method. In this step, all SNPs were coded in a linear way, i.e., the major homozygote, heterozygote, and minor homozygote were coded into 1, 2, and 3, respectively (SNPs on X chromosome were coded as 1 and 3 for major and minor allele homozygote, and also 3 for female heterozygotes). In addition, the $MAOA$ VNTR was coded as 1 for the 3 repeat and 3 for the 4 repeat in males and 1 for 3 repeat homozygotes and 3 for others in females. Finally, the regression model was verified by permutation. Permutation tests were done 1000 times by shuffling amygdala volume data across subjects. In each iteration, selection of significant snps from the ANOVA tests, regression model estimation with a forward stepwise method, and $r^2$ calculation were carried out on the shuffled data. The probability of getting a larger $r^2$ in the shuffled data than in the real data was defined as $p$-value of the model.

Volumes of bilateral amygdala were retracted from the standard output of the FreeSurfer analysis. Then the mean bilateral amygdala volume was calculated. A preliminary analysis showed that both gender and ICV were significantly associated with amygdala size (for gender, $F(1,415) = 48.06, p = 1.59 \times 10^{-11}$; for ICV, $F(1,415) = 114.23, p = 1.03 \times 10^{-23}$). Therefore, to control for the confounding effects of gender and ICV, a regression analysis was conducted, with gender and ICV as independent variables, and amygdala size as the dependent variable. The residual for each subject was normally distributed ($skewness = 0.456$, $kurtosis = 0.747$, Kolmogorov–Smirnov test = 0.039, $p = 0.136$) and was used as an index of amygdala volume in subsequent association analyses. To avoid the possible confounding effect of emotion state on amygdala structure, the associations between Beck Anxiety Inventory (BAI) and Beck Depression Inventory (BDI) scores with amygdala size were tested separately. The associations did not reach significance [for BAI, $F(1,415) = 0.74, p = 0.39$; for BDI, $F(1,415) = 2.67, p = 0.10$]. Therefore, the BAI and BDI scores were not considered in subsequent analyses.

**Statistical Analysis**

The statistical procedure was comprised of three major analyses. First, analysis of variance (ANOVA) was conducted for each of these loci to detect variants which met the inclusion criterion ($p < 0.05$, uncorrected, to control for Type II error). Second, these loci were then entered into a regression model to estimate their overall contribution to amygdala size. In the regression model, all loci with significant main effects based on the ANOVA results were included with a forward stepwise method. In this step, all SNPs were coded in a linear way, i.e., the major homozygote, heterozygote, and minor homozygote were coded into 1, 2, and 3, respectively (SNPs on X chromosome were coded as 1 and 3 for major and minor allele homozygote, and also 3 for female heterozygotes). In addition, the $MAOA$ VNTR was coded as 1 for the 3 repeat and 3 for the 4 repeat in males and 1 for 3 repeat homozygotes and 3 for others in females. Finally, the regression model was verified by permutation. Permutation tests were done 1000 times by shuffling amygdala volume data across subjects. In each iteration, selection of significant snps from the ANOVA tests, regression model estimation with a forward stepwise method, and $r^2$ calculation were carried out on the shuffled data. The probability of getting a larger $r^2$ in the shuffled data than in the real data was defined as $p$-value of the model.

**Results**

The mean bilateral amygdala volume across subjects was 1700.5 mm$^3$ ($SD = 177.7$ mm$^3$). ANOVA was used to screen the 99 loci that passed quality control procedures for associations with the amygdala size. Nine SNPs showed main effects on amygdala volume with uncorrected $p < 0.05$. Specifically, individuals who were major allele homozygotes for rs7997012 ($HTR2A$), rs7984966 ($HTR2A$), rs939334 ($HTR3D$), rs10917509 ($HTR6$), or rs363226 ($SLC18A2$), or minor allele homozygotes for rs1487275 ($TPH2$), rs6792482 ($HTR3D$), rs11676829 ($HTR5B$),
or rs12249377 (HTR7), tended to have larger amygdala size than the remaining groups. (For details, see Table 1, and online Supplementary Table S2).

These nine SNPs were added to a regression model using the forward stepwise procedure to estimate their overall contribution to amygdala size. Five of them made significant and unique contributions to the final model, while the other four SNPs were not included because of collinearity with other SNPs (see Table 2). The regression model accounted for 8.2% (6.8% adjusted) of the variance in amygdala size \( F(5,417) = 7.33, p = 1.3 \times 10^{-8} \). The confidence interval of \( R^2 \) estimated by bootstrap for 1000 times was 0.04–0.16, and that for adjusted \( R^2 \) was 0.03–0.15.

Finally, Monte Carlo permutation analyses were carried out to test the model. Figure 1 shows the permutation results. Based on 1000 permutation tests, the probability of attaining the \( R^2 \) or adjusted \( R^2 \) found in the model reached significance \( (p = 0.043 \) and 0.044, respectively). These results indicate that genes in the serotonin system contribute substantially to individual variance in amygdala volume.

### Discussion

The current study combined the advantages of both the candidate gene approach and dense genotyping technology. Our theory-driven method detected a group of biological relevant genes based on prior knowledge and thus avoided the heavy comparison correction necessary in GWAS. However, unlike candidate gene studies on single genes, our system level approach took into account the polygenic nature of amygdala structure. Given the innervation of serotonergic fibers in the amygdala (Bauman and Amaral, 2005) and the role of serotonergic genes on amygdala structure (Meyer-Lindenberg et al., 2006; Frodl et al., 2008; Zetzsche et al., 2008), we used dense gene chips to cover all tag SNPs of the serotonergic biological pathway in order to test the additive effect of potential genes. Results suggest that such a system level approach could bridge the gap between the small contributions of single genes and the considerable heritability of amygdala volume revealed by twin studies. Specifically, serotonergic genes collectively accounted for 8.2% of variance in amygdala volume. Although associations between these specific SNPs and amygdala structure have not been reported before, direct and indirect evidence have linked these genes to amygdala structure and amygdala-related psychological disorders. In the following paragraphs, we discuss each of these genes.

The TPH2 gene encodes TPH protein which is involved in the rate-limiting biosynthesis of serotonin. Postmortem studies have revealed the expression of TPH2 mRNA in the amygdala (Zill et al., 2007). Raphe neurons of Tph2 knockout mice were completely devoid of 5-HT, indicating that brain 5-HT synthesis across the lifespan is exclusively maintained by TPH2 (Gutknecht et al., 2009). Previous imaging genetic studies also found associations between several TPH SNPs and the amygdala, such as rs4570625 with the structure (Inoue et al., 2010) and function (Furmark et al., 2009) of the amygdala, and rs17110563 with bipolar disorder (Cichon et al., 2008). Thus far, however,
no study has linked rs1487275 genotype to any emotion-related behavior or psychiatric disease, although its effect on amygdala structure was identified in the current study. Therefore, future studies should explore such potential associations in healthy or clinical samples.

SLC18A2 encodes VMAT2 that transports free serotonin from cellular cytosol into synaptic vesicles (Eiden et al., 2004). Rodent studies have reported early expression of VMAT2 in amygdala (Lebrand et al., 1998) and that mice lacking one copy of the VMAT2 gene develop with significantly reduced serotonin (Fon et al., 1997). Convergent studies have linked VMAT2 gene to brain development and amygdala-related psychiatric diseases. For instance, an increase in cell death in the superficial layers of the cingulate and retrosplenial cortices during early postnatal life in Vmat2 knockout mice (Stankovski et al., 2007) and a delayed maturation of the upper cortical layers in the Vmat2(sert-cre) and Tph2(+/−) mice (Narboux-Nême et al., 2013) were reported. Moreover, VMAT2 heterozygous mice exhibit 'depression-like' phenotype (Fukui et al., 2007). In human studies, patients with bipolar disorder showed higher binding of VMAT2 (Zubieta et al., 2001), patients with major depression also showed elevated VMAT2 density (Zucker et al., 2002) and structural change of VMAT2 (Zalsman et al., 2011) in platelets. Our finding of an association between SLC18A2 variation and the structure of the amygdala seems in accordance with the above previous results.

HTR2A, HTR3D, HTR5B, HTR6, and HTR7 encode different serotonin receptors. Studies indicated the expression of HTR2A (McDonald and Mascagni, 2007), HTR3 (Morales et al., 1998), HTR6 (Marazziti et al., 2012) in the amygdala. Their effects on amygdala structure might be partly accounted for by the distribution of serotonin receptors in the amygdala, and the modulation effect of serotonin receptors on different developmental processes (Gaspar et al., 2003), such as neurogenesis, apoptosis, axon branching, and dendritogenesis. Accumulating pharmacological studies have linked these receptors to the function of the amygdala and related psychiatric diseases. For example, HTR2 agonist has been found to increase neuronal firing of the amygdala (Stein et al., 2000) and to increase anxiety-like behavior (Pockros-Burgess et al., 2014); HTR2A antagonist can have an antidepressant-like effect (Quesseveur et al., 2013); HTR3 agonist attenuates antidepressants' effect (Nakagawa et al., 1998), whereas HTR3 antagonist as well as HTR6 and HTR7 have an antidepressant effect (Wesolowska and Nikiforuk, 2007; Mnie-Filali et al., 2011; Gupta et al., 2014). A recent study also showed that social isolation stress could result in up-regulation of HTR5B, suggesting a close link between HTR5B and emotion and its neural substrates such as the amygdala (Maekawa et al., 2010). In addition to pharmacological studies, at least one molecular genetic study found a significant association between rs7997012 variation (HTR2A) and the therapeutic response to antidepressant treatments in major depression patients (Lin et al., 2014). In sum, previous studies have consistently shown that the above serotonin receptors play a major role in mood disorders, which are likely related to amygdala dysfunction. Moreover, HTR6 was indicated to mediate brain development in MAOA-deficient mouse embryos (Wang et al., 2014) and HTR7 signaling was reported to regulate neuronal morphology (Kobe et al., 2012).

### TABLE 2 | Regression model for amygdala volume with genetic data.

| Regressor | Gene       | B    | T     | p     |
|-----------|------------|------|-------|-------|
| rs10917509| HTR6       | −21.16 | −2.56  | 0.01  |
| rs11678829| HTR5B      | 48.88 | 3.00  | 0.00  |
| rs6792482 | HTR3D      | 30.62 | 2.73  | 0.01  |
| rs963226  | SLC18A2(VMAT2) | −58.70 | −2.73  | 0.01  |
| rs7984966 | HTR2A      | −65.77 | −2.30  | 0.02  |

*Gene* is the corresponding gene for each SNP; *B* is the regression coefficient, *T* and *p* are t-test results. Effects of gender and ICV are controlled prior to this analysis.

![FIGURE 1 | Permutation results for the genetic model: the dashed line represents the empirical distribution of $R^2$ obtained from the randomized data, and the solid vertical line represents $R^2$ obtained from the actual data.](image-url)

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system, an excess of serotonin affects interneuron migration (Riccio et al., 2009) and neocortical pyramid neuronal migration (Riccio et al., 2011). High levels of serotonin are also suggested to have neuroprotective effects on cortical neurons (Stankovski et al., 2007), while lack of brain serotonin is suggested to affect postnatal development and serotonergic neuronal circuitry formation (Migliarini et al., 2013). In summary, the association that we found between serotonergic genes and amygdala structure might result from the developmental role of serotonin.

Further research is required to support our findings for several reasons. First, some serotonergic genes, which have been found in several studies to impact amygdala structure, were not found to impact amygdala morphology in the present study (e.g., HTR1A). One possible explanation is that HTR1A may be important in amygdala structure and function in Caucasians (for whom most previous studies were on), but not Chinese. We were not surprised at the negative result in our Han Chinese sample, as several studies have reported that the same genetic variation can result in divergent psychological outcomes, depending on the population (Long et al., 2013; Wang et al., 2013). For example, a recent study genotyped the HTR1A polymorphism in European Americans and Koreans, and reported a significant interaction between HTR1A genotype and culture in the locus of attention (Kim et al., 2010). Moreover, the association between the HTR1A gene polymorphism (rs6295) and bipolar disorder in the Caucasian sample (Sullivan et al., 2009) was not found in the Korean population (Kim et al., 2014). Therefore, our sample of pure Han Chinese helps to prevent the confounding effect of population, but we should be cautious as it also limits the generalization of our results to other populations. Another possible explanation is that we may have missed the causative SNPs by using only tag SNPs to sample the genetic diversity of these genes. Further studies are required to test this association in other populations and to genotype more SNPs.

Second, in the current study, we focused on the role of serotonergic genes in healthy young adults to avoid the confounding effects of neurological diseases and age (Filippini et al., 2009). However, it should be noted that both developmental mechanisms and adult chronic disease may affect amygdala size, but through different mechanisms. Studies involving larger sample sizes and older adults (to explore possible effects of aging) and subjects with chronic disease are needed. Third, considering that amygdala dysfunction accompanying structural abnormality also underlies emotion related psychiatric disorders (Ubl et al., 2015) and reflects the effect of serotonergic genes (Hariri et al., 2002), further studies are needed to test the functional indices of the amygdala. Also, studies on amygdala subregions using higher resolution images could provide more information regarding the effects of serotonergic genes on the amygdala. Fourth, the current study could not identify the specific serotonin receptor(s) that transduced the effects. More direct biological evidence is required in further studies to elucidate the relationship between the serotonergic receptors and the downstream amygdala structural change.

Last but not the least, all associated SNPs in our study were located in non-coding regions. This result is consistent with the view that non-coding regions which were once labeled as “junk DNA” actually may play important functional roles (Birney et al., 2007). Some studies indicate that intron variants are involved in gene expression (Zhang et al., 2007) or mRNA secondary structure formation (Nackley et al., 2006). To explore the specific roles of the SNPs screened in our study, more systematic studies using animal models and other techniques (e.g., optogenetics) are required. Third, although genes from the serotonin system accounted for 8.2% of the variance in amygdala volume, there is still much more “missing heritability” (8.2% vs. 63–65% heritability) to be accounted for. Future studies regarding amygdala structure should incorporate other genetic systems, environmental factors, genetic epistasis, and gene-environmental interactions.

Conclusion

Our system-level approach indicated that several genes within the serotonin system had small effects on amygdala structure, and these genes together accounted for a sizable portion of the missing heritability of amygdala volume. The system-level analysis may enhance our understanding of the genetic basis of human amygdala structure and amygdala-related emotional behaviors and psychiatric diseases.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnana.2015.00129

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