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Human amygdala volume is predicted by common DNA variation in the stathmin and serotonin transporter genes

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Despite the relevance of changes in amygdala volume to psychiatric illnesses and its heritability in both health and disease, the influence of common genetic variation on amygdala morphology remains largely unexplored. In the present study, we investigated the influence of a number of novel genetic variants on amygdala volume in 139 neurologically healthy individuals of European descent. Amygdala volume was significantly associated with allelic variation in the stathmin (STMN1) and serotonin transporter (SLC6A4) genes, which have been linked to healthy and disordered affective processing. These results were replicated across both manual and automated methods of amygdala parcellation, although manual tracing showed stronger effects, providing a cautionary note to studies relying on automated parcellation methods. Future studies will need to determine whether amygdala volume mediates the impact of stathmin and serotonin transporter gene variants on normal and dysfunctional emotion processing.

Introduction

The amygdala is central to many aspects of emotional and cognitive processing[1] and is arguably the brain region most consistently implicated in neurodevelopmental and neuropsychiatric disorders.[2] Human research has consistently demonstrated the importance of the amygdala to emotion processing[3] and cognition (for example, in memory formation[4,5]). There is consistent evidence that damage to the amygdala results in disruption to emotional cognitive processing in humans, including the recognition and experience of emotions such as fear.[6–8]

Converging lines of evidence suggest that the amygdala is structurally and functionally altered in psychiatric disorders. Individuals with spider[9] and social[10] phobia show increased amygdala responding to presentations of feared stimuli, whereas individuals with depression show reduced amygdala responding in response to sad stimuli.[11] Amygdala activation to emotional faces has also been shown to predict symptom reduction in major depressive disorder.[12] Further to this, alterations in amygdala volume have been associated with a number of disorders, including unmedicated depression,[13] obsessive-compulsive disorder[14] and autism, where amygdala volume is significantly reduced in both individuals with autism and their unaffected siblings.[15]

Although large-scale twin studies have shown that amygdala volume is highly heritable,[16,17] its molecular genetic architecture remains to be determined. Here, we examined the influence of common DNA variation in a sample of neurologically healthy adults, emphasizing genes that are highly expressed in the human amygdala, focusing on those

encoding the serotonin transporter, oxytocin receptor and stathmin protein. Within the gene encoding the serotonin transporter (SLC6A4), we investigated three variants: the insertion/deletion polymorphism within the serotonin transporter gene-linked promoter region (5-HTTLPR), a single-nucleotide polymorphism (SNP) within 5-HTTLPR (rs22531) and a variable number tandem repeat within the second intron of this gene (STin2). Within the oxytocin receptor (OXTR) and stathmin 1 (STMN1) genes, we focused our search on two previously studied SNPs (rs53576 and rs182455, respectively).

The 5-HTTLPR consists of a long (l) and a short (s) allele, with the s allele showing a 43-bp reduction (initially described as a 44-bp reduction)[18,19] in length to that of the l allele, and reduced mRNA transcription levels,[20] which may in turn lead to reduced efficiency of the serotonin transporter. The presence of at least one s allele has been repeatedly associated with increased amygdala function at rest[21] and in response to emotional stimuli.[22] This association between amygdala hyperactivity in s allele carriers has been confirmed by two meta-analyses.[23,24] In addition, Scherk et al.[25] found that right amygdala volume was significantly larger in those carrying at least one s allele. We also examined rs25531, a SNP that is thought to alter the function of the 5-HTTLPR variants.[26] The presence of a G (but not an A) nucleotide is thought to alter and reduce the transcriptional efficacy of the l allele to function more similarly to that of an s allele,[18,26,27] although inconsistencies have been reported.[28,29] As s allele carriers show altered amygdala morphometry, the presence of a G nucleotide within rs25531 may also be associated with changes in amygdala volume.

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STin2, a 16–17 bp variable number tandem repeat polymorphism, presents with two major alleles corresponding to 10- and 12-repeat units, and has been found to be in linkage disequilibrium with the 5-HTTLPR polymorphism in different populations.[30–32] The 12-repeat allele has been reported to be a transcriptional enhancer,[33] showing reduced luciferase expression in embryonic and murine stem cells.[34,35] Paradoxically, Bah *et al.*[36] found that 12-repeat homozygotes displayed lower serotonin transporter availability in the brain, despite the aforementioned evidence of increased transcription. DNA variation in STin2 has been associated with a number of affective disorders,[37,38] and recent evidence suggests a combinatorial interaction between 5-HTTLPR and STin2.[39,40] Given these associations, it remains to be tested if STin2 variation is associated with amygdala volume.

The oxytocin peptide has been shown to be important in regulating social behavior, with oxytocin demonstrated to have a role in the effects of social stress, emotion recognition and social memory.[41] Because of this, and the high density of oxytocin receptors within the amygdala,[42] we investigated the relationship between DNA variation in the oxytocin receptor gene and amygdala volume. We focused on a SNP (rs53576) within the oxytocin receptor gene, which has been associated with prosocial temperament and the response of the amygdala during facial emotion processing, as well as a reported effect on amygdala volume in male participants.[43]

The search for the genetic basis of brain function has predominantly focused on genetic variants implicated in neural transmission. An alternative approach is the targeting of genes that regulate neural plasticity. Stathmin, a regulator of microtubule formation, is highly expressed in the amygdala[44–46] and is crucial for fear processing in mice.[46] Shumyatsky *et al.*[46] found that stathmin knock-out mice exhibited deficient long-term potentiation in the amygdala, and an inability to recognize danger in innately aversive environments. Interestingly, these mice displayed similar deficiencies in threat assessment to mice that had their basolateral amygdala lesioned.[45] Expanding this work to humans, Brocke *et al.*[47] investigated two purportedly functional SNPs within the control region of the gene encoding for stathmin. They reported an influence of a SNP within stathmin (rs182455) on amygdala-mediated responding during an emotionally modulated acoustic startle paradigm. Given the strong role of stathmin in amygdala structure and function in animals, it is possible that the effect of rs182455 on emotion processing in humans is mediated by amygdala morphometry.

The aim of the present study was to investigate the influence of the above common genetic variants on amygdala volume, as determined using manual and automated (Freesurfer) parcellation protocols. The use of automated software packages to quantify amygdala volume is increasing with the availability of large databases of high-resolution magnetic resonance images (MRIs), and because of collaborative efforts such as the ENIGMA consortium (http://enigma.loni.ucla.edu/). Although direct comparisons between manual tracing and automated segmentation protocols show high overlap and significant positive correlation,[48] it is unclear whether manual and automated methods are equally sensitive to the small effect sizes of genetic influences on amygdala volume.

We hypothesized that amygdala volume would be increased in s allele carriers of the 5-HTTLPR polymorphism based upon previous findings of increased risk for mental illness and increased amygdala function and volume in s allele carriers. We predicted that amygdala volume would vary as a function of allelic variation in rs53576 of the oxytocin receptor gene. As the 10-repeat allele of STin2 has been linked to a number of mental illnesses, we predicted the presence of the 10-repeat allele would be associated with reduced amygdala volume. Similarly, we predicted that the presence of a C allele of stathmin rs182455, which has been related to a reduction in cognitive-affective processing,[49] would be associated with reduced amygdala volume.

Materials and methods

Participants. One hundred and thirty-nine right-handed participants (seventy-two male, mean age 22 years, range 18–40 years) were recruited from the University of Queensland. All participants were of European ancestry to avoid any possible population stratification artifacts. Participants were all right-handed and were screened for any lifetime history of head trauma, neurological or psychiatric disorders, and active or recent use of psychoactive medications or drugs. Thirteen participants failed genotyping for at least one gene and were removed from subsequent analyses. Written informed consent was obtained from all participants before study participation. The study was approved by the Medical Research Ethics Committee of the University of Queensland.

MRI acquisition and volume measurement protocols. High-resolution T1-weighted structural MPRAGE images were collected at a voxel resolution of 0.9 mm isotropic on a 1.5-T Siemens (Siemens Medical Solutions, Erlangen, Germany) Sonata ($N = 82$) and a 3-T Siemens TRIO ($N = 57$). Image preprocessing was conducted using the FMRIB Software Library.[50] Images were stripped of extra-cerebral tissues using the Brain Extraction Tool,[51] and then reoriented in space to align with the MNI ICBM152 template using FMRIB's Linear Image Registration Tool.[52] The resulting realignment matrix was then applied to the original MPRAGE images, which were then imported into the ANALYZE 10 software package (Mayo Clinic, Rochester, MN, USA) for manual tracing.

Amygdalae were manually traced by the same investigator (DS) who was blind to genotype, based on a previously validated protocol,[53] with slight modifications defining the boundary between the amygdala and the hippocampus.[54] Intra-class correlation coefficients (absolute agreement) for right and left amygdalae were 0.94 and 0.94, respectively, for intra-rater reliability; and 0.97 and 0.91 for inter-rater reliability against an experienced amygdala tracer (VL) and based on 10 randomly selected images.

Manual tracing of the amygdala boundaries was performed on coronally displayed MRI slices and proceeded in a caudal to rostral direction. Amygdala boundaries were defined based upon neighboring gray- and white-matter structures such as

the hippocampus. Appearing initially as gray matter above the temporal horn, the amygdala was bounded by a thin strip of white matter at its superior-lateral edge, separating it from the claustrum and the tail of the caudate; the angular bundle and entorhinal cortex bounded the amygdala medially; the semilunar gyrus defined the superior-medial boundary; and the hippocampus and the temporal white matter defined the inferior and inferior-lateral boundaries, respectively. The anterior boundary of the amygdala, which can be difficult to observe in MRI images, was defined as either the slice posterior to the appearance of the optic chiasm, or where the lateral sulcus closes to form the entorhinal sulcus, whichever was more posterior. Volumetric estimates were obtained by summing all voxels within the left and right amygdalae within all traced slices.

To contrast against the results from the manual tracing protocol, we also computed amygdala volumes with the fully automated subcortical segmentation component of Freesurfer's (http://surfer.nmr.mgh.harvard.edu/) cortical reconstruction process, which has been shown to be more highly correlated with hand tracing than other automated protocols.[48] This also provided a measure of intracranial volume, which was used as a covariate in all analyses, because of the influence of intracranial volume on amygdala volumetric measurements.[55]

Genotyping. DNA was isolated from 2 ml saliva collected using Oragene self-collection kits (DNA Genotek, Kanata, Ontario, Canada). Primers for PCR amplification were designed using Primer3,[56] and are contained in Table 1. DNA was amplified using standardized procedures: PCR for rs22531 and STin2, and touchdown PCR for 5-HTTLPR and rs53576. PCR product was digested using *Bam*HI and *Msp*I enzymes (New England Biolabs, Ipswich, MA, USA) to cut the rs53576 and rs22531 PCR products, respectively. Following electrophoresis in 3% agarose gel, the PCR and digestion products were visualized using 5 μl of SYBR safe gel stain (Invitrogen, Carlsbad, CA, USA) or ethidium bromide where SYBR safe stain failed to visualize bands. We utilized an automated DNA fragment QIAxcel system (QIAGEN, Hilden, Germany) to confirm gel electrophoresis. The rs182455 SNP was genotyped using an Applied Biosystems (Foster City, CA, USA) Taqman® assay running on a Lightcycler

480 (Roche, Rotkreuz, Switzerland), following manufacturer's instructions.

An additive model was used to categorize genotypes for all analyses involving the 5-HTTLPR polymorphism, rs182455 and rs53576. As the s allele of the 5-HTTLPR polymorphism has been associated with risk for mental illness and altered amygdala activation, genotype was coded to reflect these associations such that ll, ls and ss were coded as 0, 1 and 2, respectively. Cases where an l allele for 5-HTTLPR co-occurred with a G SNP for rs25531 were recoded as s alleles. For STin2, we employed a 10-repeat dominant model as used by Sarosi *et al.*,[57] coding 10/10 and 10/12 genotypes as 1 and 12/12 as 2. Rare genotypes, 9/9 and 9/10, were excluded from analysis as they represented less than one percent of all cases. We used an additive model to classify rs182455, coding for the major allele, such that TT, CT and CC were coded as 0, 1 and 2, respectively. For rs53576, we combined the less common A/A and A/G genotypes for our comparison against G/G, as has been done previously by others.[58,59]

Results

A detailed description of brain volumetric measurements is provided in Table 2. The mean values for the manual tracing data are consistent with amygdala volumes measured in post-mortem brains.[60–63] Average automated values are in line with previous reports, which also note larger volume values resulting from automated parcellation methods.[48] Amygdala volumes obtained from manual and automated parcellation were significantly correlated, $r(137) = 0.543$, $P < 0.001$, as shown in Figure 1. Figure 2 presents a Bland–Altman plot[64,65] illustrating the agreement between manual and automated parcellation volumes.

The relationship between genotype and amygdala volume was measured by performing linear regressions, separately for the type of parcellation method (that is, manual and automated), with results detailed in Table 3. Kolmogorov–Smirnoff tests were used to assess the assumption of central tendency and confirmed that all dependent measures were significantly normal, all $D(136) < 0.08$, $P > 0.10$. Regression analyses were run in two steps: first, we controlled for the impact of major confounders on amygdala volumes, including

Table 1 Genotyping methods and frequencies

Gene	Variant	Alleles	Digest	Product/digest length	Primers (5′ → 3′)	Genotype frequencies (N)
SLC6A4	5-HTTLPR	Short (s)	—	486 bp	F: GGC GTT GCC GCT CTG AAT GC	SS (35)
		Long (l)		529 bp	R: GAG GGA CTG AGC TGG ACA ACC AC	SL (54)
						LL (43)
	rs25531	A	*Msp*I	174 + 168 + 125 + 62 bp	—	AA (111)
		G		342 + 125 + 62 bp		AG (14)
						GG (1)
	STin2	10 Repeat	—	279 bp	F: CAG AAT GGA GGG GGT CAG TA	10/10 (26)
		12 Repeat		312 bp	R: TGT TCC TAG TCT TAC GCC AGT G	10/12 (60)
						12/12 (43)
OXTR	rs53576	C	*Bam*HI	238 bp	F: GCC TGG TTT GAA CTG TTT CC	CC (78)
		T		168 + 70 bp	R: ATT CAC TCT GGG CCA TGA AG	CT (46)
						TT (5)
STMN1	rs182455	C	—		—	CC (43)
		T				CT (60)
						TT (26)

All frequencies are within Hardy-Weinberg equilibrium, $P > 0.05$.

Table 2 Amygdala volume, in cubic millimeters, as a function of genotype and parcellation method (mean ± s.d. mm^3)

Gene	Polymorphism	Genotype	Parcellation method	
			Manual (mm^3)	Freesurfer (mm^3)
SLC6A4	5-HTTLPR	Short/short	1231.71 ± 179.64	1669.54 ± 201.82
		Short/long	1200.66 ± 148.31	1657.72 ± 222.88
		Long/long	1258.04 ± 148.57	1775.08 ± 246.39
	STin2	12/12	1184.92 ± 137.84	1661.24 ± 201.42
		10/12	1247.87 ± 181.61	1718.85 ± 224.44
		10/10	1257.00 ± 126.61	1753.58 ± 223.15
OXTR	rs53576	CC	1230.18 ± 166.13	1701.59 ± 214.84
		CT + TT	1202.01 ± 153.47	1689.25 ± 228.64
STMN1	rs182455	CC	1180.14 ± 151.57	1701.41 ± 230.50
		CT	1214.55 ± 154.43	1651.81 ± 196.15
		TT	1304.43 ± 172.00	1796.21 ± 227.10
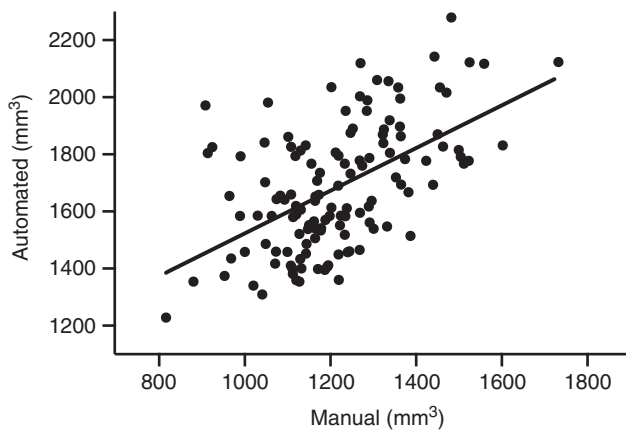
Figure 1 Correlation between amygdala volumes obtain via manual and automated parcellation methods.
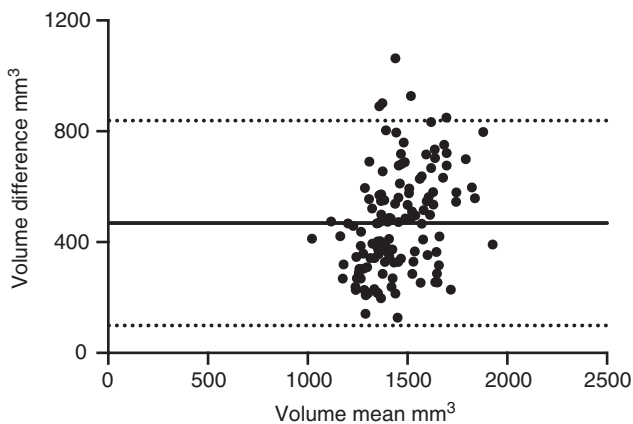
Figure 2 Bland–Altman plot of amygdala volumes obtained by performing the manual and automated parcellation methods. This plot displays the difference between manual and automated volume values against the mean of the two values for each participant, thereby providing a measure of agreement between the two methods. The solid line represents the mean difference between the two methods. Dashed lines represent the limits of agreement, which are defined as 1.96 standard deviations above and below the mean differences.

MRI field strength,[66,67] sex[68,69] and total intracranial volume.[70] We then added in the effect of genes of interest into the regression model. Both covariates and genes of interest were entered using the forced entry method, which makes no assumption as to the order of entry into the regression, and allowed the five genetic variants to be tested together. This method is advantageous when there is no strong theoretical basis for a specific order of entry, and is able to test all genetic variants together.[71]

The regression models at steps 1 and 2 were significant for volumes obtained from both of our parcellation methods (see Table 3). First, we found a significant influence of confounding variables on amygdala volumes. Although only intracranial volume predicted amygdala volumes obtained with manual tracing, amygdala volumes resulting from the automated protocol were significantly affected by intracranial volume and MRI magnet field strength, indicating a difference between the two parcellation methods.

When we examined the impact of genes of interest on amygdala volumes, we found that the stathmin rs182455 and the serotonin transporter STin2 variants were significantly associated with amygdala volumes obtained from manual tracing, whereas this effect was weaker on amygdala volumes resulting from utilizing the automated protocol.

Discussion

To our knowledge, this is the first study to explore the association between amygdala volume and key novel genetic variants implicated in normative emotional functioning as well as a range of common neuropsychiatric disorders. Furthermore, we compared the sensitivity of manual and automated (that is, Freesurfer) methods of amygdala parcellation in detecting genetic effects on amygdala volume. Our findings indicate that variation in stathmin rs182455 and, to a lesser extent, in serotonin transporter STin2 polymorphism is associated with individual differences in amygdala volumes measured with both manual and automated methods. Notably this association was stronger for the manually traced compared with the automatically segmented amygdala volumes.

The observed association between the number of C alleles and amygdala volume provides the first evidence of an influence of rs182455 on amygdala volume in healthy humans. Amygdala volumes were reduced in participants with an increasing dose of the stathmin rs182455 C allele, and

Table 3 Hierarchical regression analysis evaluating genetic influences on amygdala volume as a function of parcellation method (manual vs automated- Freesurfer)

	Amygdala parcellation method											
	Manual						Automated Freesurfer					
Measures	R	R^2	ΔR^2	F change	β	Cohen's f^2	R	R^2	ΔR^2	F change	β	Cohen's f^2
Step 1	0.470	0.221	0.221	9.439***			0.732	0.538	0.538	39.321***		
Covariates												
Sex					−0.101	0.008					−0.148	0.030
Magnet					0.105	0.014					0.501***	0.515
Intracranial volume					0.405***	0.133					0.498***	0.338
Step 2	0.591	0.349	0.129	3.760**			0.764	0.585	0.047	2.861*		
Covariates												
Sex					−0.116	0.011					−0.169*	0.037
Magnet					0.059	0.004					0.484***	0.466
Intracranial volume					0.398***	0.125					0.491***	0.314
Genetic predictors												
5-HTTLPR					0.016	0.000					0.106	0.021
STin2					−0.210*	0.009					−0.106	0.002
rs25531					0.089	0.048					0.028	0.021
rs53576					−0.103	0.013					0.028	0.001
rs182455					−0.249**	0.071					−0.139*	0.038

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

this result was confirmed by both manual and automated methods of amygdala parcellation. This finding is particularly interesting given the wide distribution of stathmin within the brain, and the amygdala in particular, and its function as a major signaling protein.[44,72,73] The wide distribution and role of stathmin suggests that there may exist other brain regions wherein morphometric or functional changes are present as a result of genetic variation in the stathmin gene. Specific to the amygdala, stathmin knock-out mice have been previously reported to display deficiencies in threat assessment that mimicked mice with lesions in the basolateral amygdala.[45] This effect of stathmin upon amygdala structure is echoed by the present findings of an association between the number of C alleles and amygdala volume, and provides the first evidence of an influence of rs182455 on amygdala volume in healthy humans. An effect of rs182455 on amygdala volume may, in turn, contribute to the previously reported genetic effects on cognitive and affective control processes,[49] and startle response to affective stimuli.[47] Future research may benefit from the use of amygdala volume as a mediating variable when investigating the relationship between stathmin rs182455 variation and cognitive and affective processing.

In addition to effects of stathmin rs182455, we also observed increased amygdala volume in the presence of the 10-repeat allele of STin2. This association, however, was only significant for manually measured amygdala volumes. This is an intriguing finding given the association of the 10-repeat allele of this marker with a number of psychiatric disorders,[37,74–76] suggesting that alteration in amygdala volume may provide a useful mediating variable between variation in STin2 and risk for mental illness. Future research is necessary to explore this potential relationship by directly testing the ability of amygdala volume variation to mediate relationships between STin2 variation and risk for or the presence of disease.

Our findings do not confirm previous reports[25,69] of an association between amygdala volume and allelic variation in the 5-HTTLPR polymorphism. This was despite the current

study having a relatively larger sample size and greater power to detect any existing effects. Similarly, we did not detect any effect of variation in the oxytocin receptor polymorphism rs53576 on amygdala volume.

The use of both automated and manual parcellation protocols allowed a direct comparison of the sensitivity of these methods to detect small genetic effects. Results for the automated parcellation method replicated those obtained from the manual parcellation, with weaker effects for rs182455 and no effect of STin2 for manual parcellation. Similarly, there was a significant effect of the nuisance variable of magnet field strength on our model for the automated parcellation but not manual tracing method. These results suggest that the application of automated protocols such as Freesurfer enable the detection of subtle genetic effects on brain structure, albeit with reduced sensitivity compared with manual tracing. This finding is particularly relevant given the increasing use of automated protocols and provides a cautionary note to future studies, in particular those seeking to combine data across multiple sites and scanner environments in order to increase sample size for genetic analyses.

The reported findings are noteworthy given that the amygdala is central to many aspects of affective and social processing and is consistently implicated in a range of common neurodevelopmental and neuropsychiatric disorders. Notably, our findings were partially replicated across both manual and automated amygdala parcellation methods, with manual tracing being more sensitive to the effect of the examined genetic variations on amygdala volumes. The present results provide insight into the potential mechanisms by which variation in these genes may influence healthy cognitive and affective functioning, and ultimately contribute to risk for mental illness. This study was limited, however, in the number of genes that were investigated. There remain many DNA variants that could potentially associate with measures of amygdala function and affective processing that have not been explored.[77–83] Future work should address this

limitation, focusing in particular on genes that regulate neural plasticity such as *STMN1* and *BDNF*.[83] A deeper understanding of the genetic influences on amygdala volume variation in normative samples will be key to inform future research investigating the association between amygdala volume and risk of developing psychiatric disorders.

Conflict of interest

The authors declare no conflict of interest.

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