LETTERS

Amygdalar and hippocampal substrates of anxious temperament differ in their heritability

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Anxious temperament (AT) in human and non-human primates is a trait-like phenotype evident early in life that is characterized by increased behavioural and physiological reactivity to mildly threatening stimuli[1–4]. Studies in children demonstrate that AT is an important risk factor for the later development of anxiety disorders, depression and comorbid substance abuse[5]. Despite its importance as an early predictor of psychopathology, little is known about the factors that predispose vulnerable children to develop AT and the brain systems that underlie its expression. To characterize the neural circuitry associated with AT and the extent to which the function of this circuit is heritable, we studied a large sample of rhesus monkeys phenotyped for AT. Using 238 young monkeys from a multigenerational single-family pedigree, we simultaneously assessed brain metabolic activity and AT while monkeys were exposed to the relevant ethological condition that elicits the phenotype. High-resolution ^{18}F-labelled deoxyglucose positron-emission tomography (FDG–PET) was selected as the imaging modality because it provides semi-quantitative indices of absolute glucose metabolic rate, allows for simultaneous measurement of behaviour and brain activity, and has a time course suited for assessing temperament-associated sustained brain responses. Here we demonstrate that the central nucleus region of the amygdala and the anterior hippocampus are key components of the neural circuit predictive of AT. We also show significant heritability of the AT phenotype by using quantitative genetic analysis. Additionally, using voxelwise analyses, we reveal significant heritability of metabolic activity in AT-associated hippocampal regions. However, activity in the amygdala region predictive of AT is not significantly heritable. Furthermore, the heritabilities of the hippocampal and amygdala regions significantly differ from each other. Even though these structures are closely linked, the results suggest differential influences of genes and environment on how these brain regions mediate AT and the ongoing risk of developing anxiety and depression.

Anxiety disorders are among the most common forms of psychopathology[6], and frequently begin during childhood and adolescence. Although all children experience acute anxiety, children with AT display extreme behavioural and physiological reactivity to novel stimuli, and in the presence of strangers inhibit their locomotor activity and vocalizations[3]. Furthermore, children with AT are maladaptively shy and chronically suffer from worry and apprehension. Some children with AT also exhibit increased pituitary–adrenal and autonomic activity[4]. Identifying neural intermediate phenotypes of AT is a critical step in elaborating how environmental and genetic factors influence the development of anxiety and emotion related psychopathology. Although AT is assumed to be partly heritable, the extent to which genetic variation influences metabolic activity in the neural circuit that underlies AT remains to be determined. We previously validated a non-human primate model of AT[7] and demonstrated that brain activity assessed across stressful and non-stressful contexts predicted AT, revealing the stable trait-like characteristics of brain metabolic activity associated with this disposition[2].

To characterize the extent to which individual differences in AT-related brain activity are heritable, we concomitantly assessed AT and regional brain metabolism in 238 young rhesus monkeys (116 males, 122 females, mean age 2.4 years, range 0.74–4.2 years) belonging to a multigenerational single-family pedigree of more than 1,500 individuals. The statistical power of an extended pedigree approach to quantitative genetic analysis is derived from the presence of substantial numbers of closely related, more distantly related and unrelated pairs that all contribute information about the effects of kinship (shared genes) on phenotypic similarity (Supplementary Information).

Similar to AT in children, AT in monkeys was assessed by using measures of threat-induced freezing behaviour and inhibited vocalizations, as well as plasma cortisol concentrations (Supplementary Information). AT and brain metabolism were assessed when monkeys freely behaved in a test cage by themselves for 30 min in a potentially threatening situation in which a human 'intruder' entered the room and stood 2.5 m from the cage[8]. During this time the intruder presented his profile to the monkey ensuring that he avoided eye contact with the animal (no eye contact; NEC). Animals with the greatest AT froze longer, vocalized less and had elevated plasma cortisol levels. The rationale underlying the use of the NEC challenge is (1) it optimally elicits the behaviours associated with the AT phenotype[8], (2) increased amygdala metabolism occurs during NEC[2] and (3) selective dorsal amygdala lesions attenuate NEC-induced behavioural and physiological responses[9].

To understand further the relation between regional brain activity and AT, monkeys were injected with FDG immediately before NEC. After NEC exposure, blood was collected for cortisol assessment and monkeys were anaesthetized and placed in a high-resolution microPET scanner to measure the FDG uptake that occurred during the NEC challenge. FDG is a glucose analogue with a half-life of 110 min that is trapped by metabolically active cells. Because the time course of FDG uptake reflects brain activity over an approximate 30-min period, and remains stably detectable in the brain, it is an ideal radiotracer to study simultaneously behaviour and brain activity elicited by exposure to ethologically relevant situations. Furthermore, FDG–PET allows measurement of brain activity within a single condition, and, unlike functional magnetic resonance imaging, it does not require a contrast

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Table 1 | Regions where regression analyses revealed that AT was significantly correlated with brain metabolism during the NEC challenge

Clusters correlated with anxious temperament				Local maxima for distinct brain regions within clusters		Location relative to anterior commissure (mm)		
Direction of correlation	Hemisphere	Cluster	Cluster volume (mm^3)	Regions within cluster	Maximum *t* value	*x*	*y*	*z*
Positive	Right	Anterior temporal lobe	850.8	Dorsal amygdala (CeA/ amygdalostriatal transition zone region)	7.68	12.500	−0.625	−8.750
				Ventral putamen	7.40	14.375	−5.625	−8.125
				Superior temporal sulcus (anterior)	7.17	18.750	−0.625	−11.250
				Temporopolar cortex	6.68	19.375	5.000	−8.125
	Left	Anterior temporal lobe	675.1	Anterior hippocampus	7.61	−12.500	−3.750	−9.375
				Ventral putamen/posterior amygdala	7.38	−11.250	−2.500	−8.125
				Mid-hippocampus	6.71	−15.625	−7.500	−10.625
				Claustrum	6.33	−14.375	3.125	−10.000
				Superior temporal sulcus	6.08	−18.750	0.625	−8.125
				Hypothalamus	5.75	−1.875	−4.375	−6.875
	Right	Posterior thalamus	11.5	Pulvinar	5.75	10.625	−15.625	1.250
Negative	Crosses midline	Parietal cortex	493.6	Intraparietal sulcus (right)	−6.85	4.375	−27.500	16.875
				Precuneus (left)	−6.69	−3.125	−29.375	15.000
				V3 (right)	−5.68	6.250	−33.750	9.375
	Left	Visual cortex	455.8	V2	−6.71	−10.000	−28.125	−3.750
				Parieto-occipital sulcus	−5.96	−3.750	−36.875	1.875
				V1	−5.57	−13.750	−31.250	−1.875
	Right	Visual cortex	287.1	V2	−7.35	6.875	−30.625	−2.500
				V3	−5.56	11.250	−31.875	−6.875
	Right	Primary visual cortex	45.9	V1	−5.98	3.125	−43.750	−3.750
	Right	Temporal parieto-occipital area	8.3	Superior temporal sulcus (posterior)	−5.78	15.625	−25.000	8.750

Data are presented with the direction of the correlation, hemisphere, brain regions involved and the volume of each AT-correlated cluster (thresholded at $P \leqslant 0.05$, Šidák corrected). Also presented are *t* values and location (in millimetres relative to the anterior commissure) of the voxel most significantly predictive of AT within each distinct brain region contained in the cluster.

with a baseline signal. These features make FDG–PET particularly useful in understanding the sustained brain responses associated with temperament, which by definition is a persistent and relatively context-independent disposition.

The results demonstrated highly significant correlations between individual differences in AT and glucose metabolism within several large clusters derived using the Šidák correction for multiple comparisons (left and right anterior temporal lobe, midline parietal cortex, left and right visual cortex, right posterior thalamus and right temporal parieto-occipital area; Table 1). The anterior temporal lobe clusters (Fig. 1) are of particular interest because they contain the amygdala, extended amygdala and anterior hippocampus, regions consistently implicated in mediating emotional behaviour, fear-related responses, and anxiety and depressive disorders[1,10–14]. Mean glucose metabolism in the left and right anterior temporal lobe clusters (residualized for age, sex and mean grey-matter probability)

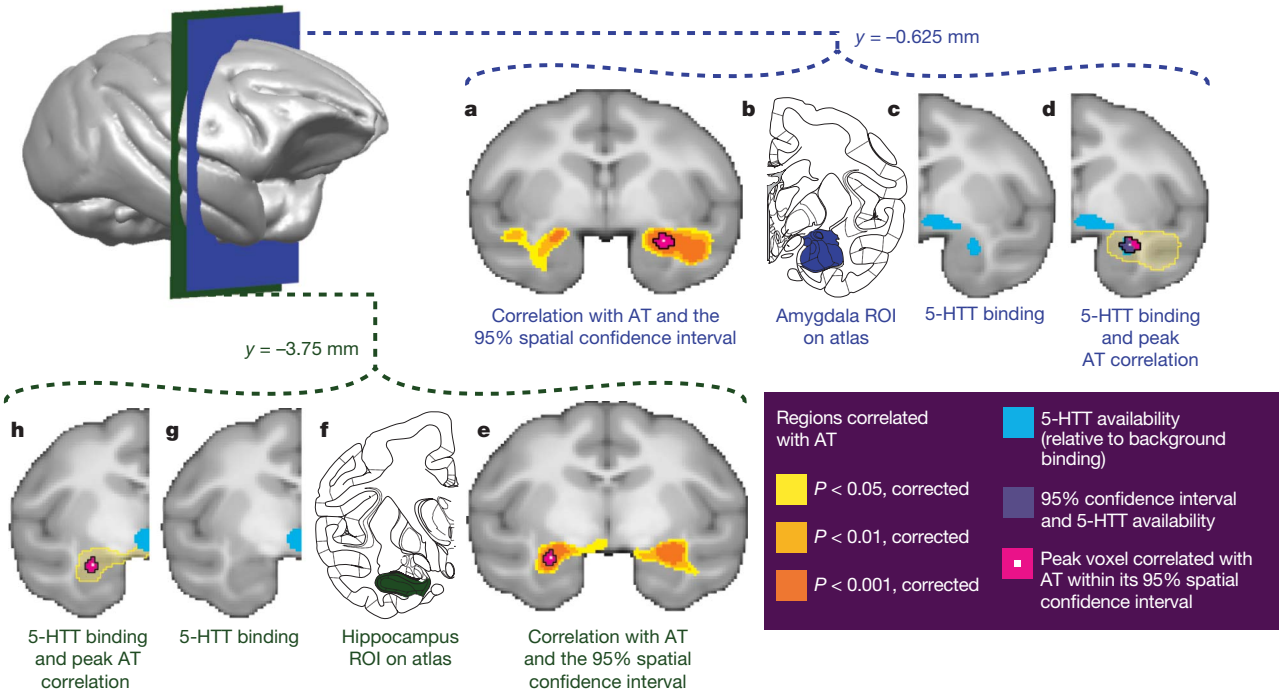

Figure 1 | Glucose metabolism in the anterior temporal lobes is predictive of AT. **a**, Amygdala and **e**, hippocampus (significance of correlations: yellow, $P < 0.05$; light orange, $P < 0.01$; dark orange, $P < 0.001$, adjusted for multiple comparisons using the Šidák correction). Pink areas represent 95% spatial confidence intervals of the peak correlations. **b**, **f**, Corresponding slices adapted from ref. 31. ROI, region of interest. **c**, **g**, 5-HTT binding differentiates the CeA region from the anterior hippocampus. **d**, The CeA, defined by the 5-HTT map, encompasses the amygdala peak. **h**, The hippocampal peak is distinct and does not overlap with the 5-HTT map.

accounted for 24.4% and 27.5% of the variance in AT, respectively. Because these clusters contain anatomically distinct brain structures, we sought to specify the regions within the right and left anterior temporal lobe clusters that most strongly predicted AT. The two peaks maximally predictive of AT were in the lateral portion of the right dorsal amygdala ($r = 0.44$, $P = 2.38 \times 10^{-13}$; Figs 1a and 2a), which contains the central nucleus of the amygdala (CeA) and the amygdalostriatal transition zone, and in the left hippocampus ($r = 0.45$, $P = 8.3 \times 10^{-13}$; Figs 1e and 2b). No effects of sex or laterality were observed in any of the reported correlations (all P values > 0.10), nor were there any significant main effects of sex on the components of AT (all P values > 0.10).

The amygdala and hippocampus work in concert in mediating emotion-modulated memory[15]; however, it is important to emphasize that these structures uniquely contribute to other aspects of emotional processing. For example, hippocampal lesions in rats and rhesus monkeys produce alterations in anxious behaviour that are distinct from the effects of amygdala lesions[16–18]. As the dorsal amygdala and anterior hippocampus are adjacent structures, calculating the spatial confidence intervals around the voxels containing the maximum t values further delineated the locations of these peaks. The confidence intervals represent volumes that with 95% certainty contain the peak correlations between metabolic activity and AT (Supplementary Information). To demarcate further the location of these peaks, the volumes contained within these confidence intervals were superimposed on a voxelwise map of [^{11}C-DASB]serotonin transporter (5-HTT) binding created from an independent sample of rhesus monkeys (Supplementary Information). This 5-HTT map, thresholded at ×250 background binding, can be used to localize precisely the CeA and differentiate it from the anterior hippocampus, because compared with surrounding regions the lateral division of the CeA has the highest density of 5-HTT binding[19]. As can be seen in Fig. 1a–d, the 95% confidence interval of the right dorsal amygdala encompasses the lateral region of the right CeA and the laterally adjacent amygdalostriatal transition zone. Anatomical studies show that the amygdalostriatal transition zone shares many similarities with the CeA, and may represent a posterior division of the ventral striatum/extended amygdala[13]. The left hippocampal 95% confidence interval does not overlap with the lateral CeA region as defined by 5-HTT binding, further confirming the spatial dissociation between the dorsal amygdala and anterior hippocampal regions predictive of AT (Fig. 1e–h).

To estimate the heritability of AT, the pedigree relationships and individual phenotype measures were used in maximum-likelihood variance component analyses computed using SOLAR (Supplementary Information). Consistent with previous findings in rhesus monkeys[20], as well as studies of human anxiety[21], AT was significantly heritable ($h^2 = 0.36$, $P = 0.015$). A voxelwise heritability analysis for brain activity was performed using SOLAR controlling for the potential confounds of age, age-squared and sex. Analyses were limited to those voxels within the FDG clusters that were significantly predictive of AT. Because previous studies demonstrated that heritable individual differences in brain structure relate to stress reactivity[22], grey-matter probability was also included as a voxelwise covariate. Glucose metabolic activity was significantly heritable in voxels in

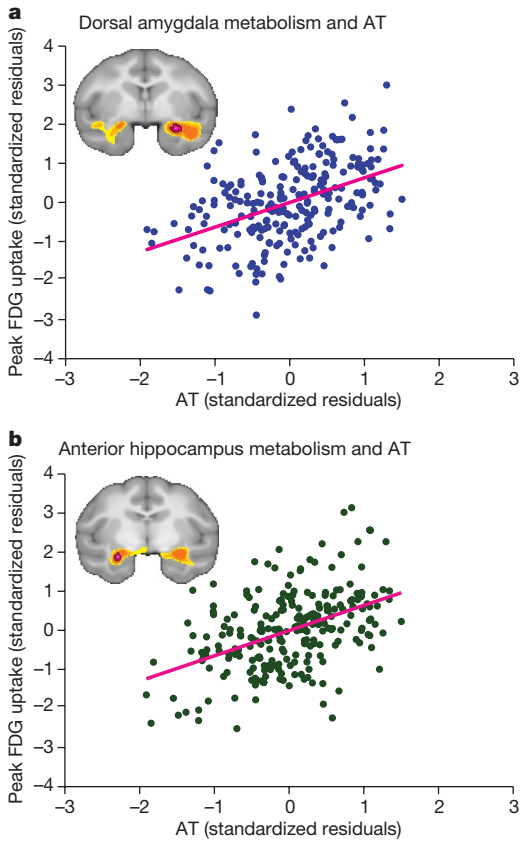

Figure 2 | Peak correlations between AT and glucose metabolism in the anterior temporal lobe. **a**, Voxel within the amygdala (Fig. 1a) reflecting the peak correlation between metabolism and AT ($r = 0.44$, $P = 2.38 \times 10^{-13}$) and **b**, voxel within the hippocampus (Fig. 1e) reflecting the peak correlation between metabolism and AT ($r = 0.45$, $P = 8.3 \times 10^{-13}$). Values for ^{18}F-labelled deoxyglucose were extracted from each animal, residualized for the effects of age, sex and grey-matter probability, and plotted against individual differences in AT.

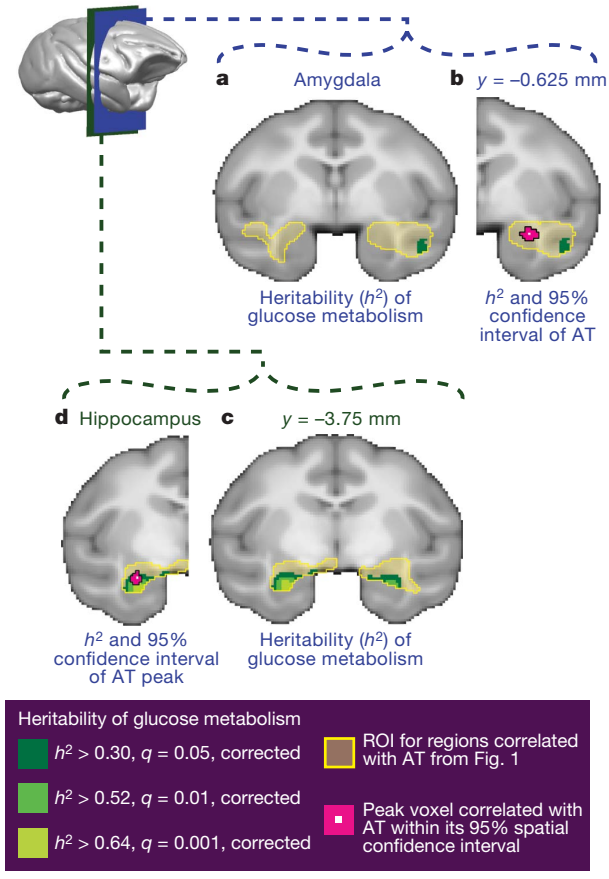

Figure 3 | Overlap between regional metabolic activity predictive of AT and regions that are significantly heritable. **a**, **b**, No significantly heritable voxels were observed in the dorsal amygdala region, although within the same slice significant heritability was detected in the superior temporal sulcus. **c**, Glucose metabolism was significantly heritable in both the right and left hippocampus, **d**, where it overlaps with the left anterior hippocampal region that correlated with AT (yellow, regions predictive of AT from Fig. 1; dark green to light green, false discovery rate: $q < 0.05$, $q < 0.01$, $q < 0.001$).

the anterior temporal lobe clusters (Fig. 3) as well as in the other clusters predictive of AT (Supplementary Information). Within the anterior temporal lobe clusters, significantly heritable regions were found bilaterally in the hippocampus (right-sided maximally significant heritable voxel: $h^2 = 0.65$, $P = 3.83 \times 10^{-5}$; left sided maximally significant heritable voxel: $h^2 = 0.76$, $P = 3.4 \times 10^{-6}$) and in the right superior temporal sulcus (maximally significant heritable voxel: $h^2 = 0.46$, $P = 5.92 \times 10^{-3}$). No significantly heritable voxels were observed in the amygdala regions predictive of AT (Fig. 3). As with any null finding, the possibility exists that significant heritability of amygdala metabolic activity could be detected with a larger sample.

The heritability estimates for the peak AT-predictive hippocampal and amygdala voxels were $h^2 = 0.52$ ($P = 0.001$) and $h^2 = 0.02$ ($P = 0.454$), respectively. To test whether the heritability of metabolic activity in these hippocampal and amygdala AT-predictive voxels significantly differed from each other, a model that allowed the two heritability estimates of these voxels to vary independently was compared with a model that constrained the heritability estimates to be equal. The observed difference in heritability between the hippocampal and amygdala peak voxels was 0.518 (95% CI 0.238–0.799). Results confirmed that the heritability of metabolic activity in the anterior hippocampal voxel was significantly greater than that in the dorsal amygdala voxel ($\chi^2 = 6.08$, d.f. = 1, $P < 0.0137$). A similar difference in heritability was found for the amygdala and hippocampal regions defined by the 95% spatial confidence intervals of the most AT-predictive peaks ($\chi^2 = 6.24$, d.f. = 1, $P < 0.0125$). For these regions the observed difference in heritability was 0.508 (95% CI 0.218–0.798). These results suggest that the heritable risk of developing AT is more likely to be related to hippocampal, and not amygdala, metabolic activity when assessed during the NEC condition. Given that the amygdala and anterior hippocampus are anatomically linked, and are both highly predictive of AT, we did not expect the heritability of these regions to be dissociable. Demonstrating differential heritability between these two closely related structures is highly valuable as it provides new insight into the neural circuits underlying AT. Additionally, it establishes a model system that can be used to investigate further the genetic and environmental mechanisms that may differentially affect amygdala and hippocampal function relevant to the development of anxiety-related psychopathology. Because heritability estimates are influenced by context, environmental variation and population characteristics including age[23,24], it is possible that greater heritability of amygdala function would be detected when examining different phenotypes, other developmental stages, or when using different models to understand other amygdala-dependent functions.

The lack of significant additive genetic effects (heritability) observed in the amygdala region may appear to be inconsistent with the numerous reported effects of single genes, most notably the repeat length polymorphism in the promoter region of the serotonin transporter gene (5HTTLPR), on emotion-related amygdala reactivity[25–28]. Most of these single-gene effects are context dependent, revealed by comparing acute changes in amygdala reactivity to a baseline state. In a previous study, with a considerably smaller sample of monkeys, we demonstrated such context-dependent effects of the 5HTTLPR on amygdala metabolic activity by comparing an activated state with a baseline condition[29]. To understand further the similarities between the current model and those demonstrating influences of single genes on amygdala reactivity, animals were genotyped for the 5HTTLPR and measured genotype analyses, sensitive to effects of single genes, were performed. Results demonstrated no significant effect of the 5HTTLPR on either AT ($P = 0.271$) or metabolism in the amygdala and hippocampal regions predictive of AT (false discovery rate $q > 0.05$). These data are consistent with a recent large-sample human study that failed to demonstrate a relation between the 5HTTLPR and resting amygdala activity as measured using perfusion imaging with arterial spin labelling, which, like FDG–PET, does not require a comparison condition[30]. These findings emphasize the importance of distinguishing between studies assessing sustained trait-like brain responses associated with AT and those investigating acute reactivity of the amygdala in relation to adult anxiety.

Although the amygdala and hippocampus have been recognized as important in emotion and psychopathology, few data exist regarding the role of these regions in the development of temperamental dispositions such as AT. Recent theories have implicated the amygdala in mediating acute fear and vigilance[11,12], whereas the hippocampus has primarily been linked to mechanisms underlying declarative memory[15]. Of interest, earlier theorists emphasized the septo-hippocampal system as being central to anxiety and specifically involved in threat-related behavioural inhibition[10]. The current findings provide support for an important role of the anterior hippocampus in the development of anxious dispositions[16,17], and suggest that the highly interconnected regions of the hippocampus and amygdala are differentially influenced by genetic and environmental factors. These data support a new model combining measures of metabolic brain activity with ethologically relevant behavioural challenges to discover genes that mediate the endophenotype underlying the risk of developing anxiety and depression.

METHODS SUMMARY

Functional and structural (magnetic resonance imaging) brain data for each animal were co-registered to a standard space based on an age-appropriate template of the rhesus monkey brain. Whole-brain linear regression analyses examined the relations between FDG uptake and AT. To account for potential confounds, age and sex were included in the regression model as covariates. Grey-matter probability was also included as a voxelwise covariate to account for the possibility that structural differences affected the relation between brain metabolic activity and AT. The resulting three-dimensional *t*-map was corrected for multiple comparisons using the Šidák equation $(1 - (1 - \alpha)^{1/n})$, which is similar to the Bonferroni method and determined the statistical threshold of $P = 0.05$, corrected ($t > 5.47$). To estimate the heritability of AT, phenotypic covariance/correlation among pairs of relatives was modelled as a function of expected pairwise kinship values to estimate the magnitude of additive genetic variance relative to that of the observed phenotypic variance. Age, age-squared and sex were included in the mean effects model as covariates to control for these potential confounds. The resulting heritability data were corrected for multiple comparisons based on the total volume of all the clusters correlated with AT using a false discovery rate ($q = 0.05$). Measured genotype analyses use the same variance-components approach as the heritability analyses, and were implemented using SOLAR. Measured genotype analyses simultaneously estimate the effect of specific genotypic differences among animals and the overall effect of pairwise kinship, thus testing for the effect of a single polymorphism while accounting for background allele sharing across the genome owing to genealogical relatedness. Full details of the methods and associated references are presented in the Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions N.H.K., S.E.S., J.R. and A.S.F. designed the study. S.E.S. oversaw data collection. J.A.O., A.S.F. and N.H.K. analysed the imaging data. T.R.O., A.S.F. and J.B. developed analytical tools. R.J.D. provided theoretical assistance. J.R. and W.S. performed the genotyping and maintained the pedigree record. J.R., W.S., T.D.D. and J.B. performed the genetic analyses. J.A.O., N.H.K., A.S.F., J.R. J.B. and R.J.D. wrote the paper.

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