Alzheimer's risk variant in *CLU* is associated with neural inefficiency in healthy individuals

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Abstract

Genome wide association studies identify rs11136000 in the *CLU* gene, which codes for Apolipoprotein J/Clusterin, as a significant risk variant for Alzheimer's disease (AD). However, the mechanisms by which this variant confers susceptibility remain relatively unknown. Eighty-five healthy Caucasian participants underwent functional magnetic resonance imaging (fMRI) during a working memory task and were genotyped for *CLU* rs11136000/*APOE* loci. Here we show that young individuals with the *CLU* rs11136000 risk variant (C) have higher activation levels in memory-related prefrontal and limbic areas during a working memory task. We also found subtle reductions in grey matter in the right hippocampal formation in carriers of the risk variant. We suggest that this pattern of multimodal imaging results may reflect incipient structural differences and inefficient functional activation. This study supports accumulating evidence suggesting that genetic risk for AD affects the neural networks associated with memory in healthy individuals.

1. Background

Functional neuroimaging studies suggest that genetic variants that confer risk for Alzheimer's disease (AD) may influence the efficiency of neural systems that support cognition. Altered, and particularly elevated, brain activation has been observed in carriers of the *APOE* e4 allele at rest and during tasks [1-13]. Several studies support a hypothesis that increased activation reflects the recruitment of additional neural resources to deal with task complexity, possibly to compensate for preclinical changes in the biology of these networks [14]. Until recently, the *APOE* e4 allele was the only confirmed risk allele for sporadic AD. Studies published in the last 5 years, however, have identified several other risk variants.

The rs11136000 single nucleotide polymorphism (SNP) [15-18] on the *CLU* gene is of particular interest for neuroimaging, considering its association with AD and similarities to *APOE*, which are both implicated in molecular pathways such as lipid metabolism [19]. We have previously shown that carriers of the CC risk genotype have increased activation in a working memory (WM) related network, which may reflect compensatory activation in response to increasing task complexity [20]. Another study suggested that the *CLU* variant may modulate functional connectivity between memory regions (right dorsolateral prefrontal cortex and right hippocampus) during episodic memory recall and recognition [21]. The *CLU* variant may also affect neural activity during attention [22] and resting-state alpha-rhythm activity in older healthy subjects [23]. Related neuroimaging studies also suggest that the *CLU* variant may affect white matter microstructure [24] and hippocampal blood flow [25].

Since the replication of imaging genetic studies is of crucial importance, we probe memory-related activation in an independent cohort of young, healthy individuals. Based upon our previous results [20], we hypothesize that *CLU* risk is associated with compensatory neural activity in a working memory network, particularly when WM demand is high. We previously showed that the right dorsolateral prefrontal cortex, posterior cingulate and hippocampal formation were associated

with *CLU* risk so we anticipate that these regions will be implicated in our new sample. We also conduct whole-brain analysis of functional activation differences between genotype groups to assess the specificity of any changes in the candidate regions. We perform voxel-based morphometry of the anatomical scans to test the hypothesis that elevated activation reflects incipient structural changes. Because of the known progression of AD-related brain pathology we would expect focal reductions in grey matter in the risk allele-carriers.

2. Methods and Materials

2.1 Participants: Eighty five right-handed Caucasian volunteers aged 19-47 were genotyped for rs11136000 on the *CLU* gene. The sample was independent from our previous study [20]. All participants had or were completing a bachelor's degree and had completed >15 years of education. No participants reported any current mental illness [26] or use of psychotropic medication. After the complete study description was provided to the subjects, written informed consent was obtained. The study was approved by the local ethics committee and Cardiff University. Of our sample, 14 were TT homozygotes, 46 were CT heterozygotes and 25 were CC homozygotes. No deviation from Hardy-Weinberg Equilibrium (HWE) was observed (p=.389). There were no associations between genotype and any demographic variables (Table 1). *APOE* isoform frequencies were (ε2 = 7.6%, ε3 = 73.5%, ε4 = 18.8%) and chi-squared test suggested that ε4 allele was not over-represented within any of the *CLU* genotype groups (p=.281).

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2.2 DNA extraction and genotyping: Genomic DNA was obtained from saliva using Oragene OG-500 saliva kits. *CLU* rs11136000 was genotyped using custom SNP genotyping arrays from Illumina (Illumina, Inc., San Diego, CA). *APOE* status was imputed using IMPUTE2 [27] and 1000 genomes data (phase 1 release 3) as the reference dataset. Imputed genotypes for rs7412 and rs429358 both had probabilities greater than 0.8. Quality Control was implemented in PLINK [28] . Individuals were excluded for ambiguous sex, cryptic relatedness up to third degree relatives by identity of descent, genotyping completeness less than 97%, and non-European ethnicity admixture detected as outliers in iterative EIGENSTRAT analyses of an LD-pruned dataset [29]. These standard quality control procedures remove a) samples whose phenotype data was inconsistent with genetic data, b) related

individuals, c) duplicate samples, and d) individuals of non-European ethnicity. All 85 individuals included in this study had genotype data available for rs11136000, rs7412 and rs429358.

2.3 Functional imaging task: During functional MRI scanning, subjects completed an n-back task as previously described [30, 31].The n-back task consisted of three conditions of increasing complexity (0, 1 & 2 back). Participants were presented with a sequence of numbers (1, 2, 3 or 4) and were asked to recall the number which they had seen at either one (1 back) or two (2 back) sequences before. During zero back, subjects were required to recall the number they were presently viewing (numbers; 1-4). The task consisted of six blocks of each of the three conditions. Each block lasted 24 seconds in which 10 stimuli were presented per block, at a rate of 1 per 2.4 seconds. The n-back task was continuous, and we measured accuracy as the number of correct trials divided by the number of total trials.

Gradient echoplanar imaging data were acquired for each subject using a 3T GT HDx system at Cardiff University Brain Research Imaging Centre (CUBRIC), School of Psychology, Cardiff University (parameters: 35 slices, slice thickness; 3mm/1mm gap, acquisition matrix; 64 x 64; FOV; 220mm, TR 2000ms, TE 35ms, flip angle 90°, acceleration (ASSET) factor; 2). High resolution three-dimensional T1-weighted images were also acquired using a three dimensional fast spoiled gradient echo sequence (FSPGR) with 172 contiguous sagittal slices of 1 mm thickness (TR 7.9s, TE 3.0ms, TI 450ms, flip angle 20°, FOV 256 x 256 x 176mm, matrix size 256 x 256 x 192 to yield 1mm isotropic voxel resolution images).

Functional image processing: Image processing and statistical analyses were conducted using statistical parametric mapping methods as implemented in SPM8 (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/). Briefly, images were realigned to a mean image, slice time corrected, spatially normalized to a standard stereotactic space (a brain template created by the Montreal Neurological Institute (MNI)) with volume units (voxels) of 2 x 2 x 2 mm, smoothed

with an 8 mm FWHM (full width at half maximum) Gaussian filter and ratio normalized to the whole-brain global mean. A first-level fixed-effects model was then computed for each participant. Regressors were designed for each subject, created from the time course of the three experimental conditions (0, 1, and 2 back; between jittered fixation periods (4-8 seconds ISI)) and convolved with a canonical hemodynamic response function. For each subject, statistical contrast images of two > zero, zero, one and two back > implicit baseline (fixation) were obtained.

Structural imaging processing: The VBM preprocessing and statistical analysis were also performed with SPM8 (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/). All structural images were checked for artefacts, structural abnormalities and pathologies. Customized T1 templates and prior images of GM, WM and CSF were created from all participants. For the segmentation, we followed the steps provided by the SPM8 guidelines (light bias regularisation (0.001), 60mm bias FWHM cut-off, warping regularisation of 4, affine regularisation to the ICBM European brain template (linear registration), sampling distance of 3). Finally, the images were smoothed [32] with a Gaussian kernel of 8mm (FWHM), whereby the intensity of each voxel was replaced by the weighted average of the surrounding voxels.

Statistical Inferences: To test for rs11136000 genetic association, individual contrast images of 2 > 0 back and grey matter were analysed using the general linear model in a second level fixed effect analysis (Each individual's number of rs11136000 risk alleles [coded 0, 1 or 2]) were entered into a multiple regression: TT/CT/CC; controlling for *APOE* status, age, gender, n-back performance and intracranial volume), hypothesising that the C allele would be associated with higher levels of neural activity during the n-back task (TT < CT < CC) and reduced grey matter density (TT > CT > CC). Statistical inferences were set at $p<.05$ corrected for multiple comparisons across the whole brain using the family wise error rate (FWE; [33]) at the voxel level, which has been shown to appropriately control for false positives in imaging genetic studies [34, 35]. ROIs were defined a priori based on coordinates taken from our prior study and thresholded at $p_{FWE} <.05$ and $p <.05$

uncorrected. The ROIs from our discovery sample were the right dorsolateral prefrontal cortex, right posterior cingulate and right hippocampal formation (Lancaster et al. 2011). Corresponding ROIs for the right dorsolateral prefrontal cortex (consisting of right BA9/BA46), right posterior cingulate and right hippocampal formation were provided by the Wake Forest University PickAtlas (www.fmri.wfubmc.edu/downloads).

3. Results

3.1 Effects of CLU rs11136000 on behavioural measures and brain function: There were no significant differences in working memory performance between *CLU* genotype groups during the n-back task (Table 1). In a whole brain analysis for the 2 > 0 back contrast, we observed a significant main effect of rs11136000 genotype in the a) right middle frontal gyrus (no. of voxels; k=31, [x=32, y=-8, z=46], t= 4.95, p_{FWE}=.007) and b) left dorsolateral prefrontal cortex (no of voxels; k=9, [x=-46, y=2, z=36], t= 4.71, p_{FWE}=.016) (see Figure 1). There were no associations between accuracy during the task and activation in these regions (p>.1 in both cases). There was no effect of *APOE* genotype on activation in the 2 > 0 back contrast (p<.05, corrected across the whole brain using the family wise error rate).

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3.2 Region of Interest Analysis: We proceeded with a region-of–interest based investigation based on the regions identified [20]. We ran the same additive genetic model of rs11136000 (CC > CT > TT) in the right dorsolateral prefrontal cortex, right posterior cingulate and right hippocampal formation, separately for each condition. We set an experiment-wide p-threshold of p < .004 for correction across the twelve observations (3 ROIs × 4 contrasts). After correction for multiple observations, we observed a significant main effect of rs11136000 with the right posterior cingulate during the 2 > 0 back contrast (k=50, [X = 8, Y = -34, Z =22], t = 2.81, $p_{UNCORRECTED}$= .003) and a significant main effect of rs11136000 within the hippocampal formation during the 0-back > baseline contrast (k = 18, [x = 24, y = -16, z = -14], t = 3.95, $p_{FWE\text{-}ROI}$ = .001), which were both significant after correction across the twelve observations ($p_{CORRECTED}$=.036 and .012, respectively). A comprehensive list of results from the ROI analysis is presented in Table 2.

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3.3 Effects of CLU rs11136000 on brain structure: We performed whole brain and ROI based multiple regressions (TT > CT > CC; controlling for *APOE* and age) to explore potential effects of *CLU* rs11136000 genotype on grey matter density. The whole-brain analysis (p_{FWE} <.05) did not identify any effects of rs11136000 genotype on grey matter density. However, using the same candidate ROIs as the fMRI component of the study, we identified an association between *CLU* rs11136000 and the right hippocampal formation (k =2, [x=27, y=-30, z=-8], t=3.09; $p_{FWE\text{-}ROI}$=.036). We found no significant structural differences in the other ROIs that had been identified based on the activation differences (after correction for multiple comparisons). We did not see any effects of APOE on grey matter across the brain or ROIs (p_{FWE} <.05).

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3.4 Partial volume effects within the right hippocampus: As we observed potential rs11136000-related volumetric reduction within the right hippocampus, we aimed to determine whether the functional changes we observed within the hippocampus (for the 0 back > baseline contrast) were due to *CLU* rs11136000 differences in grey matter density. We extracted the parameter estimates for the cluster that showed significant *CLU* rs11136000 related differences [k = 2, [X =27, Y =-30, Z = -8], t =3.09, $p_{FWE\text{-}ROI}$ = .036) and added these GM density values into the model (0 back > baseline, right hippocampus). We found a partial volume effect and several voxels within the cluster reduced in significance, however several voxels (~16%) still showed significantly increased activation in rs11136000 C risk carriers after this correction for *CLU* related differences in hippocampal GM density (k = 3, [X = 28, Y=-18, Z=-14], t=3.19, $p_{FWE\text{-}ROI}$=.013). These results suggest that increased activation and reduced right hippocampal grey matter density were partially independent effects (see Figure 2). To confirm the independence between our functional and structural findings, we extracted the parameter estimates from each cluster and performed a multiple regression. We found that *CLU* rs11136000 was associated with increased hippocampal activation (zero back >

baseline), controlling for hippocampal GM density (rs11136000 effect; t=2.479, p =.015, hippocampal GM effect; t = -.941, p =.350).

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4. Discussion

Our results, like those of our previous study, support a hypothesis that healthy individuals with the *CLU* rs11136000 C genotype have increased task-related activation [20]. Similar to our previous study, we found an rs11136000 genotype-dependent increase in activation within the right prefrontal cortex and the right posterior cingulate. The increased activation in right prefrontal cortex was located in a more anterior location than our previous finding, but we note that the cluster from our previous study did extend into the right middle frontal gyrus [20]. We also observed rs11136000 dependent increased activation within the right hippocampal formation, however this was found at the 0 back > baseline contrast which may reflect alterations within an attention system, rather than working memory because the 0-back condition dif not involve a memory component. This assumption is supported by our previous finding that *CLU* (C) associated hippocampal activation was increased during all conditions, rather than during higher working memory loads [20].

We also report novel evidence suggesting that the increased activation within the prefrontal cortex associated with rs11136000 risk genotype may be bilateral (we had previously only seen it in the right hemisphere). This may be due to differences between neuropsychological tasks and the subsequent recruitment of neural resources. Numerous studies suggest the dorsolateral prefrontal cortex is recruited in n-back tasks in response to increasing working memory load [36, 37]. It is of note that increased activation within prefrontal regions during memory tasks has been linked to other AD risk factors such as *APOE* (in a considerably older cohort) [4], and mild/subjective cognitive impairment [38, 39], however little is known about how activation within the prefrontal cortex may relate to susceptibility for AD. Increases in task-related activation (as a general neuroimaging phenotype for AD susceptibility) could reflect a dysregulation of neurovascular coupling [40] or exaggerated calcium signalling [41-43], although these hypotheses would need to be formally tested. Increased activation could also reflect a compensatory neural response due to incipient changes in brain macro/microstructure [24] and/or functional connectivity [21, 44] that have previously been associated with the rs11136000 C risk allele.

We also report that rs11136000 genotype is associated with subtle reductions in hippocampal grey matter density, but this structural finding, which has not been reported before, will have to be replicated in further studies. One study links *CLU* rs11136000 to both parietal grey matter volume and n-back performance in young individuals (mean age: 22.8 years), providing additional support for the role of *CLU* rs11136000 in a working memory-network [45]. This study also showed that rs11136000 (C) was associated with increased performance (during 3-back, which we did not test), which could reflect a compensatory behavioural response. Young individuals (mean age: 23.6 years) with the *CLU* rs11136000 C allele may also have alterations in white matter microstructure. The authors of this study suggest that this structural change may reflect a developmental vulnerability that reduces resilience to AD –related neuropathology, rather than early signs of AD –related neuropathology [24].

In sum, our findings support the hypothesis that increased genetic AD risk leads to functional changes in young healthy individuals that precede any clinical phenotypes [1-4, 7, 9-11, 13, 14, 46, 47]. The functional regions associated with *CLU* risk in the present study include areas that are implicated in the early stages of AD pathogenesis, where anatomical changes can precede the onset of clinical symptoms by several decades [48]. Thus, our results conform to a model of AD pathology where early susceptibility and/or vulnerability is associated with initial increases in brain activation during complex tasks, followed by reduced activation once compensatory mechanisms have failed and the disease manifests clinically [14]. The C risk genotype on *CLU* rs11136000 may potentially modulate plasma clusterin [49, 50] and reduce the efficiency of $A\beta_{1-42}$ sequestration [51] and is associated with diminished memory performance in later life [52, 53], suggesting that *CLU* may be implicated in hallmark features of AD pathogenesis such as $A\beta_{1-42}$ plaque aggregation [54, 55]. Future work should consider how AD risk variants such as *CLU* rs11136000 may affect neural activity during healthy and pathological aging processes, and how this variation may be mediated by $A\beta_{1-42}$. Further work could also explore how additive and/or molecular pathway-specific risk variants may affect the memory related networks implicated in preclinical AD pathophysiology.

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Table 1.

rs11136000		**TT**	**CT**	**CC**	**p**
subjects		14	46	25	.389 [a]
APOE ε4 -/+		11/3	28/18	19/6	.281 [b]
sex (male/female)		5/9	19/27	10/15	.933 [b]
age		23.64(±3.7)	24.13(±4.6)	24.68(±4.3)	.566 [c]
n-back accuracy (%)	zero-back	98 (±.02)	95(±.14)	97(±.03)	.543 [c]
	one-back	85(±.03)	83(±.13)	86(±.04)	.429 [c]
	two-back	72(±.06)	70(±.13)	71(±.09)	.805 [c]
	average	85(±.03)	82(±.13)	85(±.04)	.566 [c]

Table 1 Legend.

T = Thymine, C = cytosine (CC = risk genotype), p = p-value, ± = s.d, [a] denotes p-value tested for HWE, [b]*denotes that p-value was calculated with chi-squared test,* [c] *denotes p-value was calculated with one-way ANOVA. APOE genotype was stratified based upon the absence/presence of the ε4 allele.*

Table 2.

region of interest	n-back condition	rs11136000 TT > CT > CC	correction	*k*	β (CI)
right dorsolateral prefrontal cortex	2 > 0 back	t =2.43 p =.007	uncorrected	52	.292 (±.193)
		t =2.38 p=.009	uncorrected	108	.266 (±.181)
	0 back > baseline	--	--	--	--
	1 back > baseline	--	--	--	--
	2 back > baseline	t =2.01, p = .024	uncorrected	26	.213 (±.174)
right posterior cingulate	2 > 0 back	t =2.81, **p =.003****	uncorrected	50	.131 (±.076)
	0 back > baseline	t =1.88, p = .032	uncorrected	4	.272 (±.239)
	1 back > baseline	--	--	--	--
	2 back > baseline	--	--	--	--
right hippocampal formation	2 > 0 back	t =1.98, p =.025	uncorrected	10	.075 (±.062)
	0 back > baseline	t =4.04, p=**.001****	FWE	18	.187 (±.076)
	1 back > baseline	t =1.75, p =.040	uncorrected	2	.095 (±.089)
	2 back > baseline	--	--	--	--

Table 2 Legend.

Region of interest (ROI) analysis of the three ROIs identified from the previous study [20] in each the n-back conditions. CLU rs11136000 genotype effects that are significant after correction for multiple comparisons are highlighted in bold. (*k* = cluster size; number of voxels). Uncorrected p-threshold = p <.05. ** denotes that p-value for that ROI and survived correction for multiple comparisons across the twelve observations. -- denotes non-significance (p >.05). Beta (CI) refers to the contrast estimate and the 90% confidence intervals of the estimate from the peak voxel.

Table 3.

region of interest	rs11136000 TT > CT > CC	correction	*k*	β (CI)
right dorsolateral prefrontal cortex	t =2.54, p= .007	uncorrected	23	.016 (±.011)
right posterior cingulate	t =2.17, p = .016	uncorrected	21	.022 (±.016)
right hippocampal formation	t =3.09, p = **.036**	FWE corrected	2	.031 (±.017)

Table 3 Legend.

Voxel-based morphometry data in the multiple regression (n=85). Carriers of the *CLU* rs11136000 risk variant (C) show reduced grey matter density in the right hippocampal formation: $p_{FWE\text{-}ROI}$ =.036). CLU rs11136000 genotype effects that are significant after correction for multiple comparisons are highlighted in bold. (*k* = cluster size; number of voxels). Uncorrected p-threshold = p <.05. Beta (CI) refers to the contrast estimate and the 90% confidence intervals of the estimate from the peak voxel.

Figure 1.

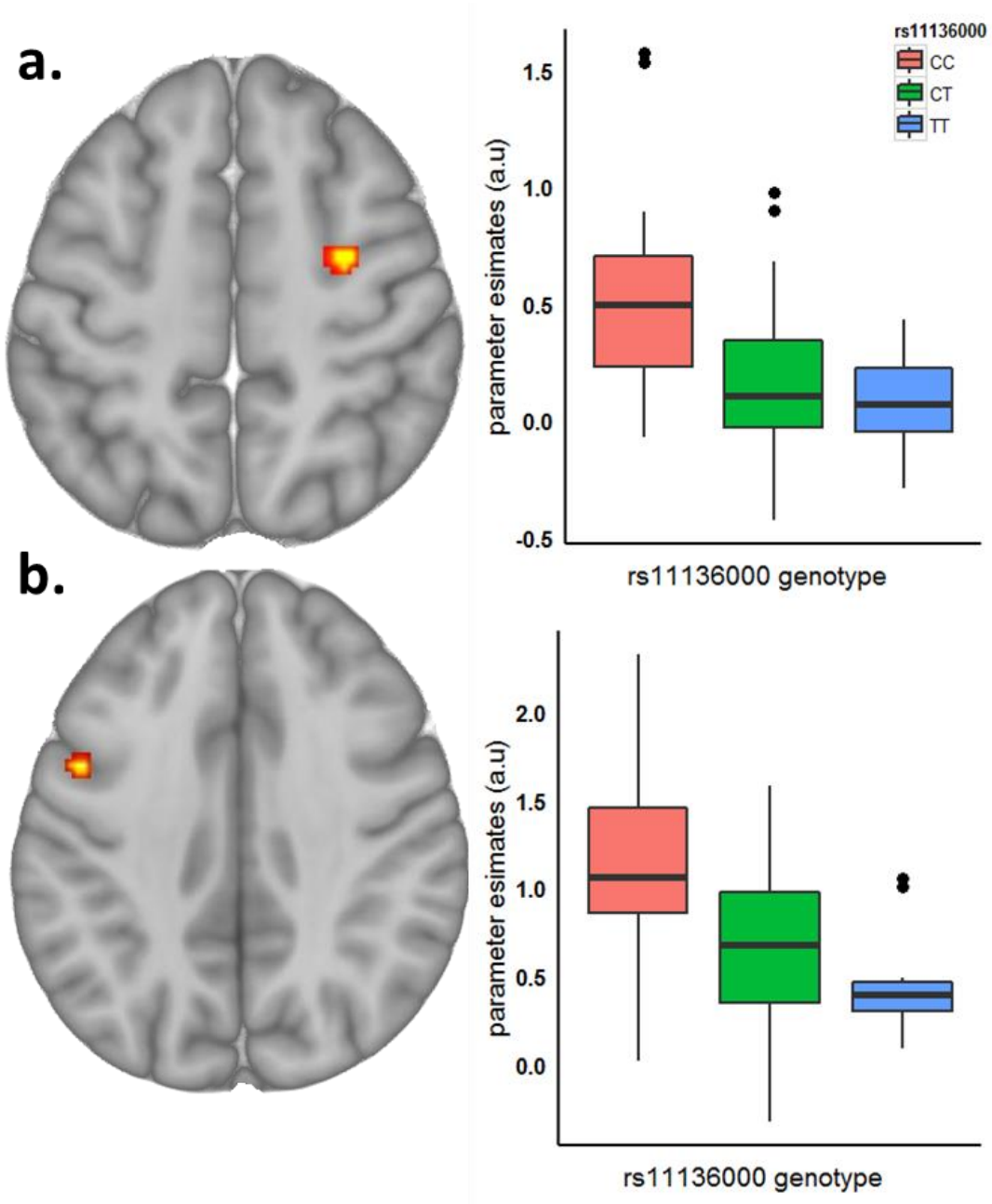

Figure 1 Legend.

Whole brain (p_{FWE}<.05 corrected) analysis for rs11136000 (TT > CT > CC) in the 2 > 0 contrast (n=85). Association between *CLU* rs11136000 risk and activity within a) the right middle frontal gyrus (1a; k = 31) and b) left dorsolateral prefrontal cortex (1b; k=9). Note: all findings remained significant after the removal of outliers. Parameter estimates (arbitrary units).

Figure 2

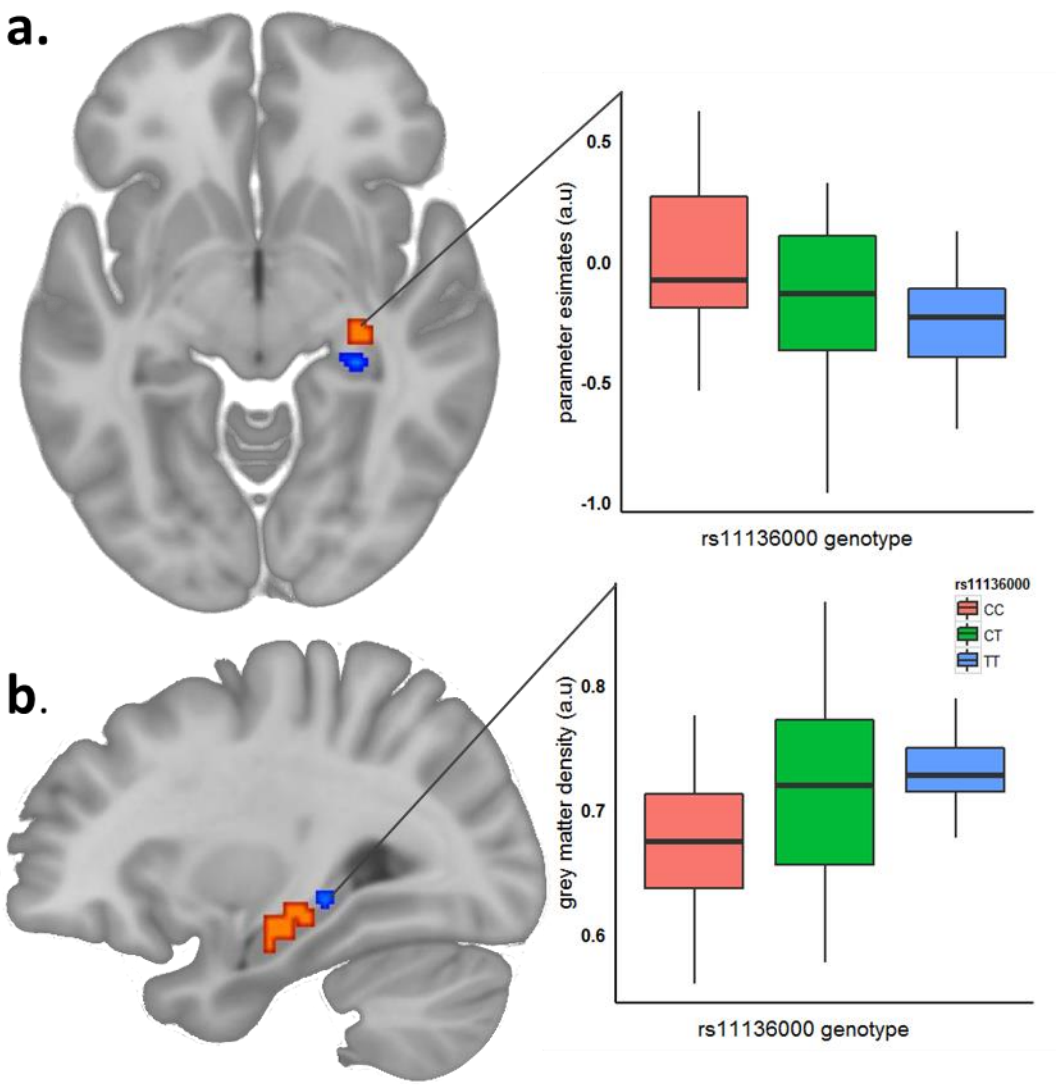

Figure 2 Legend

Right hippocampal region of interest analysis for rs11136000 in the a) 0 back > baseline contrast (CC > CT > TT) and b) grey matter density (voxel-based morphometry) analysis (CC < CT < TT). We found a) Increased activation in the anterior part of the right hippocampus during 0 back > fixation and b) reduced GM density in the posterior hippocampus. Clusters shown are p<.05 (uncorrected) for illustration purposes, both results were significant after correction ($p_{FWE\text{-}ROI}$ <.05. Please see Table 2; 0-back > fixation and Table 3; GM density) for cluster details. Note that the effects of analysis 2a. were controlled for by results obtained in analysis 2b. Parameter estimates (arbitrary units).