

ROBO1 Polymorphisms, Callosal Connectivity, and Reading Skills

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Abstract: The genetic effects on specific behavioral phenotypes are putatively mediated by specific neural functions. It remains unexplored how the axon-guidance-receptor gene *ROBO1* influences reading performance through the neural system despite the identification of *ROBO1* as a susceptibility gene for dyslexia. To address this issue, the present study recruited a group of children with a wide range of reading abilities. Two previously identified reading-related *ROBO1* polymorphisms were genotyped, and diffusion and structural MRI were acquired to measure the fiber microstructure of the corpus callosum (CC), the major white-matter tract that connects inter-hemispheric cortical regions. The results confirmed the significant influence of the *ROBO1* polymorphisms on reading scores. The fiber microstructures of the midline-CC segments around the genu and splenium were also affected by the *ROBO1* polymorphisms. Moreover, a mediation analysis further revealed that the genu could significantly mediate the effects of the *ROBO1* polymorphisms on word-list reading performance, which suggests a *ROBO1*-to-genu-to-reading pathway. The genu-linked cortical morphology, however, was not associated with either the *ROBO1* polymorphisms or reading performance. These findings offer direct evidence supporting *ROBO1*-callosum association in humans and also provide valuable insight into the functions of *ROBO1* and the gene-to-brain mechanisms that underlie human reading. *Hum Brain Mapp* 38:2616–2626, 2017. © 2017 Wiley Periodicals, Inc.

Key words: corpus callosum; diffusion MRI; mediator; reading; *ROBO1*

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INTRODUCTION

Reading is a unique human skill that is crucial for learning and career achievement in modern societies. The genetic and neural bases of reading have been active areas of research [Galaburda et al., 2006; Ramus, 2006; Wandell and Yeatman, 2013]. Putatively, the effects of specific genes on reading are mediated by specific neural functions and result in specific gene–brain–behavior pathways. To date, while a number of studies have revealed specific genetic and neural substrates of reading, the entire gene-to-brain-to-behavior pathways that underlie reading remain largely unclear.

The axon guidance receptor gene *ROBO1* is one of the genes that have been identified as a susceptibility gene for reading disability or dyslexia [Hannula-Jouppi et al., 2005]. Biologically, this gene plays a crucial role in axon growth across the midline of the central nervous system (CNS). In *Drosophila*, mutations of the *robo1* ortholog (i.e., the roundabout gene) induce abnormal axon crossing and recrossing of the CNS midline [Kidd et al., 1998]. All homozygous *Robo1* knockout mice die at birth and display a small or absent corpus callosum (CC), which is the major tract that connects inter-hemispheric cortical regions [Andrews et al., 2006; Lopez-Bendito et al., 2007; Unni et al., 2012]. Accordingly, the *ROBO1* is strongly expected to influence the CC in human brains, but direct empirical evidence is still lacking. Recently, a functional study of 10 dyslexic individuals from a Finnish family who weakly expressed the haplotype of the *ROBO1* gene revealed a correlation between auditory-response binaural suppression, which indirectly reflects midline axonal crossing, and *ROBO1* expression, which implicitly supports the effect of *ROBO1* on the human CC [Lamminmaki et al., 2012].

Regarding the neural basis of human reading, many theories have argued for the importance of inter-hemispheric communication [Galaburda et al., 1990; Habib, 2000; Van der Haegen et al., 2013], which hints at the critical role of the CC. Structural MRI studies have reported aberrant shapes and sizes of the midline-CC in dyslexic individuals, although these findings are mixed [Hynd et al., 1995; Rumsey et al., 1996; Von Plessen et al., 2002]. Investigations with diffusion MRI, which is an imaging technique that can provide measures of white matter (WM) microstructural properties, such as axonal density, diameter, and orientation and degree of myelination [Beaulieu, 2002], have further revealed abnormal CC microstructures in dyslexic populations [Frye et al., 2008; Odegard et al., 2009]. Moreover, reading abilities and callosal fiber microstructure are correlated in typically developing children [Dougherty et al., 2007].

Taken together, these findings provide strong evidence in support of the downstream role of the CC in *ROBO1* functioning and the upstream role of reading skills. Therefore, we hypothesized that the regulation of reading skills by the human *ROBO1* gene is mediated by callosal connectivity. To test this hypothesis, we studied a group of

children with a wide range of reading abilities. Specifically, in a previous association study of the *ROBO1* polymorphisms in a large population-based twin sample, significant associations between two of the *ROBO1* polymorphisms (i.e., rs4535189, and rs6803202) with performance in a reading-related task were observed; these two polymorphisms were the only ones to survive multiple-testing correction [Bates et al., 2011]. Therefore, these two polymorphisms were chosen as the *ROBO1* polymorphisms of interest in the present study and were genotyped for all of the children. Diffusion tensor imaging (DTI) was applied to measure callosal connectivity. We further explored whether the cortical morphologies and asymmetries (a phenotype trait that is thought to be related to callosal connectivity and language) of the cortical regions that are connected by the callosal regions of interest (ROIs) were related to the *ROBO1* genotype and reading score using structural MRI.

MATERIALS AND METHODS

Participants

One hundred and fifteen typically developing children (63 boys and 52 girls) were included in our study. The age range was 10–15 years (12.87 ± 1.52 years). All participants were native Mandarin speakers and regularly attended school in Beijing. Normal or corrected-to-normal vision and hearing were confirmed for each participant. The participants' parents reported no evidence of current or past major neurological or psychiatric disorders (e.g., attention deficit hyperactivity disorder). Written informed consent was obtained from the children and their parents after the details of the study were comprehensively explained. The Institutional Review Board of Beijing Normal University Imaging Center for Brain Research approved the protocol.

Reading Assessment

The participants were assessed with standardized neuropsychological tasks after the MRI scanning. The assessments included general cognitive tasks and reading tasks. The Block Design subtest of the Wechsler Intelligence Scale for Children (third edition) can provide a crude estimation for performance intelligence at best, and was used as a covariate to correct for performance intelligence [Wechsler, 1991]. We adopted the two following widely used reading tasks to evaluate the children's reading abilities: (1) character recognition (CR), and (2) word list reading (WLR). In the CR task, the participants were asked to name 150 Chinese characters without a time limit, and the final score was the number of characters that were named correctly [Lei et al., 2011; McBride-Chang et al., 2003]. In the WLR task, the participants were required to read 180 two-character words aloud as quickly as possible, and the final score was calculated as the number of the correct words in

one minute [Pan and Shu, 2014; Zhang et al., 2012]. A few participants did not successfully complete the tasks and were therefore eliminated from the behavioral association analyses (1 participant was missing CR data and 2 were missing WLR data).

Genotyping

Saliva was collected for DNA extraction from each participant. Two *ROBO1* polymorphisms, that is, rs4535189 and rs6803202, were genotyped using a MassArray system (Sequenom, San Diego, USA). Because only these two polymorphisms have previously been found to significantly affect reading-related behavioral measures, that is, phonological buffer capacity [Bates et al., 2011], we did not test other polymorphisms of the *ROBO1* gene at this stage. The sample success rates for both SNPs reached 100%, and the reproducibility of the genotyping was 100% based on a duplicate analysis of 10% of the genotypes.

According to our sample, neither polymorphisms showed deviations from Hardy–Weinberg equilibrium ($P > 0.2$). Notably, these two SNPs were in complete linkage disequilibrium in our sample ($D' = 1$): the C allele of rs4535189 was always linked to the A allele of rs6803202; and the T allele of rs4535189 was always linked to the G allele of rs6803202. The two SNPs were thus analyzed together as a single marker, and we simply divided all participants into three groups using the rs4535189 genotype: 22 C/C carriers, 63 C/T carriers, and 30 T/T carriers.

MRI Acquisition

All MRI scans were performed using the same 3T Siemens Tim Trio MRI scanner in the Imaging Center for Brain Research of Beijing Normal University. For each participant, the head was secured using straps and foam pads to minimize head movement. High-resolution 3D T1-weighted images were sagittally acquired using a magnetization-prepared rapid gradient echo (MPRAGE) sequence with the following parameters: 144 sagittal slices; echo time (TE), 3.39 ms; repetition time (TR), 2,530 ms; inversion time (TI), 1,100 ms; 1.33-mm slice thickness with no gap; acquisition matrix, 256×256 ; 1×1 mm in-plane resolution; and acquisition time, 8:07 min. Diffusion MRI was axially applied using a single-shot echo planar imaging (EPI) sequence with the following parameters: coverage of the whole brain; 62 axial slices; TR, 8,000 ms; TE, 89 ms; 30 optimal nonlinear diffusion-weighted directions with $b = 1,000$ s/mm² and one additional image without diffusion weighting (i.e., $b = 0$ s/mm²); average, 2; 2.2-mm slice thickness with no gap; acquisition matrix, 128×128 ; 2.2×2.2 mm in-plane resolution; and acquisition time, 9:08 min.

Image Processing

Diffusion measures for callosal connectivity

The diffusion MRI images were processed with the PANDA pipeline toolbox [Cui et al., 2013]. Briefly, PANDA called the modules of the FMRIB Software Library (FSL) to finish the skull-stripping, simple-motion and eddy-current corrections, diffusion tensor/parameter calculation, and spatial normalization [Jenkinson et al., 2012]. To measure the callosal microstructural properties, we generated images for the following three commonly used diffusion parameters: fractional anisotropy (FA), axial diffusivity (AD), and radial diffusivity (RD). These images were further normalized to the MNI space. Specifically, the FA, AD, and RD represent the fraction of the total diffusion that can be attributed to anisotropic diffusion, the diffusivity along the direction of the WM tracts, and the diffusivity perpendicular to the direction of WM tracts, respectively [Basser and Pierpaoli, 1996; Beaulieu, 2002]. Specifically, the AD and RD have been regarded as selectively sensitive to the specific microstructural properties; the AD is considered to be more strongly related to axonal organization, and the RD is thought to be more strongly related to the degree of myelination [Concha et al., 2006; Song et al., 2003]. Another common parameter, the mean diffusivity (MD), was not included because it is simply the sum of the AD and RD.

The midline segment is the most representative part of the CC [Aboitiz et al., 1992; Andronikou et al., 2015; Shen et al., 2015; Thompson et al., 2006]; therefore, we focused on the midline-CC in the present study. The midline mask of the CC was first manually outlined on the midline sagittal slice of the FA template in the MNI space. On this template, the boundary of the CC was evident (Fig. 1). To compensate for potential misalignments across individuals, we applied a smoothing step using a 6-mm FWHM Gaussian kernel.

Connected cortical regions in the two brain hemispheres

To determine the homologous cortical regions that were connected by the CC tracts that passed through a given region of interest (ROI) on the midline-CC, we performed fiber-tracking using the diffusion MRI data. First, the midline-CC ROI in the MNI space was mapped back to the diffusion native space for each individual using the inverse transformation of the spatial normalization. Next, we implemented the fiber-tracking using MRtrix [Tournier et al., 2012]. For each voxel, the fiber orientation distribution (FOD) was first estimated using constrained spherical deconvolution (CSD) with a maximum spherical harmonic order $l_{\max} = 6$ [Tournier et al., 2007, 2008]. For the ROI on the midline-CC, probabilistic fiber tracking was performed in the native diffusion space using the 2nd order integration over the fiber orientation distribution (iFOD2) algorithm with uniformly random seeds throughout the ROI

(step size: 0.15 mm; maximum length: 220 mm; minimum length: 10 mm) [Tournier et al., 2010]. The tracking was terminated when the brain was exited or the FOD amplitude was <0.1 . For the ROI on the midline-CC, 10,000 tracts were generated to represent the CC tracts that passed through the ROI.

For each individual, we marked the voxels that were traversed by the above mentioned ROI-seeded tracts, which resulted in a voxel-based binary map in the native diffusion space. This binary map was further transformed to the MNI space. The resultant maps of all individuals were overlaid to generate a count map at the group level. This count map was then projected onto the inner cortical surface template (i.e., white matter surface) in the MNI space using a nearest neighborhood interpolation method. This inner cortical surface template was generated using the CIVET pipeline [Ad-Dab'bagh et al., 2006; Gong et al., 2012]. After the projection, each vertex was given a value that represented the number of individuals with ROI-seeded tracts linked to that vertex. We further averaged the left and right cortical surface maps to make them symmetric because our focus was the CC-connected homologous cortical regions in the two hemispheres. The resultant surface count map was then thresholded using a nonparametric sign test ($P < 0.05$ with Bonferroni corrections for multiple testing across the vertices). For each vertex, the null hypothesis for the sign test was that there would be no ROI-seeded tracts that linked the vertex at the population level (115 individuals in total). The vertices that survived the threshold were masked on the cortical surfaces to identify the symmetric cortical regions that were putatively linked by the CC tracts that passed through the midline ROI.

Cortical morphological measures and their inter-hemispheric asymmetries

Two cortical morphological measures were analyzed, that is, cortical thickness and surface area. To extract these two measures, we used the CIVET pipeline as previously described [Gong et al., 2012]. For details, see Supporting Information. The inter-hemispheric asymmetries of the cortical thickness and surface area were quantified at each vertex using a common asymmetry index (AI): $AI = (L - R)/(L + R)$.

Statistical Analysis

To assess the effects of the polymorphisms on reading scores, we used a general linear model (GLM) with the rs4535189/rs6803202 genotype (C/C, C/T, and T/T) as a main factor, and age, gender, and Block Design performance as covariates.

Next, to identify the significant effects of the *ROBO1* polymorphisms on the midline-CC, we applied the same GLMs to each diffusion parameter (FA, RD, or AD) at the

voxel level. The Monte Carlo simulation method was applied to correct for multiple comparisons [Cox, 1996] and the midline-CC clusters that survived an FWE-corrected $P < 0.05$ were considered significant.

Finally, to determine whether the above identified clusters on the midline-CC can play a mediating role in the effects of the *ROBO1* polymorphisms on reading scores, we applied a mediation analysis by taking the rs4535189/rs6803202 genotype, midline-CC clusters, and reading scores as the predictor, mediator, and outcome, respectively. According to the standard conventions for a mediation analysis, there are four tests: (1) path *c*: the *ROBO1* polymorphism effect on reading scores, that is, the total effect of the predictor on the outcome; (2) path *a*: the *ROBO1* polymorphism effect on the midline-CC clusters; (3) path *b*: the effect of the midline-CC clusters on the reading score after controlling for the *ROBO1* polymorphism (i.e., the group factor); and (4) the $a \times b$ effect, which was referred to as the indirect effect and was indicative of whether the predictor-outcome relationship (i.e., the *ROBO1*-reading relation) was significantly reduced after controlling for the mediator (i.e., the midline-CC clusters). For these tests, age, gender, and Block Design performance were included as covariates. When all the four tests reach the level of significance, the midline-CC clusters can be considered to significantly mediate the *ROBO1*-reading relationship.

Notably, the predictor here (i.e., the rs4535189/rs6803202 genotype) was a multicategorical variable with more than two conditions (i.e., three genotypes), raising a technical difficulty for parameterizing the statistical model (e.g., dummy coding each condition). To avoid this issue, we chose to adopt the strategy of the majority of previous studies, which employed a simplified dichotomous SNP model for the mediation analysis: the dominant model (i.e., the heterozygous grouped with the minor allele homozygous) or the recessive model (the heterozygous grouped with the major allele homozygous). Here, we found only the recessive model showed a significant total effect (i.e., the path *c*; WLR, $P = 0.008$; CR, $P = 0.004$), but not the dominant model (WLR, $P = 0.829$; CR, $P = 0.247$). The recessive model was therefore applied in our subsequent mediation analysis, in which all individuals were divided into two groups: CC and T-allele carriers. The entire mediation analysis was carried out using the PROCESS macro implemented in SPSS [Preacher and Hayes, 2008], and 10,000 bootstrap samples were generated to estimate the bootstrap confidence intervals for the indirect effect. An empirical 95% confidence interval that did not include zero indicated that the indirect effect was significant at the 0.05 level [Hayes and Preacher, 2014].

For the cortical regions that were connected by the mediating midline-CC clusters, we further tested whether the cortical thicknesses and surface areas were related to the predictor (i.e., the *ROBO1* polymorphism) and the outcome (i.e., the WLR performance) of the mediation model.

For each morphological measure, GLMs were applied to each vertex within the cortical masks on both hemispheres. Age, gender, Block Design performance, and ICV were taken as covariates. Additionally, to further explore the relations between the inter-hemispheric asymmetries in cortical thickness and surface area and the two variables (i.e., the predictor and outcome variables of the mediation model), we applied GLMs to the AI values at the vertex level within the cortical masks but only for one hemisphere. To correct for multiple vertex-wise comparisons, a random field theory (RFT)-based method was applied at the cluster level [Taylor and Adler, 2003], and the cortical clusters that survived an FWE-corrected $P < 0.05$ were considered to be significant. All of these statistical procedures were implemented using SurfStat (<http://www.math.mcgill.ca/keith/surfstat/>).

RESULTS

The Effect of the rs4535189/rs6803202 Genotype on Reading Performance

The *ROBO1* rs4535189 and rs6803202 polymorphisms were in complete linkage disequilibrium. As shown in Table I, these two polymorphisms exhibited significant

effects on the two reading tests (character recognition, CR: $F_{(2,108)} = 4.465$, $P = 0.014$; word list reading, WLR: $F_{(2,107)} = 4.171$, $P = 0.018$) after controlling for age, gender, and Block Design performance. According to the post-hoc comparisons, the C/C group performed worse on the CR test than both the C/T ($P = 0.007$) and T/T groups ($P = 0.008$). Regarding the WLR test, the C/C group scored significantly lower than the C/T group ($P = 0.005$) and non-significantly lower than the T/T group ($P = 0.086$), after controlling for age, gender, and Block Design performance. There were no differences between the C/T and T/T groups in either test (CR: $P = 0.695$; WLR: $P = 0.310$). Additionally, we did not observe significant interactions of the rs4535189/rs6803202 genotype with age or gender.

The Effect of the rs4535189/rs6803202 Genotype on Callosal Connectivity

The rs4535189/rs6803202 effects on the diffusion parameters of the midline-CC are illustrated in Figure 1. There was only one observed significant cluster (FWE-corrected $P < 0.05$) for each diffusion parameter (i.e., FA, AD, or RD) after controlling for age, gender, and Block Design performance. Specifically, the clusters for FA and RD were located within the splenium of the CC with the RD cluster

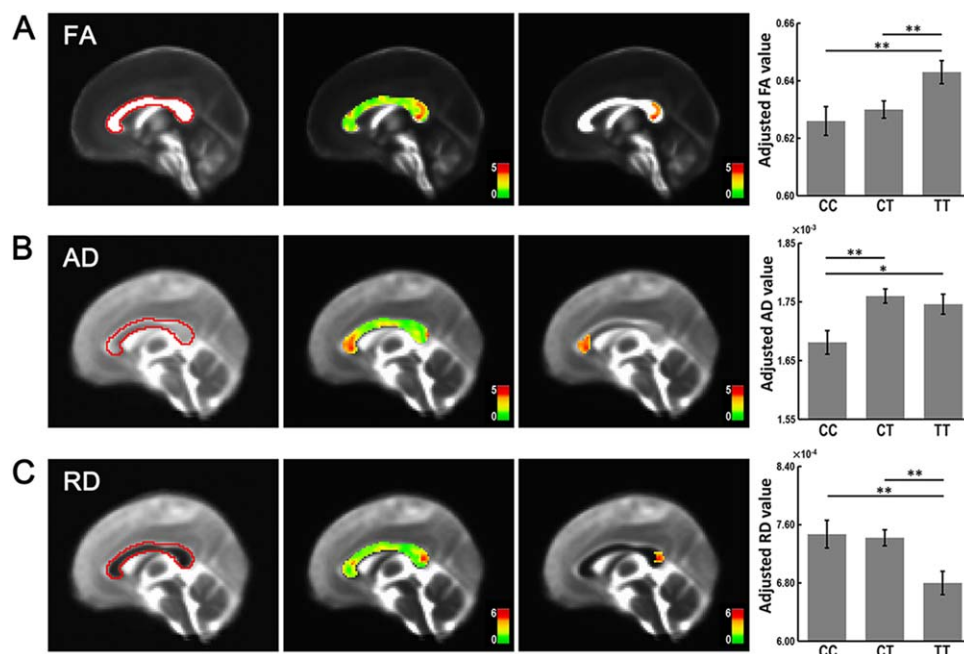


Figure 1.

The effects of the rs4535189/rs6803202 genotype on callosal connectivity. The midline boundary of the corpus callosum is marked in red (first column). Three diffusion parameters were analyzed: (A) FA, (B) AD, and (C) RD. For each parameter, the statistical F-map (second column) and the significant clusters

(third column) are displayed. For the identified clusters, the values for the three genotype groups are displayed using bar charts after adjustments for age, gender, and Block Design performance. *: $P < 0.05$; **: $P < 0.01$, according to the post-hoc comparisons.

TABLE I. Demographic information and reading performance

	CC Group N = 22	CT Group N = 63	TT Group N = 30	F value	P value	Effect size (Cohen's <i>d</i>)		
						CC vs. CT	CC vs. TT	CT vs. TT
Gender (M/F)	12/10	33/30	18/12	–	0.788			
Age	12.3 (1.7)	12.9 (1.4)	13.2 (1.5)	2.499	0.087	–0.401	–0.559	–0.208
Block Design performance	11.6 (2.3)	11.7 (3.0)	11.9 (3.2)	0.107	0.899	–0.035	–0.103	–0.065
CR	114.2 (22.9) _a	127.0 (15.2) _b	129.9 (16.5) _b	4.465	0.014*	–0.726	–0.795	–0.184
WLR	85.8 (25.1) _a	105.3 (25.0) _b	103.6 (25.6) _{a, b}	4.171	0.018*	–0.772	–0.690	0.067

Note 1. M for male and F for female.

Note 2. Data are expressed as mean (S.D.). Block Design performance is the score of block design in WISC-CR. CR, Character Recognition; WLR, Word List Reading.

Note 3. χ^2 test for gender, ANOVA test for age and Block Design performance, ANCOVA for the reading scores after controlling for age, gender, and Block Design performance.

Note 4. Means in the same row that do not share subscripts differ at $P < 0.05$ on the Tukey's post-hoc test.

Note 5. Cohen's *d* was calculated using pooled standard deviation.

more posterior (Fig. 1). In contrast, the AD cluster was located in the anterior part, that is, the genu section of the CC. To validate these results, we reran the analysis with a non-parametric statistical method, that is, the permutation test (Supporting Information Fig. 1). Moreover, we applied the Tract-Based Spatial Statistics (TBSS) analysis (confined to the midline-CC) as illustrated in Supporting Information Figure 2. These additional analyses confirmed that the rs4535189/rs6803202 genotype primarily affected the FA and RD near the posterior part of the CC, while the AD was affected in the anterior part of the CC.

Post-hoc comparisons revealed patterns of group difference among the three genotype groups. Regarding the FA cluster, the T/T group exhibited a higher FA than both the C/T ($P = 0.006$) and C/C groups ($P = 0.007$), and the latter two groups did not differ ($P = 0.538$). The RD cluster exhibited a significantly lower RD in the T/T group than in the C/T ($P = 0.002$) and C/C groups ($P = 0.011$), and no significant difference between the latter two groups was observed ($P = 0.898$). Regarding the AD cluster, the C/C group exhibited a lower value than those of the C/T ($P = 0.001$) and T/T groups ($P = 0.017$), and there was no significant difference between the C/T and T/T groups ($P = 0.513$).

The Mediation Model

As described in the section of Methods, the recessive model, which combined the T/T and C/T carriers as a single group of T-allele carriers, was applied in the entire mediation analysis. In this analysis, the predictor, mediator, and outcome were the rs4535189/rs6803202 genotype, midline-CC clusters, and reading scores, respectively. A significant path *c* (i.e., the total effect) was observed for both WLR ($\beta = 0.198$, $P = 0.008$, 95% confidence interval = [0.053, 0.342]) and CR ($\beta = 0.191$; $P = 0.004$, 95% confidence interval = [0.064, 0.319]). Among the three candidate midline-CC clusters above, only the AD cluster

reached the significance level for the path *a* ($\beta = 0.305$, $P = 0.001$, 95% confidence interval = [0.121, 0.489]), but not the FA ($P = 0.157$) and RD cluster ($P = 0.261$). Therefore, only the AD cluster around the genu was entered into the testing for the path *b*. As illustrated in Figure 2, the AD cluster showed a significant path *b* with the WLR score ($\beta = 0.166$, $P = 0.027$, 95% confidence interval = [0.019, 0.314]) but not with the CR score ($P = 0.175$). Regarding the candidate mediation model from the *ROBO1* genotype, to the AD cluster, to the WLR score, the direct effect from the *ROBO1* polymorphism to the WLR (i.e., the path *c'*) was found non-significant ($\beta = 0.147$, $P = 0.054$, 95% confidence interval = [–0.002, 0.296]), and the bootstrap simulation ($n = 10\ 000$) further confirmed a significant indirect effect *a* × *b* (95% confidence interval = [0.010, 0.117], $P < 0.05$). Given all these significant results, the AD cluster around the genu can be considered to significantly mediate the effect of the *ROBO1* polymorphisms on the WLR performance, as illustrated in Figure 2C.

The Morphology of Cortical Regions Linked by the AD Cluster

For the mediating AD cluster on the midline-CC, we further applied constrained spherical deconvolution (CSD) probabilistic tracking to locate the cortical regions that are linked by the CC tracks that passed through this cluster. Figure 3 illustrates the resulting cortical regions in the two hemispheres. As expected, the AD cluster was located around the genu and primarily linked the prefrontal lobe both mesially and laterally. We further evaluated whether the linked cortical morphology (cortical thickness and surface area) was also related to the *ROBO1* polymorphism or the WLR performance. The *ROBO1* polymorphism exhibited no significant effect on the intracranial volume (ICV), but there was a significant correlation between the ICV and WLR score ($r = 0.307$, $P = 0.0005$). Therefore, we took the ICV as a covariate in the statistical model for the

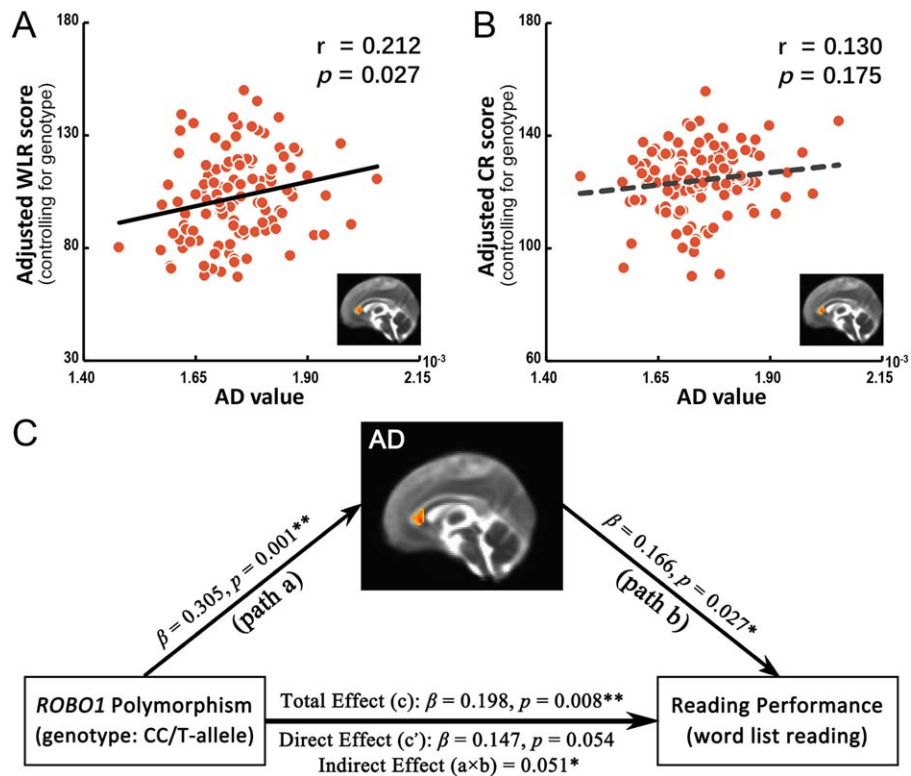


Figure 2.

The mediation results for the rs4535189/rs6803202 genotype, genu connectivity, and reading performance. The genu cluster exhibited a significant correlation with the WLR score **(A)**. However, there was no significant correlation between the AD and CR scores **(B)**. The scatter plots are displayed with adjustments for age, gender, Block Design performance, and genotype.

(C) The mediation model for the rs4535189/rs6803202 genotype, genu, and word list reading (WLR) performance. The AD in the genu cluster of the midline-CC served as a significant mediator for the rs4535189/rs6803202 genotype effects on the WLR. The path coefficients are displayed next to the arrows that indicate the links in the model. *: $P < 0.05$; **: $P < 0.01$.

morphological analysis. For the AD clusters, no cortical cluster exhibited significant relationships of cortical thickness or surface area with the *ROBO1* polymorphisms or WLR performance (Supporting Information Fig. 4).

Furthermore, we tested the associations of the inter-hemispheric asymmetries in cortical thickness and surface area with the *ROBO1* polymorphism and WLR performance. Again, within the masks of the cortical regions for

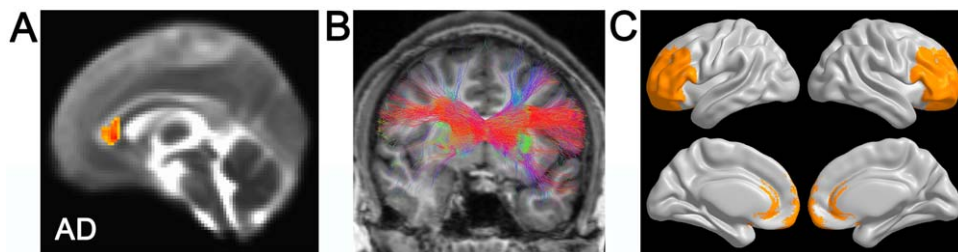


Figure 3.

The genu-cluster connected cortical regions. **(A)** The AD cluster on the midline-CC. **(B)** The constrained spherical deconvolution (CSD) fiber tracking results of the midline-CC cluster for one example subject. **(C)** The group-level symmetric cortical regions in the two hemispheres that were connected by the tracks passing through the midline-CC genu cluster are marked in orange. For details, please see Methods and Supporting Information Figure 3.

the AD clusters, there were no significant results (Supporting Information Fig. 4). Finally, we reran all thickness-related or area-related analyses by replacing the ICV covariate with the mean thickness or total surface area, respectively, in the statistical model. The results are highly consistent with our current findings (Supporting Information Fig. 5).

DISCUSSION

Using genetic, neuroimaging and behavioral data, the present study revealed a *ROBO1*-callosum-reading pathway; that is, the *ROBO1* rs4535189/rs6803202 polymorphism regulates word list reading performance by modulating the fiber microstructures of the genu of the CC. These results provide direct evidence of a *ROBO1*-callosum association in humans and also provide novel insight into the gene-to-brain mechanisms of human reading.

The Effect of *ROBO1* Polymorphism on Reading

While the *ROBO1* gene is a well-recognized candidate gene for developmental dyslexia [Galaburda et al., 2006; Hannula-Jouppi et al., 2005], investigations associating this gene with reading abilities at the SNP level remains scarce. Using a large population-based twin sample in Australia, one association study of *ROBO1* SNPs found significant associations between two *ROBO1* polymorphisms (i.e., rs6803202 and rs4535189) with a phonological memory task, and these two polymorphisms were the only two that survived multiple-comparison correction [Bates et al., 2011]. Therefore, the present study focused on these two polymorphisms as the *ROBO1* polymorphisms of interest. These two polymorphisms exhibited significant effects on the two reading tasks in our current Chinese cohort, which further supported their association with reading skills. The association of the polymorphisms with the reading tasks observed here may be related to phonological memory, which has also previously been associated with these two polymorphisms [Bates et al., 2011].

The Effect of *ROBO1* Polymorphism on the Corpus Callosum

Biologically, the *ROBO1* gene encodes a receptor that acts as molecular guidance cue during cellular migration and axonal navigation. Specifically, this receptor has been found to play a critical role in axon growth across the midline of the brain [Kidd et al., 1998; Lei et al., 2011; Wong et al., 2002]. For example, all homozygous *Robo1* knockout mice die at birth with small or absent CCs [Andrews et al., 2006; Lopez-Bendito et al., 2007; Unni et al., 2012]. However, direct empirical evidence for an association between *ROBO1* and the CC in humans is still lacking. In a Finnish family that weakly expressed a

haplotype of the *ROBO1* gene, the *ROBO1* expression level was correlated with the level of auditory cortical suppression of ipsilateral inputs [Lamminmaki et al., 2012]. Given the dependence of this suppression of ipsilateral inputs on the auditory pathway that crosses the midline (i.e., a part of the CC), this result indirectly demonstrated an influence of the *ROBO1* expression level on the human CC. In an important step forward, the present study provided the first empirical evidence for the effects of *ROBO1* on the human CC by directly measuring the CC with diffusion tensor imaging (DTI).

Intriguingly, the effects of the rs4535189/rs6803202 polymorphisms were not uniformly present across the entire CC; rather, they were localized specifically around the genu and splenium of the CC. These location-dependent findings imply complex interactions of the *ROBO1* receptor with the axonal growth region of the CC during early brain development. Putatively, the diffusion parameters (i.e., FA, AD, and RD) relate to a number of microstructural fiber properties, such as axonal density, diameter, crossing, organization, homogeneity, and myelination [Beaulieu, 2002]. Notably, it has been suggested that the AD and RD are selectively sensitive to specific microstructural properties: the AD is more strongly related to axonal organization, and the RD is more strongly related to the degree of myelination [Concha et al., 2006; Song et al., 2003]. Accordingly, the results of the present study suggest a degree of contribution of the *ROBO1* to axonal organization (e.g., axonal diameter and density) around the genu of the CC, but to axonal myelination around the splenium. That is, in addition to its important role in guiding axonal growth trajectory of callosal fibers, the *ROBO1* is also involved in structural development of these fibers, though the underlying molecular mechanism is unclear yet. The differential effects on the fiber microstructures of the genu and splenium further suggest the complexity of the functional mechanisms of the *ROBO1*. Addressing the question of how SNP-related differences in *ROBO1* expression during early development induce the different fiber microstructures of the CC in later life will require specific animal studies.

The Association Between the Genu and Reading Skills

The observed mediation pathway from the *ROBO1* polymorphism, to the genu, to the WLR performance indicated a significant association between the genu and reading skills. In fact, without considering genetic variants, multiple neuroimaging studies have revealed several reading-related white matter tracts, including the arcuate fasciculus, inferior longitudinal fasciculus, and corpus callosum [Klingberg et al., 2000; Yeatman et al., 2012; Zhao et al., 2016]. Particularly, early morphological studies have reported aberrant shape or sizes of the midline-CC in dyslexic children and adults, and the vast majority of these

observed morphological changes are located around either the genu or the splenium of the midline-CC [Hynd et al., 1995; Rumsey et al., 1996; Von Plessen et al., 2002].

Using DTI techniques, a few studies also revealed significant associations between the CC's diffusion parameters and reading performance, but mainly around the splenium of the CC [Dougherty et al., 2007; Frye et al., 2008; Odegard et al., 2009]. In contrast, our mediation results favored a role of the genu in reading performance. The discrepancy of the specific midline-CC section might relate to the differences in subjects' age range and methodology between studies. Particularly, previous studies showing a splenium-reading association were largely based on alphabetic languages, and the reading language in our present study is Chinese. It is likely that this discrepancy largely reflects the differences in languages or specific reading tests across studies.

Previous studies have suggested that the AD value within the CC is negatively correlated with the mean axonal diameter [Barazany et al., 2009] and that smaller axons conduct signals more slowly than larger axons [Rushton, 1951]. The observed positive correlation between the AD of the genu and WLR performance therefore suggested that the inter-hemispheric connectivity between the bilateral frontal lobes is decreased in good readers. The reduced inter-hemispheric connectivity around the genu in good readers may represent a greater hemispheric specialization of the frontal lobe and relevant executive functions, which play a substantial role in phonological access, a key component of reading cognition. This is compatible with the theory that better language abilities are generally associated with greater hemispheric specialization [Herve et al., 2013; Josse and Tzourio-Mazoyer, 2004].

The *ROBO1*-Genu-Reading Pathway

In cognitive neuroscience, the effects of specific genes on specific behavioral phenotypes are putatively mediated by specific neural functions [Goldberg and Weinberger, 2004; Green et al., 2008]. To date, pairwise relationships among genes, brain, and behavior (i.e., gene-behavior, gene-brain, and brain-behavior relationships) have been extensively studied in humans, and numerous valuable findings have resulted from these studies. By conceptually articulating gene-to-brain-to-behavior pathways, mediation models are more informative than pairwise relationships and provide a very useful framework for directional/causal hypotheses [Green et al., 2013]. However, the use of integrated gene-brain-behavior models to determine the neural mediation of genetic effects on behavioral phenotype remains rare, which may be partially due to the difficulty of simultaneously collecting and analyzing genetic, neuroimaging and behavioral data from the large cohorts that are typically required for mediation analyses. Another possible reason for this rarity is that mediation models are statistically more rigorous than individual pairwise relationships

because they require three concurrent relationships between three variables rather than one relationship between two variables.

To our knowledge, very few studies have explicitly demonstrated neural mediations of significant genetic effects on reading phenotypes. Using the gene-brain-behavior mediation model, the present study revealed one specific gene-brain-reading pathway; that is, the genu the CC mediates the effects of the *ROBO1* rs4535189/rs6803202 polymorphisms on the WLR performance. This pathway is of great value for elucidating the functional mechanisms of *ROBO1* because it provides answers to two important questions: (1) What neural functions are regulated by the *ROBO1* gene to modulate reading performance, and where do these functions occur?; (2) What is the behavioral consequence of the effects of *ROBO1* on the CC in humans?

It should be noted that while the gene-brain-behavior mediation model is very intuitive and adopted by the vast majority studies of imaging genetics, there could be a plasticity model, that is, the gene-behavior-brain mediation model [Bishop, 2013]. However, cross-sectional data only cannot effectively differentiate the two models by always supporting both of them, statistically. This has been a general challenge of the mediation analysis in imaging genetics. In the present study, the *ROBO1*-genu-reading model was favored more than the *ROBO1*-reading-genu model, since the AD value of callosum was found largely stable after 10 years old [Lebel and Beaulieu, 2011; Lebel et al., 2008] and therefore is less likely plastic in our participants (aged from 10 to 15 years). But still, we could not completely rule out the possibility for such a *ROBO1*-reading-genu model by using only our currently observed data, and future investigations are warranted to address this issue.

Finally, somewhat surprisingly, the cortical thicknesses, surface areas, and asymmetries in these parameters of the cortical regions that were linked by the mediating midline-CC genu cluster were not associated with either the rs4535189/rs6803202 polymorphisms or the WLR score. These findings are suggestive of a relatively independent contribution of the genu to the mediation of the *ROBO1*-reading relationship and that the cortical gray matter morphology is less involved. However, the functional activity or connectivity of the cortical gray matter may be involved.

Limitations

First, the sample size might not be small for a children imaging study, but cannot be considered large enough for a genetic analysis. Therefore, it would be important to validate our findings by replicating our analyses in a completely independent cohort with larger sample size. Next, the genu-reading correlation observed in the present study is modest ($r = 0.212$, $P = 0.027$), and cannot survive a strict Bonferroni correction ($P < 0.05/2 = 0.025$). This modest finding suggested that only a very small amount of

reading ability variance among individuals could be explained by the difference in the genu of the CC. Accordingly, the genu-related pathways observed in the present study is not likely to be the only pathway underlying the *ROBO1*-reading relationship; that is, other brain mediators or pathways may also exist. Moreover, the present study focused only on the overall reading ability, and the contributions of its specific underlying cognitive components to the *ROBO1*-callosum-reading pathways warrant further investigations. Finally, the other *ROBO1* polymorphisms remained un-genotyped in our sample. It would be intriguing to genotype the other polymorphisms and evaluate their effects on the CC and reading performance.

CONCLUSION

In summary, the present study revealed that the fiber microstructures of the genu of the CC could mediate the effects of *ROBO1* on human reading ability. This may provide valuable insight into the function of the *ROBO1* gene and the gene-to-brain mechanisms of human reading.

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