Polymorphism within a Neuronal Activity-Dependent Enhancer of *NgR1* Is Associated with Corpus Callosum Morphology in Humans

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Key Words

Nogo-66 receptor 1 · Corpus callosum · Magnetic resonance imaging · Diffusion tensor imaging · Genetic polymorphism · Bioinformatics · Epigenomics · Luciferase assay · Electrophoretic mobility shift assay

Abstract

The human Nogo-66 receptor 1 (*NgR1*) gene, also termed Nogo receptor 1 or reticulon 4 receptor (*RTN4R*) and located within 22q11.2, inhibits axonal growth and synaptic plasticity. Patients with the 22q11.2 deletion syndrome show multiple changes in brain morphology, with corpus callosum (CC) abnormalities being among the most prominent and frequently reported. Thus, we hypothesized that, in humans, *NgR1* may be involved in CC formation. We focused on rs701428, a single nucleotide polymorphism of *NgR1*, which is associated with schizophrenia. We investigated the effects of the rs701428 genotype on CC structure in 50 healthy participants using magnetic resonance imaging. Polymorphism of rs701428 was associated with CC structural variation in healthy participants; specifically, minor A allele carriers had larger whole CC volumes and lower radial diffusivity in the

central CC region compared with major G allele homozygous participants. Furthermore, we showed that the *NgR1* 3' region, which contains rs701428, is a neuronal activity-dependent enhancer, and that the minor A allele of rs701428 is susceptible to regulation of enhancer activity by MYBL2. Our results suggest that *NgR1* can influence the macro- and microstructure of the white matter of the human brain.

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Introduction

In humans, the Nogo-66 receptor 1 (*NgR1*) gene, also termed Nogo receptor 1 or reticulon 4 receptor (*RTN4R*), is located within the 22q11.2 locus. *NgR1* can bind (1) Nogo-A, (2) myelin-associated glycoprotein, and (3) oligodendrocyte myelin glycoprotein. These proteins are myelin-associated inhibitors of axonal regeneration [1–4]. Previous research has shown that *NgR1* plays a role in

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the inhibition of axonal growth and synaptic plasticity [5, 6]. *NgR1* is widely expressed in the central nervous system [7], including in neurons of the neocortex, hippocampus, amygdala, and dorsal thalamus [8].

The 22q11.2 deletion syndrome (22q11.2DS) is caused by a genomic microdeletion within chromosomal region 22q11.2, which contains as many as 35–60 genes [9]. Patients with this syndrome develop schizophrenia at a substantially increased rate of 25–30%, which is approximately 25–31 times higher than in the general population [10]. Thus, this deletion has drawn attention as a potential model for determining the key pathophysiology of schizophrenia [11, 12].

The deletion is associated with various types of structural brain alterations in the cavum septum pellucidum and cavum vergae [13, 14], polymicrogyria [15], enlarged ventricles [13, 16], a decreased volume of the total brain [14, 16], cerebellum [13], and hippocampus [17], volume changes of specific subcortical structures including the corpus callosum (CC) [18–20], reduced fractional anisotropy (FA) in areas of the frontal, parietal, and temporal lobes [21, 22], and increased FA from the posterior CC to the occipital lobes [22]. Of these alterations, abnormalities of the CC [18–20], the largest interhemispheric tract connecting the association cortices, are among the most prominent and consistently reported in 22q11.2DS. Some of these structural anomalies, including an enlarged CC, are also observed in sporadic schizophrenia [20, 23–25].

Here, we focused on rs701428, an *NgR1* single nucleotide polymorphism (SNP) that shows an association with schizophrenia in Caucasian and African-American populations [26, 27]. An association between allelic variations of this SNP and diffusion tensor imaging (DTI) metrics of the white matter tract has been reported in 22q11.2DS patients [28]. To examine the roles of *NgR1* in CC formation, we investigated a possible association between *NgR1* genetic variation and CC structure in healthy participants, using structural magnetic resonance imaging (MRI) and DTI. We also examined the physiological function of rs701428 by combining *in silico* and *in vitro* approaches.

Subjects and Methods

Participants

Fifty healthy individuals were recruited from the local community. None had a history of psychiatric disease, as determined by the non-patient edition of the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). In addition, none had a history of head trauma, neurological disease, severe medical diseases,

or substance abuse. There was no history of psychotic disorders among first-degree relatives. The participants were all physically healthy at the time of scanning. This study was approved by the Committee on Medical Ethics of Kyoto University and was performed according to the Code of Ethics of the World Medical Association. Written informed consent was obtained from each participant.

NgR1 Genotyping

Genomic DNA was extracted from venous blood samples of each participant using standard methods, with EDTA anticoagulant. The SNP rs701428 was genotyped using the LightCycler 480 system (Roche, Basel, Switzerland) and a TaqMan SNP Genotyping Assay (TaqMan SNP Genotyping Assay ID C_2785952_10; Applied Biosystems, Foster City, Calif., USA).

MRI Acquisition

All participants were scanned with a 3-tesla MRI scanner (Trio; Siemens, Erlangen, Germany). Diffusion-weighted imaging data were acquired using single-shot spin-echo echo-planar sequences. Structural MRI data were obtained using three-dimensional magnetization-prepared rapid gradient echo (3D-MPRAGE) sequences, with a 40 mT/m gradient and a receiver-only 8-channel phased-array head coil. The parameters for diffusion-weighted data were: TE (echo time) 96 ms; TR (repetition time) 10,500 ms; 96 × 96 matrix; FOV (field of view) 192 × 192 mm; 70 contiguous axial slices of 2.0 mm thickness; 81 non-colinear axis motion-probing gradients, and $b = 1,500 \text{ s/mm}^2$. The $b = 0$ images were scanned before every 9 diffusion-weighted images, hence 90 volumes in total. The parameters for 3D-MPRAGE imaging data were: TE 4.38 ms; TR 2,000 ms; inversion time 990 ms; 240 × 256 matrix; FOV 225 × 240 mm; resolution 0.9375 × 0.9375 × 1.0 mm, and 208 total axial sections without intersection gaps.

Imaging Data Preprocessing

DTI Data Preprocessing

All DTI data processing was performed using programs in the Functional MRI of the Brain (FMRIB) Software Library (FSL) version 4.1.6 (<http://www.fmrib.ox.ac.uk/fsl>). Source data were corrected for head motion and eddy currents by registering all data to the first $b = 0$ image with affine transformation. FA maps and indices of white matter integrity were calculated using the DTIFIT program of FSL. In addition, axial diffusivity (AD) and radial diffusivity (RD), measures of diffusivity parallel and perpendicular to axons, respectively, were calculated. TBSS version 1.2 in FSL was used to normalize all FA data into the MNI 152 space. The FMRIB Nonlinear Image Registration Tool (FNIRT) was used for nonlinear transformation. Normalized FA images were averaged and thinned to create a mean skeletonized FA image, taking only centers of white matter tracts common to all subjects. Voxel values of each subject's normalized FA map were projected onto the FA skeleton by searching for the local maxima along the perpendicular direction from the skeleton. Resultant skeletonized FA data were used in the following statistical analysis. The same transformations were applied to AD and RD images to create skeletonized AD and RD maps.

Structural MRI Data Preprocessing

The 3D-MPRAGE images were preprocessed using the FreeSurfer software package version 5.0.0 (<http://surfer.nmr.mgh.har>

vard.edu). The process included Talairach transformation of each subject's native brain, removal of non-brain tissue, volumetric subcortical labeling, and surface-based segmentation of gray and white matter tissue. In automatic segmentation, each voxel in normalized brain volumes was assigned a label based on an atlas containing probabilistic information about structure locations, including the CC. Subdivided cerebral white matter regions were derived from cortical parcellation.

Region of Interest Setting Using FreeSurfer

To define CC regions of interest (ROI), we used automatic segmentation in FreeSurfer [29] based on subcortical parcellation, which segments the CC into anterior, middle anterior, central, middle posterior, and posterior regions. Volumes of each region were automatically calculated during the segmentation process. The 'whole CC' was the sum of the five subregions. Masks derived from these ROI were used to measure DTI indices (FA, AD, and RD) of the whole and segmented CC. To determine DTI indices of the CC in the diffusion space, ROI derived from FreeSurfer template images were transformed from an MNI 302 space to an MNI 152 space by applying the rigid-body transformation matrix, which was calculated using FSL's FLIRT program. To check transformation quality and to confirm there were no gross transformation errors, we overlaid each CC ROI onto the FMRIB58_FA image, a mean FA template in the MNI 152 space (fig. 1).

Data Analysis

Group Comparison of Demographic Data

Minor A allele carriers are associated with schizophrenia [26]; therefore, we compared the minor A allele carrier group (A carrier group) with the homozygote wild-type G allele carrier group (G homozygous group). Demographic data and ROI volumes were analyzed by two-sample t tests using SPSS 19.0 (SPSS Inc., Chicago, Ill., USA). Statistical significance was defined as $p < 0.05$ (two-tailed) in all analyses.

Group Comparison of Volume and DTI Indices of the Whole CC

To investigate differences in whole CC volumes between the G homozygous and the A carrier group, we adjusted the volume by the intracranial volume of each patient [30]. The white matter volume of the whole brain was also measured. In addition, we calculated the mean FA, AD, and RD in the whole CC mask by multiplying the CC mask and skeletonized maps. We determined significant differences using unpaired two-sample t tests, with the statistical threshold defined as $p < 0.05$ (two-tailed).

Group Comparison of Volume and Diffusivity in CC ROI

Next, when we detected a group difference in CC total volume, we investigated volume differences in CC ROI between the G homozygous and the A carrier group to determine which CC subregion was most responsible for the volume differences observed. We determined significant differences using unpaired two-sample t tests, with the statistical threshold at $p < 0.01$ ($= 0.05/5$). We also calculated the mean FA, AD, and RD of each region using the same method as for whole CC analysis, comparing the two groups using unpaired two-sample t tests. For this analysis, the t tests were performed 15 times (5 ROI $\times$ 3 parameters); thus, after Bonferroni correction, the statistical threshold was set at $p < 0.0033$ ($= 0.05/15$).

Fig. 1. Segmented CC. **a** CC labels derived from a FreeSurfer template image. **b** Rigid-body-transformed image of a segmented CC ROI from the MNI 302 to the MNI 152 space.

Functional Analysis of the *NgR1* 3' Enhancer

Epigenomic Data

ChIP-seq and DNaseI-seq data were obtained from the ENCODE Project (<http://genome.ucsc.edu/ENCODE>). ENCODE Project data were displayed in the University of California, Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu>).

Luciferase Assays

DNA fragments corresponding to a candidate enhancer region (chr22:20224442–20228467) and a 5'-deleted region (chr22:20224442–20227395, 20228125–20228467) were polymerase chain reaction (PCR) amplified from human genomic DNA and cloned into the pGL4.50 plasmid (Promega, Madison, Wis., USA).

Fig. 2. **a** Scatter plot of whole CC volume (CC_total) versus genotype. Genotype segregates with CC volume. **b** Scatter plot of RD in the central region of the CC (RD_cCC) versus genotype.

Table 1. Demographic characteristics of the two groups

	Genotype		Statistical value (t or χ^2 test)	p value
	AA/AG	GG		
Number	36	14	–	–
Age, years	25.7±6.6	24.1±5.3	0.84	0.41 ^a
Gender, F/M	16/20	3/11	1.62	0.14 ^b
Education, years	14.0±2.9	14.3±2.8	0.28	0.78 ^a
VIQ	110.8±17.0	118.9±18.0	1.48	0.15 ^a
PIQ	115.4±17.1	122.5±13.1	1.39	0.17 ^a

Values denote means ± SD unless specified otherwise. VIQ = Verbal IQ; PIQ = performance IQ. ^aTwo-sample t test. ^b χ^2 test.

at the BamHI site using the In-Fusion HD cloning kit (TaKaRa, Otsu, Japan) to generate reference-cytomegalovirus (CMV), rs701428-CMV, and reference-S-CMV plasmids. PCR fragments were Sanger sequenced in both directions to confirm the presence of the rs701428 SNP and the absence of PCR amplification-induced mutations.

Reporter plasmids were cotransfected into mouse cortical neurons with constitutively active pRL-CMV *Renilla* luciferase (Promega) as the control plasmid, using a NEPA21 electroporator (Nepa Gene, Chiba, Japan). C57BL/6 mouse embryonic-day 16.5 embryo cortices were dissected and dissociated using a neural tis-

sue dissociation kit (P; Miltenyi-Biotec, Bergisch Gladbach, Germany). To fully dissociate cells, trituration was performed using a flame-narrowed Pasteur pipette. Dissociated mouse cortical neurons were centrifuged at 90 g for 5 min at 4°C and resuspended in a 100- μ l mixture of Opti-MEM (Invitrogen, Carlsbad, Calif., USA) and reporter plasmid. Two types of electric pulse were applied (poring pulse condition: 275 V, pulse length 0.5 ms, 2 pulses, interval between pulses 50 ms, decay 10%, rate with + polarity; transfer pulse condition: 20 V, pulse length 50 ms, 5 pulses, interval between pulses 50 ms, decay 40%, rate with +/- polarity). After electroporation, cells were immediately seeded onto polyornithine-coated 96-well plates (Nunc, Naperville, Ill., USA). The plates were preincubated overnight with polyornithine (30 mg/ml; Sigma, St. Louis, Mo., USA) in water, and washed 3 times with water before use. Neurons were maintained in Neurobasal Medium containing B27 supplement (2%; Invitrogen), penicillin-streptomycin (100 mg/ml penicillin, 100 U/ml streptomycin; Nacalai Tesque, Kyoto, Japan), and glutamine (2 mM; Life Technologies, Gaithersburg, Md., USA) and grown in vitro for 6 days. One half of the medium was replaced with fresh warm medium on the 2nd, 4th, and 6th days in vitro. Neurons 6 days in vitro were incubated for 12 h in 1 μ M tetrodotoxin (TTX; Tocris, Bristol, UK) and 100 μ M D(-)-2-amino-5-phosphonopentanoic acid (D-AP5; Tocris), and then for 6 h in 55 mM KCl. Next, cells were harvested and extracts were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega), with measurements performed using a Varioskan (Thermo Fisher Scientific, Waltham, Mass., USA). The luminescence ratio of experimental sample to control reporter was calculated for each sample and defined as the relative luciferase unit.

Electrophoretic Mobility Shift Assay

DNA binding reactions were performed in 20 µl reaction volumes containing 50 fmol end-labeled dsDNA probe and 2 µg nuclear extract in Gel Shift Assay System buffer (Thermo Fisher Scientific). The reaction mixtures were incubated at room temperature for 30 min, loaded onto 6% native polyacrylamide gels, and run in 0.5× Tris-boric acid-EDTA buffer at 130 V for 4 h. For competition experiments, cold probe (10-fold molar excess of the unlabeled oligonucleotide) was added, with the probe added last.

Nuclear extracts from control and c-MYB, MYBL1, or MYBL2-transfected 293T cells were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kits (Thermo Fisher Scientific). The upper strand sequences for double-stranded oligonucleotides were: schizophrenia risk rs701428-A allele probe: 5'-CTGAAGGAGAGTTGGGCGGGTCAGG-3', and reference-G allele probe: 5'-CTGAAGGAGAGTCGGGCGGGTCAGG-3'. The oligonucleotide probes were labeled and subjected to a gel shift assay using the Biotin 3' End Labeling and Light-Shift Chemiluminescent EMSA Kits (Thermo Fisher Scientific). To anneal complementary oligonucleotides, sense and antisense oligonucleotides were combined and incubated at 95°C for 5 min. Binding reactions containing 10× binding buffer, 1 µg poly(dI-dC), 5 mM MgCl₂, 2.5% glycerol, and 6 µg nuclear extract were incubated with biotin-labeled oligonucleotides (20 fmol per oligonucleotide), in the absence or presence of a 200-fold molar excess of unlabeled competitor, for 20 min at room temperature. For competition studies, increasing concentrations of unlabeled oligonucleotides were added to the binding reactions. Samples were then run on a native 5% polyacrylamide gel. The gel contents were transferred to nylon membranes (Hybond-N+; GE Healthcare Life Sciences, Piscataway, N.J., USA) and cross-linked to the membrane using a UV cross-linker. The membranes were blocked and visualized using the Light-Shift kit.

Results

Demographic Data

The characteristics of the participants are shown in table 1. No significant difference between the G homozygous and the A carrier group was found with regard to age, sex, handedness, education, or IQ.

Whole CC Imaging

The A carrier group had significantly larger whole CC volumes than the G homozygous group (fig. 2a), which was also confirmed using a permutation test (empirical p value = 0.026). There were no significant differences in whole CC DTI indices (table 2). We also analyzed the data with age included as a covariate, and the group difference was reproduced.

CC Subregion Imaging

The A carrier group showed a trend towards a larger anterior CC volume compared with the G homozygous group, although the difference was nonsignificant after

Table 2. Group differences in diffusivity parameters (FA, AD, and RD) in whole CC

	Genotype		d.f.	t value	p value ^a
	AA/AG	GG			
Volume	2.01	1.80	48	1.96	0.028 ^b
Diffusivity					
FA	0.682	0.667	48	1.30	0.200
AD	1.25×10 ⁻³	1.26×10 ⁻³	48	0.64	0.525
RD	3.47×10 ⁻⁴	3.65×10 ⁻⁴	48	1.39	0.172

^a Two-sample t test. ^b p < 0.05.

Table 3. Group differences in volumes in separated CC ROI

Region	Genotype		d.f.	t value	p value
	AA/AG	GG			
Whole	2.01	1.80	48	1.96	0.028 ^a
Anterior	0.534	0.467	48	2.06	0.022 ^b
Mid-anterior	0.317	0.281	48	1.17	0.124 ^b
Central	0.313	0.279	48	1.07	0.145 ^b
Mid-posterior	0.280	0.262	48	0.42	0.337 ^b
Posterior	0.566	0.513	48	1.58	0.060 ^b

^a Two-sample t test. ^b Two-sample t test, corrected by the Bonferroni method.

Table 4. Group differences in DTI indices (FA, AD, and RD) in separated CC ROI

Region	Diffusivity	Genotype		t value	p value ^a
		AA/AG	GG		
Anterior	FA	0.678076	0.6800	0.11	0.913
	AD	1.146×10 ⁻³	1.134×10 ⁻³	0.35	0.728
	RD	3.230×10 ⁻⁴	3.148×10 ⁻⁴	0.36	0.722
Mid-anterior	FA	0.635122	0.608152	1.54	0.130
	AD	1.211×10 ⁻³	1.240×10 ⁻³	1.09	0.282
	RD	3.790×10 ⁻⁴	4.152×10 ⁻⁴	1.79	0.079
Central	FA	0.697747	0.668065	2.30	0.026
	AD	1.310×10 ⁻³	1.337×10 ⁻³	1.12	0.245
	RD	3.389×10 ⁻⁴	3.797×10 ⁻⁴	3.14	0.003 ^b
Mid-posterior	FA	0.614926	0.601437	0.71	0.481
	AD	1.435×10 ⁻³	1.432×10 ⁻³	0.10	0.919
	RD	4.894×10 ⁻⁴	4.994×10 ⁻⁴	0.37	0.712
Posterior	FA	0.745049	0.727123	1.33	0.190
	AD	1.270×10 ⁻³	1.297×10 ⁻³	1.01	0.317
	RD	2.837×10 ⁻⁴	3.090×10 ⁻⁴	1.63	0.110

^a Two-sample t test, corrected by the Bonferroni method. ^b p < 0.0033 (= 0.05/15).

Fig. 3. Epigenetic annotation of the *NgR1* gene. UCSC Genome Browser screenshot showing the ~50-kbp region surrounding the rs701428 SNP in the *NgR1* gene 3' region. The lower panel represents an enlarged view of the rs701428 SNP. H3K4me1 and H3K9ac

enrichment from NT2-D1 cells, DNaseI-seq enrichment from the human prefrontal cortex, and multi-percent identity plot alignment of genomic sequences from 8 species are indicated.

Bonferroni correction (table 3). With regard to DTI indices, the mean RD in the central CC region was significantly smaller in the A carrier group than in the G homozygous group (fig. 2b; table 4).

Functional Analysis of the *NgR1* 3' Enhancer

The 3' Region of *NgR1* Harbors Regulatory Elements

Multiple species alignment of the region surrounding the rs701428 SNP was examined in the UCSC Genome Browser Multiz alignment track [31, 32]. The *NgR1* 3' region is highly conserved and is, therefore, likely to contain enhancer elements (fig. 3). Regulatory elements such as active promoters and enhancers are commonly found in open chromatin regions, which can be identified using

genome-wide DNase I hypersensitivity assays (DNaseI-seq; ENCODE Consortium) [33–35]. The rs701428 SNP in the *NgR1* 3' region was mapped within a DNase I-hypersensitive site in the human prefrontal cortex (fig. 3). To determine if this region harbors enhancers, we examined histone H3 lysine 4 monomethylation (H3K4me1) and lysine 9 acetylation (H3K9ac), markers of enhancers (ENCODE Consortium) [36–38]. H3K4me1 and H3K9ac enrichment suggests that the *NgR1* 3' region is an active regulatory element (fig. 3).

The presence of overlapping H3K4me1, H3K9ac, and DNase I hypersensitivity peaks suggests the presence of an enhancer in the *NgR1* 3' region. To investigate if this region has potential enhancer activity, chr22:20224442–

Fig. 4. The region surrounding rs701428 acts as a neuronal activity-dependent enhancer modulated by Mybl2. **a** Schematic representation of *NgR1* 3' enhancer reporter constructs. **b** The *NgR1* 3' enhancer is downregulated by neuronal activation induced by KCl, and enhanced by blockade of neuronal activation by TTX and D-AP5. * $p = 0.0079$, ** $p = 0.0017$. **c** A representative Myb-binding motif within rs701428. Alleles of rs701428 are shown: the reference G allele (green; colors refer to the online version only) and the variant A allele (red). Mybl2 overexpression decreases transcriptional repression by neuronal activity of the *NgR1* 3' enhancer with the schizophrenia risk rs701428-A allele (* $p = 0.049$; **d**) but not the reference-G allele (**e**), or with deletion of a region containing rs701428 (**f**). Data are presented as means $\pm$ SD, with each experiment conducted in triplicate.

Fig. 5. DNA fragments containing the rs701428 schizophrenia risk A allele but not the reference G allele show specific binding to Myb family transcription factors. **a** Myb family transcription factor binding was examined using EMSA. 293T cells were transfected with vectors containing c-Myb, Mybl1, Mybl2, or no insert [negative control (NC)]. Nuclear extracts were prepared and an EMSA performed using reference G allele or schizophrenia risk A allele

probes. A background band due to endogenous factors expressed in 293T cells is observed with the rs701428-A allele probe. **b** Specific binding of Mybl2 to the rs701428-A allele probe but not to the reference G allele probe. Mybl2-transfected nuclear extracts were incubated with labeled rs701428-A or -G allele probes. Binding was competed using a 200-fold molar excess of an unlabeled A or G allele competitor.

20228467 was PCR amplified and cloned into a luciferase reporter gene. It has been reported that *NgR1* expression is downregulated by neuronal activity [39]. To examine the effect of neuronal activity on the putative *NgR1* 3' enhancer, we transfected 3' enhancer reporter constructs with or without the rs701428 SNP into primary cortical neurons. Substantial increases in luciferase activity were observed for both constructs in the presence of the sodium channel blocker TTX and the NMDA receptor antagonist D-AP5, which blocks neuronal activity (fig. 4b). Conversely, neuronal depolarization by elevated potassium chloride (KCl) levels drastically decreased the transactivation activity of the *NgR1* 3' region.

The rs701428 SNP may alter transcription factor recruitment to the *NgR1* 3' enhancer. We used JASPAR [40] to screen for potential transcription factor binding sites in the region surrounding rs701428 and found a binding affinity for the myeloblastosis (MYB) family for the schizophrenia risk rs701428-A allele but not for the reference G allele (fig. 4c). The MYB family contains three

members (c-MYB, MYBL1, and MYBL2), which bind DNA with similar specificity [41, 42]. MYBL2 overexpression specifically decreased the effects of neuronal activation on the transactivation activity of the 3' *NgR1* enhancer with the schizophrenia risk rs701428-A allele (fig. 4d). In contrast, neither the 3' *NgR1* enhancer containing the reference rs701428-G allele nor that with a deletion of a 5' 700-bp region containing rs701428 was affected by MYBL2 overexpression (fig. 4e, f). To examine if the MYB transcription factor family binds to the region with the schizophrenia risk rs701428-A allele, we performed an electrophoretic mobility shift assay (EMSA). The labeled schizophrenia risk rs701428-A allele probe, but not the labeled reference-G allele probe, formed a binding complex with c-MYB, MYBL1, and MYBL2 (fig. 5a, arrow). Furthermore, MYBL2 binding to the labeled rs701428-A allele probe was diminished in the presence of a 200-fold excess of unlabeled rs701428-A allele probe but not unlabeled reference-G allele probe (fig. 5b, arrow).

Discussion

In the present study, the rs701428 polymorphism located in the 3' region of the *NgR1* gene was found to be associated with CC structural variation in the human brain. Minor A allele carriers of this SNP, which was reported to be the risk allele for schizophrenia in a previous study [26], had larger whole CC volumes and lower RD in the central CC region than major G allele homozygous participants. Furthermore, we demonstrated that the *NgR1* 3' region containing rs701428 is a neuronal activity-dependent enhancer, and that the schizophrenia risk allele A of rs701428 renders this enhancer susceptible to transcriptional regulation by MYBL2.

In 22q11.2DS, structural brain abnormalities that are likely to be developmental in nature have repeatedly been reported [14, 20, 22]. However, which genetic changes in this chromosomal region induce morphological brain abnormalities and how this occurs is not known. Our study is the first to implicate a specific gene as a candidate for the morphological changes to the brain observed in this syndrome, as well as a possible mechanism. Since the genetic polymorphism investigated in our study is associated with schizophrenia [26, 27], the putative molecular mechanism identified may, in part, be involved in the neurodevelopmental manifestation of schizophrenia pathology.

NgR1, a receptor for Nogo-A, represses synaptogenesis and axonal sprouting [43] and is downregulated by neuronal activity [39, 44, 45]. This *NgR1*-mediated mechanism is thought to be important for restricting synaptic plasticity and maintaining preformed neuronal wiring [5, 39]. We showed that the 3' region of the *NgR1* gene, containing rs701428, is a neuronal activity-dependent enhancer, and that the minor (schizophrenia risk) allele of rs701428 disinhibits repressive activity of this enhancer via MYBL2 binding. These results suggest that the risk allele may dysregulate *NgR1* expression and consequently disturb the regulatory process of restricting synaptic plasticity, a requirement for normal neural circuitry development.

Our MRI analyses found that minor (schizophrenia risk) allele carriers have larger CC volumes. This finding is compatible with previous MRI studies reporting enlarged CC volumes in 22q11.2DS patients [18]. CC enlargement in rs701428 minor allele carriers may be reasonably expected, considering the putative role of *NgR1* in neural circuitry formation [46]. *NgR1* suppresses excessive branching of axon fibers [5]. The expression of *NgR1* is downregulated by neuronal activation [39]. This mechanism plays pivotal roles in the neuronal activity-dependent regulation of axonal structures. Dysregulated *NgR1* expres-

sion in minor allele carriers may disrupt normal synaptic pruning and may result in abnormal hypertrophic white matter tracts. As the CC is the largest white matter tract in the human brain, our MRI data, as well as previous MRI studies on 22q11.2DS, may have captured a more general process of white matter wiring abnormalities.

In schizophrenia, structural MRI studies are not unanimous with regard to the structural abnormalities of the CC. Both increased and decreased CC volumes in schizophrenia patients have previously been documented [47]. The white matter alteration in schizophrenia might be caused by the interaction between genetic variation within the 22q11.2 region and variation in other risk genes for schizophrenia, such as neuregulin 1 (*NRG1*), *ERBB4*, and disrupted-in-schizophrenia 1 (*DISC1*). Dysfunction of these genes has been reported to alter myelination, similarly to *NgR1* [48–50]. In humans, variation of *ERBB4* was demonstrated to be associated with subcortical microstructure [51]. Therefore, the differential contributions of these multiple genes among individuals may result in the white matter structural variations of schizophrenia, reflecting the heterogeneity of the disease. Our data show that the putative neurodevelopmental molecular mechanism mediated by *NgR1* may partially explain the white matter pathology of schizophrenia.

Furthermore, this *NgR1* mechanism may also show regional specificity in addition to a process involving axonal development and synapse formation at the whole brain level. Indeed, our data show that minor allele carriers have decreased RD in the central region of the CC. DTI studies on 22q11.2DS are in agreement with this finding, showing a genetic effect localized in specific CC regions [21, 22]. Our result, however, is inconsistent with that of Perlstein et al. [28], who investigated the effect of the same SNP, rs701428, and reported that the G allele is associated with reduced RD. The discrepancy might be due to the difference in the white matter regions reported to be associated with the allelic variation. In addition, it should be noted that Perlstein et al. investigated this association in 22q11.2DS individuals; a variation of *NgR1* might have a complex genetic interaction with other genes in the deleted 22q11.2 region which are important for neuronal differentiation and function [11, 52].

A major limitation of our study is its relatively small sample size. Another is that we focused on CC structure and not on the other brain abnormalities reported in 22q11.2DS. Our present results are highly preliminary until replicated with larger sample numbers.

In summary, our study shows that *NgR1* can influence the macro- and microstructure of the white matter of the

human brain. A frequently reported structural anomaly in 22q11.2DS, namely CC enlargement, can be partially affected by disrupted regulation of *NgR1*. Furthermore, a 22q11 mouse model study is expected to reveal the possible contribution of *NgR1* to CC structural variation.

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Statement of Ethics

Our study was approved by the Committee on Medical Ethics of Kyoto University and was conducted in accordance with the Code of Ethics of the World Medical Association.

Disclosure Statement

The authors declare no conflict of interest.

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