ORIGINAL ARTICLE

Rare genetic variants in *CX3CR1* and their contribution to the increased risk of schizophrenia and autism spectrum disorders

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CX3CR1, a G protein-coupled receptor solely expressed by microglia in the brain, has been repeatedly reported to be associated with neurodevelopmental disorders including schizophrenia (SCZ) and autism spectrum disorders (ASD) in transcriptomic and animal studies but not in genetic studies. To address the impacts of variants in *CX3CR1* on neurodevelopmental disorders, we conducted coding exon-targeted resequencing of *CX3CR1* in 370 Japanese SCZ and 192 ASD patients using next-generation sequencing technology, followed by a genetic association study in a sample comprising 7054 unrelated individuals (2653 SCZ, 574 ASD and 3827 controls). We then performed *in silico* three-dimensional (3D) structural modeling and *in vivo* disruption of Akt phosphorylation to determine the impact of the detected variant on CX3CR1-dependent signal transduction. We detected a statistically significant association between the variant Ala55Thr in *CX3CR1* with SCZ and ASD phenotypes (odds ratio = 8.3, $P = 0.020$). A 3D structural model indicated that Ala55Thr could destabilize the conformation of the CX3CR1 helix 8 and affect its interaction with a heterotrimeric G protein. *In vitro* functional analysis showed that the CX3CR1-Ala55Thr mutation inhibited cell signaling induced by fractalkine, the ligand for CX3CR1. The combined data suggested that the variant Ala55Thr in CX3CR1 might result in the disruption of CX3CR1 signaling. Our results strengthen the association between microglia-specific genes and neurodevelopmental disorders.

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INTRODUCTION

Both schizophrenia (SCZ) and autism spectrum disorders (ASD) are highly polygenic neurodevelopmental disorders. SCZ and ASD share etiological, clinical and biological features.[1,2] The prevalence and recurrence risks to relatives are nearly identical, and heritability is estimated to be a minimum of 80% for each disorder.[3] Genetic contributions have played a major role in the etiology of both disorder; however, these genetic components are still unclear. As thousands of trait- and disease-associated common genetic variants of small effect may explain less than half of the total variation responsible for increased risk of developing either disorder,[4–6] a significant excess of rare, disruptive variants (with a frequency < 1%) have provided additional genetic risk of these complex disorders or trait variability.[7–11] In particular, many genes with rare variants that are common to both SCZ and ASD are related to synapse morphogenesis.[12–15]

Recent evidence implicates synaptic formation as an important factor in the pathogenesis of SCZ and ASD.[12,16] It is becoming clear that microglia, which are the resident macrophages and phagocytes of the central nerve system, contribute to major aspects of the structural shaping and functional modulation of the connectivity of the developing and healthy brain.[17–21] Aberrant

functions of microglia have potential implication for SCZ and ASD.[22–25] Altered gene expression of microglia has been found in the brains of SCZ[26,27] and ASD patients.[28] Transcriptomic studies of post-mortem brains have demonstrated dysregulation of microglial gene expression in SCZ[29] and ASD.[30] Microglial synapse morphogenesis is dependent on the receptor CX3C chemokine receptor 1 (CX3CR1, also known as fractalkine receptor, OMIM: 601470), which is solely expressed by microglia in the brain[19] and has been proposed as a key mediator of neuron–microglia interactions.[31] Mice lacking *Cx3cr1* exhibit deficits in synaptic pruning[19,20,32,33,34] as well as in social interaction and increased repetitive-behavior phenotypes, which is considered to be the core symptoms of ASD.[19,34] These findings strongly support the notion that *CX3CR1* is a plausible candidate risk gene for SCZ and/or ASD.

To date, two *CX3CR1* polymorphism, Var249Ile (rs3732379) and Thr280Met (rs3732378), have been reported to affect the activity of the CX3CR1 protein. These polymorphisms are associated with several neuroinflammatory disorders such as HIV-1 infection,[35] multiple sclerosis,[36] amytrophic lateral sclerosis,[37] age-related macular degeneration[38] and coronary atherosclerosis.[39] However, the effects of these common variants on disease pathogenesis are quite small.[35–38] There has been no published report of causal

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genetic variants of *CX3CR1* in cases with neurodevelopmental disorders including SCZ and ASD. We therefore hypothesized that a rare variant of the microglia-specific *CX3CR1* gene would contribute to some pathophysiological mechanisms of these neurodevelopmental disorders.[40] To increase statistical power and detect shared risk, we combined SCZ and ASD samples in a study cohort.[5,15]

We then performed *in silico* three-dimensional (3D) structural modeling and *in vivo* disruption of Akt phosphorylation to determine the impact of the detected variant on CX3CR1-dependent signal transduction based on the following: (i) CX3CR1 is a seven transmembrane domain G protein-coupled receptor (GPCR), the signaling systems of which are involved in many disease and major therapeutic targets.[41] (ii) The chemokine fractalkine, the sole ligand for CX3CR1, is known to activate the Akt signaling pathways through CX3CR1.[42–46]

In this study, we present the genetic association of a variant in *CX3CR1* with SCZ and ASD, followed by *in silico* 3D structural modeling of the predicted conformational change of the variant receptor and its *in vivo* disruption of the Akt signaling. Our findings provide the genetic evidence of the association of a microglia-specific gene with neurodevelopmental disorders.

MATERIALS AND METHODS

Study samples

Two independent Japanese sample groups were used in this study. The targeted-resequencing discovery cohort comprised 370 SCZ (mean age ± s. d., 49.7 ± 14.8 years; 53% male) and 192 ASD (mean age ± s.d. = 16.3 ± 8.4 years; 77.6% male). For the genetic association analysis, the case–control sample set comprised 2283 SCZ (48.1 ± 23.8 years; 41.7% male), 382 ASD (19.6 ± 10.7 years; 77.8% male) and 3827 control subjects (43.3 ± 14.5 years; 41.6% male). All subjects were unrelated, living on the mainland of Japan, and self-identified as Japanese. All patients fulfilled the criteria listed in the *Diagnostic and Statistical Manual of Mental Disorders,* Fifth Edition (DSM-5) for SCZ or ASD. Healthy control subjects were selected from the general population and had no history of mental disorders based on questionnaire responses from the subjects themselves during the sample inclusion step. The study was explained to each participant and/or their parents both verbally and in writing. Written informed consent was obtained from the participants and from the parents for patients under 20 years old. All procedures performed in this study involving human participants were approved by the Ethics Committee of the Nagoya University Graduate School of Medicine. They were conducted in accordance with the Helsinki Declaration of 1975 and its later amendments or comparable ethical standards.

Sample preparation

Genomic DNA was extracted from peripheral blood or saliva from each SCZ, ASD and control participant using the QIAamp DNA Blood Kit or Tissue Kit (Qiagen, Hilden, Germany). The quantity of extracted DNA was estimated using the Qubit dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA, USA) on a Qubit 2.0 Fluorometer (Life Technologies) following the manufacturer's recommended protocol.

Library preparation and resequencing

We used the next-generation sequencing technology of the Ion Torrent PGM to resequence the *CX3CR1* coding regions (Ensembl Transcript ID: ENST00000399220, NCBI reference sequence NM_001337.3, NP_001328.1; 355 amino acids) via the protocols described in the Ion AmpliSeq Library Preparation User Guide (Thermo Fisher Scientific, Waltham, MA, USA, Rev.5; MAN0006735), the Ion PGM Template OT2 200 Kit (Thermo Fisher Scientific, Rev. 5; MAN0007220) and the Ion PGM Sequencing 200 Kit (Thermo Fisher Scientific, Rev. 3; MAN0007273). Custom amplification primers were designed to cover coding exons and flanking intron regions of the selected genes with Ion AmpliSeq Designer (Thermo Fisher Scientific). Sample amplification and equalization were achieved using Ion AmpliSeq Library Kits 2.0 and the Ion Library Equalizer Kit, respectively (Thermo Fisher Scientific). Amplified sequences were ligated with Ion Xpress Barcode Adapters (Thermo Fisher Scientific). Emulsion PCR and

subsequent enrichment were performed using the Ion OneTouch Template Kit v2.0 on Ion OneTouch 2 and Ion OneTouch ES, respectively (Thermo Fisher Scientific).

Data analysis

Sequence reads were run through a data analysis pipeline of the Ion Torrent platform-specific pipeline software, Torrent Suite version 4.4 (Life Technologies) to generate sequence reads filtered according to the pipeline software quality-controls and to remove poor signal reads. Reads assembly and variant identification were performed using the Ingenuity Variant Analysis software (http://www.ingenuity.com/variants) from Ingenuity Systems using Fastq files containing sequence reads and the Ion AmpliSeq Designer BED file software to map the amplicons with default parameters (call quality > 20 and read depth > 10).

Candidate variants were defined as exonic or splice-site variants with allele frequencies of ⩽ 1% in the following three public exome databases: dbSNP Build 149 (http://www.ncbi.nlm.nih.gov/projects/SNP/); the 1000 Genomes Project (http://www.1000genomes.org); and the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org). We then examined two databases as a reference for Japanese controls: the Human Genetic Variation Database (HGVD) (http://www.genome.med.kyoto-u.ac. jp/SnpDB/) and the integrative Japanese Genome Variation Database (iJGVD) (https://ijgvd.megabank.tohoku.ac.jp).[47] Prediction of significance was performed using PolyPhen-2 (last accessed Feb 2017; http://genetics. bwh.harvard.edu/pph2/)[48] and MutationTaster (last accessed Feb 2017; http://www.mutationtaster.org).[49] Additional clinical variant annotations were obtained from NCBI ClinVar (last accessed Feb 2017; http://www.ncbi. nlm.nih.gov/clinvar/)[50] and DECIPHER v9.12 (last accessed Feb 2017; https://decipher.sanger.ac.uk).[51] Localization of a protein domain was based on the human protein reference database (http://www.hprd.org/ index.html) (HPRD). When available, parents were sequenced to determine inheritance patterns. All candidate variants were confirmed by Sanger sequencing with the ABI 3130xl Genetic Analyzer (Life Technologies) with standard methods. Sequence analysis software version 6.0 (Applied Biosystems, Foster City, CA, USA) was used to analyze all sequence data. Primer sequences for validating each variant are available in Supplementary Table S3.

Genetic association analysis

The effective sample size and statistical power was computed using the web browser program, Genetic Power Calculator, developed by Purcell *et al.* (http://pngu.mgh.harvard.edu/~purcell/gpc/).[52] An ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and TaqMan assays with custom probes were used to genotype a putative deleterious variant. Custom probe sequences are indicated in Supplementary Table S4. Each 384-microtiter plate contained two non-template controls and two samples with the variant. The reactions and data analysis were performed using Genotyping Master Mix and Sequence Detection Systems, respectively, according to standard protocols (Applied Biosystems). Differences in genotype distribution between cases and controls were tested with one-sided, Fisher's exact tests.

Phenotypic analysis

The clinical features of patients with the variant that was possibly associated with SCZ and ASD phenotypes based on genetic association analysis were examined retrospectively from medical records. All comorbidities were diagnosed by experienced psychiatrists according to DSM-5 criteria.

Modeling of the 3D structure of CX3CR1-Ala55Thr

A 3D structure of CX3CR1 could be modeled using a homology modeling technique, because many 3D structures of members of the GPCR family have been solved recently. The 3D structure of CCR2 isoform B[53] was chosen as the template for this modeling (PDBcode: 5t1a), as this structure was the closest homolog to CX3CR1 that was found by the BLAST program in the 3D structural database.[54] The sequence identity between CX3CR1 and CCR2 isoform B is about 46%. The template structure and the BLAST alignment were downloaded from the HOMCOS server,[55] and manually modified to remove the region of T4 lysozome in the structure. The program MODELLER 9.16 was used for the modeling.[56] Bound structures of membrane, fractalkine and heterotrimeric G protein were modeled by 3D superimpositions. 3D structures of GPCR in complexes were superimposed

on the CX3CR1 model using the program MATRAS,[57] and bound molecules in the complexes were also transformed with their GPCRs. The membrane model used was taken from a molecular dynamics study of GPCRs.[58] The fractalkine structure was obtained from a complex of fractalkine with a viral GPCR (PDBcode: 4xt1).[59] The structure of a heterotrimeric G protein (Gα, Gβ, Gγ) was taken from a G protein complex with the β2 adrenergic receptor (PDBcode: 3sn6).[60]

Functional analysis of CX3CR1-Ala55Thr; effect on Akt phosphorylation-mediated signaling

The cDNA of human CX3CR1 was amplified from the IMAGE clone 5216452, and was inserted into the pmCherry vector (Clontech, Palo Alto, CA, USA) using Bgl2 and EcoR1. For the construction of pmCherry-Cx3cr1 Ala55Thr, site-specific mutagenesis was carried out using the KOD-Plus-Mutagenesis Kit (TOYOBO, Shiga, Japan) with pmCherry-Cx3cr1 wild-type (WT) as a template. The following primers were used for mutagenesis: (1) forward 5′-GTGTTTACCCTCACCAACAGCAAGA-3′ and (2) reverse 5′-TACCAACAAATTTCCCACCAGGCCA-3′.

Cells of the human embryonic kidney cell line, HEK293, were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 50 U penicillin–streptomycin at 37 °C with 5% CO_2 inside a humidified incubator. Cells were transiently transfected with the pmCherry-Cx3cr1 WT or Ala55Thr plasmid by using Lipofectamine 2000 (Invitrogen), and cultured for 36 h. Then, the cells were fixed with 4% paraformaldehyde for 30 min and washed with PBS. Subsequently, the cells were mounted with a fluorescence mounting medium (DakoCytomation, Glostrup, Denmark). Images were acquired on a microscope (BX51; Olympus, Tokyo, Japan) equipped with a sCMOS camera (Zyla; Andor Technology, Belfast, UK).

For western blotting, the cells were transfected with the pmCherry-Cx3cr1 WT or Ala55Thr plasmid, cultured for 36 h, and then stimulated with fractalkine (R&D systems, Minneapolis, MN, USA) for 15 min. The cell lysates were boiled in sample buffer for 5 min. The proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBS-T) and incubated for 1 h at room temperature with the primary antibody diluted in PBS-T containing 1% non-fat dry milk. After washing in PBS-T, the membrane was incubated with an HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA, USA). The ECL chemiluminescence system (GE Healthcare, Waukesha, WI, USA) was used for signal detection. Signals were detected using the Amersham Imager 600 (GE Healthcare), and were quantified using Image J software (NIH, Bethesda, MD, USA, in public domain software).

RESULTS

Screening of variation in CX3CR1 coding exons

Nucleotide sequence data have been submitted to the DNA Data Bank of Japan (DDBJ) databases (http://www.ddbj.nig.ac.jp) under the accession number DRA004490. We identified three rare missense heterozygous variants within *CX3CR1* coding regions in genomic DNA isolated from Japanese SCZ or ASD patient samples ($n = 562$). All of the single nucleotide variants (SNVs) were validated by Sanger sequencing (Figure 1; Table 1). Nonsense variants, frameshift variants and splicing-site variants were not found. Genomic DNA of the parents was available for three of seven subjects carrying these three rare missense variants. In these three pedigrees, the variants were found to be transmitted from a parent (Supplementary Figure S1). None of SNVs detected in our study were registered in ClinVar nor in the DECIPHER database.

Genetic association of CX3CR1-Ala55Thr with SCZ and ASD

Of these three SNVs, we focused on the Ala55Thr variant because it was detected in two unrelated cases in a 562-patient cohort and was present in only one of the 60,360 Exome Aggregation Consortium (ExAC) cohort (http://exac.broadinstitute.org) (Table 1), whereas the Gly112 Ala variant was registered in Japanese databases: the HGVD (http://www.genome.med.
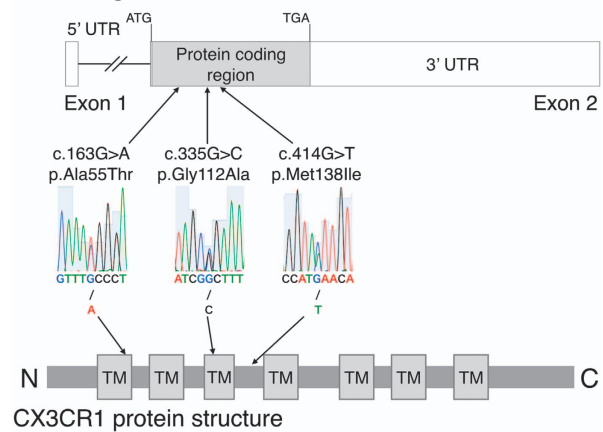

Figure 1. Genomic locations of the variants in *CX3CR1* detected in this study. *Note*: Localization of a protein domain is based on the human protein reference database (http://www.hprd.org/index.html) (HPRD). Genomic position is based on NCBI builds GRCh38 (Ensembl Transcript ID ENST000000399220). Amino acid position is based on the NCBI reference sequences NP_001328.1 and DDBJ DRA004490. UTR, untranslated regions.

kyoto-u.ac.jp/SnpDB/) and the integrative Japanese Genome Variation Database (iJGVD) (https://ijgvd.megabank.tohoku.ac.jp)[60] with approximately the same frequency as that of our cohort, and the Met138Ile variant was found in multiple Asian individuals in ExAC (Table 1).

The effective sample size and statistical power was computed using the web browser program, Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/).[61] For our sample set of cases ($n = 3227$) and controls ($n = 3827$), we computed a statistical power of $>80\%$ using the following parameters: disease prevalence of 0.01; observed rare-allele frequency of 0.0018; odds ratio for dominant effect of $\geqslant 2.3$; and type I error rate of 0.05. Differences in genotype distribution between cases and controls were tested with one-sided, Fisher's exact tests. A significant association between neurodevelopmental disorders (SCZ and ASD) and Ala55Thr (Odds ratio = 8.3, $P = 0.020$) was observed (Table 2; Supplementary Table S1). The clinical features of the five SCZ and two ASD cases, and the one healthy control case with CX3CR1-Ala55Thr are shown in the Supplementary Table S2.

Modeling of the 3D structure of CX3CR1-Ala55Thr

These genetic findings indicated the possibility that the CX3CR1-Ala55Thr variant might associate with the pathogenesis of SCZ and ASD. We therefore preformed 3D structural modeling of CX3CR1 including a membrane, the CX3CR1 ligand fractalkine, and a heterotrimeric G protein (Figure 2a). As seen for other GPCR proteins, the CX3CR1 model has seven transmembrane (TM) helices that pass through the cell membrane, and a small amphipathic α-helix called 'helix 8' in the C-terminal cytoplasmic region. Helix 8 is reported to be important for signal transduction and interacts with the heterotrimeric G protein and other diverse signaling molecules.[61,62] Ala55 is located in TM1; however, it interacts with the helix 8 and not with the membrane. Several non-polar residues of TM1 and helix 8 form a hydrophobic core: Leu51, Val52, and Ala55 of TM1, and Phe300, Tyr303, Leu304, and Leu307 of helix 8 (Figure 2b). The variant of Ala to Thr in which residue 55 is changed from a hydrophobic (alanine) to a hydrophilic residue (threonine) may weaken the hydrophobic interaction between TM1 and helix 8, and destabilize the conformation of helix 8 (Figures 2b and c). This conformational

Table 1. CX3CR1 variants identified in this study

Chr	Position (dbSNPID)	Transcript variant	Amino acid variant	SCZ (n = 370)	ASD (n = 192)	Our cohort[a] MAF	HGVD[a] MAF	iJGVD[a] MAF	ExAC (Asian)[a] MAF	ExAC (all)[a] MAF	1000 Genomes[a] MAF	In silico analysis	
												PolyPhen-2	MutationTaster
3	39266096 (rs758302878)	c.414G>T	Met138Ile	1F	—	1/1122 8.9×10^{-4}	—	—	6/8626 7×10^{-4}	6/120 720 5×10^{-5}	—	Benign	Disease causing
3	39266175 (rs201442030)	c.335G>C	Gly112Ala	1F, 1M	2M	4/1112 3.6×10^{-3}	2/1102 1.8×10^{-3}	6/4084 1.5×10^{-3}	28/8624 3.2×10^{-3}	36/120 712 3×10^{-4}	1/1008 1×10^{-3}	Probably Damaging	Polymorphism
3	39266347 (rs750585901)	c.163G>A	Ala55Thr	1M	1M	2/1122 3.6×10^{-3}	—	1/4076 2.5×10^{-4}	1/8626 1.2×10^{-4}	1/120 768 8.3×10^{-6}	—	Benign	Disease causing

Abbreviations: 1000 Genomes, the 1000 Genomes Project (http://www.1000genomes.org); ASD, autism spectrum disorders; Chr, chromosome; dbSNP, dbSNP build 149 (http://www.ncbi.nlm.nih.gov/projects/SNP/); ExAC, Exome Aggregation Consortium (http://exac.broadinstitute.org); F, female; HGVD, the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB/); iJGVD, the integrative Japanese Genome Variation Database (https://ijgvd.megabank.tohoku.ac.jp); M, male; MAF, minor allele frequency; SCZ, schizophrenia. [a] Minor allele count/total allele count. Note: Genomic position based on NCBI builds GRCh38 (Ensembl Transcript ID ENST00000399220). Amino acid position is based on NCBI reference sequence NP_001328.1.

change of helix 8 may then disturb the interaction with Gα and downstream signaling pathways.

Functional analysis of the effect of CX3CR1-Ala55Thr on Akt phosphorylation

We then investigated the effect of CX3CR1-Ala55Thr on CX3CR1 signaling. We first generated expression vectors encoding CX3CR1 WT or the Ala55Thr mutant conjugated with mCherry, and assessed their subcellular localization in HEK293 cells. There were no obvious differences in subcellular localization between CX3CR1 WT and its Ala55Thr mutant (Figure 3a). We then investigated the effects of Ala55Thr mutation on CX3CR1 signaling in HEK293 cells. It is well established that the chemokine fractalkine activates the Akt pathway through CX3CR1.[39,41,63] HEK293 cells were used for the present study because it has been reported that the level of fractalkine binding with non-transfected HEK293 cells is very low.[64] Overexpression of CX3CR1 WT in HEK293 cells increased the phosphorylation level of Akt in the presence of fractalkine. However, treatment with fractalkine did not induce Akt phosphorylation when the cells were transfected with the CX3CR1-Ala55Thr mutant (Figure 3b). These results suggest that CX3CR1-Ala55Thr mutation inhibits fractalkine-CX3CR1 signaling.

DISCUSSION

Our results of the genetic association of the Ala55Thr variant in CX3CR1 with both SCZ and ASD; the conformational change in the CX3CR1-Ala55Thr mutant compared with CX3CR1 WT that was predicted by in silico 3D structural modeling; and the down-regulation of fractalkine-CX3CR1 signaling by the Ala55Thr mutant in vivo support the hypothesis that this variant in CX3CR1 might be a plausible candidate causal variant in SCZ and ASD etiopathologies. Rare variants with a large effect may explain a part of the missing heritability.[9] In this regard, we detected one condition-related SNV through targeted resequencing of coding exons in CX3CR1 for 562 Japanese SCZ or ASD patients and subsequent genetic association analysis in a sample comprising 7054 unrelated individuals. A statistically significant association was found between Ala55Thr and SCZ, ASD and both phenotypes (odds ratio = 7.2, 13.1 and 8.3, respectively) (Table 2; Supplementary Table S1). Inheritance for cases with Ala55Thr was either from an unaffected father to son or of unknown origin (Supplementary Figure S1), suggesting variable penetrance. This finding is consistent with recent genetic studies; deleterious alleles are likely to be especially rare because of purifying selection.[9] Inherited-truncating variants especially in genes that are closely involved in neurodevelopment are highly enriched in patient populations.[13,65–68] Thus CX3CR1-Ala55Thr could increase susceptibility to the development of a neuropsychiatric disorder.

Modeling of the 3D structure of the CX3CR1 WT and its Ala55Thr mutant suggested that the Ala55Thr mutation might destabilize the hydrophobic interaction between TM1 and helix 8. The canonical mechanism of signal transduction initiated by GPCRs involves the activation of a heterotrimeric G protein by an agonist-occupied receptor.[60,63] In addition to the seven transmembrane helices, conformational dynamics of helix 8 in the cytoplasmic C terminus has been suggested to have an important role in its intracellular signal transduction.[61,62] Functional analysis using in vitro assays indicated that the Ala55Thr variant in CX3CR1 might decrease the fractalkine-CX3CR1 signaling. Its signaling impairment has been reported to influence microglial function.[69,70] On the basis of the complementary expression of fractalkine on neurons and CX3CR1 on microglia, it has been proposed that the neuron signaling to microglia is mediated through the CX3CR1 receptor.[31] Fractalkine-CX3CR1 signaling might limit microglia toxicity through induction of PI3K/Akt and ERK1/2 phosphorylation.[45,71–73] As there are no differences in

Table 2. Genetic association analysis of CX3CR1-Ala55Thr

Exon	Ref	Val	Position	Variant	Case (SCZ+ASD)				Control	
					Allele count[a]	MAF	Odds ratio	P-value[b]	Allele count[a]	MAF
2	G	A	3:39266347	p.Ala55Thr	7/6454	0.0011	8.3 (1.33–inf)	0.020	1/7652	0.00013

Abbreviations: ASD, autism spectrum disorders; MAF, minor allele frequency; Ref, reference; SCZ, schizophrenia; Val, variant. [a] Minor allele/major allele. [b]P values were calculated by one-tailed Fisher's exact test. *Note*: Genomic position is based on NCBI builds GRCh38. Amino acid position is based on NCBI reference sequence NP_001328.1.
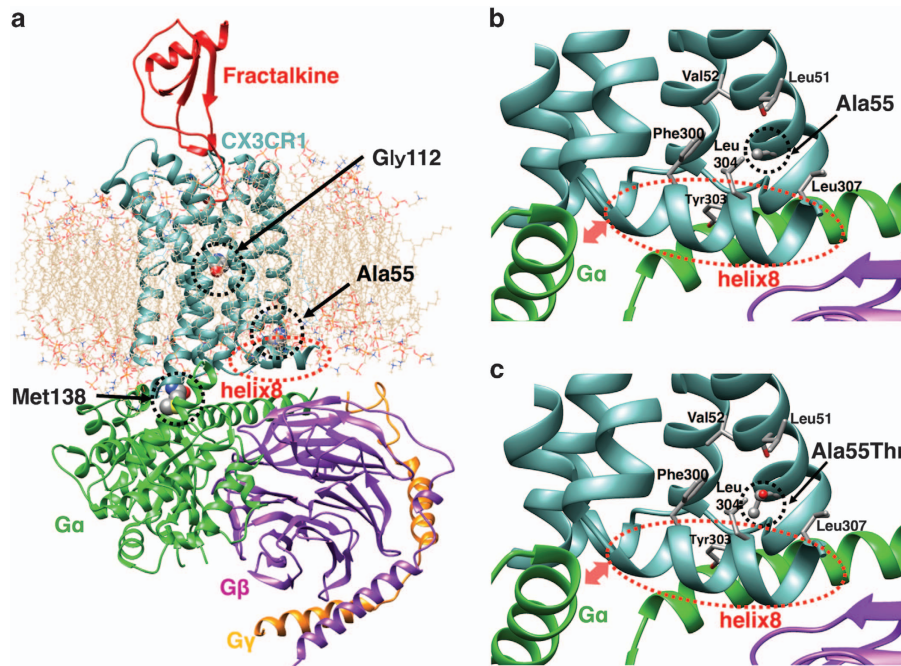
Figure 2. 3D model of the structure of the CX3CR1-Ala55Thr mutant. (**a**) 3D model of the structure of CX3CR1, fractalkine, a heterotrimeric G protein (Gα, Gβ, Gγ) and the cellular membrane. Three CX3CR1-mutated sites (Ala55, Gly112, Met138) are indicated in dotted black circles. (**b**) Enlarged view of the 55th residue of wild-type CX3CR1. (**c**) Enlarged view of the 55th residue of the CX3CR1-Ala55Thr mutant.

fractalkine and CX3CR1 expression in brain tissue between patients with and without neurodegenerative diseases,[69] it is conceivable that functional change in fractalkine-CX3CR1 signaling could contribute to the pathophysiology of neurodevelopmental disorders by modulating microglial activation.

There are several limitations of this study. First, although the data presented here support the hypothesis that rare variants in *CX3CR1* contribute to the neurodevelopmental disorders of SCZ and ASD, it is clear that multiple pathways regulate synapse connectivity in the brain, which account for complex genetic influences on SCZ and ASD pathogenesis. Of note, the Ala55Thr variant in *CX3CR1* was present in an extremely small fraction (< 0.5%) of the tested patients with neurodevelopmental disorders. The idea of performing a study that focuses on coding variants of a gene in terms of their contribution to a disease has been supported by a recent finding that rare and low-frequency coding variants contribute to the genetic architecture of a complex trait;[11] however, the promoter, untranslated regions or intronic regions of *CX3CR1* that our variation screening did not cover, also potentially contain disease-associated regions. Second, regarding the genotype–phenotype evaluations, we could neither fully explore the impact of the novel variant nor monitor variant segregation due to limited access to the detailed clinical phenotypes of patients and to lack of DNA from patient family members. Although the father carrying CX3CR1-Ala55Thr had neither history of mental disorders nor symptom causing clinically significant impairments in social, occupational or other important areas of current functioning, possibility that the father might present personality traits, which have been indicated as the 'Broader Autism Phenotype' still remains.[74] Careful assessments of proband and family members will provide insight into the underlying genetic mechanisms and determine the impact of rare *CX3CR1* variants on psychopathology. Finally, we only demonstrated a reduction in the Akt phosphorylation-mediated signaling by CX3CR1-Ala55Thr using HEK293 cells. Additional factors produced by microglia may ameliorate the disturbed function of CX3CR1 *in vivo*. Further analysis performed in microglia will be needed to assess the exact molecular mechanisms and networks affected by *CX3CR1* variants in SCZ and ASD.

In conclusion, individuals with the Ala55Thr variant of *CX3CR1* might increase the susceptibility to developing the neurodevelopmental disorders such as SCZ and ASD. To our knowledge, our findings provide the first genetic evidence in a microglia-specific gene for association with neurodevelopmental disorders. A deeper understanding of genetic risk factors and disease pathobiology may lead to major health benefits through the
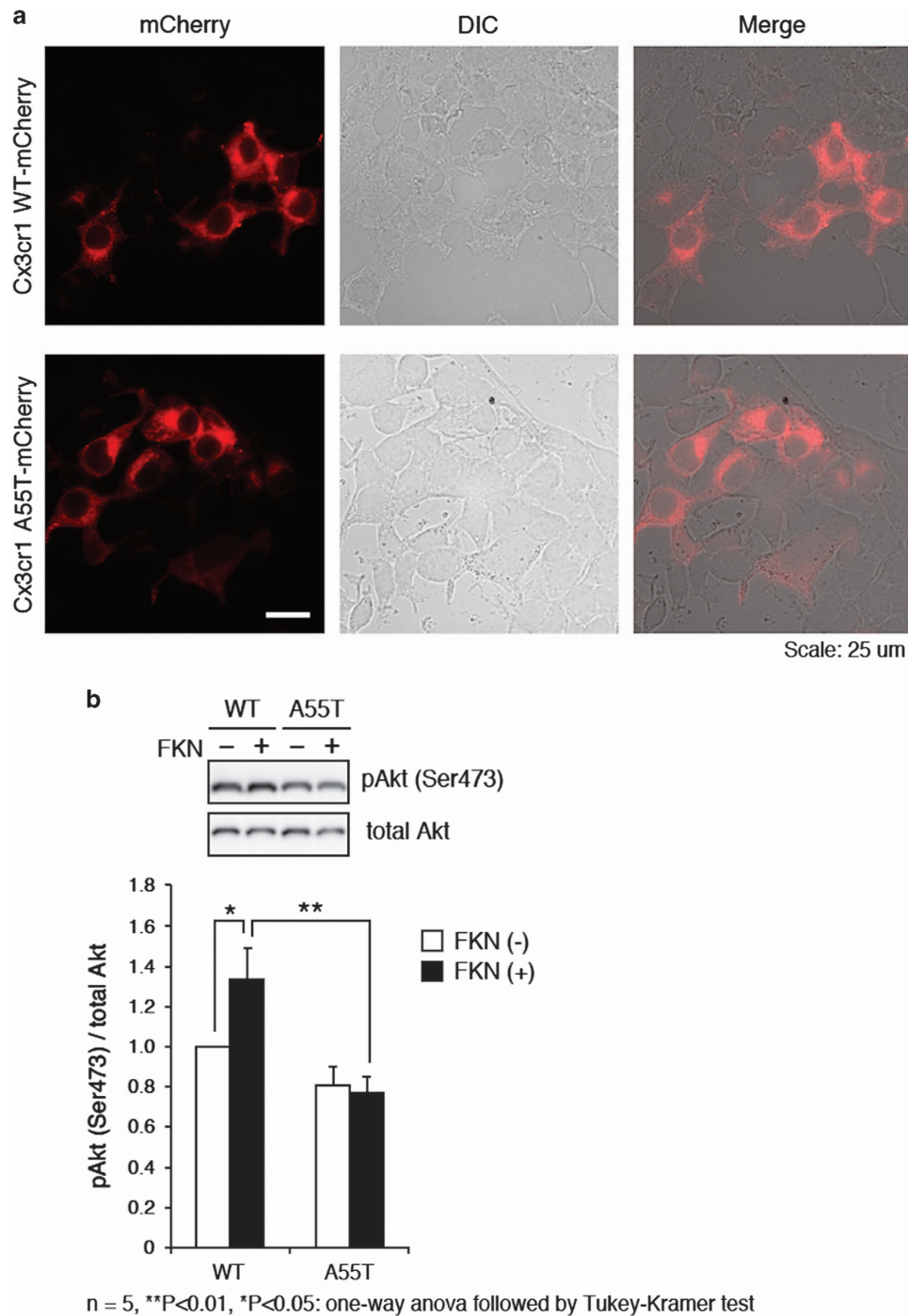
Figure 3. Effect of CX3CR1-Ala55Thr on Akt phosphorylation-mediated signaling. (**a**) Subcellular localization of CX3CR1 wild-type (WT) or its Ala55Thr mutant. mCherry-fused CX3CR1 WT or mutant-expressing vectors were transfected into HEK293 cells and cultured for 36 h before immunofluorescent analysis. DIC, differential interference contrast. Scale bar, 25 μm. (**b**) Ala55Thr mutation in CX3CR1 inhibits Akt phosphorylation upon fractalkine (FKN) treatment. HEK293 cells transfected with an mCherry-conjugated CX3CR1 WT or Ala55Thr-expressing vector were cultured for 36 h, and then the cells were stimulated with the CX3CR1 ligand fractalkine for 15 min. Cell lysates were analyzed by western blotting using the indicated antibodies. The data are expressed as means ± s.e.m. and statistical significance was tested with analysis of variance followed by Tukey–Kramer's multiple comparison test. **$P < 0.01$, *$P < 0.05$. $n = 5$.

development of methods for the prevention, diagnoses and treatment of such diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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