BRAIN
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Functional variant rs2270363 on 16p13.3 confers schizophrenia risk by regulating *NMRAL1*

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Recent genome-wide association studies have reported multiple schizophrenia risk loci, yet the functional variants and their roles in schizophrenia remain to be characterized. Here we identify a functional single nucleotide polymorphism (rs2270363: G>A) at the schizophrenia risk locus 16p13.3. rs2270363 lies in the E-box element of the promoter of *NMRAL1* and disrupts binding of the basic helix–loop–helix leucine zipper family proteins, including USF1, MAX and MXI1.

We validated the regulatory effects of rs2270363 using reporter gene assays and electrophoretic mobility shift assay. Besides, expression quantitative trait loci analysis showed that the risk allele (A) of rs2270363 was significantly associated with elevated *NMRAL1* expression in the human brain. Transcription factors knockdown and CRISPR-Cas9-mediated editing further confirmed the regulatory effects of the genomic region containing rs2270363 on *NMRAL1*. Intriguingly, *NMRAL1* was significantly downregulated in the brain of schizophrenia patients compared with healthy subjects, and knockdown of *Nmral1* expression affected proliferation and differentiation of mouse neural stem cells, as well as genes and pathways associated with brain development and synaptic transmission. Of note, *Nmral1* knockdown resulted in significant decrease of dendritic spine density, revealing the potential pathophysiological mechanisms of *NMRAL1* in schizophrenia. Finally, we independently confirmed the association between rs2270363 and schizophrenia in the Chinese population and found that the risk allele of rs2270363 was the same in European and Chinese populations.

These lines of evidence suggest that rs2270363 may confer schizophrenia risk by regulating *NMRAL1*, a gene whose expression dysregulation might be involved in the pathogenesis of schizophrenia by affecting neurodevelopment and synaptic plasticity.

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Introduction

Schizophrenia affects around 0.5–1% of the general population worldwide and the clinical symptoms of most cases manifest in late adolescence or early adulthood.[1,2] Epidemiological studies show a 2–3-fold increase in mortality rate of patients with schizophrenia,[3] which imposes huge economic and mental burden on the patient's family and society.[4,5] The heritability of schizophrenia was estimated at about 81%,[6] indicating that genetic factors play a dominant role in schizophrenia pathogenesis.[6–8] Although multiple schizophrenia risk loci have been identified by genome-wide association studies (GWASs) in recent years,[9–13] identifying potential pathogenic genetic variants in the reported risk loci and elucidating their roles in schizophrenia remain major challenges in the post-GWAS era.

Most of the disease-associated risk variants are located in non-coding regions,[13] suggesting that they may be involved in the pathogenesis of schizophrenia by influencing transcriptional regulation. Functional genomics studies help to identify risk SNPs with regulatory effects or functional consequences in the reported risk loci.[14,15] In our previous study, we identified 132 schizophrenia risk single nucleotide polymorphisms (SNPs) that disrupt binding of transcription factors.[14] To prioritize and distil the most promising functional variants for further mechanistic dissection and functional characterization, we interrogated these 132 transcription factor binding-disrupting SNPs in detail with a series of analyses, including expression quantitative trait loci (eQTL) analysis, genomic location mapping, RegulomeDB annotation[16] and differential gene expression analysis ('Material and methods' section, Supplementary Fig. 1). Our analyses suggested that rs2270363 [located in the E-box element of the *NMRAL1* promoter and disrupted binding of basic helix–loop–helix leucine zipper (bHLHZ) proteins, including USF1, MAX and MXI1][14,17] may represent a potential causal variant at the schizophrenia risk locus 16p13.3. Elucidating the role and regulatory mechanism of rs2270363 will not only help to demonstrate genetic mechanisms of complex diseases, but also facilitate understanding of the pathophysiology of diseases, bringing opportunities for the discovery of new therapeutic targets and treatments.

To elucidate the role of rs2270363 in schizophrenia pathogenesis, in this study, we systematically characterized the regulatory mechanisms of rs2270363 with a series of analyses and experiments. We firstly confirmed the association between rs2270363 and schizophrenia in the Chinese population. We then validated the regulatory effect of rs2270363 with reporter gene assays and our electrophoretic mobility shift assay (EMSA) demonstrated that rs2270363 affects transcription factors (including USF1) binding. We further showed that the A allele (risk allele) of rs2270363 was significantly associated with elevated *NMRAL1* expression in the human brain. Moreover, we found that rs2270363 and its binding transcription factors (i.e. USF1 and MAX) could regulate *NMRAL1*, a gene whose expression level was significantly downregulated in brains of schizophrenia cases compared with controls. To explore the potential role of *NMRAL1* in schizophrenia pathogenesis, we investigated the role of *NMRAL1* in neurodevelopment (proliferation and differentiation of neural stem cells). Finally, we examined the effect of *NMRAL1* on density of dendritic spines. Our study demonstrated that the functional risk variant rs2270363 (located in the *NMRAL1* promoter) might contribute to schizophrenia susceptibility by regulating expression of *NMRAL1*, a gene whose expression downregulation affected neurodevelopment and density of dendritic spines (Supplementary Fig. 2).

Materials and methods

Expression quantitative trait loci analysis

We explored whether the 132 identified transcription factor binding-disrupting SNPs were associated with gene expression in the human brain using eQTL data from the CommonMind Consortium (CMC) and Lieber Institute for Brain Development (LIBD).[14,18,19] Briefly, brain tissues (the dorsolateral prefrontal cortex) of 467 subjects (CMC) and 412 subjects (LIBD) were used for eQTL analysis and detailed information can be found in the original publication.[18,19]

Mapping of SNP locations

We then inspected the genomic locations of these 97 SNPs, as previous studies have demonstrated the pivotal roles of functional variants in the promoter region (a core regulatory region for gene expression) in disease susceptibility. For example, a functional variant in the promoter of *MDM2* attenuates the p53 tumour suppressor pathway and accelerates tumour formation,[20] and a functional variant in the microRNA-146a promoter confers risk of systemic lupus erythematosus by modulating microRNA-146a expression.[21] In addition, a functional variant in the *CHRNA1* promoter has also been reported to confer disease risk by regulating *CHRNA1* expression in thymus.[22] SNPs are considered to be located in the promoter region in this study if they meet following two criteria: (i) located 2 kb upstream and 200 bp downstream of the

transcription start site; and (ii) marked with H3K4me3 (according to data from UCSC genome browser). Promoter regions were determined as upstream 2 kb and 200 bp downstream of the transcription start site and marked with H3K4me3 (a marker for active promoters) in UCSC genome browser (http://genome.ucsc.edu/).

RegulomeDB annotation

We used RegulomeDB to further annotate and prioritize the most possible functional SNPs located in promoter regions.[16] RegulomeDB is a well-curated database that annotates SNPs according to multiple high-throughput data sets, including eQTL, chromatin immunoprecipitation sequencing, matched transcription factor motif, matched DNase footprint and so on. RegulomeDB assigns a numerical rank score to each SNP and the rank score reflects the possibility of functionality. The RegulomeDB rank scores range from 1 to 7 (1a–f, 2a–c, 3a, 3b, 4, 5, 6, 7) and SNPs with lower RegulomeDB scores are more likely to be functional.[16] Detailed information about the RegulomeDB scores and supporting data are provided in Supplementary Table 1.

Differential gene expression analysis in schizophrenia cases and controls

We performed expression analysis to explore if the eQTL genes of the five high-confidence functional SNPs (located in promoter regions and having a RegulomeDB score < 2) showed differential expression in schizophrenia cases compared with controls using the expression data from CMC ($n = 258$ cases and $n = 279$ controls).[18]

Schizophrenia cases and controls used for genetic analysis in this study

A total of 3718 schizophrenia cases and 7829 healthy controls were included in this study. Detailed information about the included subjects has been described in our previous studies.[23,24] Briefly, schizophrenia cases were diagnosed according to the *Diagnostic and Statistical Manual of Mental Disorders*, Fourth Edition (DSM-IV) criteria, with the use of Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). The diagnoses were completed independently by at least two experienced psychiatrists. All available information, including age of onset, symptoms, medication and hospitalization history, medical records, interviews with the patient, communications with family members and all other related information were evaluated to reach a consensus diagnosis. Controls were recruited from local volunteers and screened for potential psychiatric disorders. Individuals with a family history of mental disorders, drug and alcohol abuse, head injuries and epilepsy were excluded in this study. The ages of cases and controls were 42 ± 15.75 and 36 ± 9.59 years, respectively; 49.1% of cases and 65.2% of controls were males, respectively. Informed consent was obtained from all participants and this study was approved by the internal review board of Kunming Institute of Zoology, Chinese Academy of Sciences.

Genotyping

Whole venous blood was collected with an anticoagulant vacuum tube. DNA was extracted using the phenol/chloroform method or QIAamp DNA Blood Mini Kit (Qiagen, Cat. No. 51104). Genotyping was performed using the SNaPshot method as previously described.[23,25] Detailed information about genotyping assays is provided in the Supplementary material.

Genetic association analysis and meta-analysis

The association between rs2270363 and schizophrenia was assessed with PLINK software (version 1.07).[26] We performed a meta-analysis (using PLINK) by combining our results with a previously study.[27] In total, 60 136 cases and 86 647 controls were included in the meta-analysis (detailed information about the studies is provided in Supplementary material). A fixed-effect model was used for meta-analysis and a heterogeneity test (I^2 statistic) was performed using the PLINK software.

Cell culture

HEK-293T, SH-SY5Y and SK-N-SH cell lines were purchased from the Kunming Cell Bank (Kunming institute of Zoology, CAS), and cultured in Dulbecco's modified Eagle medium (DMEM) with 10% foetal bovine serum (FBS), 1% penicillin/streptavidin (Gibco, Cat. No. 15140-122). HEK-293T cells are derived from kidney and are mainly used for virus packaging. SH-SY5Y and SK-N-SH cells are neuroblastoma cell lines that are mainly used in the research of brain diseases and cancer.[28–31] The mouse neural stem cells (mNSCs) and rat primary cortical neurons were isolated and cultured as previously described.[23,32–34] No mycoplasma contamination was detected in this study.

Dual-luciferase reporter gene assays

The 861-bp DNA fragments containing rs2270363 (A allele) were amplified with specific primers and were inserted into the pGL3-Enhancer vector or pGL4.11 vector (Supplementary Fig. 3) using the TreliefTM SoSoo Cloning Kit (TSINGKE, Cat. No. TSV-S1). Polymerase chain reaction (PCR)-mediated point mutation was used to obtain the vector containing the G allele of rs2270363. HEK-293T, SH-SY5Y and SK-N-SH cells were used in luciferase reporter gene assays. Luciferase reporter gene assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Cat. No. E1980). More detailed information is provided in the Supplementary material.

EMSA

Nuclear extracts of SH-SY5Y cells were extracted with the nuclear and cytoplasmic protein extraction kit (Beyotime, Cat. No. P0028) and quantified by bicinchoninic acid (BCA) protein assay kit (Pierce, Cat. No. 23227). Single-strand oligonucleotides (35 bp) containing the G and A alleles of rs2270363 were synthesized and the sequences are provided in the Supplementary Table 2. More detailed information about the EMSA is provided in the Supplementary material.

Knockout of the genomic region containing rs2270363

The sgRNA (designed using https://zlab.bio/guide-design-resources; Supplementary Table 3) were cloned into the pSpCas9(BB)-2A-Puro (PX459) V2.0 vector. HEK293T cells (cultured in six-well plates) were transfected with 2.0 μg recombinant plasmid using Lipofectamine™ 3000 (Invitrogen, Cat. No. L3000-015). After transfecting for 48 h, 2 μg/ml puromycin (Sigma, Cat. No. 540222) was added to kill the untransfected cells for 2 days. The selected cells were then re-plated at a low density and allowed to grow for about 7 days. A single colony was picked and plated into 24-well plates for scale-up culture, and the culture medium was replaced every 2 days.

Knockdown experiments

The sequences of short hairpin RNAs (shRNAs) are provided in Supplementary Table 3. The regions targeted by the designed shRNAs are provided in Supplementary Fig. 4. Detailed information about knockdown assays is provided in the Supplementary material.

Overexpression experiments

The coding sequences of human *NMRAL1* was cloned into plenti-CAG-IRES-GFP (Addgene, Cat. No. 69047). 1× FLAG was fused at the 3′-end of *NMRAL1* gene. The primers used for cloning are provided in Supplementary Table 4. Detailed information about knockdown assays is provided in the Supplementary material.

Western blotting

The harvested cells were lysed with Radio-Immunoprecipitation Assay (RIPA) buffer (Thermo Scientific, Cat. No. 89901) containing phenylmethylsulfonyl fluoride (PMSF) buffer (100×; Beyotime, Cat. No. ST506-2), and supernatants were boiled at 95°C for 5 min. Equal amounts of total proteins were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE; SurePAGE™, Bis–Tris, 4–20%, Cat. No. M00657) and transferred onto polyvinylidene difluoride (PVDF) membranes. These membranes were blocked in Protein Free Rapid Blocking Buffer (5×; EpiZyme, Cat. No. PS108) for 30 min, probed with primary antibodies and incubated overnight at 4°C, then incubated with goat anti-rabbit secondary antibodies (Proteintech, Cat. No. SA00001-2, 1:5000) after washing with phosphate-buffered saline with Tween (PBST). The dilutions of the primary antibodies were as follows: FLAG (Sigma, Cat. No. F7425-2mg, 1:1000), β-actin (CST, Cat. No. 13E5, 1:1000).

Quantitative real-time PCR

TRIzol reagents (Life Technologies, Cat. No. 15596018) were used to extract total RNA, and cDNA were synthesized using the PrimeScript RT reagent kit with gDNA Eraser (Takala, Cat. No. RR047B). Quantitative real-time polymerase chain reaction (qPCR) were performed using SYBR-green fluorescence quantification system (TB Green® Premix Ex Taq™ II, Takala, Cat. No. RR820B). Relative mRNA expression levels were normalized to *ACTB* (human) or *Actb* (mouse and rat), and fold change were determined using the $2^{-\Delta\Delta Ct}$ method.[35] All qPCR primer sequences are listed in Supplementary Table 5.

RNA sequencing

Detailed information is provided in the Supplementary material. Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were then performed using Metascape (https://metascape.org).[36]

5-Ethynyl-2′-deoxyuridine proliferation and Cell Counting Kit-8 assays

EdU (5-ethynyl-2′-deoxyuridine) incorporation and Cell Counting Kit-8 (CCK-8) assays were conducted as previously described.[23,37,38] Detailed procedures of EdU and CCK-8 assays are provided in Supplementary material.

Spontaneous differentiation of mouse neural stem cells

The mNSCs were plated into 24-well plates [pre-coated with Laminin (Sigma, 20 µg/ml, Cat. No. L2020-1 mg)] at a density of 2×10^5 cells/well. The next day, proliferation medium was replaced by differentiation medium.[39,40] After differentiating for 72 h, cells were fixed for immunofluorescence staining.

Analysis of density of dendritic spines

Morphologies of dendritic spines were analysed as previously described.[34,41,42] Briefly, rat primary cortical neurons were isolated from brains of embryonic rat (E17.5–E18.5) and cultured for 14–15 days and then were co-transfected with 3.0 µg recombinant pSicoR-Ef1a-mCh-Puro plasmid and 1.0 µg Venus control plasmid using Lipofectamine™ 3000 (Invitrogen, Cat. No. L3000-015). Three days post-transfection, the neurons were fixed with 4% paraformaldehyde (with 4% sucrose) for immunofluorescence staining. NeuronStudio was used for dendritic spine analyses.[34,43] Three subtypes of spines (stubby, thin and mushroom) of the secondary or tertiary dendrites of each neuron were used for analysis (length of 50–125 µm per dendrite).

Immunofluorescence staining

Detailed information about immunofluorescence staining assays and corresponding antibodies is provided in the Supplementary material.

Data availability

The data that support the findings of this study are available in CMC, LIBD, ENCODE, PsychENCODE, UCSC genome browser, SZDB, RegulomeDB, GTEx Portal and the Human Protein Atlas. These data were derived from the following resources available in the public domain: CMC, https://www.nimhgenetics.org/resources/commonmind; LIBD, http://eqtl.brainseq.org/; ENCODE, https://www.encodeproject.org/; PsychENCODE, http://resource.psychencode.org/; UCSC genome browser, http://genome.ucsc.edu/; SZDB, http://www.szdb.org/; RegulomeDB, https://www.regulomedb.org/; GTEx Portal, https://www.gtexportal.org/home/; the Human Protein Atlas, https://www.proteinatlas.org/. Other data can be available from the corresponding author upon reasonable request.

Results

Prioritization of rs2270363 as a potential causal variant

Our functional genomics identified 132 SNPs that disrupted binding of transcription factors,[14] implying that these SNPs are functional or potential causal variants. To further prioritize the most promising and possible functional SNPs from the 132 transcription factor binding-disrupting SNPs, we utilized eQTL data and performed additional analyses (Supplementary Fig. 1).[18] In brief, eQTL analysis distilled 97 SNPs (among the 132 transcription factor binding-disrupting SNPs).[14] SNP location mapping showed that 30 of 97 SNPs are located in the promoter region and marked with H3K4me3 (a marker for active promoters). RegulomeDB functional annotation further prioritized five high-confidence (RegulomeDB rank score <2) functional SNPs in the promoter region, indicating that these SNPs may represent authentic functional variants (Supplementary Table 6). Finally, differential expression of the eQTL genes (Supplementary Table 7) of the five SNPs

(located in the promoter region) were examined in schizophrenia cases and controls.[18] The expression of the eQTL genes of rs223387 did not show significant change in schizophrenia cases and controls (Supplementary Table 8). We noticed that rs2269524 is in high linkage disequilibrium ($r^2 > 0.95$) with rs1801311 (Supplementary Table 9), a functional SNP whose regulatory mechanisms in schizophrenia have been recently reported.[24] Thus, we did not investigate rs1801311 in this study. Two eQTL genes (*FAM109B* and *LINC00634*) of rs2269524, two eQTL genes (*NDUFA2* and *SRA1*) of rs3822346 and two eQTL genes (*NMRAL1* and *CORO7*) of rs2270363 showed differential expression in schizophrenia cases compared with controls (Supplementary Table 8). However, we noted that three SNPs (rs2269524, rs1801311 and rs3822346) are far away from their differentially expressed eQTL genes and are not located in the promoter regions of their differentially expressed eQTL genes (Supplementary Fig. 5), suggesting that they are unlikely to regulate the expression of these genes by affecting the promoter activity. For example, our previous study showed that rs1801311, which is located in the first exon of *NDUFA6*, may confer schizophrenia risk by regulating the expression of the distal gene *NAGA* (an eQTL gene of rs1801311) rather than *NDUFA6*.[24] Interestingly, rs2270363, which is located in the *NMRAL1* promoter region, was associated with *NMRAL1* expression in the human brain, and *NMRAL1* expression was downregulated in schizophrenia cases compared with controls. This suggests that rs2270363 may regulate the expression of *NMRAL1* by modulating promoter activity. These lines of evidence suggested rs2270363 as a plausible causal variant for schizophrenia at the 16p13.3 risk locus.

Confirmation of association between rs2270363 and schizophrenia in the Chinese population

To further confirm the association between rs2270363 and schizophrenia, we performed a genetic association study in a large Chinese sample (3718 cases and 7829 controls). We found that rs2270363 was also significantly associated with schizophrenia in the Chinese population ($P = 3.17 \times 10^{-4}$, $OR_A = 1.11$; Table 1), with the same risk allele (A) as in Europeans. Of note, the *P*-value of rs2270363 in PGC2 (34 241 cases, 45 604 controls and 1235 parent affected-offspring trios) was 3.33×10^{-5} ($OR_A = 1.05$),[13] and in EAS + EUR (56 418 cases and 78 818 controls), the *P* was 4.59×10^{-6} ($OR_A = 1.05$).[27] Further trans-ancestry meta-analysis (by combing our association result with previously reported associations in Europeans and East Asians) showed that rs2270363 reached genome-wide significance level ($P = 3.98 \times 10^{-8}$, $OR_A = 1.05$, 60 136 cases and 86 647 controls; Table 1).[27] These data provide robust evidence supporting the association between rs2270363 and schizophrenia.

Validation of the regulatory effect of rs2270363 with reporter gene assays and EMSA

Our previous functional genomics study identified rs2270363 as a functional SNP at the 16p13.3 schizophrenia risk locus (Fig. 1A and

B).[14] DNase-Seq data showed that rs2270363 is located in open chromatin regions, with histone H3K4me3 modification (a marker for active promoters; Fig. 1B). Consistently, the genomic region containing rs2270363 was also marked with H3K4me3 modification in primary cultured neuronal cells derived from olfactory neuroepithelium (CNON).[17] In addition, the genomic region containing rs2270363 was also marked with H3K4me3 (a marker for promoter) and H3K27ac (a marker for enhancer) signals in human microglia, neurons and oligodendrocytes (Supplementary Fig. 6).[44] Data from ENCODE showed that rs2270363 is located in the promoter region (marked with H3K4me3) in multiple human brain tissues and neural cells (including neuroblastoma cell lines; Supplementary Figs 7 and 8).[45] These results indicated that rs2270363 is located in an active regulatory region (marked with an active promoter H3K4me3 signal), implying the potential functionality of rs2270363. Further analysis showed that rs2270363 lies in the E-box motif (CANNTG).[46] USF1, MAX and MXI1 are members of the bHLHZ protein family, which bind to the E-box sequences.[46–49] Of note, ChIP-Seq data clearly showed that transcription factors MAX, USF1 and MXI1 could bind to the genomic region containing rs2270363 in neural cells (Fig. 1B), and motif analysis revealed that rs2270363 disrupts binding of USF1, MAX and MXI1 (Fig. 1C–E). These data suggested that rs2270363 is a functional SNP with potential regulatory effects (through affecting binding of TFs).

As rs2270363 is located in the E-box element of *NMRAL1* promoter (Fig. 1F), to further validate whether rs2270363 plays a role in transcriptional regulation, we performed dual-luciferase reporter assays. We found that the genomic sequence (861 bp) containing rs2270363 exhibited a strong promoter activity in the SH-SY5Y (Fig. 1G and H), HEK-293T and SK-N-SH cell lines (Supplementary Fig. 9), and the A (risk) allele of rs2270363 conferred significant higher activity compared with the G allele (Fig. 1G and H). These results confirmed that rs2270363 is a functional SNP with regulatory effect.

We next performed EMSA to test if rs2270363 affects transcription factor binding. The A allele has one binding band while the G allele has two binding bands (Fig. 1I), and we also found that the G allele prefers to bind the transcription factor of band 2. These data indicated that different alleles of rs2270363 could bind to different transcription factors (arrowhead in the last lane in Fig. 1I). The result of the competitive experiment also indicated significant differences in binding affinities between the two alleles, i.e. the transcription factor in the binding band 2 prefers to bind the G allele (Fig. 1J). Finally, the super-shift assay showed that the transcription factor in the binding band 2 is USF1 (Fig. 1J), as a super-shift band was detected in the electrophoresis lane with anti-USF1 antibody (SS USF1, arrowhead on the right side of Fig. 1J). Interestingly, sequence conservation analysis showed that the regulatory element containing rs2270363 is not highly conserved in vertebrates (Supplementary Fig. 10), suggesting this regulatory event (in terms of regulation of *NMRAL1*) is primate-specific [as no orthologous sequences (corresponding to the genomic sequence containing rs2270363 in human) were identified in many vertebrate species (Supplementary Fig. 10)]. Taken together, these results validated that rs2270363 (which resides in the E-box binding motif of *NMRAL1* promoter) is a functional SNP and different alleles of rs2270363 could affect USF1 binding.

rs2270363 is associated with NMRAL1 expression in the human brain

We have validated the regulatory effect of rs2270363 and demonstrated the regulatory mechanisms of this functional SNP. To

Table 1 rs2270363 is associated with schizophrenia

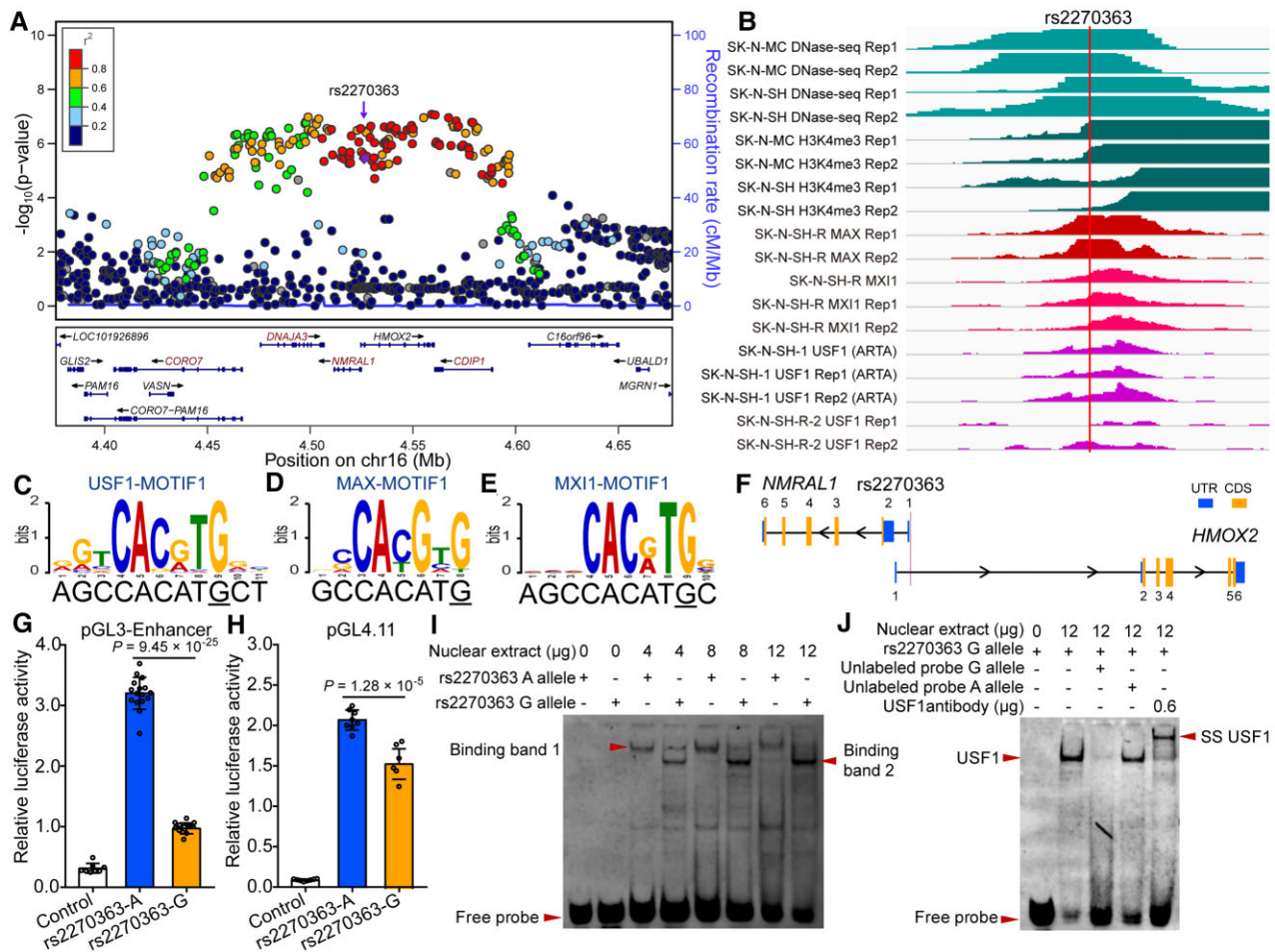| SNP ID	Sample	Sample size (cases/controls)	A$_{12}$	OR[a]	P
rs2270363	This study	3718/7829	A/G	1.11	3.17×10^{-4}
	European	33 640/43 456	A/G	1.05	1.35×10^{-4}
	East Asian	22 778/35 362	A/G	1.05	1.07×10^{-2}
	Combined	60 136/86 847	A/G	1.05	3.98×10^{-8}

[a]Odds ratio (OR) is based on A$_1$.

Figure 1 Validation of regulatory effects of rs2270363 at the 16p13.3 risk locus. (**A**) The Locus zoom plot showed the associations between variants in genomic region (300 kb) containing rs2270363 and schizophrenia in the PGC2 + CLOZUK study. (**B**) ChIP-Seq data showed that transcription factors USF1, MAX and MXI1 could bind to genomic sequence containing rs2270363 (data from ENCODE). (**C–E**) rs2270363 disrupts binding motif (i.e. position weight matrix, PWM) of USF1, MAX and MXI1. (**F**) SNP rs2270363 is located in the promoter of *NMRAL1* gene. (**G** and **H**) Dual-luciferase reporter gene assays showed that rs2270363 affected promoter activity in SH-SY5Y cells regardless of its orientation. The vector containing A allele of rs2270363 exhibited significant higher luciferase activity compared with G allele. (**I**) EMSA showed that rs2270363 affected binding of transcription factors. One binding band (band 1, marked with arrowhead) was detected for the A allele, while two binding bands (band 1 and 2) were detected for the G allele. (**J**) Super-shift and competitive experiments for EMSA. Super-shift assay showed that rs2270363 binds to USF1 (the super-shift band was detected in the lane with added USF1 antibody) and competitive experiments revealed that the G allele had stronger binding affinity compared with the A allele. $n = 8$ for control group in **G**, $n = 16$ for experimental group in **G**, $n = 8$ for **H**. Unpaired two-tailed Student's *t*-test was used to test if the difference reached significance level ($P = 0.05$). Data are presented as mean ± SD.

further identify the potential target gene of rs2270363, we conducted eQTL analysis and found that rs2270363 was significantly associated with the expression of *CORO7*, *DNAJA3*, *NMRAL1* and *CDIP1* (Fig. 2A and B) in the human brain (CMC eQTL dataset).[18] The A allele of rs2270363 is associated with higher expression of *DNAJA3*, *NMRAL1* and *CDIP1* and with lower expression of *CORO7* in CMC data set (Fig. 2C–F). These eQTL results were successfully replicated in the LIBD brain eQTL data set (Supplementary Fig. 11A– E).[19] These results suggest that rs2270363 might confer schizophrenia risk by regulating *CORO7*, *DNAJA3*, *NMRAL1* and *CDIP1*.

rs2270363 regulates NMRAL1 expression

To further investigate if rs2270363 could regulate expression of its eQTL genes (i.e. *NMRAL1*, *CORO7*, *DNAJA3* and *CDIP1*), we deleted a 9-bp genomic sequence containing rs2270363 using CRISPR/ Cas9-mediated genome editing. Due to the low transfection efficiency of SH-SY5Y, we failed to obtain edited (i.e. rs2270363

deletion) SH-SY5Y monoclonal cells. However, we successfully knocked out a 9-bp fragment (containing rs2270363) in HEK-293T cells (Fig. 3A). We also examined whether the expression of four eQTL genes of rs2270363 varied in tissues or cell types, and found that *NMRAL1*, *CORO7*, *DNAJA3* and *CDIP1* are widely and abundantly expressed in different tissues and cell types (Supplementary Figs 12A–D and 13A–D). Importantly, all four genes are expressed in SH-SY5Y and HEK 293 cells (HEK 293T cells are derived from HEK 293 cells, which are human renal epithelial cell lines; Supplementary Fig. 13A–D), suggesting that the regulatory effect of rs2270363 might be similar in HEK293T and SH-SY5Y cells. We found that rs2270363 deletion resulted in a significant increase of *NMRAL1* expression (Fig. 3B). However, expression of *CORO7*, *DNAJA3* and *CDIP* did not show significant change (Fig. 3C, Supplementary Fig. 11F and G). Besides, rs2270363 resides in the first intron of *HMOX2* (Fig. 1F; the genomic sequence surrounding *NMRAL1* promoter overlaps with the first intron of *HMOX2*), which is widely expressed in different tissues and cell types
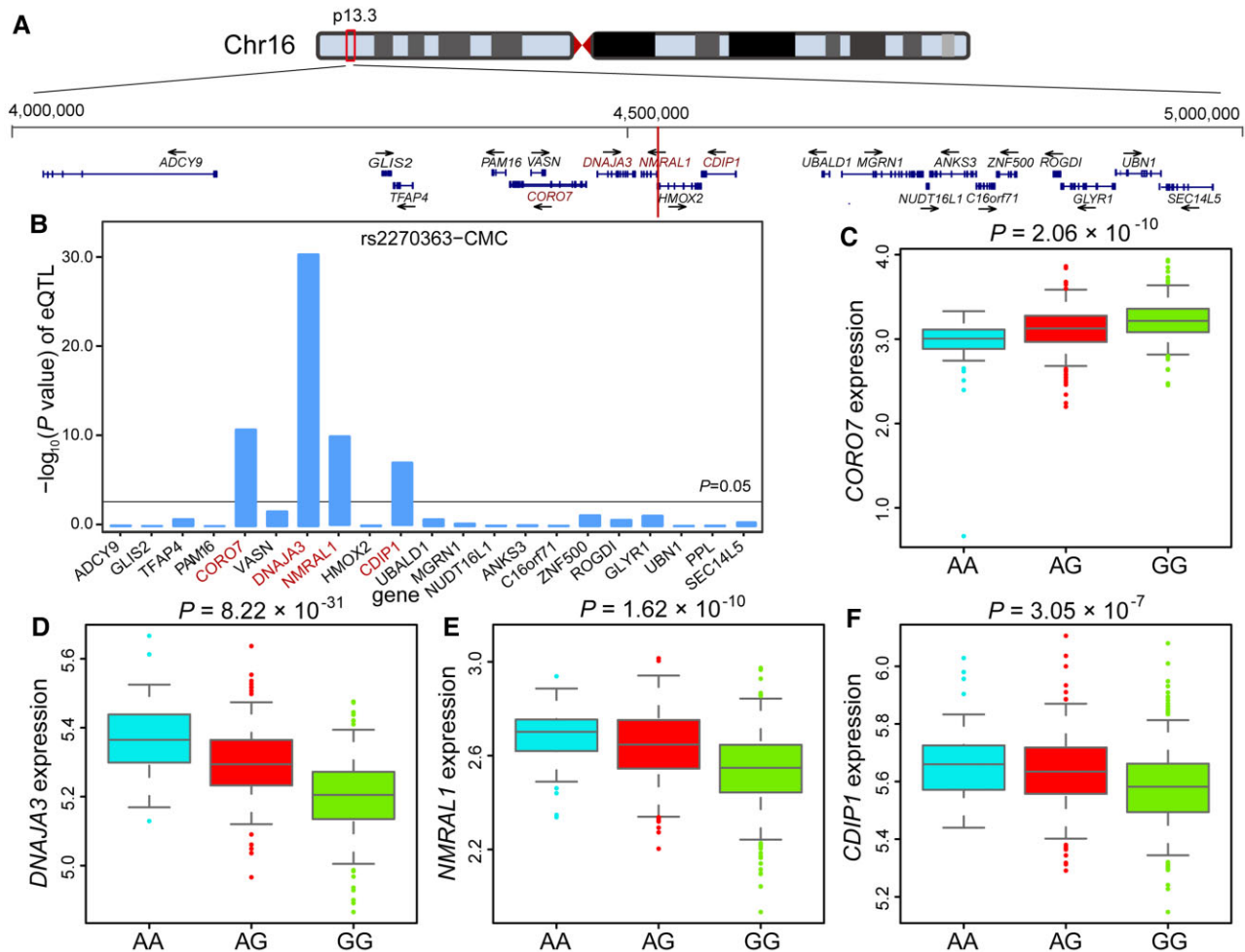
Figure 2 rs2270363 was associated with NMRAL1 expression in the human brain. (A) Genomic location of rs2270363 (1 Mb). rs2270363 lies in the promoter region of *NMRAL1*. (B) eQTL analysis showed the associations between rs2270363 and expression of its nearby genes (1 Mb window) in the human brain (data from CMC, $n = 478$).[18] (C–F) The box-plot of eQTL results. The A allele is associated with lower *CORO7* expression and higher expression of *NMRAL1*, *DNAJA3*, *CDIP1* (data from CMC, $n = 467$).[18]

(Supplementary Figs 12E and 13E). Therefore, we also investigated the effect of rs2270363 deletion on *HMOX2* expression and found that deletion of the genomic sequence containing rs2270363 (9 bp) did not affect *HMOX2* expression level (Supplementary Fig. 11H). These results indicated that rs2270363 can regulate the expression of *NMRAL1* and prioritized *NMRAL1* as the potential target gene of rs2270363.

USF1 and MAX regulate *NMRAL1* expression

To further explore if USF1 and MAX (whose binding were disrupted by rs2270363) regulate *NMRAL1* expression, we knocked down USF1 and MAX in SH-SY5Y cells. Knockdown of USF1 or MAX led to significant downregulation of *NMRAL1* (Fig. 3D–G), indicating that rs2270363 regulates *NMRAL1* expression by disrupting binding of USF1 and MAX (Fig. 1B, I and J).

Dysregulation of *NMRAL1* expression in schizophrenia cases

We have shown that the functional SNP rs2270363 might confer risk of schizophrenia by modulating *NMRAL1* expression. To further explore if *NMRAL1* expression in brains of schizophrenia

cases was dysregulated, we examined *NMRAL1* expression and the result showed that *NMRAL1* was significantly downregulated in brains of schizophrenia cases compared with controls ($P = 3.98 \times 10^{-3}$, 258 cases and 279 controls, CMC expression data from SZDB2.0; Fig. 3H).[50] In addition, we also examined other eQTL genes of rs2270363 (*CORO7*, *DNAJA3* and *CDIP1*). Among these three genes, only *CORO7* showed a significant downregulation in schizophrenia cases compared with controls (Fig. 3I). *DNAJA3* and *CDIP1* did not show significant change (Supplementary Fig. 14). Nevertheless, *CORO7* showed a less-significant level of differential expression ($P = 2.46 \times 10^{-2}$) compared with *NMRAL1* (Fig. 3I), and rs2270363 is located in the *NMRAL1* promoter (but not in *CORO7* promoter). These results collectively prioritized *NMRAL1* as the most plausible candidate risk gene for schizophrenia at the 16p13.3 risk locus.

Integrative analysis supports *NMRAL1* as a schizophrenia risk gene

To further explore if *NMRAL1* is a schizophrenia risk gene, we conducted Sherlock integrative analysis by integrating GWAS summary statistics and brain eQTL data.[18,27,51] Sherlock integrative analysis supported that *NMRAL1* is a schizophrenia risk gene
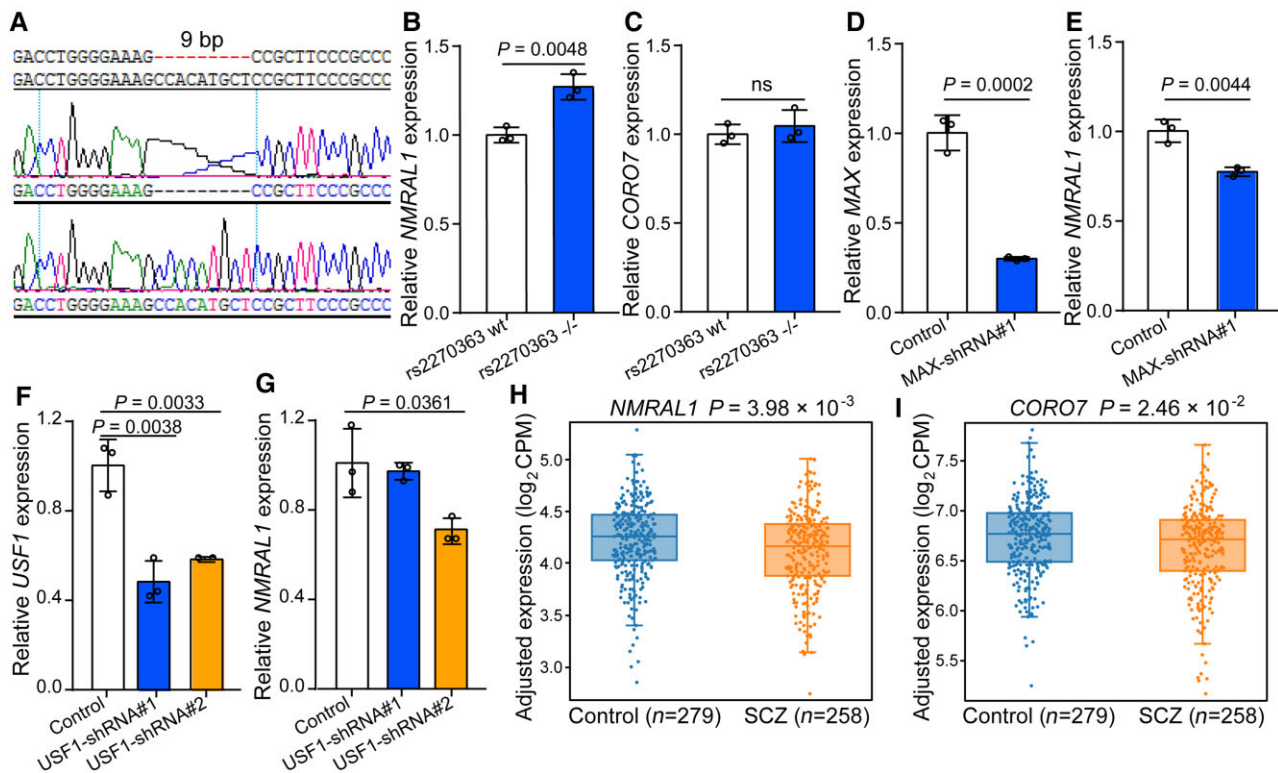
Figure 3 rs2270363 regulates NMRAL1 expression. (**A**) CRISPR-Cas9-mediated deletion of the 9-bp sequence containing rs2270363. (**B**) Deletion of rs2270363 (9 bp) led to upregulation of *NMRAL1*. However, *CORO7* expression was not altered (**C**). (**D** and **E**) MAX knockdown altered *NMRAL1* expression in neuronal cells (SH-SY5Y). (**F** and **G**) USF1 knockdown affected *NMRAL1* expression in neuronal cells. Unpaired two-tailed Student's *t*-test was used to determine if the difference reached significance level. $n = 3$. Data are presented as mean $\pm$ SD. ns = not significant. (**H** and **I**) Expression of *NMRAL1* and *CORO7* showed significant downregulation in the brains of schizophrenia cases compared with controls (http://www.szdb.org/).[50] Schizophrenia cases, $n = 258$; controls, $n = 279$.

whose expression perturbation might have a role in schizophrenia ($P = 1.58 \times 10^{-6}$). In line with this results, *SMR* analysis also showed that *NMRAL1* is a schizophrenia risk gene.[52,53] Collectively, these convergent lines of evidence prioritized *NMRAL1* as the most plausible candidate at the 16p13.3 risk locus.

NMRAL1 affects proliferation and differentiation of mouse neural stem cells

We provided lines of evidence that support *NMRAL1* may be a novel schizophrenia risk gene whose expression is regulated by rs2270363. To further investigate the role of *NMRAL1* in schizophrenia, we utilized the neural stem cell model (a model that is widely used to investigate the role of schizophrenia risk gene in the brain).[54–56] We first validated the identity of the isolated mNSCs using three well-characterized makers for NSCs (PAX6, NESTIN and SOX2; Fig. 4A). We then knocked down *Nmral1* expression in mNSCs using shRNAs (Fig. 4B). EdU incorporation assays showed that the number of EdU-positive cells was significantly increased in *Nmral1* knocked-down mNSCs (Fig. 4C and D). Consistently, CCK-8 assays also revealed that *Nmral1* knockdown promoted the proliferation rate of the mNSCs (Fig. 4E). As the risk allele (A) of rs2270363 was associated with elevated *NMRAL1* expression (Fig. 2E), we also explored the effect of *NMRAL1* overexpression on proliferation of mNSCs (Supplementary Fig. 15A and B). Both EdU and CCK-8 assays showed that *NMRAL1* overexpression inhibited the proliferation rate of the mNSCs (Supplementary Fig. 15C–E). These results demonstrated that *Nmral1* could regulate the proliferation of mNSCs.

In addition, we examined the effect of *Nmral1* on differentiation of mNSCs. Immunofluoorescence results showed that *Nmral1* knockdown affects the differentiation of mNSCs (Fig. 5A–D). The proportion of glial fibrillary acidic protein (GFAP)-positive (a marker for glia cells) cells was significantly decreased in *Nmral1* knocked-down mNSCs compared with controls (Fig. 5A and C). By contrast, the proportion of MAP2-positive (a marker for mature neurons) cells was significantly increased in *Nmral1* knocked-down mNSCs compared with controls (Fig. 5B and D). Using qPCR, we again found that the expression level of *Gfap* was significantly downregulated in *Nmral1* knocked-down mNSCs compared with controls, while the expression of *Map2* and *Tuj1* (a marker for newly generated post-mitotic neurons) were significantly increased (Fig. 5E–G). Finally, we examined the effect of human *NMRAL1* overexpression on differentiation of mNSCs. We found that *NMRAL1* overexpression did not affect differentiation of mNSCs (Supplementary Fig. 16).

Collectively, these results demonstrate the important roles of *Nmral1* in neurodevelopment, a key process that was frequently reported to be dysregulated in schizophrenia.[57–60] Our findings also support the neurodevelopmental hypothesis of schizophrenia.[58,60]

NMRAL1 regulates schizophrenia-related biological processes and signalling pathways

To identify genes and pathways regulated by *Nmral1* (in mNSCs), we performed a transcriptome analysis to determine the differentially expressed genes (DEGs). We identified 991 DEGs (|fold change| > 1.5, $P_{adj} < 0.01$). Among them, ~45% (441 genes) were
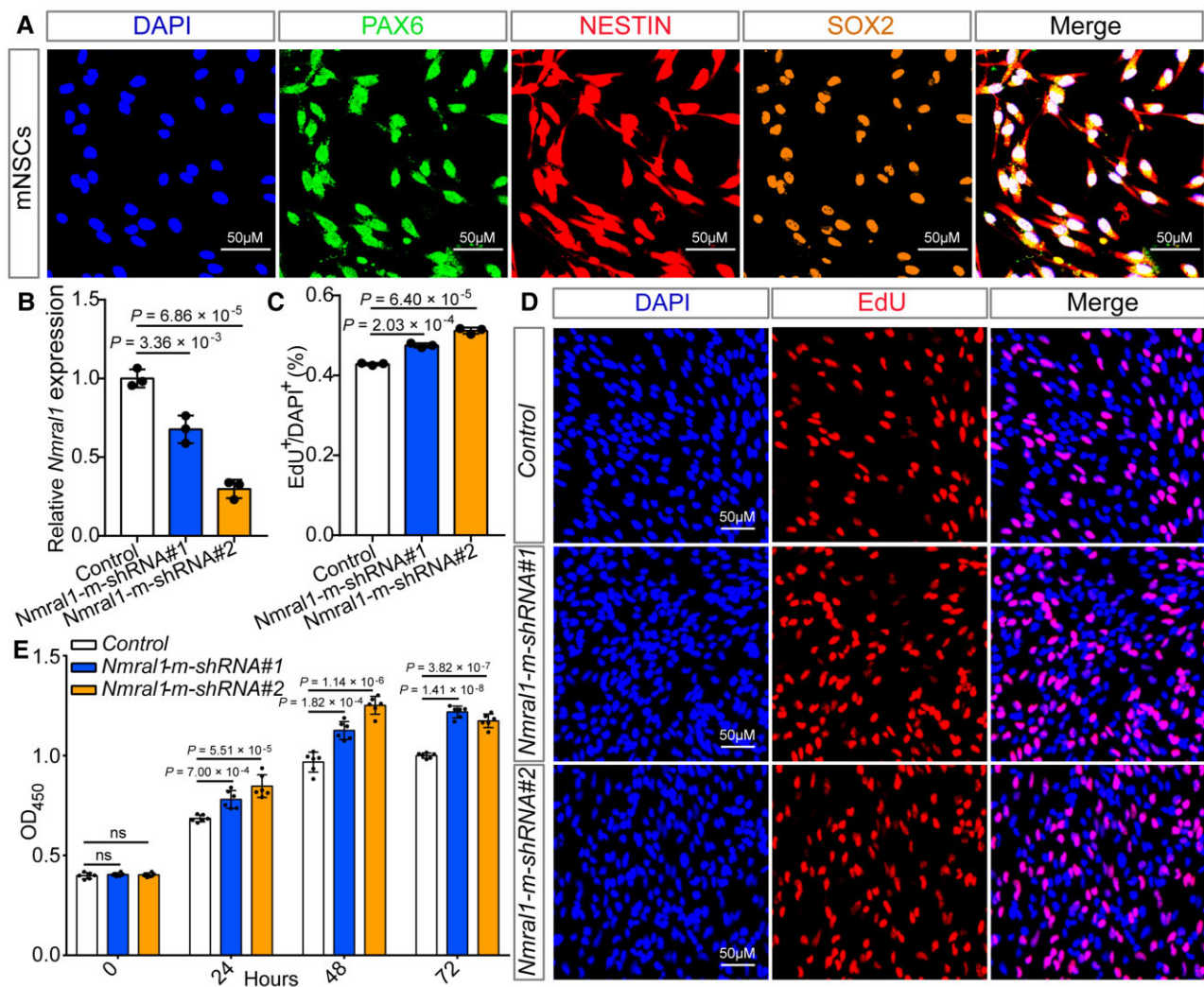
Figure 4 *Nmral1* knockdown promotes proliferation of mNSCs. (**A**) Immunofluorescence staining using three well-characterized markers (SOX2, PAX6 and NESTIN) for NSCs validated the identity of the isolated mNSCs. (**B**) *Nmral1* expression was efficiently knocked-down by the shRNAs for mouse. (**C** and **D**) The results of EdU incorporation assay. (**C**) The quantification results of the EdU incorporation assay. (**D**) Immunofluorescence staining for EdU incorporation assay. Red indicates EdU-positive cells and DAPI was used to stain the nucleus (blue). (**E**) The results of CCK-8 assay. Data were measured at four different time points: 0, 24, 48 and 72 h. Unpaired two-tailed Student's *t*-test was used to compare if the difference was significant. $n = 3$ for **B–D**; $n = 6$ for **E**. Data are presented as mean $\pm$ SD.

upregulated and ~55% (550 genes) were downregulated in *Nmral1* knocked-down mNSCs (Fig. 6A). We validated the mRNA alternations of several genes in *Nmral1* knocked-down mNSCs using qPCR (Supplementary Fig. 17A–E).

GO (biological processes) analysis showed that these DEGs were significantly enriched in neurodevelopment processes, including gliogenesis, head development and nervous system development (Fig. 6B). In addition, the DEGs were also showed enrichments in cell adhesion, proliferation, migration and differentiation pathways (Fig. 6B). Of note, modulation of chemical synaptic transmission process was also enriched among the DEGs (Fig. 6B). GO analysis (cell components) slowed that the DEGs were enriched in synapse component, further supporting the potential role of *NMRAL1* in synaptic transmission (Fig. 6C).

KEGG enrichment analysis indicated that the DEGs were significantly enriched in schizophrenia-associated signalling pathways (Fig. 6D), including calcium signalling pathway,[61–63] extracellular matrix (ECM)–receptor interaction[64,65] and cyclic adenosine

monophosphate (cAMP) signalling pathway.[66,67] The downregulated genes in *Nmral1* knocked-down mNSCs were mainly enriched in the cAMP and calcium signalling pathways (Supplementary Fig. 17F) and the upregulated genes were mainly enriched in the ECM–receptor interaction signalling pathway (Supplementary Fig. 17G). Collectively, our transcriptome analysis provides further evidence that supports the important role of *Nmral1* in neurodevelopment, and also suggests that *Nmral1* may confer risk of schizophrenia through regulating schizophrenia-associated biological processes and signalling pathways.

Nmral1 regulates density of dendritic spines

The pathophysiology of schizophrenia remains elusive. However, synaptic dysfunction has been repeatedly reported in subjects with schizophrenia.[68–70] In addition, the density of dendritic spines (layer 3 pyramidal neurons in the dorsolateral prefrontal cortex) was significantly decreased in schizophrenia cases
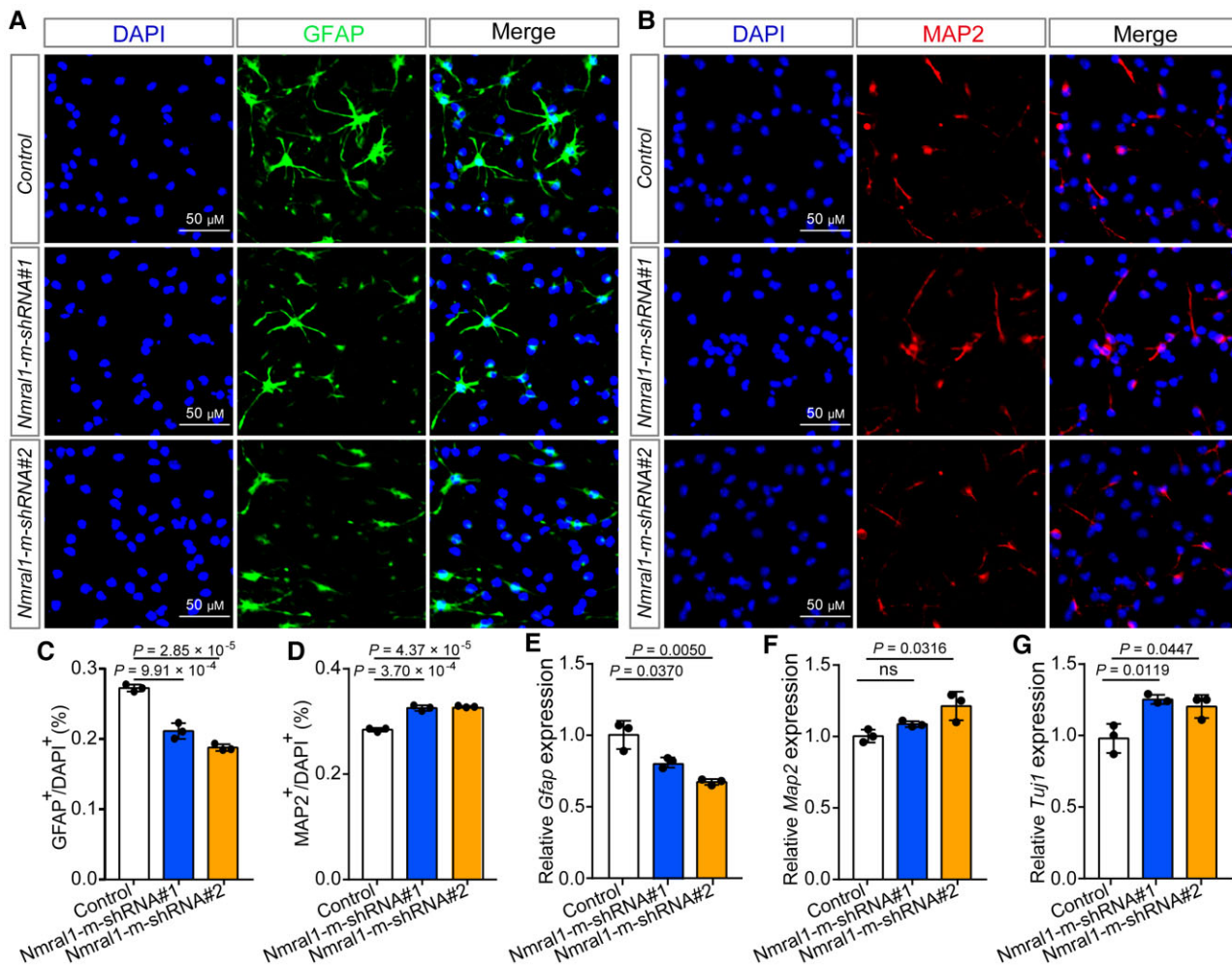
Figure 5 *Nmral1* **knockdown affects differentiation of mNSCs.** (**A** and **B**) Representative immunofluorescence staining images for glia cells and neurons differentiated from mNSCs. GFAP-positive cells were marked with green, MAP2-positive cells with red and the nuclei were stained with DAPI. (**C**) Quantification for the proportion of GFAP-positive glia cells. (**D**) Quantification for the proportion of MAP2-positive neurons. (**E**–**G**) qPCR showed the relative expression level of *Gfap*, *Map2* and *Tuj1* in control and *Nmral1* knocked-down mNSCs. Unpaired two-tailed Student's *t*-test was used to compare if the difference was significant. $n = 3$, data are presented as mean $\pm$ SD. ns = not significant.

compared with controls.[68,71,72] Of note, GO analysis showed that *Nmral1* regulates pathways associated with synaptic transmission (Fig. 6B) and the DEGs were enriched in synapse-associated components, including post-synapse, pre-synapse, plasma membrane receptor complex and parallel fibre to Purkinje cell synapse (Fig. 6C). These results suggest the potential role of *NMRAL1* in morphogenesis of dendritic spines. Therefore, we knocked-down *Nmral1* in rat primary cortical neurons. Dendritic spines analysis showed no significant changes for the density of thin and stubby spines (immature spines). However, *Nmral1* knockdown resulted in significant decrease of density of mushroom spines (Fig. 7A–C), which can form stable and mature synaptic connection.[73–75] These results revealed the important role of *NMRAL1* in dendritic spine development. Besides, these findings also suggested that *NMRAL1* might contribute to schizophrenia susceptibility by affecting the density and function of dendritic spines.

In summary, these lines of evidence suggest that rs2270363 may confer schizophrenia risk by modulating expression of *NMRAL1*, a gene which has a pivotal role in neurodevelopment and dendritic

spine morphogenesis (two featured characteristics of schizophrenia pathophysiology).

Discussion

In this study, we demonstrated the complex regulatory mechanisms of rs2270363, a functional schizophrenia risk SNP that disrupts binding of three bHLHZ transcription factors (USF1, MAX and MXI1)[76–83] and was significantly associated with schizophrenia. Previous study has shown changes of USF activity during the conversion from proliferation to differentiation.[48] Moreover, the binding strength of USF1 to the sequence containing rs2270363 was enhanced in all-*trans* retinoic acid-induced differentiation of SK-N-SH cells (Fig. 1B; data from ENCODE). GO (cellular components) analysis also showed that the DEGs were enriched in synaptic transmission-associated components, including post-synapse, pre-synapse, plasma membrane receptor complex and parallel fibre to Purkinje cell synapse (Fig. 6C), indicating the important role of *NMRAL1* in the central nervous system. MAX can form different dimers (MAX/MXI1, MAX/MAX, MAX/MAD and MAX/MYC) and play different roles in proliferation and
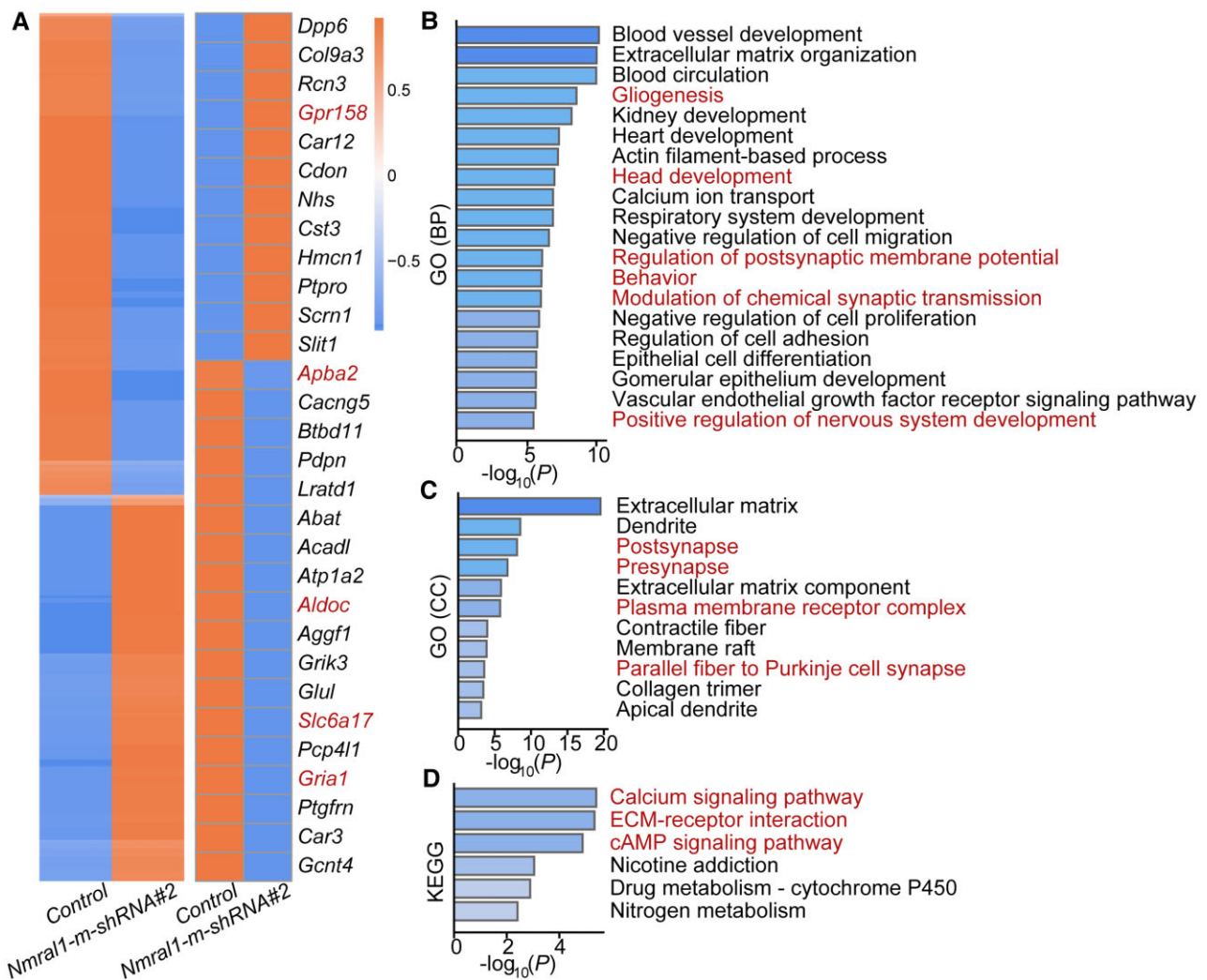
Figure 6 Nmral1 regulates schizophrenia-associated biological processes and signalling pathways. (A) Expression heat map of all the DEGs ($n = 991$) identified in *Nmral1* knocked-down mNSCs compared with controls (*left*). Heat map plot of the top 30 DEGs (*right*). Five genes (marked by red colour) were selected for qPCR verification. **(B)** GO (biological processes) analysis of all the DEGs. **(C)** GO (cellular components) analysis of all the DEGs. **(D)** KEGG analysis of all the DEGs. Red indicates schizophrenia-associated biological processes, cellular components and signalling pathways for **B–D**.

differentiation.[47,77,83] The MAX/MYC heterocomplex was gradually replaced by the MAX/MAD heterocomplex during phorbol 12-myristate 13-acetate-induced differentiation.[47] In addition, there is a competitive combination between MAX homodimers or heterodimers and USF.[84,85] These results indicated that the binding of bHLHZ proteins to rs2270363 is a dynamic process during neurodevelopment. Here, we found that *MAX* or *USF1* knockdown resulted in downregulation of *NMRAL1* in SH-SY5Y cells, suggesting that MAX and USF1 are involved in the regulation of *NMRAL1* expression (and this regulation is likely mediated by rs2270363, as rs2270363 disrupts binding of MAX and USF1). In addition, our reporter gene assays showed that the transcriptional activity of the A allele of rs2270363 was significantly stronger than the G allele, which was consistent with the observation that the A allele corresponds to higher expression of *NMRAL* in eQTL analysis. We also validated the regulatory effect of rs2270363 with EMSA, which revealed that different alleles of rs2270363 affect USF1 binding affinity.

In addition to elucidating the regulatory mechanisms of rs2270363, our study also showed that rs2270363 may confer schizophrenia risk through regulating expression of *NMRAL1*, a gene

associated with risk of schizophrenia[14,52,53,86] and with unknown function in the human brain. *NMRAL1* (also known as *HSCARG*) encodes NmrA like redox sensor 1, an NmrA-like transcriptional regulator whose subcellular localization and protein conformation is affected by changes in cellular metabolism status (e.g. cellular redox status, ammonia concentration and immune status).[87–90] NMRAL1 is an nicotinamide adenine dinucleotide phosphate (NADPH) sensor protein that preferentially binds to NADPH.[87,91] Previous studies have revealed that NMRAL1 is also involved in many biological processes, including cellular antiviral response (by negatively regulating the interferon response factor 3-mediated expression of interferon beta),[89,92–94] redox homeostasis,[88,95] DNA damage response,[96] innate immunity and cancer[91] (e.g. NMRAL1 suppresses polyubiquitination of NF-κB essential modulator by interacting with the deubiquitinase USP7 to inhibit tumour necrosis factor and interleukin 1-induced NF-κB activation).[93]

For the first time, we showed that *NMRAL1* plays pivotal roles in neurodevelopment and dendritic spine morphogenesis. Our findings support the neurodevelopmental hypothesis of schizophrenia and suggest that rs2270363 may exert functional impacts through
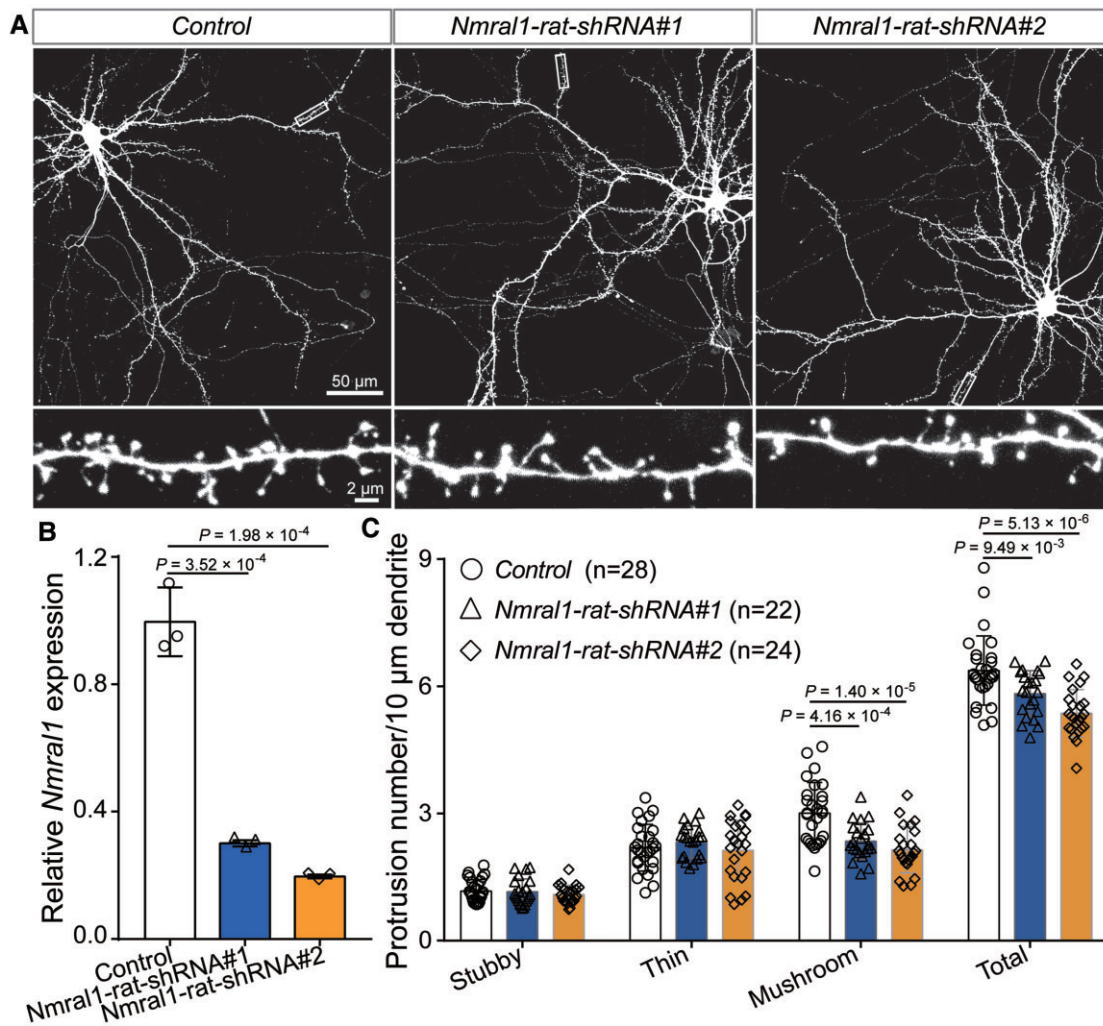
Figure 7 *Nmral1* knockdown decreased dendritic spine density. (A) Representative immunofluorescence staining images for neuronal morphologies and dendrites. **(B)** *Nmral1* expression in rat primary neurons was efficiently knocked-down by the shRNAs. **(C)** Dendritic spine density analysis for stubby, thin and mushroom spines. *P*-values were calculated using two-tailed Student's *t*-test. $n = 28$ for control; $n = 22$ for *Nmral1-rat-shRNA#1*; $n = 24$ *Nmral1-rat-shRNA#2*.

regulating expression of *NMRAL1*, a gene whose expression perturbation affects neurodevelopment and dendritic spine density. Although the pathogenesis of schizophrenia remains to be elucidated, lines of evidence suggested the pivotal role of dendritic spine deficit (dendritic spine pathology) in schizophrenia. Our study not only identified *NMRAL1* as a new schizophrenia risk gene, but also demonstrated the potential role of *NMRAL1* in schizophrenia pathogenesis (i.e. *NMRAL1* may confer risk of schizophrenia by affecting neurodevelopment and dendritic spine morphogenesis). Considering that the risk allele of rs2270363 was associated with higher *NMRAL1* expression and *NMRAL1* overexpression inhibited proliferation rate of the mNSCs, it is possible that *NMRAL1* overexpression is a potential pathogenic mechanism for schizophrenia. However, we mainly investigated the role of *NMRAL1* knockdown on neurodevelopment in the present study. More work is needed to explore if *NMRAL1* overexpression has a potential pathogenic role for schizophrenia.

We also explored the transcript expression of *NMRAL1* at differential transcript expression and differential transcript usage levels using PsychENCODE data.[97] However, no differential transcript expression or differential transcript usage of *NMRAL1* was detected (false discovery rate < 0.1) between schizophrenia and healthy controls.

Considering the important role of *NMRAL1* in neurodevelopment, we also explored if this gene was associated with other neurodevelopmental disorders, including autism spectrum disorder (18 382 cases and 27 969 controls),[98] attention deficit and hyperactivity disorder (20 183 cases and 35 191 controls) and bipolar disorder (20 352 cases and 31 358 controls).[99,100] Genetic variants near *NMRAL1* (including rs2270363) did not show suggestive associations with autism spectrum disorder, attention deficit and hyperactivity disorder and bipolar disorder (Supplementary Fig. 18). Further differential expression data (using expression data from PsychENCODE) showed that *NMRAL1* did not show differential expression in brains of autism spectrum disorder cases ($n = 51$) compared with controls ($n = 936$; $P = 0.14$).[97] However, *NMRAL1* was significantly upregulated in brains of bipolar disorder cases ($n = 222$) compared with controls ($n = 936$; $P = 0.00017$).[97] These results suggest that *NMRAL1* may also have a role in bipolar disorder.

In addition to the above evidence, the biological function of *NMRAL1* also supports the potential role of this gene in

schizophrenia. NMRAL1 has pivotal roles in regulating immunity. For example, NMRAL1 negatively regulates innate immunity by downregulating the activities of RIG-I like receptor (RLR) and NF-κB pathways.[91] Dysregulation of immune system has been frequently observed in schizophrenia.[101,102] In fact, the genetic variants in the major histocompatibility complex region showed the strongest associations with schizophrenia,[103] suggesting the important role of immunity-associated genes in schizophrenia. Besides, NMRAL1 regulates redox homeostasis, an important biological process that has been frequently reported to be dysregulated in schizophrenia.[104] Elevated oxidative stress was repeatedly reported in schizophrenia.[105,106] A recent study by Steullet et al.[107] also revealed that oxidative stress-driven parvalbumin interneuron impairment is a common mechanism in models of schizophrenia. Based on the above observations, the oxidative stress hypothesis of schizophrenia and the immune hypothesis of schizophrenia have been proposed.[102,106] It is possible that NMRAL1 participates in neurodevelopment by regulating redox homeostasis and NF-κB pathways. Nevertheless, more work is needed to demonstrated the role of NMRAL1 in schizophrenia.

Pinpointing the causal (or functional) variants from the reported schizophrenia risk loci remains a major challenge in the post-GWAS era. As a core regulatory region, the promoter plays a pivotal role in regulating gene expression. Multiple previous studies have shown that genetic variants in the promoter region contribute to disease susceptibility by affecting the expression level of risk genes. For example, rs11200638, a major genetic risk factor for wet age-related macular degeneration which lies in the HTRA1 promoter, modulates promoter activity and HTRA1 expression through affecting transcription factors AP2α and serum response factor (SRF) binding.[108] A lung cancer risk-associated SNP rs4142441, located in the promoter of OSER1-AS1, regulates tumour suppressor gene OSER1-AS1 expression.[109] rs213237 affected the ZNF323 promoter activity and confers schizophrenia risk by regulating ZNF323 expression.[110] A SNP in the MDM2 promoter was reported to attenuate the p53 tumour suppressor pathway and accelerate tumour formation in humans.[20] These findings highlighted the crucial role of functional variants in promoter region in disease susceptibility. In this study, we identified a functional variant (rs2270363) in the NMRAL1 promoter. We demonstrated the regulatory mechanism of this variant in regulating expression of NMRAL1. Our study not only provides a feasible framework to locate and identify the functional variants from the reported risk loci, but also demonstrates the regulatory mechanisms of rs2270363 and the potential pathophysiology role of NMRAL1 in schizophrenia.

Based on the reporter gene and eQTL results [i.e. the risk allele (A) of rs2270363 was associated with elevated expression of NMRAL1; Figs 1 and 2], NMRAL1 was predicted to be upregulated in schizophrenia cases compared with controls. However, expression analysis showed that NMRAL1 was downregulated in schizophrenia cases compared with controls. This inconsistency might be due to the following reasons. First, considering that the individuals used for gene expression and eQTL analysis were different, the number of subjects with the rs2270363 AA genotype in schizophrenia cases might be less than in controls in gene expression analysis, which will result in the observation of downregulation of NMRAL1 in schizophrenia cases. In fact, we examined the genotypic frequency of rs2270363 in tissue samples (258 cases, 279 controls) used for differential expression analysis.[18] The genotypic frequency of AA (risk allele of rs2270363 is A) was 5.86% in schizophrenia cases and 8.60% in controls, the genotypic frequency of AG was 40.23% in schizophrenia cases and 32.62% in controls and the genotypic frequency

of GG was 53.91% in schizophrenia cases and 58.78% in controls (Supplementary Table 10). The genotypic frequency of AA (risk allele of rs2270363 is A, which was associated with elevated NMRAL1 expression) is less in schizophrenia cases (5.86%) compared with controls (8.60%). As the A allele of rs2270363 is associated with elevated NMRAL1 expression, larger genotypic frequency of AA in controls might result in the observation of NMRAL1 downregulation in schizophrenia cases (compared with controls). Second, gene expression regulation is a complex process involving many factors (including multiple genetic variants and transcription factors). It is possible that other functional variants and rs2270363 act synergistically to regulate NMRAL1 expression. Finally, antipsychotics medication might also affect gene expression. Thus, the NMRAL1 expression level observed in schizophrenia cases might not reflect the genetic effects of rs2270363 accurately. More work is needed to investigate if NMRAL1 was dysregulated in schizophrenia cases.

Of note, rs2270363 was not among the top hits in the schizophrenia GWASs. The P of rs2270363 in recent two large-scale GWASs [i.e. PGC2 (34 241 cases, 45 604 controls and 1235 parent affected-offspring trios) and EAS + EUR (56 418 cases and 78 818 controls)] was 3.33×10^{-5} ($OR_A = 1.05$) and 4.59×10^{-6} ($OR_A = 1.05$), respectively.[13,27] However, we noticed that the P-value of rs2270363 decreases with increasing sample size, suggesting that rs2270363 is a true risk variant. In addition, we found that the P-value of the index SNP (i.e. rs6500602) of the risk locus 16p13.3 (where rs2270363 is located) reached the genome-wide significance level in EAS + EUR ($P = 2.39 \times 10^{-9}$). Consistent with this, rs2270363 also showed genome-wide significant association with schizophrenia in our meta-analysis ($P = 3.98 \times 10^{-8}$, 60 136 cases and 86 647 controls; Table 1). These results indicate that rs2270363 is a true risk variant for schizophrenia and demonstrate that integrating genetic and functional findings will facilitate the identification of bona fide risk variants for schizophrenia. Besides, we noticed that the effect size of rs2270363 was small (OR_A in European samples was 1.05), indicating the complexity of the genomic landscape of schizophrenia. Further studies considering both single and multiple variants are needed to elucidate the complex genetic aetiology of schizophrenia. In summary, our study demonstrates that rs2270363 may confer schizophrenia risk by modulating the expression of NMRAL1 (by dynamic binding to the bHLHZ proteins), a schizophrenia risk gene whose expression dysregulation might have pivotal roles in schizophrenia by affecting neurodevelopment and synaptic plasticity (Supplementary Fig. 19).

Acknowledgements

Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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