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Promoter variant rs2301228 on the neural cell adhesion molecule 1 gene confers risk of schizophrenia in Han Chinese

Wen Zhang [a], Mei-Sheng Xiao [a], Shuang Ji [b], Jinsong Tang [c], Ling Xu [a,d], Xiao Li [a,d], Ming Li [e], Hui-Zhen Wang [a,f], Hong-Yan Jiang [g], Deng-Feng Zhang [a,d], Jicai Wang [g], Shuliang Zhang [h], Xiu-Feng Xu [g], Li Yu [i], Ping Zheng [b], Xiaogang Chen [c], Yong-Gang Yao [a,*]

[a] Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan, China
[b] State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, China
[c] Institute of Mental Health, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China
[d] Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming, Yunnan, China
[e] Lieber Institute for Brain Development, Johns Hopkins Medical Campus, Baltimore, MD, USA
[f] School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, China
[g] Department of Psychiatry, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China
[h] Coal Mine Mental Hospital of Yunnan Province, Honghe, Yunnan 652402, China
[i] Laboratory for Conservation and Utilization of Bio-resource & Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming, Yunnan, China

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ABSTRACT

Background: Schizophrenia is recognized as a disorder of the brain and neuronal connectivity. The neural cell adhesion molecule 1 (*NCAM1*) gene plays a crucial role in regulating neuronal connectivity.
Methods: We conducted a two-stage association analysis on 17 *NCAM1* SNPs in two independent Han Chinese schizophrenia case–control cohorts (discovery sample from Hunan Province: 986 patients and 1040 normal controls; replication sample from Yunnan Province: 564 cases and 547 healthy controls). Allele, genotype and haplotype frequencies were compared between case and control samples. Transcription factor binding site prediction and luciferase reporter assays were employed to assess the potential function of promoter SNPs. We detected developmental changes at the transcriptional level of *NCAM1* during neuron differentiation in *Macaca mulatta* neural progenitor cells (NPC). Serum levels of NCAM1 were measured in 72 cases and 88 controls.
Results: A promoter variant, rs2301228, was found to be associated with schizophrenia at the allelic level and was validated in a replication cohort. Luciferase reporter assays demonstrated that risk allele rs2301228-A significantly down-regulated *NCAM1* gene transcription compared to the G-allele. Concordantly, schizophrenia patients had a significantly lower level of serum NCAM1 compared to healthy donors. During the NPC neuronal differentiation, *NCAM1* mRNA was significantly increased, suggesting a critical role of this gene in neural development.
Conclusions: Our results provide direct evidence for *NCAM1* as a susceptibility gene for schizophrenia, which offers support to a neurodevelopmental model and neuronal connectivity hypothesis in the onset of schizophrenia.

1. Introduction

Schizophrenia is one of the most severe and prevalent psychiatric disorders with a lifetime prevalence of about 1% worldwide (Jablensky, 1997). It is clinically heterogeneous and has a high heritability of approximately 80% (Sullivan et al., 2003). Schizophrenia is recognized as a disease of the brain and neuronal connectivity. The neural cell adhesion molecule 1 (NCAM1), also known as CD56, is a member of the immunoglobulin superfamily (Jorgensen and Bock, 1974). It is widely expressed in neural cells of the central and peripheral nervous system (Schmid et al., 1999). Three isoforms of NCAM1 protein, named NCAM-120, NCAM-140, and NCAM-180 based on their molecular weights, are responsible for several important neural functions including brain connectivity, neurite outgrowth, cell–cell adhesion, synaptic plasticity, learning and memory (Eckhardt et al., 2000; Angata et al., 2004; Weinhold et al., 2005; Hildebrandt et al., 2009).

Due to the crucial role of NCAM1 in regulating neuronal connectivity, one would naturally speculate that genetic variants of the *NCAM1* gene could confer susceptibility to schizophrenia. Previous genetic association studies reported that several single nucleotide polymorphisms (SNPs) in the *NCAM1* gene were associated with schizophrenia, but the findings were inconsistent among different studies (Vicente et al., 1997; Atz et al., 2007; Sullivan et al., 2007; Xu et al., 2008). The *NCAM1* SNPs were also reported to be associated with other complex diseases such

* Corresponding author at: Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223 China. Tel./fax: +86 871 65180085.
E-mail addresses: ygyaozh@gmail.com, yaoyg@mail.kiz.ac.cn (Y.-G. Yao).

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as bipolar disorder (Arai et al., 2004; Atz et al., 2007), Alzheimer's disease (Furney et al., 2011), neural tube defects (Deak et al., 2005), temperament and suicidal behavior (Giegling et al., 2010, 2011), suggesting an important role of NCAM1 in maintaining normal function of the neural system. *NCAM1* knockout mice also exhibit several schizophrenia related symptoms such as reduced spatial learning ability, mild cognitive impairment and social behavioral deficits (Cremer et al., 1994; Albrecht et al., 2010; Albrecht and Stork, 2012). Altered expression of *NCAM1* was frequently observed in the cerebrospinal fluid of schizophrenia patients (Vawter, 2000; Brennaman and Maness, 2010). Furthermore, recent research utilizing induced pluripotent stem cells (iPSC) revealed neural connectivity deficits during neuronal development in schizophrenia patients associated with significantly reduced *NCAM1* mRNA levels (Brennand et al., 2011).

Although available studies indicated an active involvement of *NCAM1* in schizophrenia, the independent replication studies and functional assessments are still insufficient to fully understanding the role of *NCAM1* in schizophrenia. Moreover, very few SNPs were studied in previous reports, thus could not represent the whole gene and would increase the possibility of false negative conclusions. In this study, we conducted a two-stage genetic analysis of the association between genetic variants of *NCAM1* and schizophrenia in two Han Chinese case–control cohorts. Functional characterizations of the related SNPs were also performed to explore the underpinning of the genetic association.

2. Materials and methods

2.1. Participants

The discovery cohort was composed of 986 unrelated Han Chinese patients with schizophrenia (608 males: mean age ± standard deviation (SD), 38.6 ± 13.6 years; 378 females: 38.0 ± 13.9 years) and 1040 matched healthy controls (734 males: mean age ± SD, 36.8 ± 14.3 years; 306 females: 38.5 ± 13.8 years) from Hunan Province, South Central China. The replication cohort was composed of 564 unrelated Han Chinese patients with schizophrenia (311 males: mean age ± SD, 37.0 ± 13.7 years; 253 females: 33.1 ± 12.9 years) and 547 matched healthy controls (318 males: mean age ± SD, 37.1 ± 9.5 years; 229 females: 36.0 ± 9.7 years) from Yunnan Province, Southwest China. Another 72 schizophrenia patients (53 males: mean age ± SD, 45.4 ± 11.8 years; 19 females: 39.5 ± 14.9 years) and 88 healthy controls (45 males: mean age ± SD, 40.8 ± 12.2 years; 43 females: 37.5 ± 10.6 years) were recruited from Luxi County, Yunnan Province for serum ELISA assays. The patients were clinically diagnosed according to Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) and had at least a two-year history of schizophrenia. A review of case records to ensure diagnostic accuracy was performed independently by two senior clinicians prior to blood sample collection. The schizophrenia patients and related controls from Hunan Province were also described in our previous study aimed to validate reported risk alleles from a previous genome-wide association study (GWAS) (Ma et al., 2013) and to discern the mtDNA haplogroup effect on schizophrenia (Zhang et al., 2014). The control cohort from Yunnan Province was composed of adult individuals who visited the local hospital for physical examinations and had no neuropsychiatric disease. Written informed consents conforming to the tenets of the Declaration of Helsinki were obtained from all participants or the appointed guardians of the patients (for those who were unable to provide informed consent at the time of blood collection) prior to this study. The institutional review board of Kunming Institute of Zoology approved this study.

2.2. Promoter sequencing and SNP genotyping

Based on previous reports (Barton et al., 1990; Hirsch et al., 1991), a fragment encompassing approximately 1.5 kb upstream of the transcriptional start site of *NCAM1* (Fig. 1), which contains most of the cis-regulatory elements for gene transcription, was amplified by PCR and directly sequenced using an ABI PRISM 3730 Genetic Analyzer (Perkin-Elmer Applied Biosystems). Seven common SNPs were identified in the promoter region after sequencing, which is consistent with data from the 1000 Genomes Project Consortium (The, 1000 Genomes Project Consortium, 2012). In addition, 10 SNPs (including 9 reported disease-associated variants from the literature (Arai et al., 2004; Atz et al., 2007; Sullivan et al., 2007; Giegling et al., 2010, 2011; Arnett et al., 2011; Furney et al., 2011) and one tag SNP; Table 1) in the *NCAM1* gene region were selected and genotyped by SNaPshot assays as described in our previous studies (Wang et al., 2012; Bi et al., 2014; Li et al., 2014). In brief, multiplex PCR was performed in a total volume of 8 μL containing 20–50 ng genomic DNA, 0.4 mM dNTPs, 0.2–0.5 μM of each primer, 2.0 mM $MgCl_2$ and 1.0 U of FastStart Taq DNA Polymerase (Roche Applied Science). The PCR conditions consisted of a denaturation cycle at 94 °C for 2 min; 40 amplification cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. PCR products were purified at 37 °C for 40 min with 1.0 U of shrimp alkaline phosphatase (SAP) and 0.5 U of Exonuclease I (TaKaRa Biotechnology Co. Ltd., Dalian, China), followed by an incubation at 96 °C for 10 min to ensure enzymatic inactivation. The single base extension reaction was conducted in a volume of 10 μL reaction solution, containing 4 μL purified multiplex PCR product, 5 μL SNaPshot Multiplex Ready Reaction Mix and 0.4–0.8 μM pooled SNP-specific oligonucleotide primers. Single-base extension reactions were carried out for 25 amplification cycles, each cycle with the following conditions: 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. The product was purified at 37 °C for 40 min, followed by an inactivation procedure at 75 °C for 20 min. Approximately 4 μL of purified product was mixed with 9 μL of Hi-Di™ formamide, and were analyzed by the ABI PRISM™ 3730xl DNA analyzer (Applied Biosystems). The SNaPshot results were read by GeneMarker software (Holland and Parson, 2011).

The SNaPshot assay was also used to genotype these positive SNPs identified at the discovery stage in the validation sample. All sequencing and SNaPshot chromatograms were independently read by two experienced researchers (WZ and XL read the discovery stage data; WZ and HZW read the validation stage data). The PCR condition and primers are shown in Table S1.

2.3. Transcription factor binding site prediction and luciferase assays

The MultiSearchSites tool PROMO (Messeguer et al., 2002; Farré et al., 2003) (http://alggen.lsi.upc.es/) was employed to predict changes in potential transcription factor binding site (TFBS) for the promoter SNPs. Only human transcription factors and human TFBS were selected for prediction. The matrix dissimilarity rate was restricted to 0%.

Ten luciferase reporter vectors were constructed by cloning different lengths of the promoter region containing different haplotypes of promoter SNPs into pGL3-Basic vector (Promega, Madison City, WI, USA) (Fig. 2). Three mutant vectors (with a change of allele A to G of rs2301228) were constructed on the basis of vectors 1–3 by using QuickChange II site-directed mutagenesis kit (Stratagene, Agilent Technologies, La Jolla, CA, USA) according to manufacturer's instruction. The primers used for promoter reporter vectors are shown in Table S1. PCR fragments with different haplotypes of *NCAM1* promoter region were digested by *Xho*I-*Hind*III. After gel purification, the digested fragments were ligated into a pGL3-basic luciferase vector. All inserts were confirmed by direct sequencing.

To discern the potential effects of *NCAM1* promoter variants with different alleles/haplotypes, two cell lines (HEK293T and U251) were used for transient transfection. Cells were seeded in 24-well plates at a density of 1×10^5 cells per well and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad City, CA, USA) supplemented with 10% fetal bovine serum. After overnight culture, cells were co-transfected with 500 ng of each reporter vector and 50 ng of *Renilla* luciferase pRL-TK plasmid (Promega) using Lipofectamine 2000

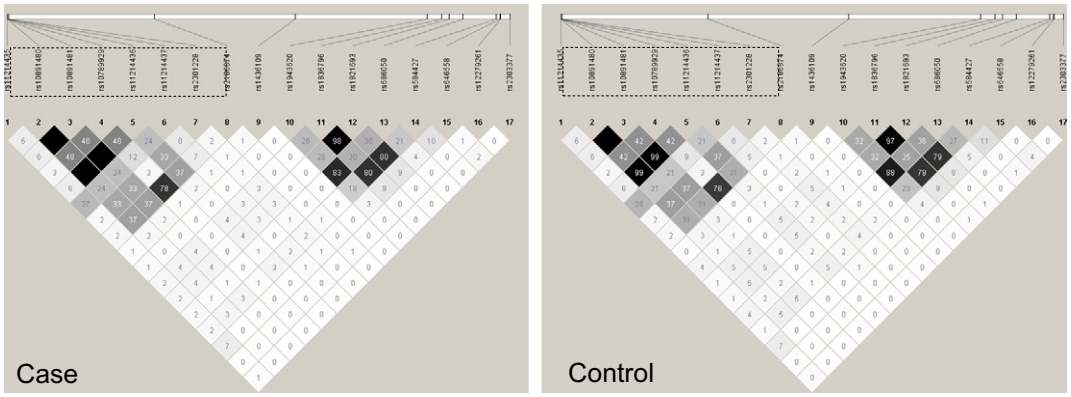

Fig. 1. Schematic structure and linkage disequilibrium (LD) of *NCAM1* gene SNPs in schizophrenia patients and normal controls from Hunan Province, China. Results were performed by Haploview 4.2 (Barrett et al., 2005). The value in each square refers to $r^2 \times 100$. The blacker squares represent higher LD. The individual squares show the r^2 value for each SNP pair. SNPs in dashed boxes are located in the *NCAM1* promoter region.

transfection reagent (Invitrogen). All transfection assays were performed in triplicates. HEK293T cells were harvested after 24 h and U251 cells were harvested after 48 h based on the cell status in 150 μL passive lysis buffer (Promega) for 1 h. Luciferase activity was immediately detected by the Dual-Luciferase Reporter Assay System (Promega) using a GloMax 96 Luminometer (Promega) and luciferase assay kit (Promega) following our previous study (Xiao et al., 2011). The experiments were repeated at least three times in both cell lines.

2.4. mRNA quantification in rhesus monkey neural progenitor cells and neurons

Neural progenitor cells (NPC) of *Macaca mulatta* were differentiated from embryonic stem cells (ESCs). In brief, NPCs were directionally differentiated into neurons according to the protocol described in the previous study with minor modifications (Brennand et al., 2011; Zhao et al., in press). In detail, embryoid bodies were differentiated from ESCs and then transferred to culture dishes coated with 1% Agar. Colonies were maintained in suspension in N2 medium (DMEM/F12 (Invitrogen), 1 × N2 (Invitrogen)) for 5–7 days and then plated in polyornithine (PORN)/laminin-coated plates. Visible rosettes formed within 1 week and were manually dissected and cultured in NPC medium (DMEM/F12, 1 × N2, 1 × B27, RA (1 μM; Sigma-Aldrich), 1 mg/mL laminin (Invitrogen) supplemented with 20 ng/mL FGF2 (Invitrogen)). The identity of NPCs was confirmed by the expression of neural stem cell markers Nestin and Sox2 using immunofluorescence assays.

NPCs were maintained at a high density, grown on PORN/laminin-coated plates in NPC medium and split approximately 1:4 every week with Accutase (Millipore). For neuronal differentiation, NPCs were dissociated with Accutase and plated at low density in neural

Table 1
Allele and genotype frequencies of 17 SNPs in the discovery cohort (986 cases and 1040 controls from Hunan Province) and 2 SNPs in the replication cohort (564 cases and 547 controls from Yunnan Province).

SNP	Allele	No. of alleles			Genotype	No. of individuals			HWE *P*-value
		Case	Control	*P*-value[a]		Case	Control	*P*-value[a]	
Discovery sample (986 cases and 1040 controls)									
rs11214435	C/T	268/1704	256/1824	0.224	CC/CT/TT	25/218/743	17/222/801	0.316	0.249/0.839
rs10891480	T/G	591/1381	645/1435	0.472	TT/TG/GG	95/401/490	105/435/500	0.762	0.481/0.596
rs10891481	A/G	591/1381	645/1435	0.472	AA/AG/GG	95/401/490	105/435/500	0.762	0.481/0.596
rs10789929	C/T	347/1625	337/1743	0.236	CC/CT/TT	30/287/669	35/267/738	0.217	0.929/0.239
rs11214436	T/G	591/1381	642/1438	0.536	TT/TG/GG	95/401/490	105/432/503	0.825	0.481/0.543
rs11214437	T/C	724/1248	679/1401	**0.007**	TT/TC/CC	139/446/401	115/449/476	**0.027**	0.504/0.633
rs2301228	G/A	247/1725	308/1772	**0.035**	GG/GA/AA	17/213/756	18/272/750	0.055	0.759/0.403
rs2186874[b]	A/G	329/1643	326/1754	0.383	AA/AG/GG	30/269/687	31/264/745	0.615	0.681/0.372
rs1436109[c]	A/C	235/1737	237/1843	0.604	AA/AC/CC	16/203/767	10/217/813	0.416	0.721/0.435
rs1943620[c]	A/G	874/1098	968/1112	0.156	AA/AG/GG	183/508/295	240/488/312	**0.028**	0.230/0.130
rs1836796[c]	A/C	502/1470	577/1503	0.100	AA/AC/CC	61/380/545	92/393/555	0.076	0.737/0.162
rs1821693[c]	A/G	499/1473	571/1509	0.121	AA/AG/GG	61/377/548	89/393/558	0.122	0.812/0.226
rs686050[c]	G/C	952/1020	1030/1050	0.429	GG/GC/CC	224/504/258	263/504/273	0.348	0.558/0.453
rs584427[c]	A/C	518/1454	595/1485	0.096	AA/AC/CC	65/388/533	84/427/529	0.239	0.686/0.907
rs646558[c]	T/G	490/1482	484/1596	0.240	TT/TG/GG	73/344/569	57/370/613	0.211	0.137/0.952
rs12279261[c]	C/T	124/1848	132/1948	0.939	CC/CT/TT	2/120/864	7/118/915	0.244	0.582/0.412
rs2303377[c]	T/C	832/1140	933/1147	0.087	TT/TC/CC	177/478/331	222/489/329	0.152	0.918/0.198
Replication sample (564 cases and 547 controls)									
rs2301228	G/A	137/991	172/922	**0.015**	GG/GA/AA	4/129/431	11/150/386	**0.029**	0.276/0.535
rs1943620	A/G	524/604	486/608	0.337	AA/AG/GG	103/318/143	102/282/163	0.201	0.001/0.303
Combined sample (1550 cases and 1587 controls)									
rs2301228	G/A	384/2716	480/2694	**0.0017**	GG/GA/AA	21/342/1187	29/422/1136	**0.006**	0.515/0.154
rs1943620	A/G	1398/1702	1454/1720	0.571	AA/AG/GG	286/826/438	342/770/475	**0.018**	0.003/0.365

HWE: Hardy–Weinberg Equilibrium.
[a] *P*-values were calculated by the Chi-square test. A *P*-value <0.05 was marked in bold.
[b] Tag SNP in the *NCAM1* gene region.
[c] SNPs were reported to be associated with different diseases (Arai et al., 2004; Atz et al., 2007; Sullivan et al., 2007; Giegling et al., 2010, 2011; Arnett et al., 2011; Furney et al., 2011).

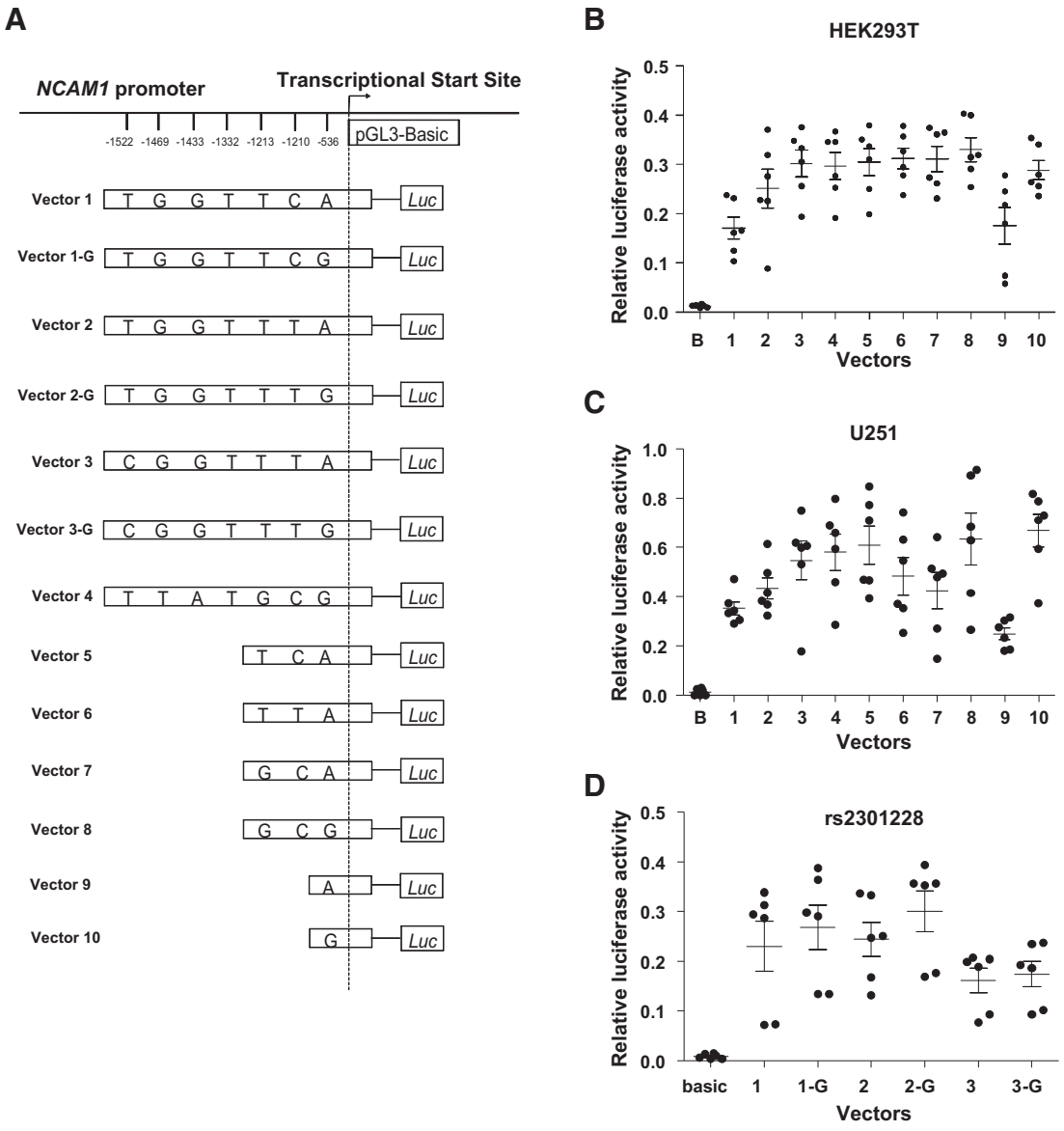

Fig. 2. Luciferase reporter assay for *NCAM1* promoter variants. Relative luciferase activities for 10 vectors carrying different haplotypes and/or alleles (A) of *NCAM1* promoter variants were measured in HEK293T cells (B) and U251 cells (C). Relative luciferase activities for vectors 1–3 and their mutants (with a change of A to G for rs2301228) were measured in HEK293T cells (D). The physical order of the 7 SNPs in *NCAM1* promoter region are marked with the respective location of each position relative to transcriptional start site: rs11214435, −1522; rs1089140, −1469; rs1089141, −1433; rs12279261, −1332; rs11214436, −1213; rs11214437, −1210; and rs2301228, −536. Data are shown as mean ± standard error (SE) and are based on three independent experiments.

differentiation medium (DMEM/F12-Glutamax, 1 × N2, 1 × B27, RA (1 μM; Sigma-Aldrich), 20 ng/mL BDNF (R&D), 20 ng/mL GDNF (R&D), 1 mM dibutyryl-cyclic AMP (Sigma), 200 nM ascorbic acid (Sigma) in PORN/laminin-coated plates). We harvested four dishes of neurons differentiated from NPCs as indicated by the expression of β3-tubulin. Total RNA was isolated from the cells using TRIZOL (Invitrogen, 15596-018) and cDNA was synthesized by using the M-MLV Reverse Transcriptase (Promega, M170A) according to the manufacturer's instruction. The relative mRNA expression levels of two major *NCAM1* transcripts of rhesus monkey were quantified by real-time quantitative PCR (qPCR). *NCAM1* mRNA levels were normalized to a housekeeping gene, *GAPDH*. The qPCR was performed on the platform of iQ2 system (BioRad Laboratories) with SYBR® Premix Ex Taq™ II kit (TaKaRa, DRR081A). The following primers were used for qPCR analysis: for the long transcript of rhesus *NCAM1*, forward primer 5′-CGACCAGAGAAGCAAGAG-3′/reverse primer 5′-GCACTTCAAGGTAC ATGGA-3′; for the short transcript of rhesus *NCAM1*, forward primer 5′-AGCAGCGAAGAAAAGACT-3′/reverse primer 5′-GCACTTCAAGGTAC ATGGA-3′; for housekeeping *GAPDH* gene, forward primer 5′-ATGGGG AAGGTGAAGGTCGG-3′/reverse primer 5′-TCCTGGAAGATGGTGATG GG-3′.

2.5. Serum NCAM1 ELISA

Serum NCAM1 levels were measured in 72 schizophrenia patients and 88 normal controls. The sera were three-fold diluted after sampling and immediately subjected to ELISA assay using RayBio® Human NCAM-1 ELISA kit (RayBiotech, Inc., ELH-NCAM1-001) following the manufacturer's protocol. In brief, 100 μL of 2.5-fold serial diluted standards and samples were added into a 96-well plate coated with anti-Human NCAM1. The plate was incubated for 2.5 h at room temperature with gentle shaking. After washing, 100 μL of 1× biotinylated antibody was added to each well and incubated for 1 h. The plate was washed again, and 100 μL of Streptavidin solution was added and the plate was incubated for 45 min. After washing, 100 μL of tetramethylbenzidine one-step substrate reagent was added into

each well and incubated for 30 min in the dark, followed by addition of 50 μL stop solution. The absorbance was immediately measured at 450 nm. All the above incubations were conducted at room temperature with gentle shaking.

2.6. Statistical analyses

Power calculations were performed using Quanto software (Gauderman, 2002). Considering an average population minor allele frequency (MAF) of 10%, the power to detect an odds ratio as low as 1.5 for a risk allele/genotype/haplotype was above 90% for the comparison between schizophrenia patients and controls in the discovery cohort (Fig. S1). The allele, genotype and haplotype frequencies of all SNPs were compared between case and control groups by the Chi-square test. Linkage disequilibrium pattern between SNPs were inferred by Haploview software (Barrett et al., 2005). Haplotypes consisting of all 17 SNPs were reconstructed by PHASE software (Stephens et al., 2001). Gene-based testing for the *NCAM1* gene in schizophrenia was performed by VEGAS (Liu et al., 2010), using available GWAS datasets from the Psychiatric Genomics Consortium (PGC) retrieved from Ricopili (http://www.broadinstitute.org/mpg/ricopili/). Meta-analysis for association of rs2301228 with schizophrenia using PGC data (Ripke et al., 2013) and our current data was conducted by program Review Manager 5.2 (RevMan 5.2, The Cochrane Collaboration, http://ims.cochrane.org/revman). Unpaired two-tailed Student's *t*-test was conducted to evaluate the difference of serum NCAM1 levels between case and control groups. The comparison of demographic characteristics (e.g., age, sex and so on) between cases and controls were assessed using one-way ANOVA, and there were no differences between groups.

3. Results

3.1. Association of NCAM1 SNPs/haplotypes with schizophrenia

At the discovery stage for analysis of 986 patients and 1040 normal controls from Hunan Province, we identified 7 common SNPs and 3 rare variants in the promoter region of the *NCAM1* gene. Together with 10 selected SNPs that were located in the *NCAM1* gene region and were genotyped by SNaPshot, we found that rs11214437 in the promoter region showed a significant difference at both allelic ($P = 0.007$) and genotypic levels ($P = 0.027$) between the case and control samples. The major allele of rs2301228 in the promoter region was also significantly enriched in patients ($P = 0.035$, odds ratio [OR] = 1.214, 95% confidence interval [CI] = 1.014–1.453), and the genotype of this SNP presented marginal significance between cases and controls ($P = 0.055$) (Table 1). Furthermore, a previously reported schizophrenia-associated intronic SNP, rs1943620 (Sullivan et al., 2007), also showed a significant difference at the genotypic level ($P = 0.028$; Table 1). No gender and age effect was observed for each SNP.

In the replication sample, we were able to successfully genotype two (rs2301228 and rs1943620) of the three SNPs (rs11214437 failed to be successfully genotyped by SNaPshot) that were identified in the discovery sample. We confirmed the association of rs2301228 with schizophrenia ($P = 0.015$, OR = 1.349, 95% CI = 1.06–1.718). When the discovery and validation samples were combined, the significance was strengthened (rs2301228, $P = 0.0017$, OR = 1.26, 95% CI = 1.091–1.456) and was maintained following multiple comparison correction for the 17 SNPs analyzed in this study (Bonferroni corrected $P = 0.029$) (Table 1). No heterogeneity was detected in the combined samples (rs2301228, $P = 0.494$).

We estimated the linkage pattern of the 17 SNPs based on the discovery sample from Hunan Province and found a similar pattern for the cases and controls (Fig. 1). A total of 152 haplotypes in cases and 159 haplotypes in controls were reconstructed by PHASE software (Stephens et al., 2001). Given the statistical power issue, only haplotypes with a frequency above 1% in either group were considered for further analysis (Table 2). We found that haplotype frequency distribution was highly biased between case and control samples

Table 2
Haplotype frequency of 17 SNPs of the *NCAM1* gene in 986 schizophrenia cases and 1040 normal controls from Hunan Province, China.

Haplotype[a]	Case[b]	Control[b]	*P*-value[c]	OR
TGGTTTAGCACGGCGAC	160 (0.081)	123 (0.059)	0.216	1.405
CGGTTTAGCGCGCCTAT	101 (0.051)	95 (0.046)	0.411	1.128
TGGTTCAGCACGGCGAC	97 (0.049)	141 (0.068)	0.432	0.711
TGGTTCAGCACGGCGAT	97 (0.049)	138 (0.066)	0.720	0.728
TTATGCGGCGAACAGAC	84 (0.043)	136 (0.065)	0.050	0.636
TGGTTCAGCGAACAGAC	70 (0.035)	93 (0.045)	0.136	0.786
TTACGCAACGAACAGAC	62 (0.031)	23 (0.011)	2.0×10^{-4}	2.903
TTACGCAACGAACAGAT	49 (0.025)	67 (0.032)	0.160	0.766
TGGTTCAGAGCGCCTAT	46 (0.023)	42 (0.020)	0.494	1.159
TTATGCGGCACGGCGAC	42 (0.021)	36 (0.017)	0.355	1.236
CGGTTTAGCGCGCCTAC	38 (0.019)	47 (0.023)	0.460	0.850
TTACGCAACACGGCGAC	35 (0.018)	37 (0.018)	0.992	0.998
TGGTTTAGCACGGCGAT	35 (0.018)	56 (0.027)	1.000	0.653
TTACGCAACACGGCTAT	34 (0.017)	30 (0.014)	0.472	1.199
TGGTTTAGCGCGCCTAT	33 (0.017)	33 (0.016)	0.827	1.056
TGGTTCAGCGAACAGAT	33 (0.017)	39 (0.019)	0.627	0.891
CGGTTTAGCGCGCCGAC	33 (0.017)	5 (0.002)	9.0×10^{-5}	7.063
TGGTTCAGCACGGCGGC	32 (0.016)	27 (0.013)	0.389	1.254
CGGTTTAGCACGGCGAT	31 (0.016)	20 (0.010)	0.081	1.645
TTATGCGGCGCGCCGAC	30 (0.015)	12 (0.006)	0.108	2.662
TGGTTTAGCGAACAGAT	28 (0.014)	12 (0.006)	0.240	2.482
TGGTTCAGAGAACAGAC	28 (0.014)	27 (0.013)	0.738	1.095
TTACGCAACACGGCTAC	27 (0.014)	30 (0.014)	0.843	0.949
TTACGCAACGCGCCTAT	25 (0.013)	34 (0.016)	0.330	0.773
TGGTTCAGAACGGCGAC	25 (0.013)	25 (0.012)	0.850	1.055
TTACGCAACGCGCCTAC	24 (0.012)	3 (0.001)	0.001	8.530
TGGTTTAGAACGGCGAC	24 (0.012)	19 (0.009)	0.346	1.336
TGGTTCAGCGCGGCGAT	24 (0.012)	15 (0.007)	0.106	1.696
TGGTTCAGCGCGCCGAT	22 (0.011)	19 (0.009)	0.520	1.224
TTATGCGGCGAACAGAT	21 (0.011)	19 (0.009)	0.626	1.168
TGGTTTAGCGAACAGAC	21 (0.011)	38 (0.018)	1.000	0.578
TTATGCGGCGCGCCTAT	20 (0.010)	17 (0.008)	0.510	1.243
TGGTTTAGCACGGCTAT	18 (0.009)	25 (0.012)	0.369	0.757
TGGTTTAGCGCGCCGAC	18 (0.009)	29 (0.014)	0.153	0.652
TTATGCGGCACGGCGAT	9 (0.005)	21 (0.010)	1.000	0.450
CGGTTTAGCGCGCCGAT	7 (0.004)	27 (0.013)	0.036	0.271
Others[d]	489 (0.248)	520 (0.250)	–	–
Global *P*-value[c]	–	–	3.73×10^{-15}	–

[a] The alleles are presented according to their physical order as shown in Fig. 1.
[b] The frequency of each haplotype is presented in brackets.
[c] *P*-values were calculated by the Chi-square test with Bonferroni correction.
[d] These haplotypes with a frequency less than 1% were aggregated in both case and control samples.

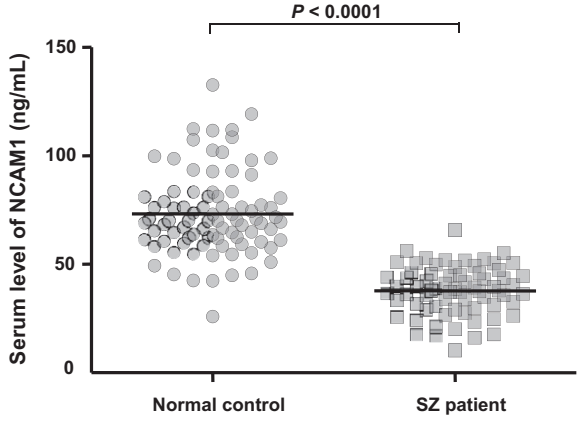

Fig. 3. Serum NCAM1 levels in schizophrenia patients and healthy controls. Sera were collected from patients with schizophrenia (n = 72) and healthy controls (n = 88). The levels of serum NCAM1 were measured by ELISA. Unpaired Student's *t*-test was performed for case–control comparison.

(global $P = 3.73 \times 10^{-15}$), among which three haplotypes conferred significant susceptibility to schizophrenia even after adjustment for multiple comparisons. Intriguingly, these three haplotypes all carried the risk allele "A" of rs2301228.

3.2. Potential transcription factors binding and luciferase activity of promoters with different haplotypes

We evaluated whether the risk SNP rs2301228 in the promoter region of *NCAM1* may affect potential transcription factor binding sites. The "G" allele of rs2301228, together with its surrounding nucleotides, was predicted to be a c-Ets-1 binding motif (CAGGAA**G**). The risk allele "A" of rs2301228 would lead to a collapse of this binding motif (changed to CAGGAA**A**). This *in silico* prediction result suggested that different alleles of rs2301228 may affect *NCAM1* expression.

To further assess the putative functional role of *NCAM1* promoter SNPs associated with schizophrenia, we constructed 10 reporter vectors containing different alleles/haplotypes of the *NCAM1* promoter region and performed luciferase assays. Among them, vectors 1–4 contained 4 different haplotypes of all 7 SNPs, vectors 5–8 harbored 4 haplotypes of 3 SNPs, vectors 9 and 10 carried different alleles of rs2301228, which were closest to the transcriptional start site, respectively (Fig. 2). The luciferase activity was significantly reduced in vector 9 (contains risk "A" allele of rs2301228) comparing with vector 10 (contain "G" allele of rs2301228) in both HEK293T and U251 cells, and the result was reproducible (Fig. 2A and B). In both cell lines, the rs2301228-G allele containing vectors with longer inserts also showed

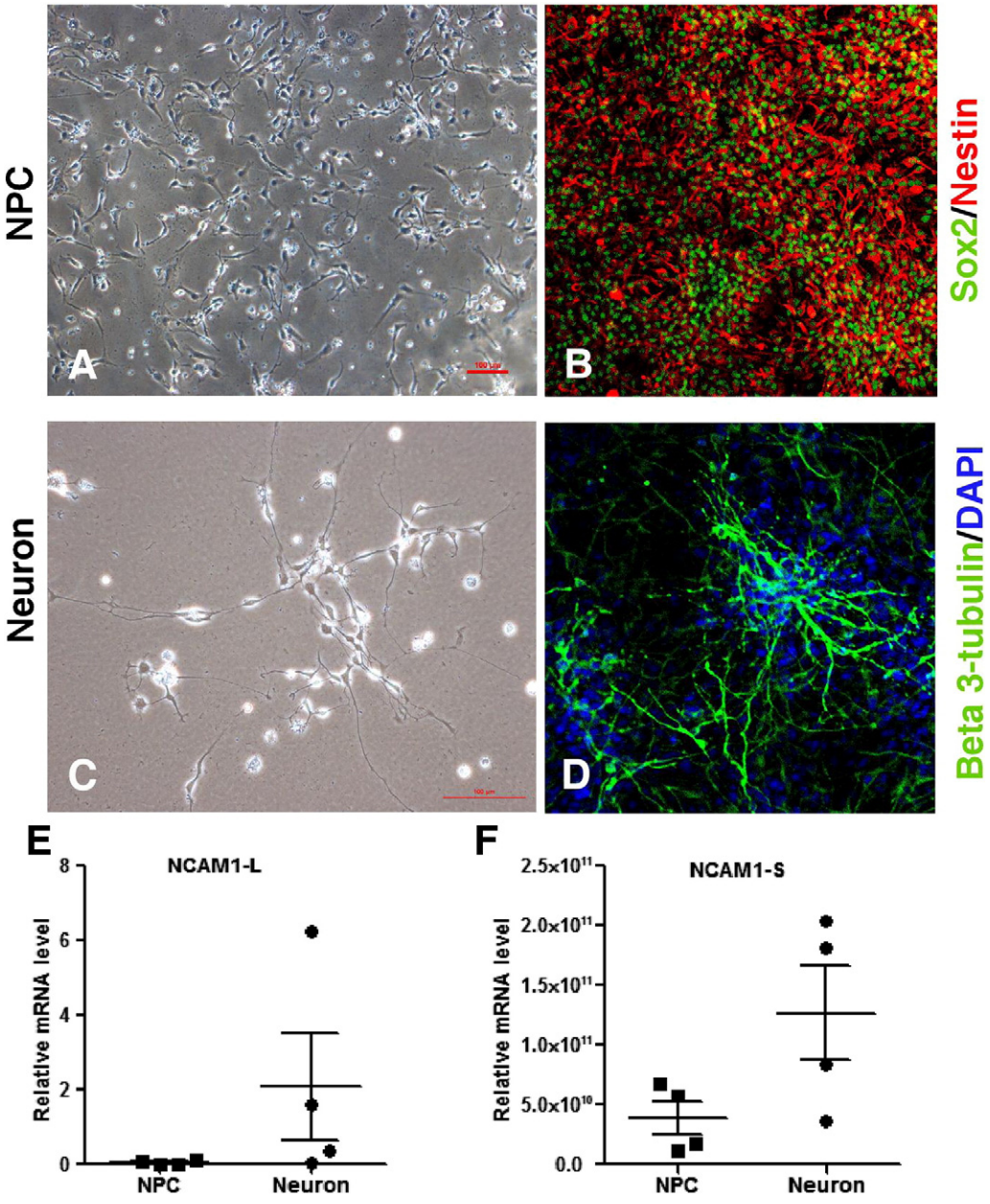

Fig. 4. Characterization of *Macaca mulatta* neural progenitor cells (NPC) and neuronal differentiation. (A) Bright field of NPCs. (B) Immunofluorescence staining shows expression of two common neural stem cell markers Sox2 and Nestin in NPCs. (C) Bright field of neurons differentiated from NPCs. (D) Immunofluorescence staining showing expression of common neuron marker ß3-tubulin. mRNA expression levels of *NCAM1* long (E) and short (F) transcripts in neural progenitor cells and neurons of *M. mulatta*. NCAM1-L: *NCAM1* long transcript, a homolog of transcript encoding NCAM-180 in humans. NCAM1-S: *NCAM1* short transcript, a homolog of transcript encoding NCAM-140 in humans. Data are represented as mean ± SE.

a trend of increased luciferase activity compared with corresponding rs2301228-A allele carriers (Fig. 2A and B). When the rs2301228-A allele was mutated into G in vectors 1–3, the luciferase activity was moderately enhanced (Fig. 2C). These results indicate an active role of rs2301228 in regulating *NCAM1* transcription.

3.3. Altered expression of NCAM1 in patients with schizophrenia and NPC differentiation

ELISA tests demonstrated that the schizophrenia patients had significantly lower levels of serum NCAM1 as compared to normal controls (Fig. 3) ($P < 0.001$). In order to further characterize whether expression of *NCAM1* was altered during neural development, we performed neuronal differentiation of NPCs in *M. mulatta* and compared the expression levels of *NCAM1* transcripts in NPCs and their derivatives. Under neuronal differentiation conditions, NPCs (positive for Nestin and Sox2 staining; Fig. 4) were predominantly differentiated into neurons (β3-tubulin positive, ~90% of the total derivatives). We quantified the mRNA levels of both long and short transcripts of *NCAM1* in NPCs and their neural derivatives (mainly neurons). As shown in Fig. 4, both transcripts of *NCAM1* were temporally regulated during neuron differentiation. *NCAM1* mRNA was expressed at relatively low levels in NPCs, but was highly expressed in neurons (Fig. 4). This developmental expression pattern, together with previous iPSC studies of schizophrenia patients (Brennand et al., 2011), indicates the importance of *NCAM1* expression levels in neural development and neuronal connectivity regulation in primates.

4. Discussion

Neurodevelopmental dysfunction and brain disconnectivity are two widely recognized hypotheses for the pathogenesis of schizophrenia (Schmitt et al., 2011; Rapoport et al., 2012). NCAM1, a key factor in neural development and regulation of neuronal connectivity (Hildebrandt et al., 2007; Cox et al., 2009; Hildebrandt et al., 2009), is purported to play an important role in schizophrenia. Previous studies found decreased NCAM1 levels in the hippocampus of schizophrenia postmortem brains (Barbeau et al., 1995). Recent studies utilizing iPSCs from schizophrenia patients also revealed a transcriptional down-regulation of *NCAM1* in the neuronal development process of schizophrenia, and this alteration was related to decreased neuronal connectivity (Brennand et al., 2011).

In this study, three common SNPs of *NCAM1* were identified to be nominally significantly associated with schizophrenia in the discovery sample from Hunan Province. Further analysis of two of these three SNPs showed that the promoter variant rs2301228 could be validated in the replication cohort from Yunnan Province. This association was even more robust when the discovery and replication samples were combined ($P = 0.0017$; Bonferroni corrected $P = 0.029$). To further validate our results, we re-analyzed the association of the *NCAM1* gene with schizophrenia using available GWAS datasets of PGC (Ripke et al., 2013). Positive associations were observed at the gene level (Table S2). Moreover, there are numerous SNPs showing nominally significant associations ($0.05 > P > 1.0 \times 10^{-7}$) with schizophrenia in the PGC datasets (Fig. S2). We performed a meta-analysis for rs2301228 using both PGC data (Ripke et al., 2013) and our own data. The association of rs2301228 with schizophrenia was significant in the combined sample ($P = 0.005$, Fig. S3), supporting a putative role of this SNP in conferring susceptibility to schizophrenia. Interestingly, rs2301228 was previously reported to confer susceptibility to other psychiatric disorders, i.e., temperament and suicidal behavior (Giegling et al., 2010, 2011). *In silico* prediction revealed that risk allele rs2301228-A would result in a loss of TFBS comparing to the G allele. Furthermore, the risk allele rs2301228-A was demonstrated to reduce promoter activity in the luciferase assay (Fig. 2). Note that the genotyping of rs11214437 failed in SNaPshot assay for the replication samples, and this may have led to an inability to detect association. SNP rs11214437 was in strong linkage disequilibrium with rs2301228 ($|D'| = 1$, although r^2 is low which is largely due to the difference of MAF between two sites). We speculate that association with this site would likely attribute to the genetic hitchhiking effect of rs2301228.

Three susceptible haplotypes were recognized, all carrying the rs2301228-A allele, indicating that the susceptibility to schizophrenia conferred by these haplotypes might be caused by risk allele A of rs2301228. Indeed, we found that mutagenesis of the risk allele A to G could moderately enhance luciferase activity (Fig. 2C). Collectively, these results suggest that rs2301228 could be involved in schizophrenia, possibly through altering the transcription of *NCAM1*.

Recent studies have identified several proteins and/or metabolites in patient sera as potential biomarkers for schizophrenia (Levin et al., 2010; Schwarz et al., 2012; Yang et al., 2013). In this study, we found that serum NCAM1 protein was significantly reduced in schizophrenia patients as compared to that of controls ($P < 0.0001$; Fig. 3). This observation implicates serum NCAM1 as a potential biomarker for schizophrenia. Unfortunately, no allele/genotype effect of rs2301228 was

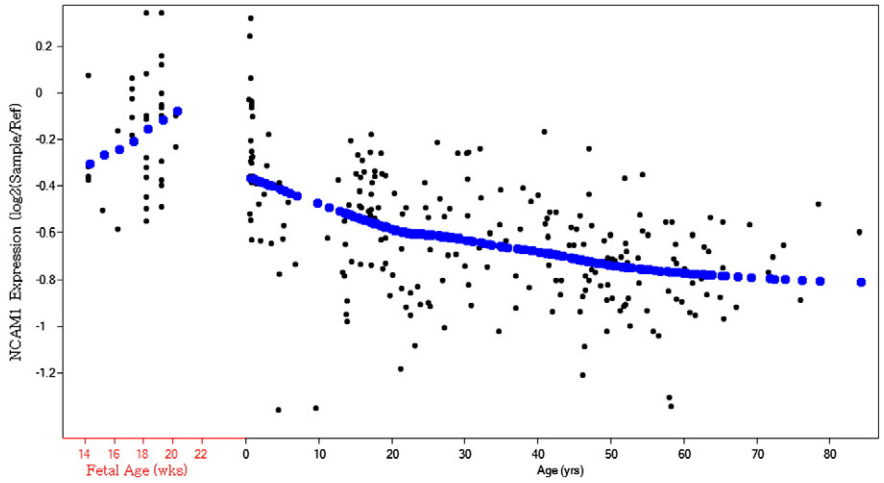

Fig. 5. Correlation analysis of *NCAM1* expression with age using a well-characterized gene expression database BrainCloud. The BrainCloud is comprised of 261 postmortem dorsolateral prefrontal cortexes (DLPFC) of normal subjects, including 113 Caucasian subjects and 148 African American individuals across the lifespan (Colantuoni et al., 2011). The raw expression data and demographic information such as RIN, race, sex, and age were obtained. Statistical analysis was conducted using linear regression, with RIN, race, sex, and age as covariates. We observed a *P* value < 0.00001.

found on serum NCAM1 levels based on the analysis of 72 cases and 88 controls; however, this may be due to the very low frequency of GG genotype carriers in the analyzed samples. Spatial and temporal dynamics of gene expression are important in the regulation of human brain development (Colantuoni et al., 2011; Kang et al., 2011). We found that *NCAM1* transcription exhibited a temporal regulation pattern during neuronal differentiation by using *M. mulatta* NPCs. In particular, both transcripts were more highly expressed in neurons as compared to NPCs (Fig. 4). Furthermore, reanalysis of data from BrainCloud (Colantuoni et al., 2011) also showed that *NCAM1* expression levels are significantly increased in patients with younger age (fetal and juvenile samples) and decreased with aging (linear regression $P < 0.00001$; Fig. 5). These observations, together with previous studies utilizing iPSCs of schizophrenia patients (Brennand et al., 2011) and the schizophrenia-like behavioral changes in *NCAM1* knockout mice (Cremer et al., 1994; Albrecht et al., 2010; Albrecht and Stork, 2012), suggested an active role of *NCAM1* in schizophrenia. Altered expression of this gene may ultimately contribute to risk of schizophrenia and other related neuropsychiatric diseases.

5. Conclusions

In summary, we identified a promoter SNP of the *NCAM1* gene which confers susceptibility to schizophrenia, partially through altering the promoter activity. The reduced NCAM1 protein level was associated with schizophrenia and expression of this gene increased during the differentiation of rhesus monkey NPCs to neurons. These results provide further evidence supporting the neuronal connectivity hypothesis of schizophrenia and indicate the importance of *NCAM1* expression level in neuropsychiatric diseases. Future studies, with independent samples, higher coverage of the *NCAM1* gene region, and functional assays are essential to validate these results and to further elucidate the mechanistic underpinnings of altered *NCAM1* expression in patients with schizophrenia.

6. Limitations

There is one limitation in this study. *NCAM1* is a large gene, which covers 317 kb of genomic DNA. Although we analyzed 17 SNPs in this study, these SNPs may not fully represent the whole gene, and a more comprehensive investigation would have involved the use of at least 58 tagging SNPs. In addition, rare variants in this gene may also have an effect on schizophrenia.

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Contributors

WZ, PZ, XC and YGY designed the study; JT, HYJ, JW, SZ, XFX, LY and XC collected the samples and clinical information; WZ, MSX, SJ, LX, XL and HZW performed the experiments; WZ, MSX, ML and DFZ performed the data analyses; WZ, ML and YGY wrote the manuscript. All the authors read and revised the manuscript.

Conflict of interest

The authors report no biomedical financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.schres.2014.09.036.

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