Alterations of serine racemase expression determine proliferation and differentiation of neuroblastoma cells

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Abstract

Genetic deletion of serine racemase(SR) causes development disability of neuronal dendrites whereas increased expression of SR is found in differentiated cells in culture. These observations suggest roles of SR in cell proliferation and differentiation. Very recently,we have characterized increased SR expression when a neuroblastoma cell line,N2A,undergoes differentiation. However,whether and how the expression levels of SR determine cell destiny remains unclear. We herein indicated that valproic acid(VPA) or all-trans retinoic acid induced N2A differentiation which was mitigated by knockdown of SR. Knockdown of SR increased N2A proliferation,promoted cell cycle entry,and modulated expression of cell cycle-related proteins in N2A cells. Tumor formation in nude mice was used to further examine the effect of SR expression on cell destiny. N2A cells stably expressing scramble shRNA(*Srr*^{wt}-N2A) or *Srr* shRNA (*Srr*^{kd}-N2A) were subcutaneously injected into nude mice,respectively. The tumors growing from *Srr*^{wt}-N2A,significantly mitigated by intraperitoneal injection of VPA,were smaller than the tumors growing from *Srr*^{kd}-N2A which were not affected by VPA. With R2 program,the levels of SR expression were predicted to be positively correlated with survival probability in neuroblastoma patients. This study unveils that alteration of SR modulated proliferation and differentiation of neuroblastoma cells and the mechanism. The results further suggest that induction of SR expression is a novel strategy to treat neuroblastoma.

Introduction

Serine racemase(SR) is a pyridoxal-5'-phosphate(PLP)-dependent enzyme which catalyzes racemization to preferentially produce D-serine and α , β -elimination reaction to generate pyruvate and ammonia(1). D-serine is an endogenous coagonist of N-methyl-D-aspartate receptor(NMDA-R),fine-tuning its activity(2, 3). Elaborate maintenance of D-serine participates in essential physiology including synaptic plasticity,neurotransmission,and cell migration(4-6). Thus,alterations of SR expression and D-serine levels are involved in neuropsychiatric disorders.

Reduced levels of SR and D-serine leading to hypoactivation of NMDA-R activity has been linked with schizophrenia(7, 8). A few studies suggest that genetic deletion of SR causes development delay of dendrites and instability of synapse which underlie the pathology of schizophrenia. For example,deletion of SR in mice produces less elaborate dendrite arborization and decreased spine density of pyramidal neurons in primary somatosensory cortex and of the neurons in the hippocampal formation(9, 10). These observations suggest that deficiency of SR postpones the development of newborn neurons. Conversely,SR is increased in differentiated cells or tissues. For example,SR is increased in confluent keratinocyte cultures compared with growing phase of cultures(11). P19 cells,a line of pluripotent embryonal carcinoma,express SR when induced differentiation into neurons or glia by all-trans retinoic acid;this differentiation is not overcome by inhibiting synthesis of D-serine(12). Emerging study indicates that SR is increased in colorectal adenoma and more significantly in advanced stage(13). Thus, alteration of SR expression may mediate cell proliferation and differentiation.

Neuroblastoma is considered to derive from undifferentiated neural crest cells(14). Originating from sympathoadrenal nervous system,neuroblastoma is an extracranial tumor which accounts for more than 7% of malignancies in pediatric patients and ~15% of all pediatric deaths due to cancers(15). Although overall survival has improved, around 36% of patients due to the characteristics of metastasis and undifferentiation-related malignancy remain difficult to be cured(16). For these portions of pediatric patients,overall survival rates remain below 40% in spite of multimodal therapies including radiotherapy,myeloablative chemotherapy,immunotherapy,and aggressive surgery(17). The detailed molecular events driving neuroblastoma are unclear but the genes regulating proliferation and differentiation of neural crest stem cells are thought to underlie the initiation and progression of neuroblastoma(18). For example,mutation of the anaplastic lymphoma kinase(ALK) gene has been found in 50% familial and 5-15% of sporadic cases of neuroblastoma(19-21). Amplification of MYCN- which has homology to but distinct with *v-myc*(22, 23) - is found in ~25% cases and correlates with high-risk neuroblastoma(14). Together with DNA diploidy and 11q alterations, amplification of MYCN is used to indicate poor prognosis(14). Either ALK mutation or amplification of MYCN is involved in the occurrence of neuroblastoma because these mutations cause uncontrolled cell proliferation and inability to differentiation(24, 25). Thus,the investigations of how neuronal fate is determined and of what factors contribute to the proliferation and differentiation of neuroblastoma cells are warranted regarding the challenges with neuroblastoma therapy.

Very recently,we found that valproic acid(VPA) induced SR expression in the context of differentiated condition but failed to increase SR in undifferentiated milieu(26). Thus, we aim to explore whether and how SR modulates differentiation of N2A cells. With *in vitro* cultures,we found that SR mediated the differentiation effect of VPA on N2A cells whereas knockdown of SR promoted its proliferation. The effects resulted from alteration of the expression of cell cycle-related proteins. With nude mice to develop neuroblastoma *in vivo*,stable knockdown of SR increased tumor proliferation and VPA mitigated the proliferation of tumors only under the context of normal SR expression. This study highlights a novel role of SR in differentiation of neuroblastoma cells and further suggests that intervention by inducing SR expression may be an invaluable therapy against neuroblastoma.

Materials And Methods

The following materials were used in this study:PrimeSTAR[®] HS DNA polymerase (cat# R010A),pMD18-T vector(cat# 6011),PrimeScript[™] RT reagent kit with gDNA eraser,KpnI,and XbaI(Takara Bio,Dalian,China). Trizol reagent,lipofectamine 2000,pcDNA3.1(cat#V79520)(Invitrogen,Carlsbad,CA,USA). SYBR[®] Green PCR master mixture(Life Technology,NY,USA). DMEM(cat# 12430-047),0.05% trypsin(cat# 25300-054),and Opti-MEM media(cat# 31985-070)(Gibco,Shanghai,China). FBS(cat# 04-001-1ACS)(Biological Industries,Israel). Penicillin-Streptomycin(cat# C0222), phenylmethanesulfonyl fluoride(PMSF,cat# ST505),protease inhibitor cocktail(cat# P1010), and BCA protein assay kit(cat# P0012)(Beyotime Biotechnology,Shanghai, China. Neuro-2a(N2A)(cat# CCL-131,RRID:CVCL_0470,ATCC,Manassas,VA,USA). Valproic acid(cat#HY-10585),DMSO,and D-serine(cat#HY-100808)(MedChemExpress, Shanghai,China).

The antibodies used in this study included:mouse monoclonal anti-SR(cat# 612052,RRID:AB_399439) (BD,Biosciences,San Jose,CA,USA);rabbit monoclonal anti-pH3(phospho-histone H3)(cat# 53348T,RRID:AB_2799431),rabbit monoclonal anti-CyclinD1(cat# 55506T),mouse monoclonal anti-P27(cat#3698T, RRID:AB_2077832),and rabbit monoclonal anti-phospho-Rb(Ser807/811) (ca #8516T,RRID:AB_11178658)(Cell Signaling Technology,Beverly,MA,USA). Mouse monoclonal anti-P21(cat# sc-271610,RRID:AB_10647231) and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase(GAPDH)(cat# sc-47724,RRID: AB_627678)(Santa Cruz Biotechnology,Santa Cruz,CA,USA). Mouse monoclonal anti-neuronal class III β -tubulin(Tuj1)(cat# AT809)(Beyotime Biotechnology,Shanghai,China) Rabbit monoclonal anti-Ki67(cat# ab16667,RRID:AB_302459,Abcam,Cambridge,MA, USA). Peroxidase-conjugated goat anti-mouse IgG(H+L) antibody(cat# ZB-2305, RRID:AB_2747415) and horseradish-labeled goat anti-rabbit IgG (H+L) antibody(cat# ZB-2301,RRID: AB_2747412)(ZSGB-Bio,Beijing,China). Alexa fluor[®] 488-conjugated secondary antibody(RRID:AB_143160,Thermo Fisher Scientific Inc,USA). Alexa fluor[®] 594-conjugated secondary antibodies (RRID:AB_141672, Thermo Fisher Scientific Inc,USA). Mounting medium containing DAPI(RRID:AB_2336790,Vector Laboratories, Inc,CA, USA).

Study approval

The procedures to manipulate animals and to reduce animal discomfort were approved by the Institutional Review Board of Wenzhou Medical University(approval number# wyd2020-0297).

Cell culture

Neuro-2a(N2A) cells were cultured in Dulbecco's Modified Eagle's Medium(DMEM) supplemented with 10% fetal bovine serum(FBS). Under differentiated condition,the media were switched into DMEM plus 1% FBS containing 1mM VPA. The cultures were maintained in a humidified 5% CO₂ incubator at 37 °C.

Real-time quantitative reverse-transcription PCR

N2A cells were cultured in 6-well plates at the density of 400,000 cell/well,in DMEM plus 10% FBS. After 24 hours,the culture media were switched to DMEM plus 1% FBS containing 1mM VPA for inducing differentiation. At different timepoints subject to treatment,the cells were used for RNA extraction with Trizol reagent. Reverse transcription reaction was conducted to synthesize cDNA by use of a PrimeScript[™] RT reagent kit with gDNA eraser(Takara Bio). Twenty nanograms of cDNA was used as a template to amplify *Srr* and *actin* mRNA. The primers were used in qRT-PCR to amplify *Srr* and *actin*: *mSrr*,forward primer,5'-ACCCAGTGCTAACAAGCTC-3',reverse primer,5'-ACTACGG CTTTGGGCTTCTC-3';*m β -actin*,forward,5'-GAGGA GATTACTGCTCTGGCTCCTA-3',reverse,5'-TCATCGTACTCCTGCTTGCTGAT-

3'. Similarly, the Power SYBR® Green PCR master mixture (Life Technology, NY, USA) was used as the reaction dye for the qRT-PCR reaction and all PCR reactions were conducted in a 96-well ABI plate format in ABI7500 (Applied Biosystems, USA).

Western blot

N2A cells were harvested and homogenized in Super RIPA buffer. Proteins were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. After sequential incubation with primary and secondary antibody, the membranes were developed with chemiluminescence system kit (ThermoFisher). Images of immunoblots were obtained with FluorChem E Systems (ProteinSimple, Santa Clara, MA, USA) and the optic densities of the immunoblots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Construction of SR-expressing plasmid

Trizol reagents were used to extract RNA from N2A cell. With the extracted RNAs as a template, cDNA was synthesized with reverse transcription reaction kit (PrimeScript™ RT reagent kit with gDNA eraser). By use of an enzyme mixture of an Ex Taq polymerase® (cat# RR001A, Takara Bio) and a high-fidelity Taq polymerase (PrimeSTAR® HS DNA polymerase, Takara Bio) at the ratio of 1:1, PCR reaction was used to amplify *Srr* cDNA product with the forward primer, 5'-ATGTGTGCTCAGTACT GCATCT-3', and the reverse primer, 5'-CTAAACAGAAACCGTCTG GTAAGGAG-3'. The PCR products were analyzed with DNA electrophoresis and ~1kbp DNA fragment was purified from agarose gel. With the purified product as a template, PCR reaction was used to synthesize product incorporating desired restriction sites and the Kozak sequence to enhance translation. The forward primer was 5'-AAGATCTCGAG GCCACCATGTGTG CTCAGTACTGCATCT-3', with BglII restriction site and the Kozak consensus sequence underlined, respectively. The reverse primer was 5'-CAGAAAC CGTCTGGTAAGGAGTACCCATACGACGTCCCAGACTACGCTTAACTGCAGTCTAGA-3', with PstI and XbaI restriction site underlined, respectively. After PCR reaction, the product was ligated to pMD18-T vector. After screening targeted clone with sequencing result, the bacterial clone was amplified and used to extract plasmid. The plasmid was restricted by sequential restriction of KpnI and XbaI and ligated to similarly restricted pcDNA3.1. The ligated product was transformed into DH5α *E. coli* bacteria and the plasmid was extracted as a SR-expressing plasmid.

Establishment of a SR-knockdown N2A cell line

Initially, three plasmids expressing *Srr* shRNA were transiently transfected into N2A cells and *Srr* mRNA and SR protein levels were examined so as to screen knockdown efficacy. The plasmids screened

included:sh336,GCTCAGTACTGCATCTCCTTT;
sh445,GGCGCAATCTCTTCTTCAAAT;sh597,GCTCTCACCTATGCTGCTAAA. The expression of each shRNA was driven by a U6 snRNA promoter in pGPU6/GFP/Neo (GenePharma,Shanghai). Interference plasmid sh445 reached maximal effect. Stable transfection of these GFP-containing plasmids(sh445) was used for generating SR-knockdown cell line. N2A cells were cultured in 6-well plates at the density of 400,000 cell/well,in DMEM plus 10% FBS,until 60-70% confluency. The culture media were switched into DMEM and transfected with pGPU6/GFP/Neo(GenePharma, Shanghai),expressing *Srr* shRNA(sh445,GGCGCAATCTCTTCTTCAAAT) or scramble RNA(NC,TTCTCCGAACGTGTCACGT) at 2µg/well. After four hours,the media were switched to DMEM plus 10% FBS supplemented with penicillin(100 unit/mL) and streptomycin(100 µg/mL)(PS) for another 24 h. The transfected N2A cells were gently trypsinized,suspended,and selected with fluorescence-activated cell sorting (FACS), basing on GFP fluorescence. The selected N2A cells by FACS were cultured in DMEM plus 10% FBS supplemented with PS and G418(300 µg/mL) for three days. G418 was added to the culture to exclude N2A cells dislodging the transfected plasmids. The selection by addition of G418 and FACS were repeated at least five times to obtain high-purified SR-knockdown N2A cells.

Neurite outgrowth assay

For determination of the effect of VPA on neuronal differentiation,the neurons containing the neurite length more than two-fold neuronal soma diameter were considered as differentiated neurons and were counted and quantified(LEICA DMI8 Fluorescence Microscope). The measurement of neurite length was conducted in triplicate culture preparations and the values were averaged from between 150-200 cells in either group.

Cell cycle analysis by fluorescence-activated cell sorting(FACS)

N2A cells were cultured in 6-well plates at the density of 400,000 cells/well,in DMEM plus 10% FBS. After 24 h,the culture media were switched into DMEM plus 1% FBS and the cells were subject to vehicle or VPA treatment for 48 h. After treatment,the cells were subject to gentle trypsinization and subsequent centrifugation. The supernatants were discarded and the precipitates were washed with PBS. With addition of ice-cold 70% ethanol,the cells were dispersed evenly for storage at -20 °C until use. Before cell cycle analysis,the cells were washed and subject to propidium(PI) staining. With a cell cycle and apoptosis analysis kit(C1052,Beyotime Biotechnology),cell cycle was analyzed with BD FACSCalibur basing on PI staining intensity(BD Biosciences,San Jose,CA,USA).

5-ethynyl-2'-deoxyuridine labeling

5-ethynyl-2'-deoxyuridine(EdU) labeling was used for monitoring N2A proliferation. N2A cells were cultured on glass slides sitting in 12-well plates and treated by VPA(1mM) in DMEM plus 1% FBS for 48 hours. EdU was added at a final concentration,10 μ M,for 2 hours. N2A cells were fixed in 4% paraformaldehyde at room temperature for 10 minutes,then washed with PBS three times. After wash,the neurons were blocked with 3% bovine serum albumin and permeabilized with 0.2% Triton-X100 at room temperature for 20 minutes. After wash,the neurons were incubated with developing solution(Click-iT™ EdU imaging kit) at room temperature for 30 minutes in the dark. The cells were coverslipped,and images were captured with a fluorescence microscope(LEICA DMI8 Fluorescence Microscope).

Establishment of neuroblastoma model with nude mice

Nude mice were purchased from Charles River Laboratories (Beijing) and fed in a standard pathogen-free animal facility for one week before experiment at the age of six weeks. The modified N2A cells as described above were gently digested by trypsin to single cell suspension and washed. The cells were made into 10^7 cells/mL cell suspension. By route of subcutaneous injection,0.1mL cell suspension was bilaterally injected into nude mice at the junction of hindlimb and abdomen. After 3.5 weeks,the mice developing approximately equal sizes of tumors were evenly assigned to groups either subject to intraperitoneal injection of vehicle or VPA(250 mg/kg). After two weeks,the mice were euthanized and tumors were immediately excised,using for evaluating the weights and sizes. The isolated tissues from tumor were also used for qRT-PCR and immunofluorescence.

Utilization of R2 program

The program of R2:Genomics Analysis and Visualization Platform(<http://r2.amc.nl>) with Kaplan scanning was used to predict the relationship between SR expression and the prognosis of neuroblastoma patients. Neuroblastoma Oberthuer(ArrayExpress:E-TABM-38),Neuroblastoma Versteeg(GEO:GSE16476 88/122),and Neuroblastoma SEQC(GEO:GSE62564),publicly available datasets,were used for the analysis. In brief,R2 program calculated the optimal cutoff expression for *Srr* by dividing the patients in a good and bad prognosis cohort. According to the expression of *Srr*,samples within a dataset were sorted and divided into two groups,high and low level of expression groups,on the basis of a cutoff expression value. All cutoff expression levels and their resulting groups were analyzed for survival. The cutoff level was reported and used to generate Kaplan-Meier graphs.

Statistics

All data were presented as means $\pm$ SEM unless stated. Data were examined with Shapiro-Wilk test for normality distribution. For parametric data, Student's *t-test* was used to compare the differences between two groups. In experiments with more than two groups,the data were subject to one-way ANOVA,followed

by Tukey's *post-hoc* test. When $p < 0.05$, differences were regarded as significance. Survival rates were estimated by the Kaplan-Meier method, and the log-rank test was used to assess survival difference.

Results

Elevation of SR expression was coupled with neuronal differentiation

In previous study, we have indicated that VPA increases SR expression in DMEM plus 1% FBS but not in DMEM plus 10% FBS compared with sham treatment (26). With staining by use of a neuronal marker, β III-tubulin, differentiated neurons were identified by growing neurites longer than two-fold neuronal soma (Figure.1A). In DMEM plus 1% FBS, VPA treatment (1mM) induced N2A differentiation when the treatments were applied between 24, 48, 72, and 96 hours, respectively (Figure.1A) whereas in DMEM plus 10% FBS, neurites did not grow. The percentages of cells undergoing differentiation in DMEM plus 1% FBS were increased when extending the treatment of VPA. For example, VPA induced 13.98% ($p < 0.0001$ vs. sham treatment), 47% ($p < 0.0001$), 61.88% ($p < 0.0001$), and 67.10% ($p < 0.0001$) of N2A cells to differentiate at 24, 48, 72, 96 hours of treatments, respectively (Figure.1B). So far, we indicated VPA induced the differentiation of N2A cells but the function of SR expression involving in neuronal differentiation is unclear.

Knockdown of SR reduced neuronal differentiation

Thus, we next analyzed the effect of knocking down SR on neuronal differentiation. By using shRNA, the effects of interference on *Srr* mRNA and SR protein levels were tested after transient transfection of plasmids expressing *Srr* shRNAs, i.e., sh336, sh445, and sh597, respectively. The plasmids knocked down *Srr* mRNA to 44.8% ($p = 0.002$), 36.9% ($p = 0.006$), and 40.9% ($p = 0.006$) and SR protein levels to 48.68% ($p = 0.001$), 43.98% ($p = 0.001$), and 64.97% ($p = 0.007$) of those from transfection of plasmid expressing scrambled shRNA (Supplemental Figures.1A-1C). Comparing the knockdown effects caused by the three plasmids, the plasmid expressing *Srr* shRNA (sh445) achieved maximal knockdown effect on *Srr* mRNA (63.1%) and SR protein (56.02%) (Supplemental Figures.1A-1C). Therefore, transfection of the plasmid expressing sh445 and subsequent application of G418 were used to generate stable SR-knockdown cell line, namely, *Srr*^{kd}-N2A whereas the cells stably expressing scrambled shRNA were used as a control cell line, i.e. *Srr*^{wt}-N2A (Supplemental Figures.1D-1F). With the stably-transfected cell lines, significantly lower percentage of *Srr*^{kd}-N2A cells differentiated under the induction of VPA at the same length of treatment, compared with *Srr*^{wt}-N2A ($p < 0.0001$ for 24, 72, and 96 hours of treatments, respectively; $p = 0.01$ for 48 hours of treatment) (Figures.2A and 2B). With extension of treatment, higher percentage of *Srr*^{wt}-N2A cells underwent differentiation (Figures.2A and 2B), similar to the above findings (Figures.1A and 1B). Similarly, all-trans retinoic acid (RA) induced N2A differentiation and the percentages of the differentiation were increased when extending the treatment (Supplemental Figures.2A and 2B). Consistent with the differentiation effect, SR expression was gradually increased

when the treatment time was extended(Supplemental Figures.2C-2E). Similar with VPA,RA induced higher percentages of differentiation in *Srr*^{wt}-N2A compared with those in *Srr*^{kd}-N2A cell(Supplemental Figures.2F and 2G).

The differentiation defect in *Srr*^{kd}-N2A was rescued by SR overexpression (Figures.3A-3D). For example,overexpression of SR-expressing plasmid induced 45.99+/-2.85% of *Srr*^{kd}-N2A cells to differentiate whereas transfection of vehicle plasmid induced 10.03+/-1.58% of *Srr*^{kd}-N2A cells to differentiate under VPA induction ($p<0.0001$)(Figures.3A-3D). To our surprise,addition of D-serine(20 μ M) did not rescue the differentiation deficit:20 μ M D-serine induced 9.58+/-1.50% of *Srr*^{kd}-N2A to differentiate in comparison with 9.27+/-0.74% of cells to differentiate under sham treatment($p=0.755$)(Supplemental Figure.3). Notably,overexpression of SR in *Srr*^{wt}-N2A did not promote differentiation(Figures.3E-3H),i.e.,overexpression of SR-expressing plasmid induced 21.63+/-1.90% of *Srr*^{wt}-N2A cells to differentiate whereas transfection of vehicle plasmid induced 23.38+/-2.04% of *Srr*^{wt}-N2A cells to differentiate ($p=0.254$) (Figures.3E-3H).

Knockdown of SR promoted cell proliferation and cell cycle entry

Reports indicate that the proteins promoting cell cycle exit may increase cell proliferation at lower level of expression. Thus,we explored whether knockdown of SR modulates cell proliferation. *Srr*^{kd}-N2A and *Srr*^{wt}-N2A cells were labelled with a proliferation marker,EdU. As expected,higher percentage of *Srr*^{kd}-N2A cells compared with *Srr*^{wt}-N2A were labeled with EdU staining under VPA treatment($p=0.005$) (Figure.4B). We further subject the cells to cell cycle analysis. Higher percentages of *Srr*^{kd}-N2A cells(20.08+/-1.15%) relative with *Srr*^{wt}-N2A cells(12.91+/-0.91%) were retained in S phase($***p<0.0001$)(Figure.4A). Higher percentages of *Srr*^{kd}-N2A cells(30.8+/-2.59) relative with *Srr*^{wt}-N2A cells(23.92+/-4.34) were retained in G2/M phase($p=0.01$) (Figure.4A). Correspondingly,lower percentage of *Srr*^{kd}-N2A cells stayed in G0/G1 phase(49.12+/-2.7%) compared with *Srr*^{wt}-N2A cells in G0/G1 phase (63.18+/-2.7%; $p<0.0001$)(Figure.4A). VPA treatment reduced the percentage of *Srr*^{wt}-N2A cell and *Srr*^{kd}-N2A cells in S phase(10.44+/-1.06% for *Srr*^{wt}, $**p=0.03$;7.94+/-0.47% for *Srr*^{kd}, $###p<0.0001$ vs.non-treatment,respectively) and G2/M phase(13.21+/-0.75% for *Srr*^{wt}, $***p<0.0001$;19.33+/-1.96% for *Srr*^{kd}, $###p<0.0001$ vs.non-treatment,respectively). VPA treatment increased the percentages in G0/G1 phase compared with non-treatment,respectively(76.35+/-1.67% for *Srr*^{wt}, $***p<0.0001$;72.74+/-2.2% for *Srr*^{kd}, $###p<0.0001$ vs.non-treatment,respectively)(Figure.4A). We further examined whether knockdown of SR modulated expression of cell-cycle related proteins.

Knockdown of SR modulated expression of cell-cycle related proteins

We further analyzed the expression of cell cycle-related proteins. Consistent with EdU labeling, cell proliferation marker, pH3 and Ki67, were increased in VPA-treated *Srr^{kd}*-N2A compared with VPA-treated *Srr^{wt}*-N2A ($p=0.005$ for pH3 comparison; $p=0.029$ for Ki67 comparison) (Figures. 5E and 5G). CyclinD1 and p-Rb, the proteins promoting G1/S transition, were increased in VPA-treated *Srr^{kd}*-N2A compared with those of VPA-treated *Srr^{wt}*-N2A ($p=0.003$ for CyclinD1 comparison; $p=0.0032$ for p-Rb comparison) (Figures. 5B and 5F). By contrast, P21 and P27, the proteins blocking G1/S transition, were decreased in VPA-treated *Srr^{kd}*-N2A compared with VPA-treated *Srr^{wt}*-N2A ($p<0.0001$ for P21 comparison; $p=0.003$ for P27 comparison) (Figures. 5C and 5D). In DMEM plus 10% FBS, the proteins level of CyclinD1, P21, P27, pH3, pRB, Ki67 were not different between *Srr^{kd}* and *Srr^{WT}* N2A cells, respectively (Supplemental Figure. 4).

SR expression was linked with efficacy against neuroblastoma

To further test the effect of knockdown of SR on neuronal proliferation and differentiation, we subcutaneously injected *Srr^{wt}*-N2A and *Srr^{kd}*-N2A into nude mice. After 3.5 weeks following transplantation, the mice were divided into groups subject to intraperitoneal injection of vehicle or of VPA. The tumors were isolated and analyzed on the 2nd week after VPA or vehicle injection. The volumes of tumors developed from *Srr^{wt}*-N2A (NC: $1.45 \pm 0.40 \text{ cm}^3$) were less than those from *Srr^{kd}*-N2A (sh445: $2.15 \pm 0.37 \text{ cm}^3$) ($p=0.02$) (Figures. 6A and 6D). Similarly, the weights of tumors developed from the former (NC: $0.98 \pm 0.1 \text{ g}$) were less than those from the latter (sh445: $1.24 \pm 0.2 \text{ g}$) ($p=0.03$) (Figures. 6B and 6D). Compared with vehicle, VPA injection significantly reduced the tumor sizes from *Srr^{wt}*-N2A (NC VPA: $0.68 \pm 0.23 \text{ cm}^3$ vs. NC: $1.45 \pm 0.40 \text{ cm}^3$, $p<0.0001$) but did not have effect on *Srr^{kd}*-N2A-derived tumors (sh445 VPA: $2.18 \pm 0.38 \text{ cm}^3$ vs. sh445: $2.15 \pm 0.37 \text{ cm}^3$, $p=0.561$) (Figures. 6A and 6D). Regarding the weights, VPA injection significantly reduced the tumor weights from *Srr^{wt}*-N2A (NC VPA: $0.42 \pm 0.11 \text{ g}$ vs. NC: $0.98 \pm 0.1 \text{ g}$, $p<0.0001$) but did not have effect on *Srr^{kd}*-N2A-derived tumors (sh445 VPA: $1.32 \pm 0.24 \text{ g}$ vs. sh445: $1.24 \pm 0.2 \text{ g}$, $p=0.25$), compared with vehicle (Figures. 6B and 6D). In the tumors, knockdown of SR obviously reduced SR expression and VPA treatment induced SR expression in the tumors (Figures. 6C and 6E).

High SR expression was associated with better prognosis with R2 analysis

To predict the relationship between SR expression and the prognosis in neuroblastoma patients, the program of R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>) with Kaplan scanning was used. With this method, we found that high expression of SR was associated with good prognosis whereas low expression of SR was coupled with low probability of survival (Figure. 7). For example, the patients with high expression of SR have above 95% probability of survival during the 24-168 months follow-up whereas the patients with low expression of SR have only around 60% probability to survival

during the 24 months follow-up;the survival rate dramatically reduced to around 55% between 48-216 months follow-up in the group with low level of SR expression(Figure.7).

Discussion

In the whole,we found that *in vitro*,induced expression of SR mediated differentiation of neuroblastoma cells whereas knockdown of SR mitigated differentiation and increased proliferation under VPA induction. These effects were confirmed by use of a neuroblastoma model. In transplantation experiment,VPA reduced the sizes and weights of *Srr*^{wt}-N2A-derived neuroblasotma but did not have effect on *Srr*^{kd}-N2A-derived tumors. With R2 program,SR expression was linked with high survival whereas low expression of SR was coupled with low level of survival.

Induction of differentiation has shown efficacy in neuroblastoma therapy. For example,application of histone deacetylase inhibitors(HDACi) has emerged as a potential strategy to treat neuroblastoma because of their capacities to inhibit tumor proliferation,or induce differentiation,apoptosis and cell cycle arrest in neuroblastoma (27-30). VPA is a HDACi and a cell differentiator as well. Our recent study suggests that the differentiation effect accounts for SR expression since under undifferentiaton condition,VPA do not increase SR expression(26). The effect of VPA on SR expression is dependent on its differentiation effect rather than the effect of HDAC inhibition. Further, we indicated all-trans retinoic acid,a differentiator of neural precursor cells,increased SR expression,consolidating that SR expression is associated with differentiation. In combination with the observation that knockdown of SR promoted proliferation and cell cycle entry,we conclude that SR expression mediates N2A differentiation. We indicated that SR expression modulated cell proliferation and differentiation of neuroblastoma cells;the coupling between elevation of SR expression and the differentiation was in coincidence with previous reports(11, 12). Particularly,differentiation of P19 cells is coupled with SR expression but is not overcome by inhibiting D-serine synthesis(12). In this study,addition of D-serine did not rescue differentiation deficit in SR-knockdown neurons. We consider that unknown proteins interacting with SR may mediate the differentiation deficit. At least,we clarified that modulation of cell cycle-related protein accounted for the differentiation defect under the context of SR knockdown.

Cell cycle is determined by balanced expression of cell cycle-promoting proteins such as cyclin,cyclin-dependent kinases(CDK) and cell cycle-blocking proteins such as P27^{Kip1} and P21. In neuroblastoma cells,P27^{Kip1} plays roles in inducing cell cycle exit and differentiation via diverse mechanisms(31) by interacting with cyclin and CDK, blocking entry into cell cycle or stabilizing NG2,a transcriptional factor,promoting differentiation(31). Compared with *Srr*^{wt}-N2A cells,*Srr*^{kd}-N2A contained lower level of P27^{Kip1} *versus* higher level of CyclinD1,a G1/S transitional cyclin,under the induction of VPA. The expression pattern suggests that increased expression of SR promote cell cycle exit which was depended on regulation of cell cycle-related proteins. However, the mechanism by which SR modulates the expression of cell cycle-related proteins is yet unclear.

Upregulation of SR is connected with neurodegenerative diseases such as Alzheimer' disease(32-36),amyotrophic lateral sclerosis(37),and diabetic retinopathy(38-40) in which excitotoxicity is a common mechanism whereas downregulation of SR has been majorly connected with schizophrenia(7, 8),due to that deficiency of SR impacts dendrite development during early stage of neuronal development. The mechanism by which deficiency of SR mediates neuronal development delay underlying schizophrenia is unclear. Basing on this study,we propose that deficiency of SR regulates the expression of cell cycle-related proteins and affects neuronal development,thereby leading to less elaborate dendrite arbor in SR-deficient schizophrenia model.

In summary,we indicated that SR expression determined whether N2A underwent proliferation and differentiation and further dissected the mechanism. Further,we indicated that VPA treatment mitigated growth of neuroblastoma by inducing SR expression which suggests that induction of SR expression may be a novel strategy to treat neuroblastoma.

Declarations

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Data availability

All the data were contained in the main text and in the supplemented materials.

Conflict of interest

All the authors have found none to disclaim.

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Figures

Figure 1

VAP induced N2A differentiation. **A**, N2A cells were coated on coverslip sitting in 12-well plate at the density of 80,000 cells/well, cultured in DMEM plus 10% FBS. After 24 hours, the culture media were switched into DMEM plus 1% FBS containing 1mM VPA. The cells were fixed with 4% paraformaldehyde at different timepoints (24-96 hours) of treatment and subject to immunofluorescence with β III-tubulin (1:200). DAPI staining was used to indicate nuclei. The images from triplicate culture preparations were captured under fluorescence microscope and the typical images were shown. Scale bar, 50 μ m. **B**, The cells were defined as differentiated neurons when the values of neurite lengths were bigger than two-fold the values of soma diameter. The percentages of differentiated cells were calculated as the ratios between differentiated neurons and DAPI-staining cells in the same field. The percentage of differentiated neurons were obtained from 15 randomly selected fields for each preparation, at least 50 cells in each field. *** $p < 0.0001$ indicated the differences between treatments vs. non-treatment, respectively. One-way ANOVA was used to compare the differences.

Figure 2

Stable knockdown of SR inhibited neuronal differentiation. The *Srr^{kd}*-N2A(sh445) or *Srr^{wt}* N2A neurons(NC) were plated in 150,000 cells/well in 6-well plates,cultured in DMEM plus 10% FBS. After 24 hours,the culture media were switched into DMEM plus 1% FBS containing 1mM VPA. After treatment by VPA between 0-96 hours,the images were acquired under fluorescence microscope. Scale bar,50 μ m.

B,The differentiated neurons were quantified as described in the materials and methods. The percentages of differentiated neurons were calculated as the ratios between differentiated neurons and total neurons. The ratios were averaged from triplicate culture preparations and 9-15 randomly selected fields were quantified for each culture preparation in each timepoint. The numbers of the percentage of differentiated neurons in *Srr^{kd}*-N2A were lower than those of *Srr^{wt}*-N2A neurons. At 24,72,and 96 hours of treatments,*Srr^{kd}* N2A vs. *Srr^{wt}* N2A, $p<0.0001$,respectively. At 48 hour of treatment,*Srr^{kd}* vs.*Srr^{wt}*, $p=0.001$. Student's *t*-test was used to compare the difference between *Srr^{kd}* and *Srr^{wt}* at each timepoint.

Figure 3

Overexpression of SR rescued the differentiation deficit in *Srr*^{kd}-N2A cells but did not promote differentiation in *Srr*^{wt} N2A neurons. **A**, The *Srr*^{kd}-N2A(sh445) were plated in 6-well plates at the density of 400,000 cells/well, cultured in DMEM plus 10% FBS. After 24 hours in culture, the cells were transiently transfected with 2 µg/well empty vector (EV) or equal amount of SR-expressing plasmid, in DMEM, for 4 hours. After the transfection, the media were switched into DMEM plus 1% FBS containing 1mM VPA. After 48 hours, the cells were harvested for immunoblotting against SR and an internal-loading control, GAPDH. **B**, Transient transfection of SR-expressing plasmid (Com) increased SR expression compared with transfection of empty vector (EV). The results were averaged from triplicate culture preparations. **p=0.009. **C and D**, Transient transfection of SR-expressing plasmid (Com) increased neuronal differentiation compared with transfection of empty vector (EV) in *Srr*^{kd}-N2A neurons, under VPA induction. The ratios were averaged from triplicate culture preparations and 9-15 randomly selected fields were quantified for each culture preparation. ***p<0.0001. **E**, The *Srr*^{wt}-N2A were plated in 6-well plates at the density of 400,000 cells/well, cultured in DMEM plus 10% FBS. After 24 hours in culture, the cells were transiently transfected with 2 µg/well empty vector (EV) and equal amount of SR-expressing plasmid to overexpress SR (OE), in DMEM, for 4 hours. After the transfection, the media were switched into DMEM plus 1% FBS containing 1mM VPA. After 48 hours, the cells were harvested for immunoblotting against SR. **F**, Transient transfection of SR-expressing plasmid (OE) increased SR expression compared with transfection of empty vector (EV). The results were averaged from triplicate culture preparations. ***p<0.0001. **G and H**, Transient transfection of SR-expressing plasmid (OE) did not increase differentiation compared with transfection of empty vector (EV) in *Srr*^{wt}-N2A neurons, under VPA induction. The ratios were averaged from triplicate culture preparations and 9-15 randomly selected fields were quantified for each culture preparation. p=0.254.

Figure 4

Stable knockdown of SR promoted cell proliferation and cell cycle entry. **A**, The *Srr^{kd}*-N2A(sh445) or *Srr^{wt}*-N2A neurons (NC) were subject to cell cycle analysis as described in the materials and methods. In G0/G1 phase, NC VPA vs. NC, ** $p < 0.0001$; sh445 VPA vs. sh445, ### $p < 0.0001$; sh445 vs. NC, ** $p < 0.001$; sh445 VPA vs. NC, ** $p < 0.001$. In G2/M phase, NC VPA vs. NC, *** $p < 0.0001$; sh445 VPA vs. sh445, ### $p < 0.0001$; sh445 vs. NC, * $p = 0.01$. In S phase, NC VPA vs. NC, $p = 0.03$; sh445 VPA vs. sh445, ### $p < 0.0001$; sh445 vs. NC, *** $p < 0.0001$; sh445 VPA vs. NC, *** $p < 0.0001$. **B**, top panel, The *Srr^{kd}*-N2A(sh445) or *Srr^{wt}*-N2A neurons (NC) were coated in coverslips sitting in 12-well plates at the density of 100,000 cells/well. After cultured in DMEM plus 10% FBS for 24 hours, the media were switched into DMEM plus 1% FBS containing 1mM VPA. After 48 hours in culture, the EdU dye (10 μ M) was added to the culture and incubated for 2 hours. The cells were fixed and stained according to the manufacturer's manual. DAPI was used to indicate nuclei. The images were acquired under fluorescence microscope. The images were typical of quadruplicate culture preparations. **Low panel**, the cells positive for EdU staining were counted and the proliferation was represented as the ratios between the numbers of EdU⁺ cells and the numbers of DAPI⁺ cells. The ratios were averaged from 9-15 randomly selected fields in each culture preparations. ** $p = 0.005$.

Figure 5

Stable knockdown of SR modulated expression of cell cycle-related proteins. The *Srr*^{kd}-N2A(sh445) or *Srr*^{wt} N2A neurons(NC) was plated in 6-well plates at the density of 400,000 cells/well. After cultured in DMEM plus 10% FBS for 24 hours, the media were switched into DMEM plus 1% FBS containing 1mM VPA. After 48 hours in culture,the cells were harvested for immunoblotting. Knockdown of SR in *Srr*^{kd}(sh445)-N2A decreased SR,***p<0.0001(A); increased CyclinD1,**p=0.003(B); decreased P21,***p<0.0001 (C);decreased P27,**p=0.003(D);increased pH3,**p=0.005 (E);increased p-RB, **p=0.0032(F);increased Ki67,*p=0.029(G),relative to those in *Srr*^{wt} N2A neurons. Student's *t*-test was used to compare the differences.

Figure 6

Stable knockdown of SR promoted tumor growth and VPA mitigated the growth in *Srr*^{wt}-N2A-derived tumors but not in *Srr*^{kd}-N2A(sh445)-derived tumors. Establishment of neuroblastoma model with nude mice was conducted as described in the materials and methods. Two weeks after injection of VPA or PBS, the mice were subject to euthanization. The tumors were excised and the volumes were measured with a caliper (**A**) and the weights were evaluated by use of an analytic balance (**B**). **A**, The volumes of the sh445-derived tumors (sh445, 2.15 ± 0.37 cm³) were bigger than those of the *Srr*^{wt}-derived tumors (NC, 1.45 ± 0.40 cm³) (*p=0.02). VPA injection reduced tumor sizes in NC (NC VPA, 0.68 ± 0.23 cm³) compared with PBS injection (NC) (**p<0.0001) whereas VPA injection did not reduce tumor sizes in sh445 mice (sh445 VPA, 2.18 ± 0.38 cm³) compared with PBS injection (sh445) (p=0.561). **B**, The values of the weights of the sh445-derived tumors (sh445, 1.24 ± 0.20 g) were bigger than *Srr*^{wt}-derived tumors (NC, 0.98 ± 0.10 g) (*p=0.03). VPA injection reduced tumor in NC (NC VPA, 0.42 ± 0.11 g) compared with PBS injection (NC) (**p<0.0001) whereas VPA injection did not reduce tumor in sh445 mice (sh445 VPA, 1.32 ± 0.24 g) compared with PBS injection (sh445) (p=0.25). The values were averaged from tumors isolated from five mice in each group. One-way ANOVA was used to compare the differences. **C**, The excised tissues from tumors were also subject to qRT-PCR to evaluate the levels of *Srr* mRNA. *Srr* mRNA levels in sh445-derived tumor were lower than NC (**p=0.002). VPA injection increased *Srr* mRNA levels in NC (**p=0.001) or in sh445-derived tumors (#p=0.015), relative to PBS injection, respectively. The results were averaged from tissues isolated from three mice. One-way ANOVA was used to compare the differences. **D**, The tumors isolated from PBS-injection *Srr*^{wt} N2A-derived neuroblastoma mice (NC), VPA-injection *Srr*^{wt} N2A-derived neuroblastoma mice (NC VPA), PBS-injection sh445 N2A-derived neuroblastoma mice (sh445), and VPA-injection sh445 N2A-derived neuroblastoma mice (sh445 VPA) were displayed. **E**, The isolated tissues from tumors were made into paraffin-embedded sections and were immunoblotted against SR for the tissues. DAPI was used to indicate nuclei and SR staining and DAPI

staining were merged in the last column. Omitting the primary antibody did not produce staining. Scale bar,50 μ m.

Figure 7

The potential relationship between SR expression and the prognosis of neuroblastoma patients. Utilization of R2 program as described in the materials and methods was used to predict the relationship

between SR expression and the prognosis of neuroblastoma patients. High level of SR expression(n=22) was associated with good prognosis in neuroblastoma patients whereas low level of SR expression(n=66) with bad prognosis in neuroblastoma patients. Survival rates were estimated by the Kaplan-Meier method, and the log-rank test was used to assess survival difference. **p=0.0016.

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