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Original Article

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Valproic Acid Induces Telomerase Reverse Transcriptase Expression during Cortical Development

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The valproic acid (VPA)-induced animal model is one of the most widely utilized environmental risk factor models of autism. Autism spectrum disorder (ASD) remains an insurmountable challenge among neurodevelopmental disorders due to its heterogeneity, unresolved pathological pathways and lack of treatment. We previously reported that VPA-exposed rats and cultured rat primary neurons have increased Pax6 expression during post-midterm embryonic development which led to the sequential upregulation of glutamatergic neuronal markers. In this study, we provide experimental evidence that telomerase reverse transcriptase (TERT), a protein component of ribonucleoproteins complex of telomerase, is involved in the abnormal components caused by VPA in addition to Pax6 and its downstream signals. In embryonic rat brains and cultured rat primary neural progenitor cells (NPCs), VPA induced the increased expression of TERT as revealed by Western blot, RT-PCR, and immunostainings. The HDAC inhibitor property of VPA is responsible for the TERT upregulation. Chromatin immunoprecipitation revealed that VPA increased the histone acetylation but blocked the HDAC1 binding to both *Pax6* and *Tert* genes. Interestingly, the VPA-induced TERT overexpression resulted to sequential upregulations of glutamatergic markers such as Ngn2 and NeuroD1, and inter-synaptic markers such as PSD-95, α-CaMKII, vGluT1 and synaptophysin. Transfection of *Tert* siRNA reversed the effects of VPA in cultured NPCs confirming the direct involvement of TERT in the expression of those markers. This study suggests the involvement of TERT in the VPA-induced autistic phenotypes and has important implications for the role of TERT as a modulator of balanced neuronal development and transmission in the brain.

Key words: telomerase reverse transcriptase, valproic acid, autism, excitatory/inhibitory imbalance, glutamatergic neuronal differentiation

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INTRODUCTION

The prenatally VPA-exposed rodent is a valid environmental factor animal model of autism spectrum disorder (ASD) widely utilized for its clinical relevance. However, despite the substantial studies conducted using this animal model, the factors and pathways that can delineate the pathophysiology of ASD are still unknown. A druggable target must be determined to effectively carry out the investigations involving this model and to help devise a treatment for the heterogeneous and seemingly insurmountable ASD. Previously, we have elucidated that VPA affects the glutamatergic differentiation of prenatal rat brains through Pax6 [1], with a possible involvement of other components, which can be essential for characterizing the mechanisms of ASD.

Telomeres are regions of DNA repeat sequences located at each end of a chromosome [2]. During cell division, the enzymes duplicating DNA are unable to continue their duplication until the chromosomes' ends [3]. As a result, the length of telomeres is decreased by the division of cell [4]. In lively dividing cells, however, decreased length of telomeres is constantly restored by telomerase. Telomerase is an RNA-dependent polymerase which lengthens telomeres in DNA strands by adding TTAGGG telomere repeat sequence to the chromosome's ends. This addition prevents shortening of the chromosomal ends even after multiple rounds of replication. Telomerase is composed of two protein components including reverse transcriptase activity (TERT, telomerase reverse transcriptase) and an RNA template (TERC, telomerase RNA component) [5, 6]. The activity and expression of telomerase in neural progenitor cells (NPCs) are reported as relatively high, and declines when NPCs differentiate to neuronal fates. There is a decrease in the RNA component of TERT observed before the initiation of neuronal differentiation while the expression level of TERT is constantly elevated during brain development in mice, hinting an involvement of telomerase activity in nervous system development [7].

Neuronal differentiation during embryonic brain development is governed by a number of mechanisms, one of them is the molecular downstream of Pax6 [8]. Pax6 regulates brain development by preventing depletion of NPCs, and also regulates the process of differentiation into glutamatergic neuron through inducing Tbr2, Ngn2, NeuroD1 and Tbr1 [9-12]. Pax6 is expressed in NPCs during cortical neurogenesis, making Pax6 a widely used marker for NPCs [13]. Heins et al. [14] first determined that the overexpression of Pax6 in cortical NPCs deters their proliferation and induces neuronal differentiation. However, detailed analysis of the cell cycle concluded that Pax6 also plays an essential part in the maintenance of proliferative NPCs during corticogenesis [15]. These showed how Pax6 controls the balance between NPC proliferation and differentiation into glutamatergic neuron in the cortex [16].

Valproic acid (VPA) is observed to influence the proliferation and differentiation of neuronal cells. VPA acts as an inhibitor for class I and II HDACs through activating transcription from diverse promoters and causes hyperacetylation of histones. Interestingly, this inhibition of HDAC curbs proliferation and promotes neuronal differentiation of adult hippocampal neuronal progenitor cells [17]. VPA also directly or indirectly inhibits GSK-3β activity altering the Wnt/β-catenin signaling, and β-catenin, as a result, affects the regulation of the ERK pathway. VPA is generally considered as a neuroprotective drug through its regulation of the mentioned pathways, including LOX, PPARs, and PTEN pathways [18].

This study is aimed to examine the effect of VPA administration in the expression of TERT during early embryonic development and in cultured NPCs. Moreover, the mechanism regulating TERT expression in NPCs and the alteration of synaptic protein expression by regulating TERT expression are also investigated. We hypothesize that the expression of TERT is upregulated by VPA during development through histone deacetylase inhibition. Transfection of *Tert* siRNA is utilized to further confirm these results. The end of this study will describe whether TERT is involved in excitatory neurotransmission which has major implications in the manifestation of neurodevelopmental disorders, most especially, autism spectrum disorders.

MATERIALS AND METHODS

Materials

The materials used in this study's experiments are listed below along with their respective suppliers: Lithium chloride, Sodium butyrate, Trichostatin A, and Valproic acid from Sigma (St. Louis, MO); ECL™ reagents from Amersham Life Science (Arlington Heights, IL); Trizol® reagent, SuperScript™II Reverse Transcriptase, *Tert* siRNA [TERT-RSS313560 (rat), TERT-MSS211505-7 (mouse)] from Invitrogen (Carlsbad, CA); Protein G Agarose from Millipore (Billerica, MA); TDZD-8 (4-Benzyl-2-methyl-1,2,4,-Thiadiazolidine-3,5-dione) from Calbiochem (La Jolla, CA); Taq polymerase from Takara (Shiga, Japan).

Antibodies from the following companies: β-actin antibody from Sigma (St. Louis, MO); Tuj-1 antibody from Covance (Princeton, NJ); TERT antibody from Santa Cruz Biotechnology (Santa Cruz, CA); vGluT1, PSD-95, α-CaMKII, NeuroD1, Mash1, BRG1, Synaptophysin antibody from Abcam (Cambridgeshire, England); GAD, Nestin, GFAP, Pax6 and Ngn2GFAP antibody from Millipore (Billerica, MA); HDAC1, Histone H3, Acetyl-Histone H3,

GSK3β and phospho-GSK3β antibody from Cell signaling (Boston, MA).

Animals

Time-determined pregnant SD (Sprague-Dawley) rats were obtained from DaeHan BioLink (Daejeon, Korea). Animals were kept in a room with a 12 hour: 12 hour circadian light cycle (starting from 06:00) with constant humidity (55±5%) and constant temperature (22±2°C). Animals were treated and maintained in accordance with the Principle of Laboratory Animal Care (NIH publication No. 8023, revised 1978) and were approved by the Animal Care and Use Committee of Konkuk University, Korea (KU12115, 12016). All efforts were undertaken toward minimizing the number of animals used and their suffering.

Sodium valproate injection to pregnant rats

The sodium valproate was prepared in 0.9% saline solution (100 mg/ml).Timed pregnant rats were subcutaneously injected with 400 mg/kg valproate on gestational day 12. A subcutaneous injection of saline was used as a control.

Primary neural progenitor cell (NPC) cultures

The cortical progenitor cells from embryos were prepared following by previous reports [19, 20] with minor modifications [21]. NPC cultures were prepared from E14 embryos of SD rats.

Transient siRNA transfection

siRNA transfection was performed using lipofectamine 2000 (Invitrogen, Carlslab, CA). Briefly, *Tert* siRNA (Invitrogen, TERT-RSS313560) was mixed with lipofectamine in OPTI-MEM media without serum and antibiotics. On 6-well plate, 20 pmol of siRNA was used for 1×10^6 cells. Three hour after NPC subculture, confluent NPCs was transfected with the mixture of siRNA-lipofectamine. Three hour after adding siRNA-lipofectamine mixture, NPCs were rescued by changing the medium. A control siRNA (Invitrogen) having similar GC contents with *Tert* siRNA was used as a control.

Western blotting

After transfection, NPCs were lysed with SB (2x SDS-PAGE sample buffer) as previously reported [22]. Isolated cortical tissues were homogenized with RIPA buffer, and the extracted lysates were mixed with SB. Protein concentrations were calculated by BCA assay and 50 μg of proteins for each sample was run in the 10% SDS-PAGE. Polyvinyl alcohol (1%, in PBS) was used for blocking the nitrocellulose membranes containing transferred proteins. The blocked membranes were treated with primary antibody and incubated for 16 hour at 4°C. After incubation for primary antibody, a peroxidase-conjugated secondary antibody (Santa Cruz, CA), with the same species as the primary antibody used, was incubated to the membranes for 2 hour at RT (room temperature). Each band of interest was detected using the ECL™ reagent and visualized by Bio-Rad image analyzer (Bio-Rad, Hemel Hampstead, UK). The loading control used was β-actin for all proteins of interest.

RT-PCR

From NPCs or isolated cortical embryonic brain, RNA was extracted with Trizol reagent (Invitrogen, Carlslab, CA). An aliquot of 1 μg from the total RNA was transformed into cDNA by reverse transcription reaction. The DNA bands of interest were amplified using PCR and the PCR products for amplified DNA of interest were run in 1.0% agarose gel. After electrophoresis, agarose gel was stained by ethidium bromide, and visualized by image analyzer. All primers were purchased from Invitrogen, and the sequence of primers used in this study are as below:

Tert (rat), 5' GTGGTACCAACCCCATCAGG (forward), 5' TCTTTAAGAAGCGGCGCTCA (reverse)

Gapdh (rat), 5' TCCCTCAAGATTGTCAGCAA (forward), 5' AGATCCACAACGGATACATT (reverse)

Immunocytochemistry

NPCs were grown on cover slip, and fixed with 4% PFA (paraformaldehyde) at 4°C for 2 hour. The cells were immersed in 0.3% Triton X-100 for 15 min at RT and blocked with a blocking buffer for 30 min at RT. The cells were incubated for 16 hour at 4°C while being treated with primary antibodies. Secondary antibodies conjugated fluorescent protein were treated to the cells for 2 hour at RT. After washing, the NPCs onto cover slip was mounted in Vectashield and visualized by confocal microscope (TCS-SP, Leica, Heidelberg, Germany).

Immunohistochemistry

Embryonic brains were fixed with 4% PFAand dehydrated with sucrose before sectioning by cryostat (CM 3050, Leica Instruments). The chosen sections were immersed in 0.1% Triton X-100 for 30 min at RT and blocked for 1 hour with 1% BSA in PBS at RT. These sections were incubated for 16 hour at 4°C after being treated with primary antibodies. After washing, the sections were incubated with secondary antibodies for 2 hour at RT. After washing, the sections were mounted in Vectashieldand visualized by confocal microscope (TCS-SP, Leica, Heidelberg, Germany).

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Immunoprecipitation

Immunoprecipitation using NPCs was performed by following report [22]. NPCs were lysed with LB buffer for 15 min and the lysates were centrifuged at 1500 g for 5 min. The supernatant was incubated with antibody for 24 hour at 4°C. After incubation, the sample was incubated with Protein G Agarose (Millipore) for 24 hour at 4°C. After incubation, the supernatant was discarded after centrifugation. The agarose beads in the pellet were washed using LB buffer. The pellet was used for Western blot experiments. For negative control experiments, all procedures were identically handled without any antibody.

Chromatin immunoprecipitation

We performed chromatin immunoprecipitation based on previous report [23] with minor modifications [22]. The sequence of primers used in this study are as below:

Pax6 (rat), 5'AGGACCTCGTAGAGATGATGAAAC (forward),
5'AAAAGAGTTGCTCGTGAGAGTTTT (reverse)
Tert (rat), 5'TAGTTCAGGCCCATATCTCCTAAG (forward)
5'ACCCAAGAGAATTAAGCGTTGTAG (reverse)

In utero transfection

In utero transfection was performed as previously reported [22]. Briefly, E14.5 mice embryos were used for electroporation after anesthetization with isoflurane. *Tert* siRNA was labeled using Silencer® siRNA Labeling Kit with Cy™3 dye (ThermoFisher Scientific, AM1632) before injection. The labeled *Tert* siRNA was injected into the lateral ventricles of the embryonic brain, and electroporation was conducted by a square wave electroporator (BTX). Embryos were isolated four days after electroporation, and used for immunohistochemical studies.

Statistical analysis

Data were shown as the mean±standard error of mean (S.E.M) and analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Newman-Keuls test as a posthoc test. Two-way ANOVA was performed to identify the effect of valproic acid treatment or *Tert* siRNA transfection, or the interaction between the two factors. If significant effects were found in any of the factors, posthoc comparisons were conducted using Bonferroni's post tests. When the p value was less than 0.05 ($p<0.05$), differences were determined statistically significant. All statistical analyzes were examined by PASW Statistics (18.0; SPSS Inc, Chicago, IL, USA).

RESULTS

VPA induces TERT upregulation in neural progenitor cells and embryonic brains

For *in vitro* experiments, cultured primary NPCs were exposed to 0.5 mM VPA 3 hour after subculture. In Fig. 1A, Western blot analysis revealed that VPA exposure increased the expression of

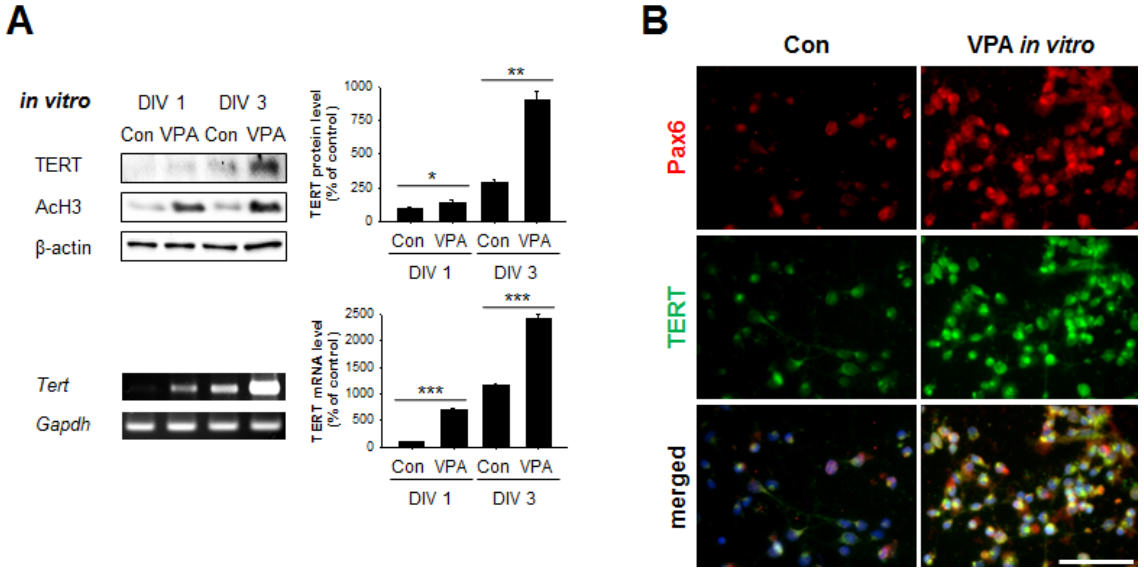

Fig. 1. VPA induced the expression of TERT mRNA and protein in cultured NPCs. (A) The expression of TERT protein was examined by Western blot, and the expression of *Tert* mRNA was examined by RT-PCRin VPA-treated NPCs. Results are mean±S.E.M. (n=3). NPCs were cultured with embryos fromthree different pregnant rats. *p<0.05, **p<0.01, ***p<0.001 vs. control. (B) Immunocytochemistry images of VPA-treated NPCs to detect Pax6 and TERT expressions. NPCs were cultured with embryos from three different pregnant rats. Scale bar is 100 μm.

TERT proteins in NPCs compared to the control samples during DIV1 [t=-3.3424, p=0.0288] and DIV3 [t=-8.2950, p=0.0012]. Accordingly, the mRNA levels of TERT in the VPA-treated NPCs were also enhanced during DIV1 [t=-22.5381, p<0.0001 vs control] and DIV3 [t=-12.0228, p=0.0003 vs control]. Moreover, immunocytochemistry confirmed these results by showing increased staining detections of Pax6 and TERT in the VPA-treated NPCs (Fig. 1B). Indeed, VPA exposure increased both the protein and mRNA levels of TERT in cultured rat NPCs. As we reported previously, the expression of nestin was also enhanced after VPA treatment in cultured NPCs [24].

For *in vivo* experiments, pregnant rats received sodium valproate subcutaneously at gestational day 12, and TERT expression in the embryonic brains was analyzed at E14 and E16 through Western blot and RT-PCR (Fig. 2A). VPA-exposed embryonic brains, specifically the mPFC, were also isolated for immunohistochemistry at E14. In congruence with the *in vitro* experiments, the expression of TERT proteins and mRNA in VPA-exposed embryonic brains were increased at E14 [Proteins: t=-5.5512, p=0.0052; mRNA: t=-4.9931, p=0.0075] and E16 [Proteins: t=-3.7486, p=0.0120; mRNA: t=-3.1814, p=0.0335] compared to control brains. Furthermore, the expressions of nestin and TERT were enhanced in the mPFC of VPA-exposed embryos as detected by immunohistochemistry (Fig. 2B). These results supplement the previous study showing increased Pax6 and nestin expressions in the brain of VPA-exposed rat embryos [1]. Thus, the *in vitro* and *in vivo* experiments of present study newly demonstrate the enhancing effects of VPA in the expression of TERT in embryonic brains and cultured NPCs.

VPA-induced histone deacetylase inhibition influenced TERT overexpression in neural progenitor cells

To investigate the biochemical pathway mediating the effects of VPA on TERT expression, we treated several chemicals on cultured NPCs at DIV1 with the following concentrations: VPA (0.5 mM), trichostatin A (TSA, HDAC inhibitor, 0.2 μM), sodium butyrate (SB, HDAC inhibitor, 0.1 mM), while Lithium Chloride (LiCl, GSK-3β inhibitor, 0.2 mM), and TDZD-8 (GSK-3β inhibitor, 5 μM). VPA, TSA and SB independently increased the expression of TERT proteins in cultured NPCs. On the other hand,

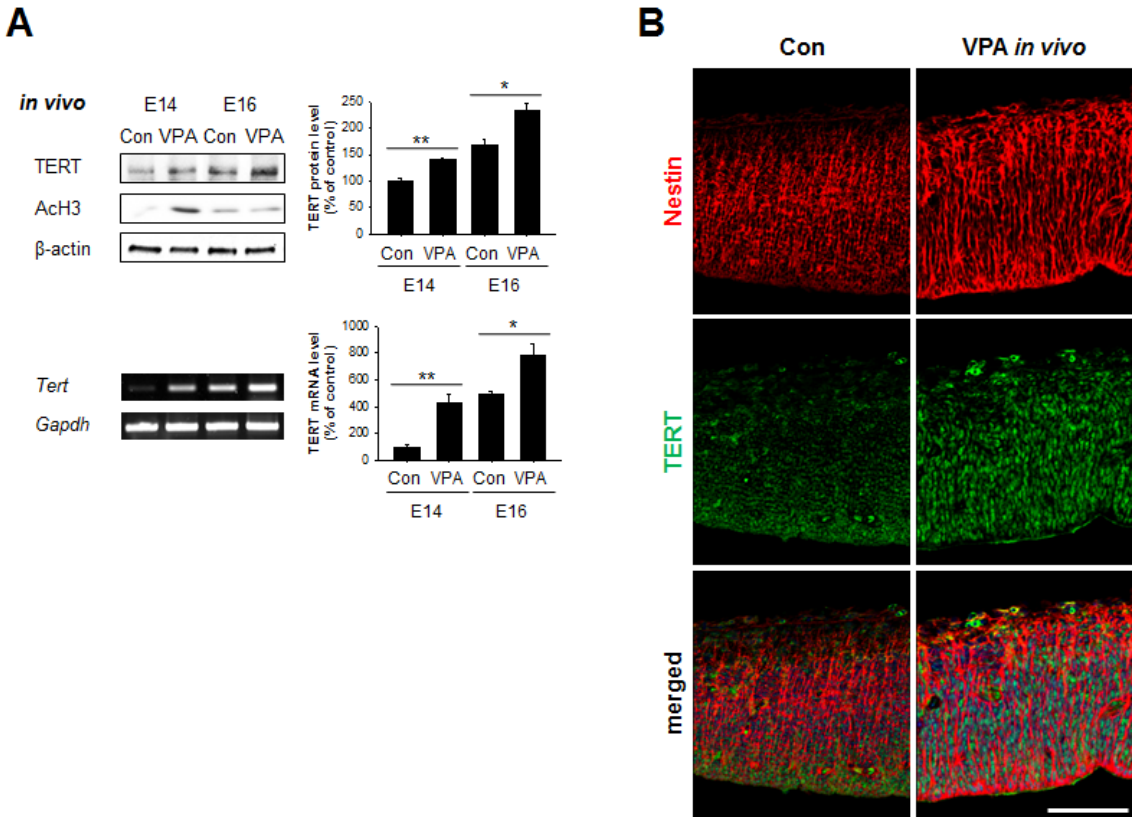

Fig. 2. Prenatal VPA exposure induced TERT expression in the mPFC region. (A) The expression of TERT protein was examined by Western blot, and the expression of *Tert* mRNA was examined by RT-PCRin embryos exposed to VPA-treatment in pregnant mothers. Results are mean±S.E.M. (n=3). Four rat embryos were randomly chosen from three different pregnant rats. *p<0.05, **p<0.01 vs. control. (B) Immunocytochemistry images of VPA-exposed embryonic brains to detect nestin and TERT expressions. One embryo was randomly chosen from three different litters. Scale bar; 100 μm.

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TDZD decreased TERT expression [$F_{(5,12)}$=592.4, p<0.0001] and LiCl did not show any effects on TERT protein level (Fig. 3). In addition, only VPA, TSA, and SB upregulated the expression of AcH3 proteins [$F_{(5,12)}$=31.48, p<0.0001]. Meanwhile, VPA, LiCl, and TDZD increased the phosphorylation of GSK-3β but not TSA or SB [$F_{(5,12)}$=21.61, p<0.0001]. These results confirm the properties of VPA as HDAC and GSK-3β inhibitor. Moreover, the HDACi property of VPA is directly involved in the increased expressions of TERT and AcH3 proteins.

VPA exposure in neural progenitor cells induces opened chromatin status and increased acetylation in Pax6 and Tert genes

We examined the binding of proteins related to chromatin opening onto the promoter regions of *Tert* or *Pax6* genes using ChIP after VPA exposure in NPCs. In Fig. 4A, *in vitro* VPA exposure (0.5 mM) induced histone H3 acetylation [*Pax6*: t=-51.54, p<0.0001; *Tert*: t=-53.60, p<0.0001] and HDAC1 protein dissociation [*Pax6*: t=55.74, p<0.0001; *Tert*: t=35.24, p<0.0001] from both *Pax6* and *Tert* genes. In addition, VPA increased the TERT proteins bound to *Pax6* gene [t=-36.22, p<0.0001], but not the proteins bound to *Tert* gene [t=-2.1365, p=0.0995]. Also, VPA exposure in NPCs increased the binding of Brg1, a chromatin remodeler, unto the promoter region of *Pax6*. We also confirmed the interaction between TERT and Brg1 proteins using IP as reported previously [22].

We then examined the binding of proteins related to chromatin opening onto the promoter regions of *Tert* or *Pax6* gene using ChIP after VPA exposure *in vivo* (Fig. 4B). Similar to *in vitro* re-

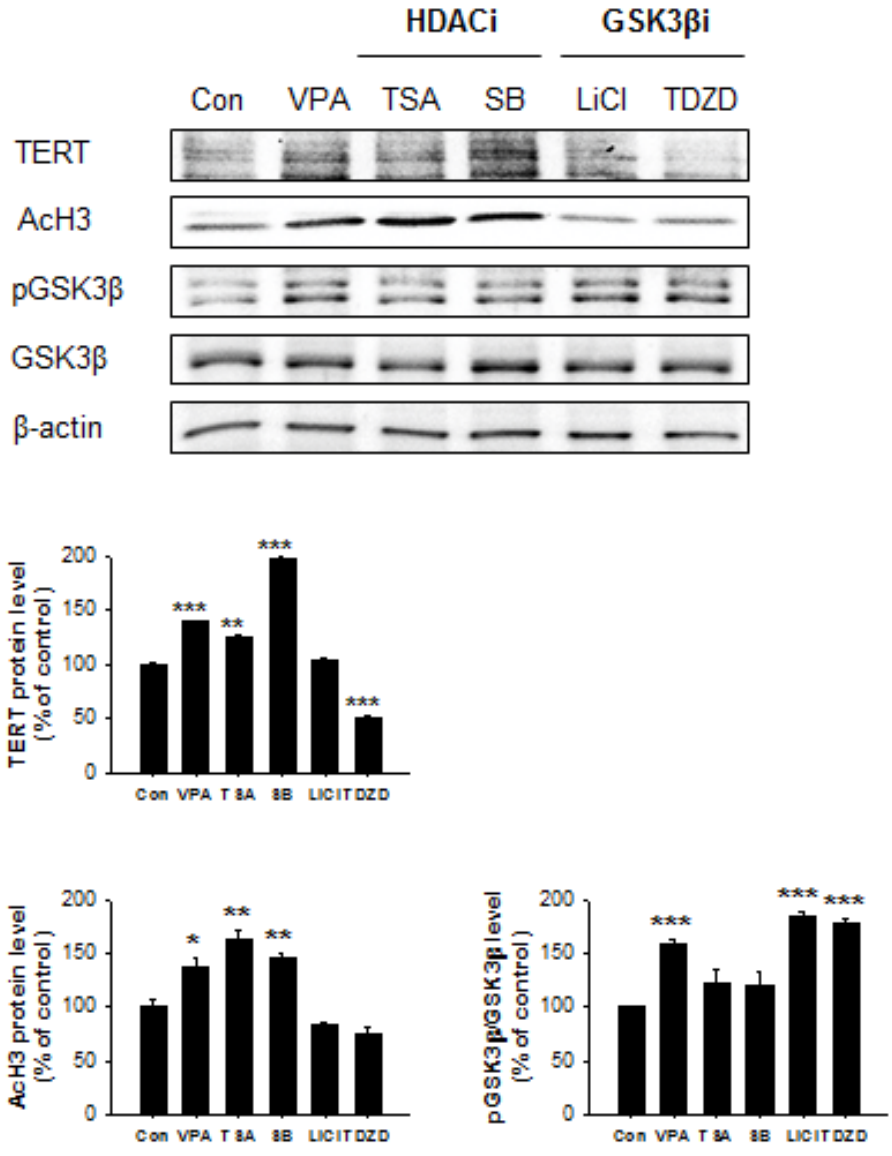

Fig. 3. The HDAC inhibitor activity of VPA increases TERT expression in NPCs. The bands and graphs show the effects of VPA and other HDACi or GSK3βi drugs in the expression of TERT, AcH3, pGSK3β and GSK3β proteins. β-actin was used as the loading control. Results are mean±S.E.M. (n=3). NPCs were cultured with embryos from three different pregnant rats. *p<0.05, **p<0.01, ***p<0.001 vs. control.

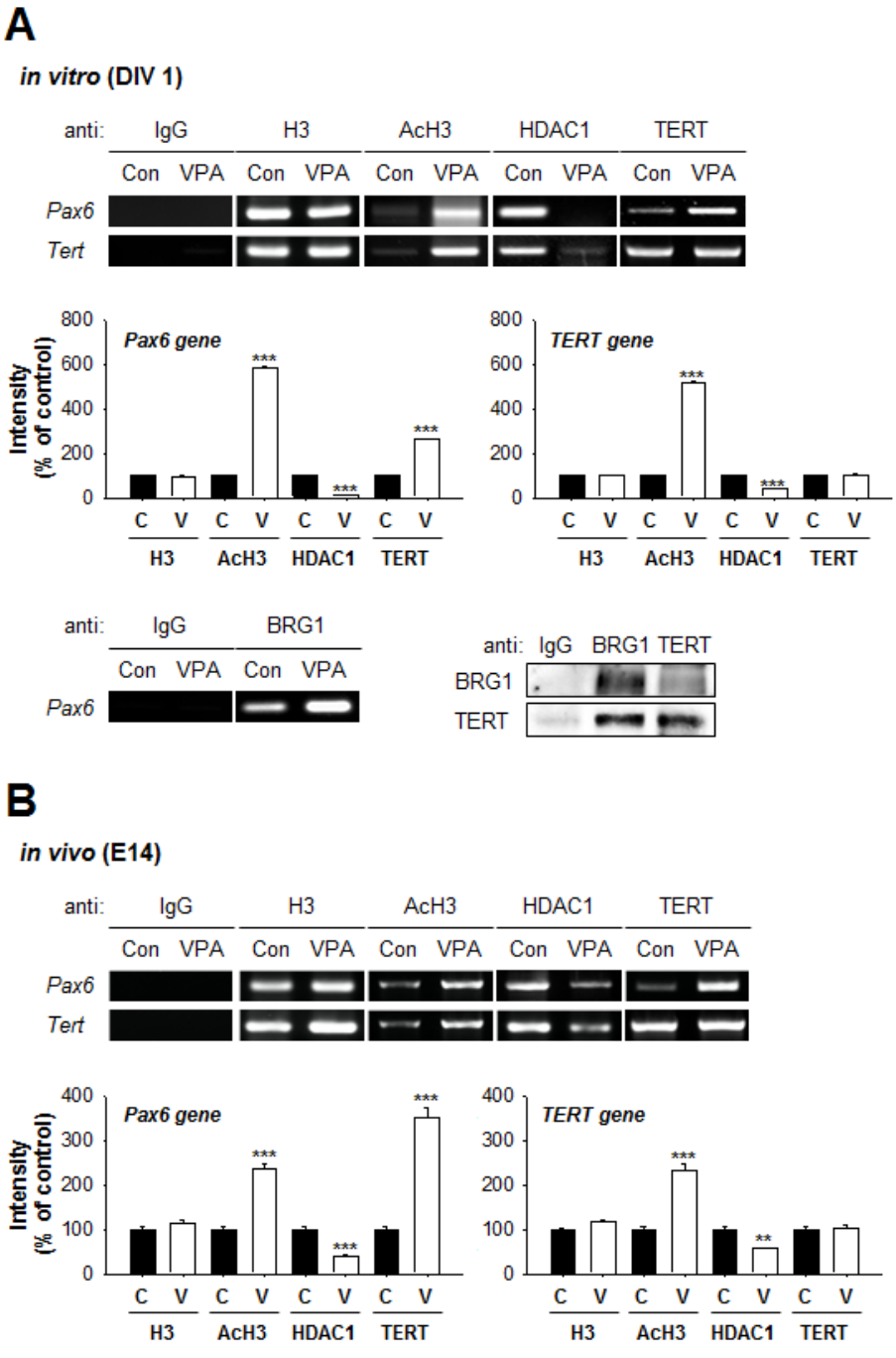

Fig. 4. VPA exposure induced opened chromatin status in *Pax6* and *Tert* genes. (A) The chromatin status markers of *Pax6* and *Tert* genes in VPA-treated NPCs were analyzed through chromatin immunoprecipitation (ChIP). NPCs were cultured with embryos from three different pregnant rats. (B) ChIP was also performed in the VPA-exposed E14 embryonic brains. Results are mean± S.E.M. (n=3). One rat embryo was randomly selected from three different pregnant rats. $^{**}p<0.01$, $^{***}p<0.001$ vs. control.

sults, VPA induced the acetylation of histone H3 [*Pax6*: t=-9.2652, p=0.0008; *Tert*: t=-8.9653, p=0.0009] and the dissociation of HDAC1 [*Pax6*: t=10.2414, p=0.0005; *Tert*: t=6.5130, p=0.0029] in the embryonic brains in both *Pax6* and *Tert* genes. Moreover, we found that VPA increased the TERT proteins bound to *Pax6* gene [t=-11.3052, p=0.0003], whereas TERT proteins bound to *Tert* gene were not changed [t=-0.3905, p=0.7160]. Both the *in vitro* and *in vivo* experiments confirm that VPA increases histone H3 acetylation bound to the promoter region of *Pax6* and *Tert* genes, and TERT protein binding to *Pax6* gene promoter, which may carry major epigenetic implications to the ASD pathophysiology.

siRNA knock-down of TERT decreases the expression of excitatory synaptic proteins

To investigate whether TERT has a critical role in VPA-mediated alterations of the expression of synaptic proteins, transient *Tert* siRNA transfection on NPCs was performed. Two-way ANOVA was performed to determine the effect of valproate treatment or

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transient *Tert* siRNA transfection. Fig. 5A depicts the relationship between protein expression and neuronal subtype differentiation.

On culture day 1, TERT, Pax6 and nestin expressions were decreased by *Tert* siRNA transfection. Meanwhile, on culture day 7, Ngn2 and NeuroD1 expressions were decreased by *Tert* siRNA transfection, whereas Mash1 expression was increased. In culture

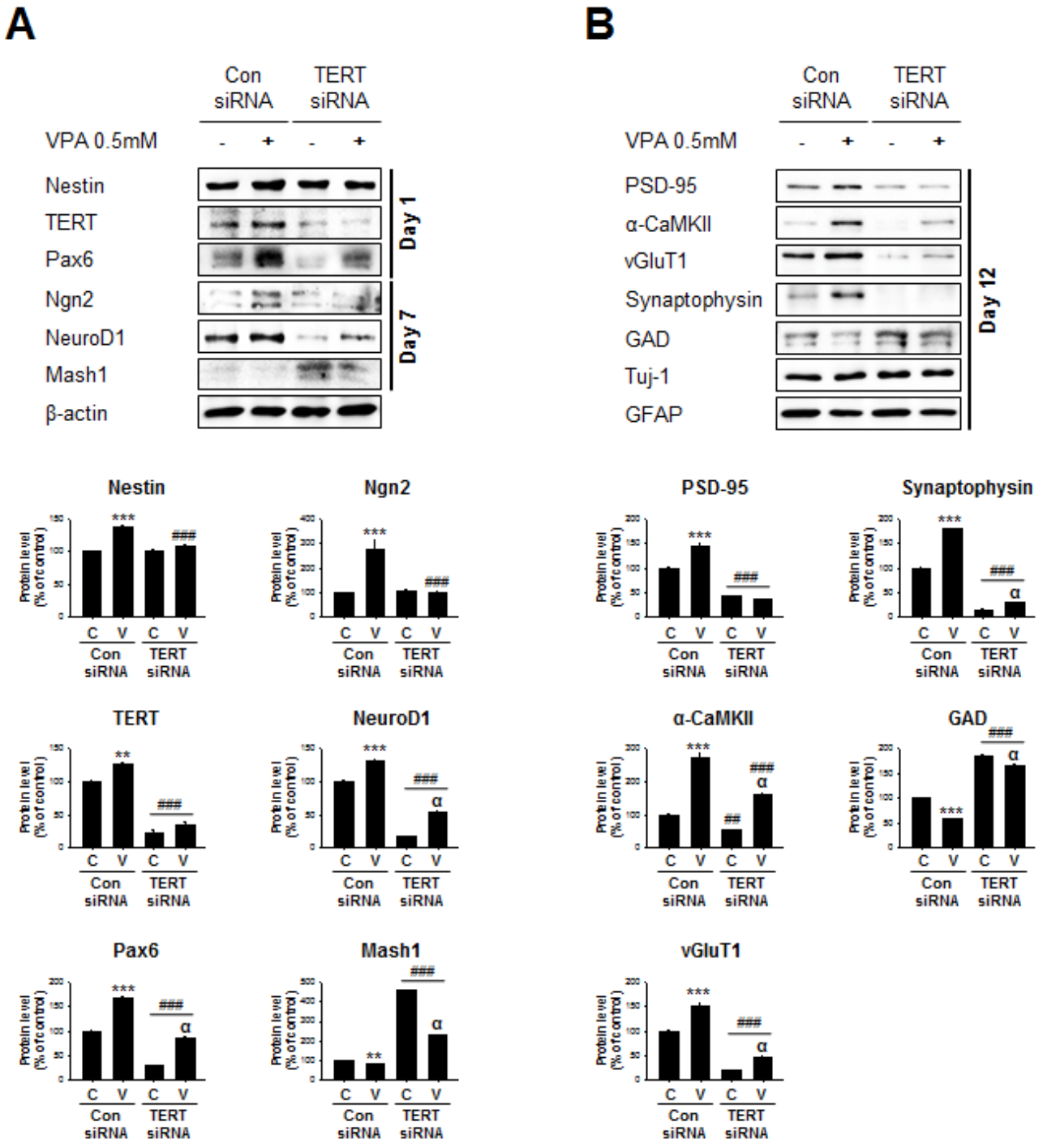

Fig. 5. Expression of excitatory/inhibitory synaptic proteins after *Tert* siRNA transfection in cultured NPCs. (A) The expression of sequential protein markers known for induction of glutamatergic differentiation was analyzed 1 and 7 days after transfection of *Tert* siRNA. (B) Twelve days later, the expressions of pre- and post- synaptic markers were analyzed. Results are mean±S.E.M. (n=3). NPCs were cultured with embryos from three different pregnant rats. $^{**}p<0.01$, $^{***}p<0.001$ for control group vs. VPA-treated group; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ for control siRNA group vs. *Tert* siRNA group; $^{\alpha}p<0.01$, $^{\alpha}p<0.001$ for *Tert* siRNA control group vs. *Tert* siRNA VPA-treated group, as determined by Bonferroni's posthoc comparisons test.

day 12, *Tert* deficiency blocked the expressions of the postsynaptic PSD95, the presynaptic vGluT1, and the synaptophysin protein, but enhanced GAD expression. *Tert* siRNA transfection decreased α-CaMKII expression levels, although still higher than the control levels. Lastly, *Tert* deficiency generated no significant effects on Tuj-1 and GFAP expressions. Below are the detailed statistical results of each protein expression as affected by *Tert* siRNA transfection and on different days of analysis.

During DIV1 (Fig. 5A), expression of nestin was affected by VPA treatment [$F_{(1,8)}$=33.86, p=0.0004] and *Tert* siRNA transfection [$F_{(1,8)}$=33.67, p=0.0004]. Post-hoc test showed that nestin was enhanced by VPA (p<0.001 vs control) which was decreased to control levels by *Tert* siRNA (p<0.001 vs Con siRNA+VPA). The interaction between VPA treatment and *Tert* siRNA transfection had a significance [$F_{(1,8)}$=79.65, p<0.0001]. In addition, expression of TERT protein was affected VPA treatment [$F_{(1,8)}$=25.79, p=0.0010] and *Tert* siRNA transfection [$F_{(1,8)}$=536.8, p<0.0001]. Post-hoc test showed that expression of TERT protein was increased by VPA treatment (p<0.01 vs control). Conversely, *Tert* siRNA reduced the expression of TERT as compared to control siRNA groups (p<0.001). On the other hand, the VPA+*Tert* siRNA group have higher TERT expression than the *Tert* siRNA only group (p<0.001). The interaction between VPA treatment and *Tert* siRNA had no significance [$F_{(1,8)}$=4.645, p=0.0633]. Interestingly, expression of Pax6 protein was also affected by VPA treatment [$F_{(1,8)}$=523.8, p<0.0001] and *Tert* siRNA transfection [$F_{(1,8)}$=814.4, p<0.0001]. Post-hoc test showed that Pax6 expression was increased by VPA treatment (p<0.001 vs control). On the other hand, *Tert* siRNA decreased Pax6 expression in the VPA-treated groups (p<0.001). Nevertheless, Pax6 expression was still at higher levels in the VPA+*Tert* siRNA groups compared to the *Tert* siRNA only group (p<0.001). The interaction between VPA treatment and *Tert* siRNA had a significance [$F_{(1,8)}$=6.015, p=0.0398].

At DIV7, the Ngn2 expression was significantly changed by VPA treatment [$F_{(1,8)}$=17.04, p=0.0033] and *Tert* siRNA transfection [$F_{(1,8)}$=17.37, p=0.0031]. Post-hoc test showed that expression of

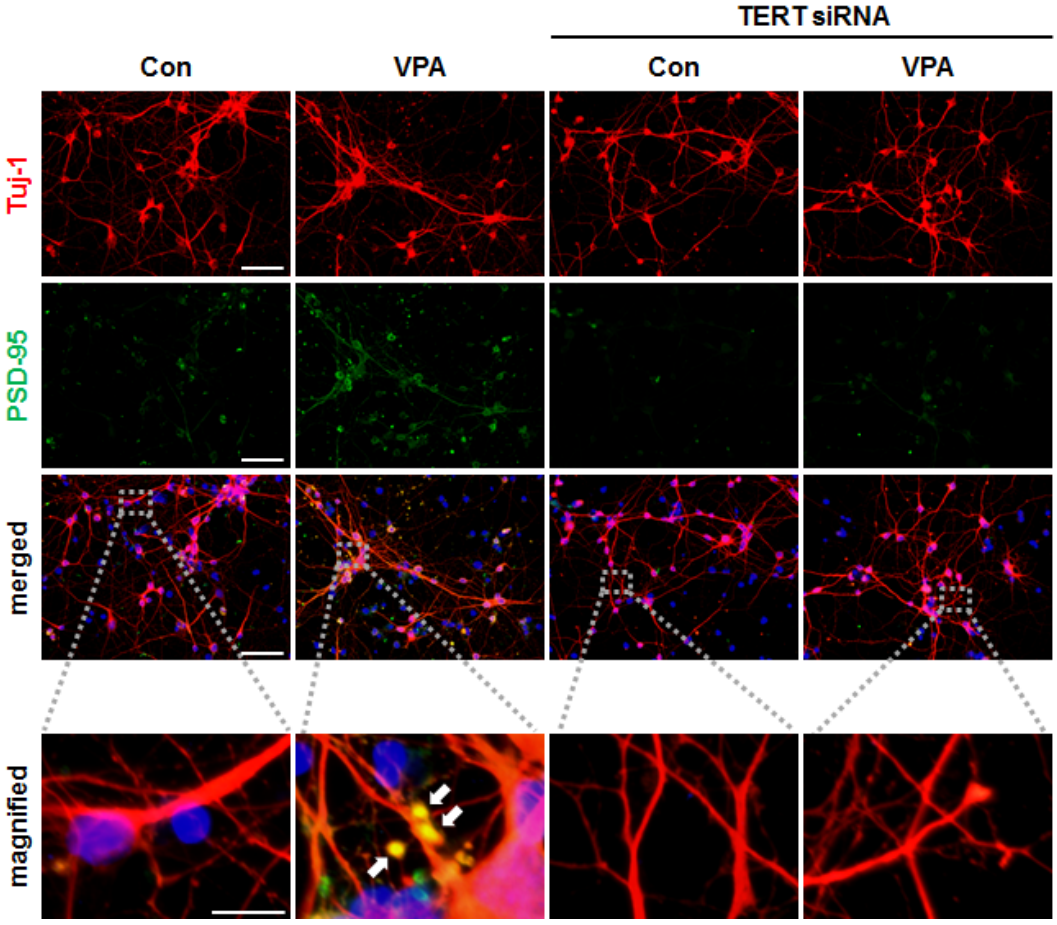

Fig. 6. The effect of TERT deficiency in the expression of PSD-95. Cultured NPCs were incubated until DIV12 after *Tert* siRNA transfection. White arrows represent localized PSD-95 puncta in neuronal axons stained with Tuj-1. Scale bar represents 100 μm. Scale bar for magnified figures represents 20 μm.

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Ngn2 was increased by VPA treatment (p<0.001 vs control) while Ngn2 was decreased by *Tert* siRNA in the VPA-treated groups (p<0.001). The interaction between VPA treatment and *Tert* siRNA had a significance [$F_{(1,8)}$=19.16, p=0.0024]. Expression of NeuroD1 was changed by VPA treatment [$F_{(1,8)}$=241.7, p<0.0001] and *Tert* siRNA transfection [$F_{(1,8)}$=1691, p<0.0001]. Post-hoc test showed that expression of NeuroD1 was increased by VPA treatment (p<0.001 vs control). *Tert* siRNA decreased NeuroD1 expression in the VPA-treated groups (p<0.001). Meanwhile, NeuroD1 was still at higher levels in the VPA+*Tert* siRNA group than the *Tert* siRNA only group (p<0.001). The interaction between VPA treatment and *Tert* siRNA had a significance [$F_{(1,8)}$=20.27, p=0.0020]. Concurrently, expression of Mash1 was affected by VPA treatment [$F_{(1,8)}$=2112, p<0.0001] and *Tert* siRNA transfection [$F_{(1,8)}$=9245, p<0.0001]. Post-hoc test showed that expression of Mash1 was decreased by VPA treatment (p<0.01 vs control). Conversely, *Tert* siRNA increased Mash1 expression in the control (p<0.001) and VPA-treated groups (p<0.05) compared to the control siRNA+VPA group. Notably, VPA treatment decreased Mash1 expression in the *Tert* siRNA group (p<0.001). The interaction between VPA treatment and *Tert* siRNA had a significance [$F_{(1,8)}$=1550, p<0.0001].

At DIV12 (Fig. 5B), the level of synaptic proteins expressed by glutamatergic and GABAergic neurons were examined. Expression level of PSD-95 protein was significantly changed by VPA treatment [$F_{(1,8)}$=14.50, p=0.0052] and *Tert* siRNA transfection [$F_{(1,8)}$=291.7, p<0.0001]. Post-hoc test showed that protein level of PSD-95 was increased by VPA treatment (p<0.001 vs control). Meanwhile, PSD-95 expression was decreased in *Tert* siRNA compared to control siRNA groups (p<0.001). The interaction between VPA treatment and *Tert* siRNA had a significance [$F_{(1,8)}$=26.96, p=0.0008]. This result was supplemented by immunostaining of neuronal axons showing increased localization of PSD-95 in the puncta (Fig. 6). The post synapse-related α-CaMKII expression was also affected by VPA treatment [$F_{(1,8)}$=402.7, p<0.0001] and by *Tert* siRNA transfection [$F_{(1,8)}$=125.1, p<0.0001]. Post-hoc test showed that expression level of α-CaMKII was elevated by VPA treatment (p<0.001 vs control). *Tert* siRNA decreased α-CaMKII expression VPA-treated groups (p<0.001). Nevertheless, α-CaMKII was still at higher levels in the VPA+*Tert* siRNA group than the *Tert* siRNA only group (p<0.001). The interaction between VPA treatment and *Tert* siRNA had a significance [$F_{(1,8)}$=22.58, p=0.0014]. The glutamatergic vGluT1 protein expression was affected by VPA treatment [$F_{(1,8)}$=101.8, p<0.0001] and *Tert* siRNA transfection [$F_{(1,8)}$=549.8, p<0.0001]. Post-hoc test revealed that protein expression level of vGluT1 was increased by VPA treatment (p<0.001). *Tert* siRNA decreased vGluT1 expression in the VPA-treated groups (p<0.001). Interestingly, vGluT1 expression was higher in the VPA+*Tert* siRNA group than the *Tert* siRNA only group (p<0.01). The interaction between VPA treatment and *Tert* siRNA had a significance [$F_{(1,8)}$=9.854, p=0.0138].

Protein expression level of synaptophysin was significantly changed by VPA treatment [$F_{(1,8)}$=711.0, p<0.0001] and *Tert* siRNA transfection [$F_{(1,8)}$=4540, p<0.0001]. Post-hoc test showed that expression level of synaptophysin was increased by VPA treatment (p<0.001 vs control). *Tert* siRNA decreased synaptophysin expression as compared to control siRNA (p<0.001). Interestingly, VPA+*Tert* siRNA group slightly had higher synaptophysin expression than the *Tert* siRNA only group. (p<0.05). The interaction between VPA treatment and Tert siRNA had a significance [$F_{(1,8)}$=361.3, p<0.0001]. Expression of GAD, which is expressed in GABAergic neurons, was affected by VPA treatment [$F_{(1,8)}$=125.0, p<0.0001] and *Tert* siRNA transfection [$F_{(1,8)}$=1219, p<0.0001]. Post-hoc test showed that GAD expression was decreased by VPA treatment (p<0.001). Conversely, *Tert* siRNA increased GAD expression compared to the control siRNA groups (p<0.001). Meanwhile, VPA slightly attenuated GAD expression in the *Tert* siRNA group (p<0.05). The interaction between VPA treatment and *Tert* siRNA had a significance [$F_{(1,8)}$=15.73, p=0.0041]. The neuron-specific Tuj-1 expression was not affected by VPA treatment [$F_{(1,8)}$=0.09567, p=0.7650] or *Tert* siRNA transfection [$F_{(1,8)}$=0.1030, p=0.7565]. The interaction between VPA treatment and *Tert* siRNA had no significance [$F_{(1,8)}$=1.370, p=0.2755]. Lastly, the glial marker, GFAP, expression was not also affected by VPA treatment [$F_{(1,8)}$=0.1450, p=0.7133] or *Tert* siRNA transfection [$F_{(1,8)}$=0.8864, p=0.3740]. The interaction between VPA treatment and *Tert* siRNA had no significance [$F_{(1,8)}$=0.0005084, p=0.9826].

In addition, we performed *in utero* transfection of *Tert* siRNA to test whether the knock-down of TERT affects neuronal subtype differentiation *in vivo*. *Tert* siRNA was labeled with fluorescent dye as described in Materials and Methods. In accordance with our previous study [22], *Tert* siRNA-transfected cells in the cortical region at E18.5 were not overlapped with Ngn2, which is a transcription factor inducing glutamatergic neuronal differentiation, but overlapped with Calbindin D, which is a marker for GABAergic neuronal differentiation (Fig. 7A, B). in contrast, embryonic brain from *Tert*-overexpressed transgenic mice showed increased expression level of Pax6 glutamatergic transcription factor at E12.5 (Fig. 7C, as previously reported in Kim et al., 2015).

DISCUSSION

The use of *in vivo* and *in vitro* studies to verify the effect of VPA on the expression of TERT supports and reassures that results

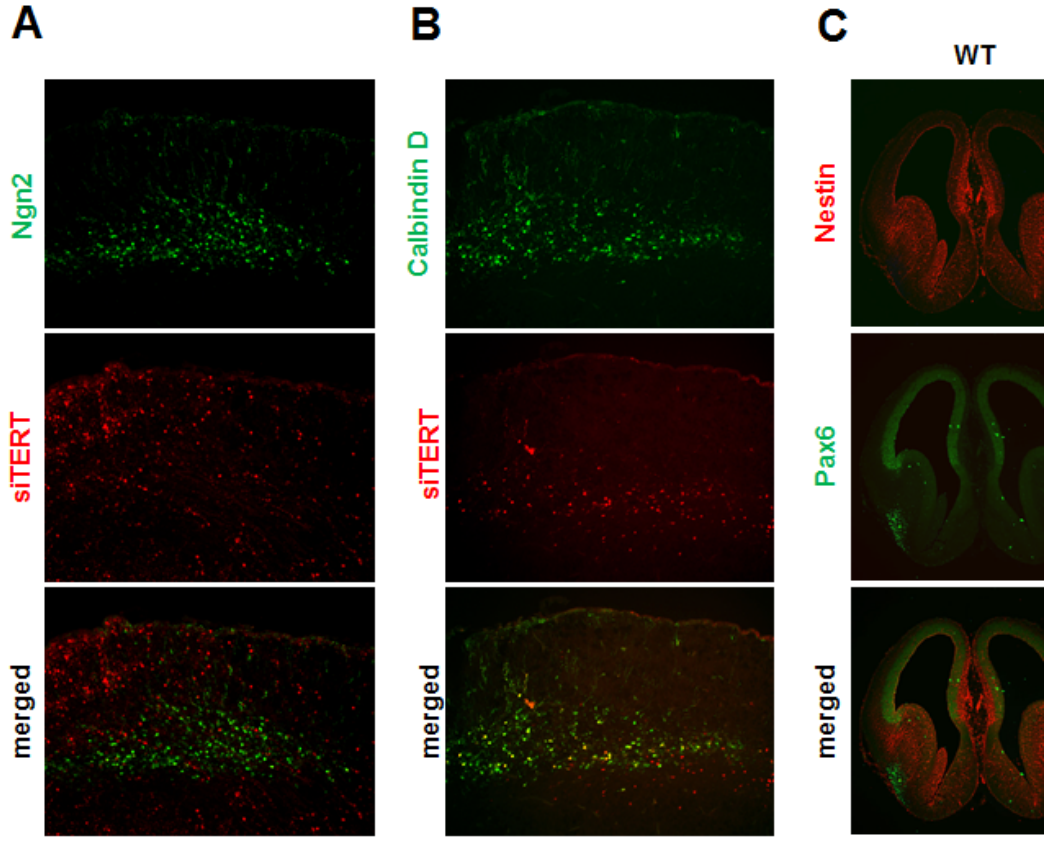

Fig. 7. Expression of transcription factors regulating neuronal subtype differentiation after modifying *Tert* expression. (A,B) In utero transfection was used for knock-down of *Tert* in the embryonic mice brain. The fluorescent-labeled *Tert* siRNA was delivered into subventricular zone of E14.5 mice brain, and electroporated mice brain was isolated at E18.5 for immunohistochemistry. Ngn2 is a transcription factor for regulating glutamatergic differentiation, and Calbindin D is a marker for processing GABAergic differentiation in neurons. (C) Brain expression of nestin and Pax6 from WT and TERT-tg mice at E12.5 (Scale bar, 500 μm).

from both studies complement one another. Here, we investigated and found that VPA induces TERT overexpression in E12-exposed embryonic rats and in cultured NPCs. At the same time, this also confirms our previous study where Pax6 was overexpressed [1]. As a result of VPA-induced TERT overexpression, the sequential expressions of glutamatergic transcription factors and glutamatergic synapse proteins were enhanced. These include the upregulations of Ngn2 and NeuroD1 at day 7 post-treatment, and glutamatergic synapse proteins such as PSD-95, α-CaMKII and vGluT1 at day 12 post-treatment. The use of *Tert* siRNA transfection in VPA-treated NPCs revealed that Pax6 is directly affected by TERT expression, showing that Pax6 is TERT's downstream signal. Moreover, the upregulation of glutamatergic and postsynaptic neuronal markers by TERT could be initiated through Pax6, which can be directly affected by VPA, independent of TERT expression.

The expression of TERT is agreed to be regulated by intricate regulatory pathways mainly at the transcriptional level, with the remodeling of chromatin and nucleosome organization identified as key factors in the physiological translation [25]. Post-transcription histone acetylation/deacetylation is implicated to be crucial in altering chromatin structure and modulating genetic makeup [25, 26]. In human normal somatic cells, histone deacetylase (HDAC) is suggested to be recruited by Sp1 and Sp3, and has associated with *Tert* promoter for localized deacetylation of nucleosomal histones and transcriptional silencing of *Tert* genes [27]. This effect can be reversed by HDAC inhibitors, for example, Trichostatin A (TSA), which activates the *Tert* promoter in normal renal cells and skin fibroblasts at a transcriptional level resulting in the activation of telomerase [28]. In this study, we observed that VPA exposure to neural progenitor cells and embryonic brains upregulate the expression of TERT. NPCs treated with VPA (0.5 mM) not only increased TERT protein expression but also showed increased staining detections of Pax6. Other HDAC inhibitors, TSA and SB, also upregulated TERT protein expression in NPCs while the GSK-3β inhibitor TDZD, but not LiCl, decreased its expression. This confirms that the HDAC inhibiting property of VPA regulates TERT

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expression.

Pax6 was upregulated in NPCs after VPA exposure, as previously reported [1]. Interestingly, in the current study, Pax6 expression was attenuated when the VPA-treated cells were transfected with *Tert* siRNA. This suggests a direct action of TERT in the expression of Pax6. However, VPA treatment may also directly contribute to the expression of Pax6 as revealed in the *Tert* knockdown of cultured NPCs (Fig. 5A). Furthermore, the VPA-induced expression of Pax6, after *Tert* siRNA transfection, could have induced the increased expressions of NeuroD1 and α-CaMKII at day 7 and day 12, respectively. Thus, we can infer that VPA can directly target Pax6, independent of TERT.

It is interesting to note how VPA can regulate TERT proteins bound to *Pax6* gene, suggesting a pathway shared by TERT and Pax6 in potentially dysregulating glutamatergic neuronal differentiation. One suggested waypoint is the interaction of Brg1 protein to both *Tert* and *Pax6* genes. Brg1 is a sort of chromatin remodeling protein required for the process of neurogenesis in vertebrates and mediates the transcriptional activities of *Ngn* and *NeuroD* genes [29]. This interaction of Brg1 protein to transcription factors would be a key process in activating the pro-neurogenic differentiation in NPCs [30]. Indeed, the immunoprecipitation of Brg1 unto *Pax6* gene was upregulated after VPA exposure, showing their interacting relationship (Fig. 4A). This interaction was also previously elucidated where Pax6 and BAF, which contains Brg1, together creates a complex, which in their downstream initiate neurogenesis in adult neural stem cells and converts postnatal glia into neurons [31].

Both telomerase and TERT are expressed in neural precursor cells of rats and mice during embryonic development. Telomerase activity levels noticeably decrease between E13 and E18 until they become undetectable [7]. This decline in telomerase activity goes along with the marked decrease of the proliferation of neuroblasts. On the other hand, TERT activity levels belatedly decline at postnatal day 5, concurring with the occurrence of synapse formation and programmed cell death of neurons [32]. This non-parallel decline of activity levels of telomere and TERT suggest that TERT may have a role in cell protection and synapse formation. In our previous study utilizing TERT-tg mice, we elucidated the effects of TERT overexpression in glutamatergic neuronal differentiation, postsynaptic maturation, and autism-relevant behaviors [22]. Here, we supplement the involvement of TERT in neurodevelopment through in vitro studies showing that *Tert* siRNA transfection in cultured NPCs decreased the expression of the regulators of glutamatergic neuronal differentiation at DIV 7 and decreased the expression of synaptic developmental markers at DIV 12. To qualify TERT expression as a valid risk factor of ASD and perhaps other neurodevelopmental disorders, other related parameters like telomerase expression, activity levels or telomere length must also be investigated.

This study further expands the relevance of the VPA model of autism as a strong environmental contributor to ASD with a significant clinical relevance [33, 34]. We already learned through various validated rodent models that VPA induces altered neuronal apoptosis [35], increased neuronal cell progenitor pool and macrocephaly phenotype [24], increased neurite outgrowth in rat primary cortical neurons [36], altered glutamate regulations in the hippocampus [37], cerebellar anomalies [38], histone hyperacetylation and MeCP2 altered expression [39, 40], and imbalance between excitation and inhibition neurotransmission [1, 41, 42], among others. In the current study, we firstly understood that VPA enhances TERT expression. Moreover, we confirmed that Pax6 is directly affected by TERT but could be independently enhanced by VPA, and possibly by other regulators. Moving forward, it is important to unify the already known effects of VPA in the brain and converge these diverse pathways to help explain the mechanisms of ASD. Moreover, we are also testing therapeutic substances that target the affected pathways in the VPA model and how they reverse the abnormalities in the brain and behavior of this model.

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REFERENCES

1. Kim KC, Lee DK, Go HS, Kim P, Choi CS, Kim JW, Jeon SJ, Song MR, Shin CY (2014) Pax6-dependent cortical glutamatergic neuronal differentiation regulates autism-like behavior in prenatally valproic acid-exposed rat offspring. Mol Neurobiol 49:512-528.
2. Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR (1988) A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci U S A 85:6622-6626.
3. Watson JD (1972) Origin of concatemeric T7 DNA. Nat New Biol 239:197-201.
4. Harley CB, Futcher AB, Greider CW (1990) Telomeres short-

en during ageing of human fibroblasts. Nature 345:458-460.
5. Oulton R, Harrington L (2000) Telomeres, telomerase, and cancer: life on the edge of genomic stability. Curr Opin Oncol 12:74-81.
6. Price CM (1999) Telomeres and telomerase: broad effects on cell growth. Curr Opin Genet Dev 9:218-224.
7. Klapper W, Shin T, Mattson MP (2001) Differential regulation of telomerase activity and TERT expression during brain development in mice. J Neurosci Res 64:252-260.
8. Georgala PA, Manuel M, Price DJ (2011) The generation of superficial cortical layers is regulated by levels of the transcription factor Pax6. Cereb Cortex 21:81-94.
9. Scardigli R, Bäumer N, Gruss P, Guillemot F, Le Roux I (2003) Direct and concentration-dependent regulation of the proneural gene Neurogenin2 by Pax6. Development 130:3269-3281.
10. Quinn JC, Molinek M, Martynoga BS, Zaki PA, Faedo A, Bulfone A, Hevner RF, West JD, Price DJ (2007) Pax6 controls cerebral cortical cell number by regulating exit from the cell cycle and specifies cortical cell identity by a cell autonomous mechanism. Dev Biol 302:50-65.
11. Bel-Vialar S, Medevielle F, Pituello F (2007) The on/off of Pax6 controls the tempo of neuronal differentiation in the developing spinal cord. Dev Biol 305:659-673.
12. Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 25:247-251.
13. Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M (2008) Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. Stem Cells 26:1663-1672.
14. Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde YA, Götz M (2002) Glial cells generate neurons: the role of the transcription factor Pax6. Nat Neurosci 5:308-315.
15. Estivill-Torrus G, Pearson H, van Heyningen V, Price DJ, Rashbass P (2002) Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. Development 129:455-466.
16. Osumi N, Kikkawa T (2013) The role of the transcription factor pax6 in brain development and evolution: evidence and hypothesis. In: Cortical development (Kageyama R, Yamamori T, eds), pp 43-61. Springer, Tokyo.
17. Kim BW, Yang S, Lee CH, Son H (2011) A critical time window for the survival of neural progenitor cells by HDAC inhibitors in the hippocampus. Mol Cells 31:159-164.
18. Kostrouchová M, Kostrouch Z, Kostrouchová M (2007) Valproic acid, a molecular lead to multiple regulatory pathways. Folia Biol (Praha) 53:37-49.
19. Benoit BO, Savarese T, Joly M, Engstrom CM, Pang L, Reilly J, Recht LD, Ross AH, Quesenberry PJ (2001) Neurotrophin channeling of neural progenitor cell differentiation. J Neurobiol 46:265-280.
20. Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A (2005) Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 3:e283.
21. Go HS, Shin CY, Lee SH, Jeon SJ, Kim KC, Choi CS, Ko KH (2009) Increased proliferation and gliogenesis of cultured rat neural progenitor cells by lipopolysaccharide-stimulated astrocytes. Neuroimmunomodulation 16:365-376.
22. Kim KC, Rhee J, Park JE, Lee DK, Choi CS, Kim JW, Lee HW, Song MR, Yoo HJ, Chung C, Shin CY (2015) Overexpression of telomerase reverse transcriptase induces autism-like excitatory phenotypes in mice. Mol Neurobiol 53:7312-7328.
23. Nelson JD, Denisenko O, Bomsztyk K (2006) Protocol for the fast chromatin immunoprecipitation (ChIP) method. Nat Protoc 1:179-185.
24. Go HS, Kim KC, Choi CS, Jeon SJ, Kwon KJ, Han SH, Lee J, Cheong JH, Ryu JH, Kim CH, Ko KH, Shin CY (2012) Prenatal exposure to valproic acid increases the neural progenitor cell pool and induces macrocephaly in rat brain via a mechanism involving the GSK-3β/β-catenin pathway. Neuropharmacology 63:1028-1041.
25. Stein GS, Montecino M, van Wijnen AJ, Stein JL, Lian JB (2000) Nuclear structure-gene expression interrelationships: implications for aberrant gene expression in cancer. Cancer Res 60:2067-2076.
26. Hou M, Wang X, Popov N, Zhang A, Zhao X, Zhou R, Zetterberg A, Björkholm M, Henriksson M, Gruber A, Xu D (2002) The histone deacetylase inhibitor trichostatin A derepresses the telomerase reverse transcriptase (hTERT) gene in human cells. Exp Cell Res 274:25-34.
27. Won J, Yim J, Kim TK (2002) Sp1 and Sp3 recruit histone deacetylase to repress transcription of human telomerase reverse transcriptase (hTERT) promoter in normal human somatic cells. J Biol Chem 277:38230-38238.
28. Takakura M, Kyo S, Sowa Y, Wang Z, Yatabe N, Maida Y, Tanaka M, Inoue M (2001) Telomerase activation by histone deacetylase inhibitor in normal cells. Nucleic Acids Res 29:3006-3011.

 https://doi.org/10.5607/en.2017.26.5.252

29. Seo S, Richardson GA, Kroll KL (2005) The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD. Development 132:105-115.
30. Urbán N, Guillemot F (2014) Neurogenesis in the embryonic and adult brain: same regulators, different roles. Front Cell Neurosci 8:396.
31. Ninkovic J, Steiner-Mezzadri A, Jawerka M, Akinci U, Masserdotti G, Petricca S, Fischer J, von Holst A, Beckers J, Lie CD, Petrik D, Miller E, Tang J, Wu J, Lefebvre V, Demmers J, Eisch A, Metzger D, Crabtree G, Irmler M, Poot R, Götz M (2013) The BAF complex interacts with Pax6 in adult neural progenitors to establish a neurogenic cross-regulatory transcriptional network. Cell Stem Cell 13:403-418.
32. Mattson MP, Zhang P, Fu W (2000) Roles for TERT and telomerase in cell differentiation and apoptosis. Landes Bioscience, Austin, TX.
33. Christensen J, Grønborg TK, Sørensen MJ, Schendel D, Parner ET, Pedersen LH, Vestergaard M (2013) Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. JAMA 309:1696-1703.
34. Roullet FI, Lai JK, Foster JA (2013) In utero exposure to valproic acid and autism--a current review of clinical and animal studies. Neurotoxicol Teratol 36:47-56.
35. Go HS, Seo JE, Kim KC, Han SM, Kim P, Kang YS, Han SH, Shin CY, Ko KH (2011) Valproic acid inhibits neural progenitor cell death by activation of NF-κB signaling pathway and up-regulation of Bcl-XL. J Biomed Sci 18:48.
36. Cho KS, Kwon KJ, Choi CS, Jeon SJ, Kim KC, Park JH, Ko HM, Lee SH, Cheong JH, Ryu JH, Han SH, Shin CY (2013) Valproic acid induces astrocyte-dependent neurite outgrowth from cultured rat primary cortical neuron via modulation of tPA/PAI-1 activity. Glia 61:694-709.
37. Bristot Silvestrin R, Bambini-Junior V, Galland F, Daniele Bobermim L, Quincozes-Santos A, Torres Abib R, Zanotto C, Batassini C, Brolese G, Gonçalves CA, Riesgo R, Gottfried C (2013) Animal model of autism induced by prenatal exposure to valproate: altered glutamate metabolism in the hippocampus. Brain Res 1495:52-60.
38. Ingram JL, Peckham SM, Tisdale B, Rodier PM (2000) Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. Neurotoxicol Teratol 22:319-324.
39. Kataoka S, Takuma K, Hara Y, Maeda Y, Ago Y, Matsuda T (2013) Autism-like behaviours with transient histone hyperacetylation in mice treated prenatally with valproic acid. Int J Neuropsychopharmacol 16:91-103.
40. Kim KC, Choi CS, Kim JW, Han SH, Cheong JH, Ryu JH, Shin CY (2016) MeCP2 modulates sex differences in the postsynaptic development of the valproate animal model of autism. Mol Neurobiol 53:40-56.
41. Fukuchi M, Nii T, Ishimaru N, Minamino A, Hara D, Takasaki I, Tabuchi A, Tsuda M (2009) Valproic acid induces up- or down-regulation of gene expression responsible for the neuronal excitation and inhibition in rat cortical neurons through its epigenetic actions. Neurosci Res 65:35-43.
42. Kim KC, Kim P, Go HS, Choi CS, Park JH, Kim HJ, Jeon SJ, Dela Pena IC, Han SH, Cheong JH, Ryu JH, Shin CY (2013) Male-specific alteration in excitatory post-synaptic development and social interaction in pre-natal valproic acid exposure model of autism spectrum disorder. J Neurochem 124:832-843.