Epigenetically Upregulated T-Type Calcium Channels Contribute to Abnormal Proliferation of Embryonic Neural Progenitor Cells Exposed to Valproic Acid

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Abstract
Valproic acid is a clinically used mood stabilizer and antiepileptic drug. Valproic acid has been suggested as a teratogen associated with the manifestation of neurodevelopmental disorders, such as fetal valproate syndrome and autism spectrum disorders, when taken during specific time window of pregnancy. Previous studies proposed that prenatal exposure to valproic acid induces abnormal proliferation and differentiation of neural progenitor cells, presumably by inhibiting histone deacetylase and releasing the condensed chromatin structure. Here, we found valproic acid up-regulates the transcription of T-type calcium channels by inhibiting histone deacetylation in neural progenitor cells. The pharmacological blockade of T-type calcium channels prevented the increased proliferation of neural progenitor cells induced by valproic acid. Differentiated neural cells from neural progenitor cells treated with valproic acid displayed increased levels of calcium influx in response to potassium chloride-induced depolarization. These results suggest that prenatal exposure to valproic acid up-regulates T-type calcium channels, which may contribute to increased proliferation of neural progenitor cells by inducing an abnormal calcium response and underlie the pathogenesis of neurodevelopmental disorders.

Key Words: Valproic acid, Embryonic cortical brain, Neural progenitor cells, Epigenetic regulation, Proliferation, T-type calcium channels

INTRODUCTION
Neurodevelopmental disorders can be caused by exposure to environmental toxicants resulting in abnormal neurological proliferation and/or differentiation. Valproic acid (VPA) is a clinically used mood stabilizer and antiepileptic drug. VPA has been suggested as a teratogen that can induce neurodevelopmental disorders, such as fetal valproate syndrome and autism spectrum disorders (Dalens et al., 1980; DiLiberti et al., 1984; Williams et al., 2001; Ornoy, 2009). VPA induces transcriptional activation by inhibiting histone deacetylase and releasing the condensed chromatin structure (Gottlicher et al., 2001). Prenatal exposure to VPA causes dysregulation of genetic transcription, which results in enhanced glutamatergic and abnormal cholinergic neuronal development and behavioral impairments, including social deficits, repetitive behaviors, hyperactivity, cognition deficits, and seizure susceptibility, in VPA rodent models (Kim et al., 2014a, 2014b, 2017, 2019). VPA also facilitates the proliferation of neural progenitor cells (NPCs), which might be the cause for the induction of anatomical changes, such as macrocephaly, observed in human patients and animal models exposed to VPA during the embryonic period (Kozma, 2001; Go et al., 2012).

The T-type calcium channel is a low-voltage activated channel that regulates calcium-dependent physiological processes at the resting membrane potential in excitable cells or non-excitable cells, such as embryonic progenitor cells (NPCs) (If-tinca and Zamponi, 2009; Louhivuori et al., 2013). T-type calcium channels consist of three subtypes—CaV3.1, CaV3.2, and CaV3.3—encoded by the genes CACNA1G, CACNA1H, and CACNA1I, respectively. T-type calcium channels mediate different actions depending on their subtype and the type of...
cell containing the channels (Ifitina and Zamponi, 2009). T-type calcium channels have been implicated in regulating cellular viability, proliferation, and differentiation (Panner et al., 2005; Oguni et al., 2010; Rodriguez-Gomez et al., 2012; Kim et al., 2018). We previously showed that the pharmacological blockade of T-type calcium channels severely affects the viability of NPCs but not that of differentiated neural cells, suggesting a critical role of T-type calcium channels in the embryonic developmental period. Consistent with this view, CaV3.1 is expressed during the embryonic period and has decreased expression in the perinatal period (Kim et al., 2018). Recent genetic studies implicated CaV3.1 and 3.2 as potential risk genes for neural developmental disorders, such as autism spectrum disorders (Splatznig et al., 2006; Strom et al., 2010) and epilepsy (Chen et al., 2003; Singh et al., 2007). However, how an abnormality in T-type calcium channels mediates the neuropathophysiological process is unclear.

In this study, we investigated whether genes encoding T-type calcium channels are epigenetic targets during prenatal exposure to VPA and assessed the role of T-type calcium channels in VPA-induced neurodevelopmental abnormalities. We found that the mRNA levels of T-type calcium channels were increased by VPA treatment. CaV3.1 was identified as the epigenetic target of VPA, as its increase was not detected in NPCs treated with valproamide, a carbamoxide derivative of VPA that does not inhibit histone deacetylase. The proliferation of VPA was increased in NPCs, and pharmacological blockade of T-type calcium channels prevented this increased proliferation. Cells that differentiated from NPCs treated with VPA exhibited abnormal calcium ion (Ca\(^{2+}\)) response to KCl-induced depolarization stimulation. Our results provide a clue for understanding the role of T-type calcium channels in the pathophysiology of neurodevelopmental disorders.

### MATERIALS AND METHODS

**Animals**

Pregnant female Sprague-Dawley rats were obtained from Orient Bio (Gyeonggi-do, Korea). Animal housing and treatments, including anesthesia, euthanasia, and administration of VPA, were carried out in accordance with the principles of laboratory animal care (NIH Publication No. 85-23, revised 1985) and were approved by the animal care and use committee of Konkuk University, Korea (KU14143).

**Prenatal exposure to VPA and preparation of cortices**

VPA was administered as previously reported (Kim et al., 2011). VPA (400 mg/kg) was subcutaneously injected into pregnant Sprague-Dawley rats on embryonic day 12.5 (E12.5). Cortices were collected from embryos on E14.5 and stored at –80°C for further analysis.

**Primary culture of cortical NPCs and treatment**

Primary NPCs were isolated from the E14.5 cortices obtained from the rat embryos and were maintained in a humidified chamber at 37°C with growth factors, as described previously (Go et al., 2012). For differentiation, NPCs were subcultured and seeded in plates or coverslips coated with poly-L-ornithine (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) and containing DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) with B27 (Invitrogen) and without growth factors. After 3 h of recovery time, drugs were added to the NPCs, and the NPCs were incubated for 24 h in a humidified chamber.

**Polymerase chain reaction (PCR)**

Total RNA was extracted from NPCs or cortices using TRizol reagent (Invitrogen). The RNA was reverse transcribed using a RevertAid Reverse transcriptase kit (K1622; Fermentas, Waltham, MA, USA). For quantitative real-time PCR, cDNA was amplified with custom-made primers using SYBR® Premix Ex Taq II (RR820A; Takara Bio, Shiga, Japan) in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Fold-changes in gene expression were quantified using the comparative threshold cycle (Ct) method. The primer sequences were as follows:

- **Cacna1g** (NM_031601): Forward - 5ʹ-CATGCCACCTTATTGAACCTTTGG-3ʹ, Reverse - 5ʹ-CGGAGGGTGTCCTTCAATAAC-3ʹ
- **Cacna1h** (NM_153814): Forward - 5ʹ-GCCCTCGAGGACTTCATCTT, Reverse - 5ʹ-GTGTACCCAGGCTGATTCTT-3ʹ
- **Cacna1i** (NM_020084): Forward - 5ʹ-ACAGGGCATACTGGAATTACC-3ʹ, Reverse - 5ʹ-TGAGAGCGGTGACACAAACT-3ʹ
- **18s rRNA** (Westmark and Malter, 2007): Forward - 5ʹ-AATCAGTTATGGTTCCTTTGG-3ʹ, Reverse - 5ʹ-TCGGCATGTATTAGCTCTAGAAAT-3ʹ

**Western blot analysis**

Tissues and cells were lysed with 2× sample buffer (4% w/v sodium dodecyl sulfate [SDS], 20% glycerol, 200 mM dithiothreitol [DTT], 0.1 M Tris-HCl, pH 6.8, and 0.02% bromophenol blue), including protease and phosphatase inhibitors. Protein samples were quantified using a standard bicinchoninic acid (BCA) analysis and an equal amount of total protein was resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 1% skim milk and incubated overnight at 4°C with primary antibodies against CACNA1G, (ab134269; Abcam, Cambridge, UK; 1:2,000), β-actin (A2066; Sigma-Aldrich; 1:50,000), Histone H3 (9715S; Cell Signaling Technology, Beverly, MA, USA; 1:2,500), or acetylated Histone H3 (06-599; Millipore, Billerica, MA, USA; 1:2,500). After washing each membrane three times with 0.1% Triton-X 100, the blots were incubated with the peroxidase-conjugated secondary antibody for 2 h at 20-25°C. After washing the blots with 0.1% Tris-buffered saline (TBS-Tween), the blots were incubated with the peroxidase-conjugated secondary antibody for 2 h at 20-25°C. After washing the blots with 0.1% TBS-Tween, bands were detected using a LAS-3000 imaging system (Fuji Film, Tokyo, Japan). The band intensity was analyzed using the Multi Gauge v3.0 software (Fuji Film).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation was performed as previously reported (Kim et al., 2014a). To collect in vitro samples, 43 µL of 37% formaldehyde was applied to each well of a six-well plate containing 1.6 mL of culture medium and cultivated NPCs, and the plate was incubated for 15 min at 20-25°C temperature. Subsequently, 225 µL of 1 M glycine was added to each well and incubated for an additional 5 min. Cells were collected, centrifuged (2,000 × g for 5 min at 4°C).
4°C), and washed twice with cold phosphate-buffered saline (PBS). For in vivo sample preparation, embryonic cortices were collected from embryos exposed to saline or VPA and homogenized in PBS. Aliquots (1.6 mL) of the homogenates received 43 µL of 37% formaldehyde and the mixtures were incubated at 20-25°C temperature for 15 min. After incubation, 225 µL of 1 M glycine was added to each mixture, incubated for 5 min, centrifuged (2,000×g for 5 min at 4°C), and washed twice with cold PBS. The collected samples were lysed with immunoprecipitation (IP) buffer comprising 150 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% IGEPA CAL-630, 1.0% Triton X-100, and protease inhibitor cocktail (4693159001; Roche, Basel, Switzerland) on ice and centrifuged (12,000×g for 1 min at 4°C). The supernatant was discarded, and pellets were washed with cold IP buffer. To shear the chromatin, each pellet was resuspended in IP buffer and sonicated on ice. After centrifugation (12,000×g for 10 min at 4°C), supernatants were collected and the samples were incubated with the designated primary antibody for 12 h at 4°C. A mixture containing 20 µL of IP buffer and 20 µL of protein A/G Agarose beads (20421; Pierce, Rockford, IL, USA) was added to each sample, which was then incubated for 45 min at 4°C on a rotating platform. The samples were washed five times with cold IP buffer and the supernatants were removed. In the respective pellets, 100 µL of 10% Chelex 100 resin (1422822; Bio-Rad, Hercules, CA, USA) was added for DNA isolation. The mixtures were boiled for 10 min at 90°C and centrifuged (12,000×g for 1 min at 4°C). Each supernatant was collected and used for PCR. Primers were designed to complement the promoter region using the Primer Design Tool (Integrated DNA Technologies, Coralville, IA, USA). The amplified PCR products were resolved in an ethidium bromide-containing agarose gel and visualized using an ultraviolet lamp. The primers sequences were as follows: Cacna1g: Forward - 5′-AGCAAAACACTCCCAGACCC-3′, Reverse - 5′-AAATCCGACTCTCCACTGC-3′, and Glyceraldehyde 3-phosphate dehydrogenase (Gapdh): Forward - 5′-CTGTCGCTCTCCTCCTACTTT-3′, Reverse - 5′-AGCTTTCTGGCCTTGTACAT-3′.

**Immunocytochemistry**

NPCs were plated on coverslips and fixed with 4% paraformaldehyde at 4°C for 15 min. Samples were permeabilized with 0.1% Triton X-100 dissolved in blocking buffer for 15 min and blocked with blocking buffer comprising 1% bovine serum albumin and 3% fetal bovine serum in PBS for 30 min at 20-25°C temperature. Coverslips were incubated overnight at 4°C with primary antibodies. After three washes, the coverslips were incubated with secondary antibodies conjugated with donkey anti-mouse or donkey anti-rabbit IgG for 1 h at 20-25°C temperature. The coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA), and cells were imaged using a model Bx61 fluorescence microscope (Olympus, Tokyo, Japan).

**Calcium imaging**

Calcium imaging was carried out in NPCs plated on coverslips. Tyrode’s solution contained 119 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 25 mM HEPES, and 30 mM glucose (pH adjusted to 7.4). To record calcium entry, coverslips were incubated with 5 mM Fluo3-AM (Invitrogen) in Tyrode’s solution supplemented with 0.02% Pluronic F-127 (Sigma-Aldrich) for 30 min at 37°C in the dark. The coverslips were placed in a perfusion chamber on a model Ti2 inverted microscope (Nikon, Tokyo, Japan). Calcium was visualized with a filter set with an excitation peak of 480 nm, 490 nm long pass mirror, 500-550 nm emission filter, and manual flip shutter. Images were captured every 1 s using a model DS-Qi2 camera (Nikon). For depolarization, 15 and 50 mM KCl were added to the perfusion chamber. F<sub>max</sub> and F<sub>base</sub> denote maximal increase after stimulation and baseline fluorescence, respectively. Fluorescence change (ΔF) was normalized using the following equation: ΔF=(F<sub>max</sub>−F<sub>base</sub>)/F<sub>base</sub>. Calcium responsive cells displayed a ΔF after depolarization>3 times the baseline F<sub>base</sub>.

**Cell viability assay**

Cell viability was measured using the standard 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based assay. After 24 h of drug treatment, MTT (Sigma-Aldrich, 200 µg/mL) was added to each well and incubated for 2 h at 37°C. After removing the medium, dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals formed by viable cells. Absorbance was measured using a microplate reader at a wavelength of 570 nm and a reference filter of 620 nm, using a SpectrMax190 device (Molecular Devices, Sunnyvale, CA, USA).

**Statistical analyses**

Statistical analyses were performed using Prism software (GraphPad, La Jolla, CA, USA). Data are presented as mean ± SEM. After confirming the normality of data in datasets, Student’s t-test, one-way ANOVA, or Kruskal-Wallis test was performed, depending on the study design and dataset. A p-value<0.05 was considered statistically significant.

**Fig. 1.** CaV3.1 mRNA and protein levels are increased in embryonic cortex exposed to VPA. Pregnant rats were exposed to VPA at E12.5, and T-type calcium channel mRNA (Cacna1g, Cacna1h, and Cacna1i) levels and CaV3.1 protein levels were analyzed in the embryonic cortex on E14.5. (A) Quantitative real-time PCR results showing mRNA levels of T-type calcium channel subtypes. Cacna1g and Cacna1h mRNA levels are significantly increased by VPA treatment, and there was an increasing trend in the mRNA level of Cacna1i (Veh, n=5, VPA, n=6, t-test, Cacna1g, t<sub>p</sub>=2.441, p=0.0373, Cacna1h, t<sub>p</sub>=2.474, p=0.0354, Cacna1i, t<sub>p</sub>=1954, p=0.0824). (B) Blots of CaV3.1, acetylated histone H3, histone H3, and β-actin and quantitative analysis of CaV3.1 protein levels. β-actin was used as the loading control for quantification of the CaV3.1 on blots (N=7 per group, t-test, t<sub>p</sub>=4.88, p=0.0004). Data are presented as mean ± SEM. *p<0.05 and **p<0.01.
RESULTS

Prenatal exposure to VPA increases T-type calcium channels in embryonic cortices

Previously, we showed that VPA induces abnormal genetic transcriptional activation by inhibiting histone deacetylase in the embryonic cortex, which results in neurodevelopmental defects (Kim et al., 2014a, 2014b). To investigate whether prenatal exposure to VPA affects transcription of genes for T-type calcium channels in the embryonic cortex, we measured the mRNA levels of Cacna1g, Cacna1h, and Cacna1i in the cortex on E14.5 after exposure to VPA on E12.5. Cacna1g and Cacna1h were significantly increased, and Cacna1i displayed a trend toward an increase, with a p-value close to significance (Fig. 1A). Consistent with the increased mRNA levels, CaV3.1 protein levels were also increased by VPA treatment along with increased acetylation in histone H3 (Fig. 1B). These results suggested that Cacna1g may be an epigenetic target gene of VPA.

VPA increases T-type calcium channels levels in cultured embryonic NPCs

NPCs are proliferative cells with the potential to differentiate into different types of neuronal cells. In our previous study, we showed that VPA induces abnormal differentiation and proliferation of NPCs, which results in impaired embryonic cortical development (Go et al., 2012; Kim et al., 2014a, 2014b). Thus, we presently tested whether the VPA-induced increase in CaV3.1 expression also occurs in NPCs. Cultured cortical NPCs were seeded and treated with VPA (0.5 mM) at day in vitro (DIV) 0. Twenty-four hours later, we measured Cacna1g, Cacna1h, and Cacna1i mRNA levels using quantitative real-time PCR. Similar to the in vivo results, VPA increased Cacna1g, Cacna1h, and Cacna1i mRNA levels (Fig. 2A). To confirm whether these increased mRNA levels induce increased CaV3.1 expression, we measured the levels of CaV3.1 protein by Western blotting. Similar to the above mentioned in vivo results, VPA increased the levels of CaV3.1 and promoted acetylation in histone H3 in cultured cortical NPCs (Fig. 2B). Consistent with the Western blotting results, immunostaining with CaV3.1 antibodies revealed an increased intensity of CaV3.1 expression in cultured cortical NPCs (Fig. 2C).

VPA up-regulates CaV3.1 expression through epigenetic modulation

To investigate whether VPA promotes CaV3.1 expression through histone modification, we treated NPCs with VPA (0.5 mM), which is a derivative of valproic acid that does not inhibit histone deacetylase. As expected, VPA significantly increased CaV3.1 expression but VPM did not (Fig. 3A). Next, to confirm that the increase in CaV3.1 protein is induced through epigenetic modulation, we treated NPCs with VPA, VPM, and two additional histone deacetylase inhibitors, trichostatin A (0.2 M) and sodium butyrate (0.1 mM). The levels of CaV3.1 were significantly increased by VPA, trichostatin A, and sodium butyrate, but not by VPM (Fig. 3B).
VPA promotes acetylation in histone H3 bound to the Cacna1g promoter region in embryonic cortices and cortical NPCs

Next, we examined the binding of acetylated histone H3 to promoter regions of the Cacna1g gene to confirm that the changed CaV3.1 expression is induced from epigenetic modulation by VPA. We performed chromatin IP with E14.5 cortices from embryos exposed to VPA and cultured cortical NPCs 24 h after VPA treatment. Increased binding of acetylated H3 to the Cacna1g promoter region was observed in both embryonic cortices and NPCs (Fig. 4). These results suggested that CaV3.1 is an epigenetic target of VPA.

T-type calcium channels mediate increased proliferation in NPCs exposed to VPA

We previously showed that VPA facilitates proliferation and promotes Pax6 expression in the embryonic cortex and NPCs, which results in macrocephaly and excitatory-inhibitory imbalance, respectively, in an animal model of autism induced by prenatal exposure to VPA (Go et al., 2012; Kim et al., 2014b). Given that T-type calcium channels are important in regulating cell cycling, proliferation, and differentiation (Panner et al., 2005; Lory et al., 2006; Oguri et al., 2010; Rodriguez-Gomez et al., 2012; Louhivori et al., 2013), the increased CaV3.1 expression may mediate increased proliferation or altered differentiation in VPA-treated NPCs. To confirm this hypothesis, we applied T-type calcium channel blockers to VPA-treated NPCs and conducted the MTT assay to investigate the effect on the number of viable cells. The blockers used were NNC55-039, which blocks CaV3.1 and CaV3.2 (Huang et al., 2004; Taylor et al., 2008) and mibebradil, which is a broad T-type calcium channel blocker and weak L-type calcium channel blocker (Martin et al., 2000). VPA treatment increased the number of viable cells compared to vehicle treatment, indicating that proliferation of NPCs was increased by VPA (Fig. 5A, 5B). NNC55-0396 blocked the increased number of viable cells in VPA-treated NPCs at 0.6 and 1.2 µM. However, at the highest concentration of 2.5 µM, NNC55-0396 treatment resulted in a reduction in the number of viable NPCs, compared to vehicle and VPA treatments (Fig. 5A), indicating the induction of cell death by a strong blockade of T-type calcium channels as previously reported (Kim et al., 2018). Similarly, mibebradil prevented an increase in the number of viable cells in VPA-treated NPCs over 2.5 µM (Fig. 5B). Next, we measured the levels of Pax6 after treatment with NNC55-0396 in VPA-pretreated NPCs to investigate the role of T-type calcium channels in the facilitated glutamatergic neuronal differentiation by VPA. T-type calcium channel blockade did not alter the increased levels of Pax6 by VPA treatment (Fig. 5C). These results suggested that T-type calcium channels may contribute to the increased proliferation, but not differentiation, of NPCs by VPA.

Differentiated NPCs exposed to VPA have increased activity-dependent calcium influx

Next, we questioned whether activity-dependent calcium influx is altered in differentiated neural cells from VPA-treated NPCs. We measured the calcium response to KCl-induced depolarization stimulation using Fluo3-AM in the differentiated neural cells from NPCs treated with either vehicle or VPA 24 hrs prior. Differentiated neural cells from VPA-treated NPCs showed a significant increase in the calcium response compared to vehicle-treated cells (Fig. 5D). These results suggest that activity-dependent calcium influx may contribute to the increased proliferation and differentiation of NPCs by VPA.
NPCs displayed an increase in calcium influx in response to 15 mM KCl-induced depolarization stimulation, compared to differentiated neural cells from vehicle-treated NPCs (Fig. 6A). Calcium responsive cells, in response to both 15 and 50 mM KCl stimulation, were also increased by VPA treatment (Fig. 6B). To confirm whether the increased activity-dependent Ca\textsuperscript{2+} influx was induced by increased levels of L-type calcium channels, we measured the levels of Cacna1c mRNA, which encodes CaV1.2, a subunit of L-type calcium channels. Cacna1c mRNA levels were not changed by either VPA or VPM treatment, suggesting that CaV1.2 is not a target of VPA and may not be the cause for the increased Ca\textsuperscript{2+} influx in response to KCl-induced depolarization stimulation (Fig. 6C).

**DISCUSSION**

In this study, we found that VPA increases the mRNA levels of all subtypes of T-type calcium channels in primary cultured rat NPCs and embryonic rat cortex. CaV3.1 protein levels were increased by other histone deacetylase inhibitors, but not by VPM, suggesting that CaV3.1 is an epigenetic target of VPA during embryonic cortical development. Pharmacological blockade of T-type calcium channels prevented an increase in VPA-induced proliferation of NPCs. Lastly, we found that activity-dependent Ca\textsuperscript{2+} influx is increased in differentiated neural cells from NPCs previously exposed to VPA. Our results may provide a clue for understanding the role of T-type calcium channels in VPA-induced neurodevelopmental impairments.

We found that T-type calcium channels are upregulated by VPA exposure during the embryonic period. T-type calcium channels have been implicated in neurodevelopmental disorders, such as autism spectrum disorders (Splawski et al., 2006; Strom et al., 2010) and absence seizure (Chen et al., 2003; Singh et al., 2007). Interestingly, gain of function mutation in these genes has been found in clinical genetic studies, and the patients with these mutated genes displayed severe motor and cognitive impairments and epilepsy (Khosravani et al., 2004, 2005; Chemin et al., 2018). Transgenic mice overexpressing Cacna1g reportedly showed a spike-wave discharge in the thalamocortical network along with behavioral arrest, which is a typical phenotype associated with absence seizure; however, the mice did not display movement impairments like ataxia or tremor (Ernst et al., 2009). Of note, prenatal exposure to VPA also induces autism-like behavioral symptoms and increased seizure susceptibility (Kim et al., 2014a, 2014b, 2017, 2019). Thus, these lines of evidence might indicate a role of T-type calcium channels in mediating normal neurodevelopment.

We found that VPA increased the number of viable NPCs, indicating that VPA increased the proliferation of NPCs. Of note, the increased proliferation of NPCs exposed to VPA was prevented by T-type calcium channel blockers, suggesting a crucial role of T-type calcium channels in mediating the abnormal proliferation of NPCs exposed to VPA. Indeed, the importance of T-type calcium channels in regulating the cell cycle and cell proliferation has been reported in proliferating cells, such as cancer cells (Hirooka et al., 2002; Pannen et al., 2005; Oguri et al., 2010). Thus, it is reasonable to speculate that calcium influx through T-type calcium channels is involved in cell-cycle regulation pathways in NPCs. We previously showed that VPA increases the proliferation of NPCs by inhibiting glycogen synthase kinase 3β (GSK3β) activity and activating β-catenin-dependent signaling (Go et al., 2012). Thus, calcium influx through T-type calcium channels may be involved in this signaling pathway. Indeed, we previously showed that pharmacological blockade of T-type calcium channels activates GSK3β and induces apoptotic cell death in NPCs (Kim et al., 2018). It is conceivable that Ca\textsuperscript{2+} entry via T-type calcium channels may regulate calcium-dependent kinases and its downstream signaling such as AKT-GSK3β (Cross et al., 1995; Yano et al., 1998), which mediates the physiological processes such as cell cycle regulation in NPCs (Toth et al., 2016). We previously showed that VPA increases proliferation in NPCs, which results in an increase in the number of neurons in the rat embryonic brain and leads to macrocephaly in offspring prenatally exposed to VPA (Go et al., 2012). Thus, we may hypothesize a possible role of T-type calcium channels in regulating the neural population in the embryonic brain and inducing macrocephaly. This hypothesis awaits further investigation.

Differentiated neural cells from VPA-treated NPCs showed increased Ca\textsuperscript{2+} influx in response to KCl-induced depolarization, suggesting that prenatal exposure to VPA causes an abnormality in activity-dependent Ca\textsuperscript{2+} entry. Given VPA promotes differentiation into excitatory neurons by upregulating Pax6 levels (Kim et al., 2014b), it is plausible that the increased number of cells responsive to KCl stimulation might be due to the facilitated differentiation by VPA. We did not see any changes in mRNA levels of Cacna1c in NPCs exposed to VPA, suggesting the expression of CaV1.2 is not a cause for the increased Ca\textsuperscript{2+} response to KCl stimulation in the NPCs. Although we cannot rule out that the up-regulation of the other subunits of L-type calcium channels in NPCs exposed to VPA may be responsible for the increased Ca\textsuperscript{2+} influx in response to KCl stimulation, the increased expression of T-type calcium channels by VPA may also be involved in the abnormally increased Ca\textsuperscript{2+} influx. The abnormal activity-dependent Ca\textsuperscript{2+} response and its related signaling in the pathophysiology of VPA-induced neurodevelopmental disorders would be intriguing.

![Fig. 6.](image-url) Fig. 6. Differentiated NPCs pretreated with VPA display increased activity-dependent calcium entry. Calcium was measured using Fluo-3AM in NPCs after 15 mM or 50 mM KCl treatment of VPA-pretreated NPCs. (A) The maximum amplitude of calcium responses of NPCs treated with vehicle or VPA to 15 mM KCl. Maximum fluorescence was normalized to baseline (F\textsubscript{ baseline}). VPA-treated NPCs showed an enhanced Ca\textsuperscript{2+} influx in response to 15 mM KCl stimulation (N=13, t-test, t\textsubscript{12}=8.55, p<0.0001). (B) The percentage of calcium responsive cells exhibiting significantly increased Ca\textsuperscript{2+} response upon stimulation with 15 or 50 mM KCl. (C) Cacna1c mRNA levels measured through real-time PCR were not significantly changed (N=4, F\textsubscript{ ratio}=0.6556, p=0.5423). Data are presented as mean ± SEM. ***p<0.001.
ing to investigate further.

In conclusion, our study suggests that up-regulation of T-type calcium channels mediates the abnormal proliferation in VPA-exposed NPCs, which might lead to neurodevelopmental disorders. Additionally, abnormal activity-dependent Ca²⁺ entry may also contribute to the pathophysiology of VPA-induced neurodevelopmental disorders. How Ca²⁺ derived from T-type calcium channels is involved in the proliferation of NPCs and how abnormal activity-dependent Ca²⁺ signaling affects the pathophysiology of VPA-induced neurodevelopmental disorders require further investigation.

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