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T-Type Calcium Channels Are Required to Maintain Viability of Neural Progenitor Cells

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Abstract

T-type calcium channels are low voltage-activated calcium channels that evoke small and transient calcium currents. Recently, T-type calcium channels have been implicated in neurodevelopmental disorders such as autism spectrum disorder and neural tube defects. However, their function during embryonic development is largely unknown. Here, we investigated the function and expression of T-type calcium channels in embryonic neural progenitor cells (NPCs). First, we compared the expression of T-type calcium channel subtypes (CaV3.1, 3.2, and 3.3) in NPCs and differentiated neural cells (neurons and astrocytes). We detected all subtypes in neurons but not in astrocytes. In NPCs, CaV3.1 was the dominant subtype, whereas CaV3.2 was weakly expressed, and CaV3.3 was not detected. Next, we determined CaV3.1 expression levels in the cortex during early brain development. Expression levels of CaV3.1 in the embryonic period were transiently decreased during the perinatal period and increased at postnatal day 11. We then pharmacologically blocked T-type calcium channels to determine the effects in neuronal cells. The blockade of T-type calcium channels reduced cell viability, and induced apoptotic cell death in NPCs but not in differentiated astrocytes. Furthermore, blocking T-type calcium channels rapidly reduced AKT-phosphorylation (Ser473) and GSK3β-phosphorylation (Ser9). Our results suggest that T-type calcium channels play essential roles in maintaining NPC viability, and T-type calcium channel blockers are toxic to embryonic neural cells, and may potentially be responsible for neurodevelopmental disorders.

Key Words: T-type calcium channel, Neural progenitor cells, AKT, GSK3β, Apoptosis, Toxicity

INTRODUCTION

T-type calcium channels are low voltage-activated calcium channels that transiently open to evoke tiny Ca^{2+} currents (reviewed in Perez-Reyes, 2003). T-type calcium channels regulate calcium influx from the extracellular region by opening the calcium channel (Cazade *et al*., 2017), or activating calcium-induced calcium release from the internal calcium source (Kitchens *et al*., 2003; Coulon *et al*., 2009). These results suggest a critical role for T-type calcium channels in regulating intracellular calcium homeostasis and maintaining cellular function (Assandri *et al*., 1999; Chemin *et al*., 2000; Cazade *et al*., 2017).

T-type calcium channels consist of three subtypes (CaV3.1, CaV3.2, and CaV3.3) with unique functions that depend on cell types and location (Iftinca and Zamponi, 2009). In proliferative cells such as cancer cells, adipocytes, and stem cells, T-type calcium channels modulate cellular proliferation and cell cycle. Moreover, blocking these channels induces apoptotic cell death, cell cycle arrest, or differentiation (Panner and Wurster, 2006; Oguri *et al*., 2010; Rodriguez-Gomez *et al*., 2012). Recently, interest in the role of T-type calcium channels in the brain has increased, due to their relevance to neurodevelopmental disorders such as autism spectrum disorder, neural tube defects, and absence seizure (Cheong and Shin, 2013; Abdul-Wajid *et al*., 2015). These previous reports indicate the importance of T-type calcium channels during brain development.

Importantly, T-type calcium channel blockers were ap-

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proved as an anti-epileptic drug, and their clinical use is expected to increase (Zamponi, 2016). Additionally, clinically used drugs for hypertension and angina have T-type calcium channel blocking potential (Kopecky *et al*., 2014). However, the role of T-type calcium channels and toxic effects of T-type calcium channel blockers are not well understood.

In this study, we investigated the expression levels of T-type calcium channels in neural progenitor cells (NPCs), cortical neurons, and astrocytes. We also confirmed the temporal expression pattern of CaV3.1 in cortical regions from embryonic day 14 to postnatal day 18. Next, to determine a role of T-type calcium channels in maintaining cell viability, we treated NPCs and differentiated neural cells including astrocytes and neurons, with multiple T-type calcium channels blockers. We found that T-type calcium channels blockers induced apoptotic cell death in NPCs via apoptosis-related pathway. Our results suggest that T-type calcium channels are required to maintain NPC viability, and T-type calcium channels blockade during the embryonic period can have toxic effects.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium/F12 (DMEM/F12), fetal bovine serum (FBS), penicillin/streptomycin, and 0.25% trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY, USA). FGF and B-27 supplement were purchased from Invitrogen (Carlsbad, CA, USA). EGF, NNC55-0396 (N0287), mibefradil dihydrochloride (M5441), ML218 (SML0385), nickel chloride (339350), and nifedipine (N7634) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

The following is a list of the catalog number, company, and concentration of the primary antibodies used in this study: anti-CACNA1G (ab134269, Abcam, Cambridge, MA, USA, 1:2000), anti-Caspase3 (9662, Cell Signaling Technology, Danvers, MA, USA, 1:2000), anti-Bcl2 (sc-492, Santa Cruz, Dallas, TX, USA, 1:2000), anti-phospho-AKT (Ser473, 9272S, Cell Signaling Technology, 1:2000), AKT (9272, Cell Signaling Technology, 1:2000), anti-phospho-GSK3β (Ser9, 9336S, Cell Signaling Technology, 1:2000), anti- GSK3β (9336, Cell Signaling Technology, 1:2000), anti-phospho-ERK1/2 (Thr202/Tyr204, 9101S, Cell Signaling Technology, 1:2000), anti-ERK1/2 (0101, Cell Signaling Technology, 1:2000), anti-phospho-JNK (Thr183/Tyr185, 9251S, Cell Signaling Technology, 1:2000), anti-JNK (9252, Cell Signaling Technology, 1:2000), anti-phospho-p38 (Thr180/Tyr182, 9211S, Cell Signaling Technology, 1:2000), anti-p38 (9211S, Cell Signaling Technology, 1:2000), anti-actin (A5316, Sigma-Aldrich, 1:50000).

Methods

Cell cultures: Primary NPCs were isolated from the cerebral cortex of Sprague-Dawley (SD) rat embryos (embryonic day 14 or E14) as described previously (Go *et al*., 2012). For differentiation, NPCs were plated into poly-L-ornithine (1 mg/ml) pre-coated multi-well plates (1×10^6/ml). NPCs were incubated in a B27 supplement-containing DMEM/F12 media. After 3 h of incubation for recovery, drugs were added to the NPCs. Samples were prepared at designated time points. Primary cortical neurons and astrocytes were prepared as previously described (Kim *et al*., 2011).

Brain preparation: Pregnant SD rats or SD rat pups were euthanized and cortices were collected from the embryos or pups, respectively, on designated days. Cortices were rapidly frozen with and samples were stored at -80°C until used for analysis. All animal procedures, including anesthesia and euthanasia, were performed in accordance to 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).

Western blot analysis: Cells were lysed with 2x sample buffer (4% w/v SDS, 20% glycerol, 200 mM DTT, 0.1 M Tris-HCl, pH 6.8, and 0.02% bromophenol blue) that contained protease and phosphatase inhibitors. BCA analysis was used to quantify protein samples and an equal amount of proteins was loaded during electrophoresis. Following electrophoresis, proteins were transferred to a nitrocellulose (NC) membrane, and blocked with 1% skim milk for 1 h. Membranes were

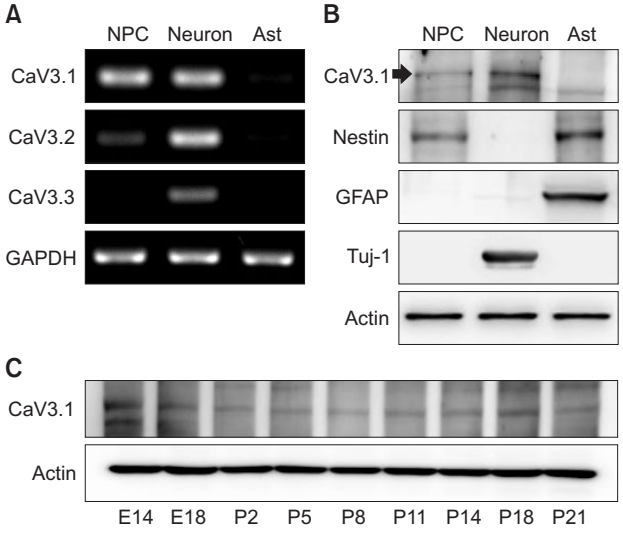

Fig. 1. Expression of T-type calcium channels in different neuronal cell types during different developmental periods. (A) mRNA expression of T-type calcium channel subtypes in NPCs, primary cortical neurons, and astrocytes. (B) Protein expression of CaV3.1 in neuronal cells or (C) in the cortex during the developmental period.

Table 1. RT-PCR primer sequences

Genes	Sequence (F)	Sequence (R)	Source
Cacna1g	CATGCCACCTTTAGGAACTTTG	CGGAGGGTGTCCTTCATAATAC	NM_031601
Cacna1h	GCCTTCGACGACTTCATCTT	GTGTCACCCAGGTAGCATTT	NM_153814
Cacna1i	ACAGGCGATAACTGGAATGG	GTAGAGCGGTGACACAAACT	NM_020084
18s rRNA	CATTAAATCAGTTATGGTTCCTTTGG	TCGGCATGTATTAGCTCTAGAATTACC	(Westmark and Malter, 2007)

washed three times for five minutes each and then incubated with the appropriate primary antibody overnight at 4°C. Membranes were then incubated with the peroxidase-conjugated secondary antibody for 2 h at room temperature. After three more five-minute washes, protein blots were developed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK), and detected with the LAS-3000 imaging system (Fuji Film, Tokyo, Japan). Band intensities were analyzed using Multi Gauge v3.0 (Fuji Film) and normalized to actin or to total protein levels.

Cell viability assay: Cell viability was measured using MTT assay. After 24 h drug treatment, MTT (Sigma-Aldrich, M2128) reagent was applied at a concentration of 0.25 mg/ml, and treated plates were incubated for 2 h. Media was then removed and 500 μl of dimethyl sulfoxide (DMSO) was applied to each well. Plates were incubated for an additional 20 min on an orbital shaker to dissolve the violet MTT formazan crystals. Absorbance was measured using a microplate reader at the wavelength of 570 nm, and a reference filter of 620 nm (SpectrMax190, Molecular Devices, Sunnyvale, CA, USA).

RT-PCR: Total RNA was extracted from NPCs using Trizol reagent. A RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Fisher Scientific, Waltham, MA, USA) was used for reverse transcription of 1 μg total RNA, and 100 ng of cDNA was used for each PCR amplification. Amplified PCR products were loaded and electrophorated in EtBr-containing agarose gel and subsequently visualized. Used primers sequences are listed below:

Immunocytochemistry: NPCs were plated on coverslips, washed with PBS three times, and were then fixed with 4% paraformaldehyde at 37°C for 20 min. After three more washes, samples were incubated in 2x sodium citrate buffer (SSC, 0.3 M NaCl, 0.03 M Na-citrate, pH 7.0) for 5 min, and again washed three times with 2x SSC buffer. Next, a 2x SSC buffer containing propidium iodide (0.03 μg/ml) was added to each well for 5 min and again washed three times using a 2x SSC buffer. Each sample was mounted with vector shield mounting solution containing DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA). Cells were visualized and imaged with a fluorescence microscope (Bx61, Olympus, Tokyo, Japan).

Statistics

Data were analyzed with a Student's t-test. *p*-values less than 0.05 were considered statistically significant. Results are expressed as mean ± standard error of the mean (SEM). The statistics were generated with GraphPad Prism 5 (La Jolla,

A

	Viability	CaV3.1	CaV3.2	CaV3.3	L-type
NNC55-0396	Dead	Block	Block	–	–
Mibefradil	Dead	Block	Block	Block	Block
ML218	Alive	–	Block	Block	–
$NiCl_2$ (<100 μM)	Alive	–	Block		–
$NiCl_2$ (>100 μM)	Dead	Block	Block	Block	
Nifedipine	Alive	–	–	–	Block

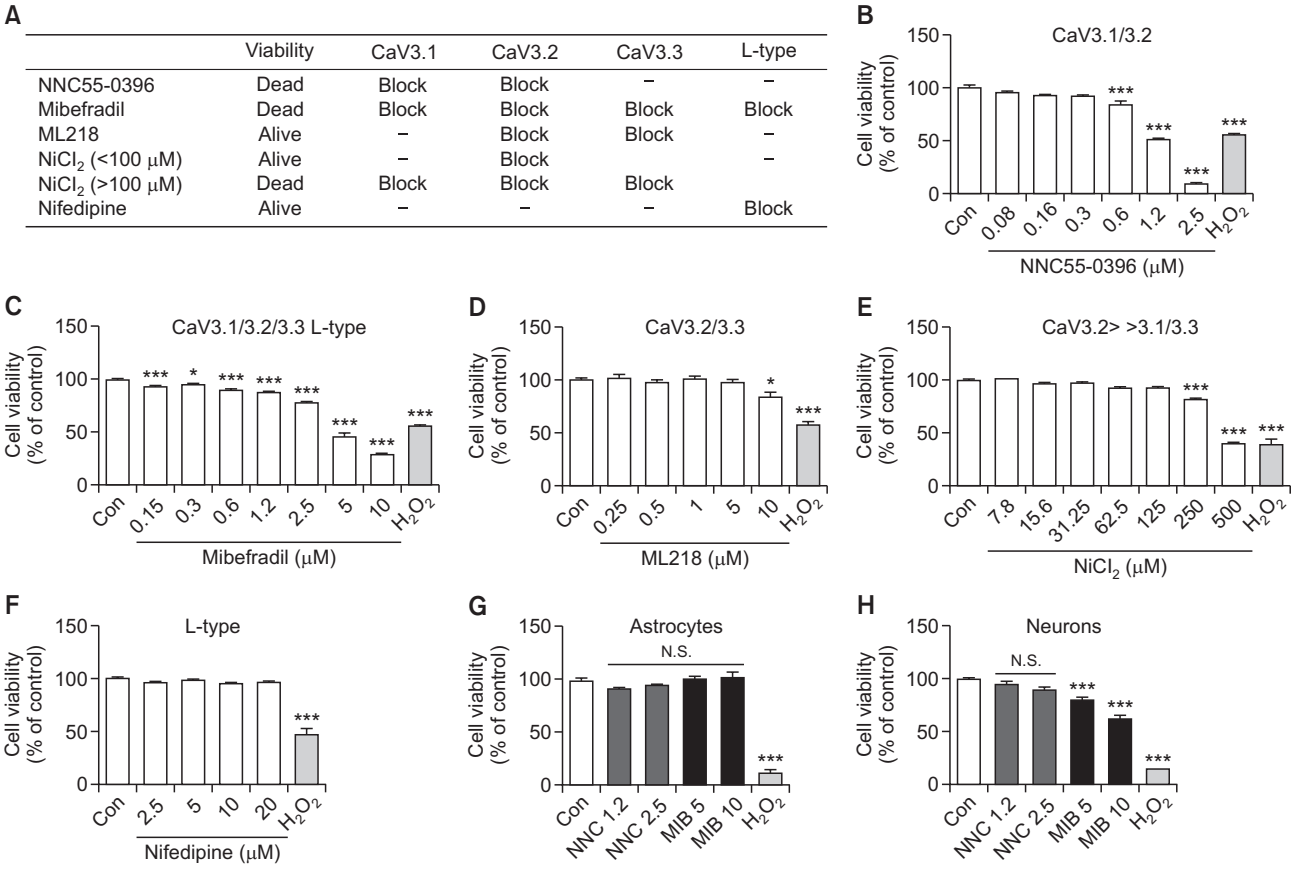

Fig. 2. Inhibition of T-type calcium channels decreases NPC viability. (A) Table summarizing the function of channel blockers and their effects on NPC viability. (B-F) Results of MTT analysis 24 h after pharmacological blockade of T or L-type calcium channels using NNC55-0396 (B), mibefradil (C), ML218 (D), $NiCl_2$ (E), and nifedipine (F), in NPCs. (G-H) MTT results from astrocytes (G) and neurons (H) 24 h after treatment with T-type calcium channel inhibitors (NNC55-0396 and mibefradil). Graphs represent the mean ± SEM. N=4-13, **p*<0.05 and ****p*<0.001.

CA, USA).

RESULTS

CaV3.1 is the dominant subtype in neural progenitor cells

First, we investigated mRNA expression of the three T-type calcium channel subtypes found in NPCs, cortical neurons, and astrocytes (Fig. 1A). In NPCs, CaV3.1 was strongly expressed, whereas CaV3.2 expression was weak. All subunits were strongly expressed in neurons and scarcely detected in astrocytes. To confirm PCR results, we used western blot analysis of CaV3.1 expression levels in NPCs, neurons, and astrocytes (Fig. 1B). Consistent with our PCR results, CaV3.1 was expressed in NPCs and neurons, but not in astrocytes. Next, we examined CaV3.1 expression levels in the cortex, from embryonic day 14 to postnatal day 21 (Fig. 1C). CaV3.1 expression was prominent during the prenatal period, transiently decreased during the perinatal period, and increased again by postnatal day 11, although this increase was to a lesser extent than that observed during the embryonic period. This suggests that CaV3.1 may be important during the embryonic period.

T-type calcium channels are required for maintenance of NPC viability

To determine the importance of T-type calcium channels in cellular viability, we performed MTT analysis after T-type calcium channel blcokers treatment in NPCs, astrocytes, and neurons. To rule out the possibility of obtaining a misleading result due to non-specific effects, we confirmed response-specificity by using various T-type calcium channel blockers, as well as an L-type calcium channel blocker (Nifedipine). The T-type calcium channel blockers used were: mibefradil (broad T-type calcium channel blocker and weak L-type calcium channel blocker) (Martin *et al*., 2000), NNC55-0396 (CaV3.1 and CaV3.2 blockers) (Huang *et al*., 2004; Taylor *et al*., 2008), ML218 (CaV3.2 and CaV3.3) (Xiang *et al*., 2011), nickel chloride (more specific to CaV3.2 at lower concentrations) (Lee *et al*., 1999; Rossier, 2016), and nifedipine (L-type calcium channel) (D'Ascenzo *et al*., 2006) (Fig. 2A). The MTT assay was performed 24 h after drug application (Fig. 2B-2H). All of the T-type calcium channel blockers, except ML218, decreased NPC cellular viability. Nickel chloride did not affect viability at concentrations below 250 μM, which are known to block CaV3.2 and 3.3, but viability was decreased at concentrations over 250 μM, which is the 50% inhibition concentration (IC50) of CaV3.1 (Rossier, 2016). The L-type calcium channel blocker, nifedipine, did not affect cell viability, even at concentrations higher than the IC50 of 5 μM (D'Ascenzo *et al*., 2006). Given that CaV3.3 mRNA expression was rarely seen in NPCs (Fig. 1A), and that both NNC55-0396 and mibefradil can block CaV3.1 and CaV3.2, simultaneous blocking of these subtypes may be responsible for the reduced viability in NPCs.

Conversely, mibefradil and NNC55-0396 did not decrease astrocyte viability, which may be due to the lack of T-type calcium channel expression (Fig. 2G). In neurons, mibefradil slightly reduced viability at concentrations over 5 μM, in which mibefradil can block L-type and all T-type calcium channels (Viana *et al*., 1997), whereas NNC55-0396 did not affect viability (Fig. 2H). This may be due to complementary mechanisms of additional calcium channels or to CaV3.3, which is not blocked by NNC55-0396.

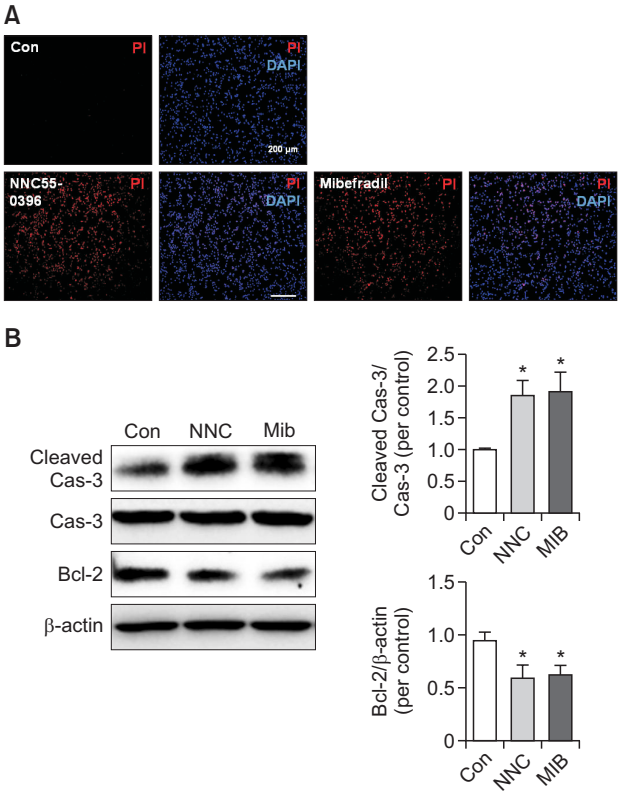

Fig. 3. Apoptotic cell death in NPCs by T-type calcium channel blockade. (A) Images of PI staining of NPCs after treatment with T-type calcium channel inhibitors (NNC55-0396 and Mibefradil). (B) Change in apoptotic cell marker proteins after NNC55-0396 and mibefradil treatment (N=5-8). Graphs represent the mean ± SEM. **p*<0.05.

T-type calcium channel blockers induce apoptosis in NPCs

We performed PI-staining to confirm NPC cell death 24 h after calcium channel blocker treatment (Fig. 3A). Treatment with NNC55-0396, or mibefradil increased the number of PI-positive cells. To further characterize the cell death, we performed western blot to detect changes in apoptotic signaling proteins (Fig. 3B). We found that NNC55-0396, and mibefradil treatment increased the expression of cleaved caspase 3, an apoptotic protein, and decreased levels of Bcl-2, an anti-apoptotic protein, in NPCs. These results suggest that T-type calcium channel blockers reduce NPC viability by inducing apoptosis.

T-type calcium channel blockers rapidly decrease AKT phosphorylation

T-type calcium channel blockers are used as anticancer drugs (Dziegielewska *et al*., 2014b), and induce apoptosis in multiple cancer cell lines via multiple signaling proteins including AKT (Ser473) (Valerie *et al*., 2013), ERK1/2 (Thr202/Tyr204) (Huang *et al*., 2015), and p38-MAPK (Thr180/Tyr182) (Dziegielewska *et al*., 2014a). We therefore investigated the phosphorylation of related signaling proteins using western blot analysis (Fig. 4). Within 10 min of treatment, mibefradil

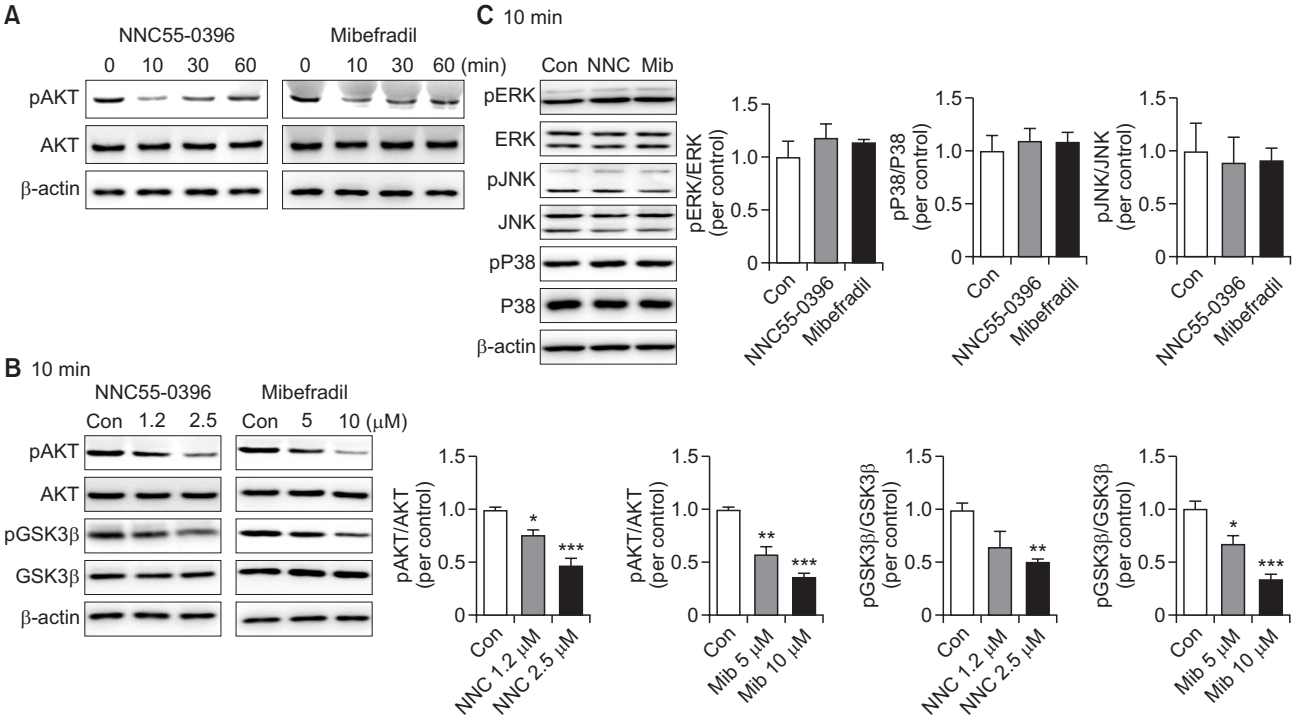

Fig. 4. Inhibition of T-type calcium channels reduces AKT and GSK3β phosphorylation. (A) NNC55-0396 and mibefradil decrease AKT phosphorylation within 10 min of treatment. (B) NNC55-0396 and mibefradil decrease AKT and GSK3β phosphorylation in a concentration-dependent manner (N=3-5). (C) Phosphorylation of ERK, JNK, and p38 did not change 10 min after T-type calcium channel blockade (N=3). Graphs represent the mean ± SEM. **p*<0.05, ***p*<0.01, and ****p*<0.001.

and NNC55-0396 decreased AKT (Ser473) phosphorylation, without affecting ERK, JNK, or p38 signaling (Fig. 4C). We also observed concentration-dependent AKT (Ser473) and GSK3β (Ser9) dephosphorylation. Since AKT dephosphorylation regulates GSK3β dephosphorylation during apoptosis (Franke *et al*., 2003), this molecular pathway may be involved in T-type calcium channel blocker-induced apoptosis in NPCs.

DISCUSSION

In this study, we identified the expression pattern of T-type calcium channels according to neural cell type and developmental period. NPCs had strong CaV3.1 mRNA expression and weak levels of CaV3.2 mRNA expression. In the cortex, high CaV3.1 expression was observed during the embryonic period, whereas expression decreased during the perinatal period, and then rebounded at postnatal day 11. These expression patterns suggest an important role for T-type calcium channels during the embryonic period. Consistent with this, we observed that T-type calcium channel blockade induced apoptotic cell death in NPCs, possibly via the AKT-GSK3β pathway. Our results demonstrate a crucial role of T-type calcium channels in maintaining the viability of NPCs, and the toxic effects of T-type calcium channels blockade.

Expression patterns of T-type calcium channels in the embryonic brain have yet to be specified. In our study, CaV3.1 and 3.2 were expressed in NPCs, and CaV3.1 expression was comparable to that of differentiated cortical neurons. Moreover, CaV3.1 expression during the embryonic stage was stronger than that in the cortex during the postnatal period, suggesting a potential role in early developmental period. Given that CaV3.1 is highly expressed in proliferative cells, and regulates proliferation, and cell cycle (Hirooka *et al*., 2002; Panner *et al*., 2005; Oguri *et al*., 2010), the high expression of CaV3.1 during the embryonic period may be attributed to active cellular proliferation that occurs during this period compared to the postnatal period. Additionally, CaV3.2 may also play an essential role in the developmental stage. Indeed, mutations were observed in both CaV3.1, and CaV3.2 genetic loci in patients with autism spectrum disorder (Splawski *et al*., 2006; Strom *et al*., 2010). Moreover, a recent report found that blockade of T-type calcium channels resulted in a neural tube closure defect (Abdul-Wajid *et al*., 2015). These studies suggest that t-type calcium channels play a vital role in early development.

In our study, we could not explain the subtype-specific roles of T-type calcium channels due to lack of subtype-specific blockers for CaV3.1, 3.2 and 3.3. Instead, we carefully used multiple T-type calcium channel blockers to understand the importance of T-type calcium channels. NNC55-0396, a highly selective T-type calcium channel blocker, was shown to block CaV3.1 currents in CaV3.1 transfected HEK 293 cells (1 μM) (Huang *et al*., 2004). However, NNC55-0396 can also block CaV3.2 currents at similar concentrations (Chen *et al*., 2010; Watanabe *et al*., 2015). Mibefradil blocks several T-type calcium channel subtypes (around 1 μM), including CaV3.1, 3.2 and 3.3, as well as L-type calcium channel by its metabolite (Martin *et al*., 2000; Wu *et al*., 2000). ML218 blocks CaV3.2 and 3.3 at concentrations of approximately 300 nM (Xiang *et al*., 2011). Ni^{2+} blocks CaV3.2 channels (13 μM) at a higher

potency than CaV3.1 (25-470 μM). In our study, we found that NPC viability was decreased by NNC55-0396, mibefradil, and a higher concentration of Ni^{2+}. However, the L-type calcium channel blocker, nifedipine, did not affect NPC viability. These results indicate that blockade of CaV3.1 together with CaV3.2, may reduce viability. Indeed, either CaV3.1 or CaV3.2 knockout mice did not show embryo lethality (Kim *et al*., 2001; Harraz *et al*., 2015), suggesting that CaV3.1 and CaV3.2 can compensate for deficiency in another subunit. Thus, in our study, CaV3.2 inhibition may have been complemented by CaV3.1, as seen with ML218 treatment, a putative CaV3.2 and 3.3 blocker, and also seen with low concentrations of nickel, which blocked both CaV3.2 and 3.3. These ambiguous issues will be clarified with subtype-specific genetic silencing experiments in our future study.

Another significant result is that neuron viability was affected by mibefradil, and not by NNC55-0396. Since mibefradil can block all types of T-type calcium channels, and L-type calcium channels at concentrations around 10 μM (Martin *et al*., 2000), complementary mechanisms between L-type and T-type calcium channels may be expected. However, T-type and L-type calcium channels are activated by different voltage currents, and therefore a complementary mechanism between them may be difficult to postulate. Instead, a complementary mechanism of T-type calcium channel subtypes may be more plausible. Neurons strongly express all T-type calcium channel subtypes including CaV3.3. Thus, CaV3.3 may compensate for the calcium influx from CaV3.1 and 3.2, blocked by NNC55-0396 in neurons. Consistent with PCR and western blot analysis that indicated the absence T-type calcium channels in astrocytes, cell viability in astrocytes was not affected by T-type calcium channel blockade. Based on these results, we hypothesize that T-type calcium channels play a crucial role in the support of cell viability in cell types that express them, as seen in cancer cells (Dziegielewska *et al*., 2014b).

To determine the mechanism underlying the T-type calcium channel blocker-induced reduction in NPC viability, we investigated changes in the phosphorylation of AKT, ERK, JNK and P38 proteins. Within 10 min of treatment, T-type calcium channel blockers rapidly decreased AKT (Ser473) phosphorylation. However, phosphorylation levels of the other proteins examined did not change. Additionally, GSK3β phosphorylation levels were also reduced within 10 min of treatment with mibefradil and NNC55-0396, in a concentration-dependent manner (Fig. 4). Given that GSK3β is a well-known substrate of AKT, these two proteins may be involved in reduced NPC viability after T-type calcium channel blockade. Indeed, GSK3β mediates apoptosis in various conditions including DNA damage, hypoxia, and endoplasmic reticulum (ER) stress (Jacobs *et al*., 2012). In a recent study, T-type calcium channel blockade and CaV3.1 and 3.2 silencing, induced apoptotic cell death via disruption of Ca^{2+} homeostasis in the ER. Specifically, NNC55-0396 treatment increased the intracellular calcium load by activating the Ca^{2+} release from the ER, a phenomenon called calcium-induced calcium release (Huang *et al*., 2015). Therefore, ER stress caused by T-type calcium channel blockade may cause de-phosphorylation of AKT and GSKβ, and subsequent apoptotic cell death in NPCs. This possibility will be addressed in our future study.

Our results suggest that T-type calcium channels play an essential role in maintaining cellular viability during the embryonic stage and that blockade of T-type calcium channels might be dangerous during this period. Recently, T-type calcium channels have become therapeutic targets for many neurological disorders including seizure, Parkinson disease, Alzheimer's disease, and neuropathic pain. Some T-type calcium channel blockers have already been used clinically as an anti-epileptic drug (Cheong and Shin, 2013; Zamponi, 2016). Additionally, some clinically used L-type calcium channel blockers can also block T-type calcium channels (Kopecky *et al*., 2014). Thus, safety issues of these drugs should be considered and addressed. Our findings shed light on a possible safety issue regarding the clinical use of T-type calcium channel blockers, especially in pregnant women. Although we could not specify the *in vivo* effects of T-type calcium channel blockers, future studies of T-type calcium channels in developmental disorders such as autism, will provide insight into the essential role of these channels in neural development.

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