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Construction and identification of the pshRNA-CACNA1G-SH-SY5Ycells targeted to silence Cav3.1 mRNA expression

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Abstract. T-type calcium channels are a class of low voltage-dependent calcium channels that may be activated following minor depolarizations of the cell membrane. Cav3.1 is the dominant subtype of the T-type calcium channel in SH-SY5Y cells. T-type channels play a key role in the regulation of the intracellular calcium concentration, which is involved in the neurotoxic effect of local anesthetics. However, there is a lack of specific inhibitors of T-type calcium channels. The existing T-type calcium channel inhibitors exhibit poor specificity and may block the high voltage-dependent calcium channels, such as the L- and N-type channels. Furthermore, there is no selectivity to the subtype of the T-type calcium channel. Therefore, the development of a specific T-type calcium channel inhibitor may contribute to the elucidation of the functions and characteristics of T-type calcium channels. The aim of this study was to silence the Cav3.1 mRNA expression in SH-SY5Y cells via the RNA interference (RNAi) method in order to construct pshRNA-CACNA1G-SH-SY5Y cells and assess Cav3.1 mRNA and protein expression by western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) to identify the constructed cell line. The results demonstrated that Cav3.1 mRNA and protein expression were significantly reduced following transfection with the SH-SY5Y cells by the supernatant liquors. The results also demonstrated that the pshRNA-CACNA1G-SH-SY5Y cells were successfully constructed. These findings may contribute to the elucidation of the functions of Cav3.1 in SH-SY5Y cells.

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Introduction

T-type calcium channels are divided into three subtypes, Cav3.1, Cav3.2 and Cav3.3, coded by α1G, α1H and α1I, respectively. They are a class of low voltage-dependent calcium channels that may be activated following minor depolarizations of the cell membrane. Furthermore, there is a window current in the T-type calcium channel, which refers to the voltage overlap between the activation and steady-state inactivation at low or resting membrane potentials (1-3). As a result, extracellular calcium ions are able to enter the intracellular compartment through a small proportion of channels that remain open under the window current. These electrophysiological characteristics are responsible for the T-type calcium channels being a key element in the regulation of neuron excitability and neurotransmitter release (4-6).

The functions of the T-type calcium channels have yet to be fully elucidated, which may be the reason for the lack of specific antagonists for this type of channel. The existing T-type calcium channel inhibitors exhibit poor specificity and may block the high voltage-dependent calcium channels, such as the L- and N-type channels (7). Furthermore, there is no selectivity to the subtype of the T-type calcium channel. Therefore, the development of a specific T-type calcium channel inhibitor may contribute to the elucidation of the functions and characteristics of this type of calcium channel.

In our previous study, all three subtypes of the T-type calcium channel, Cav3.1, Cav3.2 and Cav3.3, were detected in SH-SY5Y cells (8). However, there was some diversity in the expression levels. Cav3.1 was the dominant subtype in SH-SY5Y cells, whereas the expression of Cav3.2 and Cav3.3 was significantly lower. The aim of the present study was to silence Cav3.1 mRNA expression in SH-SY5Y cells via the RNA interference (RNAi) method in order to construct pshRNA-CACNA1G-SH-SY5Y cells and detect Cav3.1 mRNA and protein expression by western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) with the aim of identifying the constructed cell line. Findings of the present study may contribute to the elucidation of the functions of the Cav3.1 T-type calcium channel in the SH-SY5Y cells.

Materials and methods

Materials. The SH-SY5Y cell line was purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). psPAX, pMD2.G lentiviral packaging system, 293FT packaging cells and pSUPER-retro-puro plasmid were purchased from Laura Biotech Co., Ltd. (Guangzhou, China). Goat polyclonal anti-Cav3.1 and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The primer of Cav3.1 and β-actin was synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Plasmid Maxiprep kits and DNA purification kits were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). DNA polymerase, DNA ligase and PrimeSTAR HS DNA polymerase were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Restriction enzymes *Bgl*II and *Hin*dIII were purchased from New England Biolabs (Beverly, MA, USA).

Cell culture. SH-SY5Y cells were cultured in DMEM/F12 medium with 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator at 37˚C. The medium was renewed every 2 days (9).

shRNA sequence design. According to NM_018896 gene sequence, the human Cav3.1 (α1G) gene, we investigated three interference targets and designed three interference sequences and one negative sequence (Table I) (http://www.genscript.com/ssl-bin/app/rnai and https://rnaidesigner.invitrogen.com/rnaiexpress/). The sequences were synthesized by Sangon Biotech. The interference targets were as follows: 961: accaactgct cagcggggga gcacaacccc ttcaagggcg ccatcaactt tgacaacatt; 2281: ggcatcgaat accacgagca gcccgaggag cttaccaacg ccctagaaat cagcaacatc; and 3841: gtggtccttg tcatcatctt ccttaactgc atcaccatcg ccatggagcg ccccaaaatt.

Construction of pshRNA-pSUPER-retro-puro. Following annealing, the synthetic nucleotide described above formed a double chain and was combined with the enzymatically digested pSUPER-retro-puro to construct pNC-puro (NC), pshRNA-puro-CACNA1G-RNAi1 (RNAi1), pshRNA-puro-CACNA1G-RNAi2 (RNAi2) and pshRNA-puro-CACNA1G-RNAi3 (RNAi3). The reaction system of the enzymatic digestion of pSUPER-retro-puro plasmid was as follows: 4 μl NEB 10 buffer, 1 μl *Bgl*II (10 units), 1 μl *Hin*dIII (10 units), 20 μl pSUPER-retro-puro plasmid (2 μg) and 14 μl DDH_2O. The reaction system of the connection of shRNA with the pSUPER-retro-puro plasmid was as follows: 5 μl enzymatically digested pSUPER-retro-puro plasmid, 4.5 μl double-chain shRNA, 2 μl connection buffer, 1 μl T4 DNA ligase and 7.5 μl ddH_2O. The products were transferred into competent cells and the recombinant plasmids were measured with a DNA sequencing system (Sangon Biotech).

Construction of pshRNA-CACNA1G-SH-SY5Y cells. The calcium phosphate mixture was prepared as follows: 30 μl $CaCl_2$ (2M), 15 μg pSPAX2, 5 μg pMD2.G, 20 μg pNC-puro (NC) or pshRNA-puro-CACNA1G-RNAi1 (RNAi1) or pshRNA-puro-CACNA1G-RNAi2 (RNAi2) or pshRNA-puro-CACNA1G-RNAi3 (RNAi3) and 30 μl ddH_2O. The ratio of recombinant plasmid:pSPAX2:pMD2.G was 4:3:1. Six hours after the mixture was prepared at room temperature, 293T cells were cultured with this mixture for 36-48 h. The liquid supernatant of the culture solution was collected 3-4 times. The collected liquid supernatant was added to the SH-SY5Y cell culture solution and incubated at 37˚C for 3 h. The liquid supernatant was then changed and the process was repeated twice. The transfected SH-SY5Y cells were conserved at -80˚C.

Cav3.1 mRNA detected by RT-PCR. Total RNA from the transfected SH-SY5Y cells was extracted using TRIzol reagent (Takara Biotechnology Co., Ltd.). The isolated RNA was subsequently treated with RNase-free DNase to remove genomic DNA contamination. Conventional gene expression analysis was performed according to previously published protocols (10). Briefly, isolated RNA was reverse-transcribed and amplified with a commercial kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Oligonucleotide primers (Table II) were designed using Oligo 6 Primer Analysis software (Molecular Biology Insights Inc., Cascade, CO, USA). Total RNA (2 μg) was reverse-transcribed for 60 min at 42˚C followed by PCR amplification. The PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. The intensity of the bands was measured by densitometry and the relative value of Cav3.1 to the β-actin band was calculated in each sample.

Cav3.1 protein detected by western blot analysis. All the procedures were performed on ice to prevent proteolysis of the calcium channel subunits. Culture flasks or plates were quickly rinsed with chilled PBS. The cells were collected using a plastic cell scraper, removed and lysed in lysis buffer A [20.0 mmol/l Tris-HCl, 1.0 mmol/l Na_3VO_4, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 0.1 mmol/l ethylenediaminetetraacetic acid, 0.1 mmol/l ethylene glycol tetraacetic acid, 0.5 mmol/l phenylmethylsulfonyl fluoride and 0.02% protease inhibitor cocktail (pH 7.9)]. Protein samples were dissolved in 4X sample buffer [250 mmol/l Tris-HCl, 200 mmol/l sucrose, 300 mmol/l dithiothreitol, 0.01% Coomassie brilliant blue G and 8% SDS (pH 6.8)] and were subsequently denatured at 95˚C for 5 min. Equivalent amounts of protein were separated by 7.5% sodium dodecylsulfate polyacrylamide gel electrophoresis and were transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4˚C with the following primary antibodies: rabbit anti-human Cav3.1 or β-actin (1:500; Santa Cruz Biotechnology, Inc.). The membranes were washed thoroughly with Tris-buffered saline/Tween-20 and incubated for 2 h in peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:500, Santa Cruz Biotechnology, Inc.) at room temperature. The immune complexes were detected by enhanced chemiluminescence. Membranes were then exposed to X-ray film. Quantification of the protein bands was conducted by scanning the films and importing the images into Adobe Photoshop software (Adobe, San Jose, CA, USA). Scanning densitometry was used for semi-quantitative analysis of data. The Cav3.1 protein was normalized to the corresponding β-actin product.

Statistical analysis. Data are expressed as means ± standard error of the mean and were analyzed using SPSS 13.0 software

Table I. Primers of negative and interference sequences.

Primer name	Primer sequence
NC	
Up	5'-gatccccgccagcttagcactgactcttcaagagagagtcagtgctaagctggcttttta-3'
Down	5'-agcttaaaaagcgccttccgtcttgggaatctcttgaattcccaagacggaaggcgcggg-3'
shRNA1 (RNAi1)	
Up	5'-gatccccgccatcaactttgacaacatttttcaagagaaatgttgtcaaagttgatggctttta-3'
Down	5'-agcttaaaaagccatcaactttgacaacatttctcttgaaaatgttgtcaaagttgatggcggg-3'
shRNA2 (RNAi2)	
Up	5'-gatcccccgcttaccaacgccctagaaatttcaagagaatttctagggcgttggtaagcgttttta-3'
Down	5'-agcttaaaaacgcttaccaacgccctagaaattctcttgaaatttctagggcgttggtaagcgggg-3'
shRNA3 (RNAi3)	
Up	5'-gatcccccccttgtcatcatcttccttaattcaagagattaaggaagatgatgacaagggtttta-3'
Down	5'-agcttaaaaacccttgtcatcatcttccttaatctcttgaattaaggaagatgatgacaaggggg-3'

Underlined sections show the targeted sequence of the Cav3.1 gene (NM_018896).

Table II. Primer sequences of β-actin and Cav3.1.

Gene	Primer sequence	Product size (bp)
β-actin		
Forward	5'-TGGCACCCAGCACAATGAA-3'	186
Reverse	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'	
Cav3.1		
Forward	5'-GCCATCTTCCAGGTCATCAC-3'	140
Reverse	5'-ACCAGGCACAGGTTGATCAT-3'	

(SPSS, Inc., Chicago, IL, USA). The comparisons of protein and mRNA expression between the three T-type calcium channel subtypes in cultured SH-SY5Y cells were performed using repeated measures analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DNA sequence analysis of pshRNA-pSUPER-retro-puro. The DNA sequences of pshRNA-pSUPER-retro-puro vector were detected and were in complete accordance with the three designed interference sequences and the negative sequence (Fig. 1).

Cav3.1 mRNA expression detection by RT-PCR. Compared to the untransferred SH-SY5Y cells and pNC-puro cells, Cav3.1 mRNA expression in the cells of the RNAi1, RNAi2 and RNAi3 groups was distinctly decreased. However, there were no significant differences in the Cav3.1 mRNA expression between the cells in the untransferred and NC groups (Fig. 2).

Cav3.1 protein expression detection by western blot analysis. The Cav3.1 protein and mRNA expression were similar. There were no significant differences in the Cav3.1 protein expression between the cells in untransferred and NC groups. However, the Cav3.1 protein expression in the cells of the RNAi1, RNAi2 and RNAi3 groups was clearly decreased (Fig. 3).

Discussion

With the development of biomedical technology, genetic engineering has become an important method in the clinical setting and scientific research (11-14). The small double-stranded RNA molecules may silence gene expression, which is considered a sequence-specific gene inactivation system. RNAi is a phenomenon of homologous specificity mRNA degradation induced by the highly conservative double-stranded RNA. RNAi technology may specifically eliminate or shut down specific gene expressions. The RNAi method exhibits the following characteristics: i) functions at the transcriptional level of the gene silencing mechanism; ii) is of high specificity, with degradation of only the corresponding single endogenous gene mRNA; iii) is highly efficient and a relatively small amount of double-stranded RNA (dsRNA) molecular may fully suppress the corresponding gene expression via catalytic amplification; iv) the inhibitory effect of RNAi on specific gene expressions may

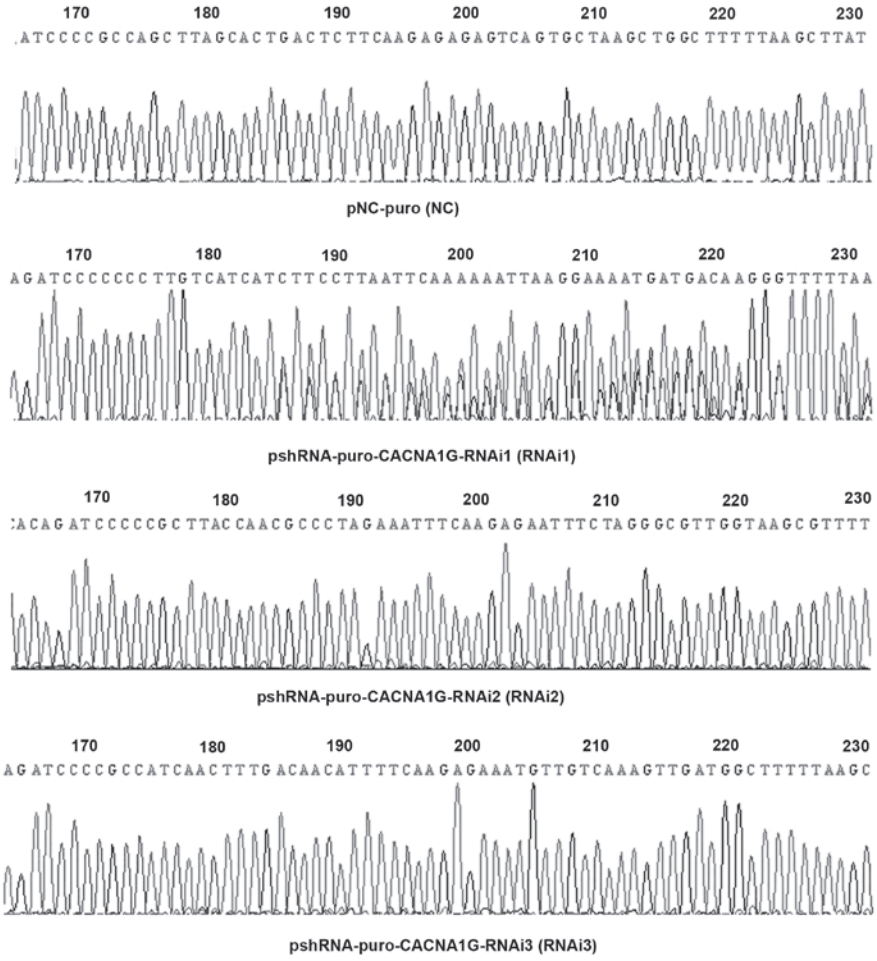

Figure 1. DNA sequence analysis of recombinant cells.

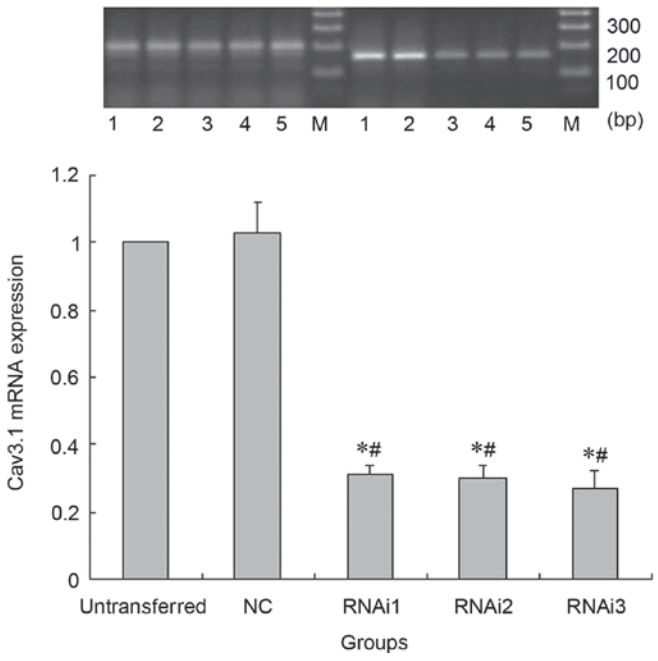

Figure 2. Expression of Cav3.1 mRNA (mean ± standard error of the mean, n=6). The intensity of the bands was measured by densitometry and the relative value of Cav3.1 to the β-actin band was calculated in each sample. Lanes 1, 2, 3, 4 and 5: untransferred SH-SY5Y cells, NC, RNAi1, RNAi2 and RNAi3, respectively. *P<0.01 vs. cells in the untransferred group, #P<0.01 vs. cells in the NC group.

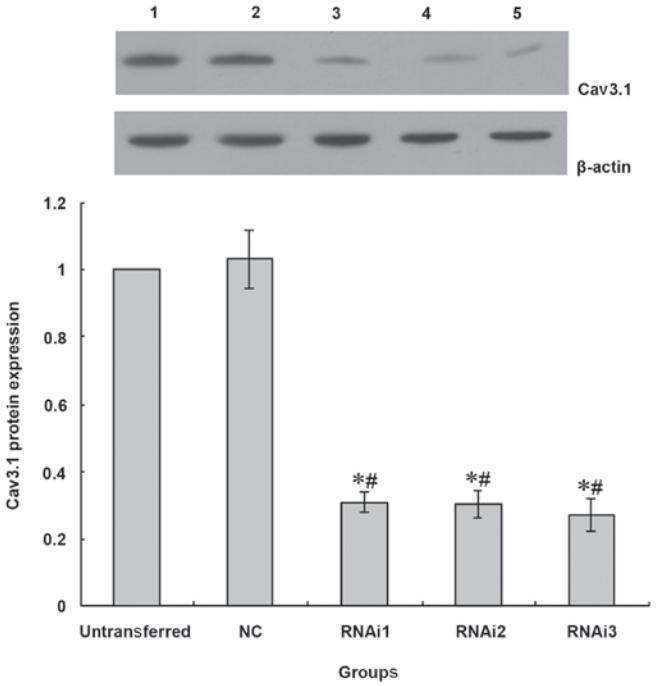

Figure 3. Expression of Cav3.1 protein (mean ± standard error of the mean, n=6). Cav3.1 protein was normalized to the corresponding β-actin product. Lanes 1, 2, 3, 4 and 5: untransferred SH-SY5Y cells, NC, RNAi1, RNAi2 and RNAi3, respectively. *P<0.01 vs. cells in untransferred group, #P<0.01 vs. cells in the NC group.

be transmitted intercellularly for a long distance; and v) the dsRNA usually is ≥21-bp long and dsRNAs >30 bp are not able to induce specific RNAi in mammals.

Several methods are adapted to RNAi. First, the chemical synthesis method is in common use (15-17). The small interfering RNA molecules (siRNAs) consisting of 21 bases with two free nucleotides in the 3' terminal are synthesized *in vitro*. Compared to the other synthesized RNA molecules, siRNAs are able to degrade the target gene with the highest efficiency. Second, siRNAs are connected to the vector. Plasmids or viruses are often used as vectors. The synthesis of the sense or antisense strand is regulated by the U6 snRNA and T7 RNA polymerase. Following annealing, the sense and antisense strands form the dsRNA that connects to the vector and interferes with gene expression. The construction of the dsRNA vector is also often used in lower organisms.

In this study, three RNAi sequence were designed according to the CACNA1G gene sequence NM_018896 and the RNAi design principle. Following annealing, the sense and antisense sequences formed dsRNA molecules and were connected to the pSUPER-retro-puro plasmid vector. The sequences of the connected to pSUPER-retro-puro plasmid vector were same as those designed by DNA sequencing. The dsRNA molecules were packaged with the lentiviral vectors and 293FT cells. The supernatant liquor of the virus was collected following centrifugation. The SH-SY5Y cells were infected with the collected supernatant liquor to construct pshRNA-CACNA1G-SH-SY5Y cells and Cav3.1 protein or mRNA were detected with western blot analysis or RT-PCR. The results demonstrated that Cav3.1 protein and mRNA expression significantly decreased following infection of the SH-SY5Y cells by the supernatant liquors. These data suggest that the pshRNA-CACNA1G-SH-SY5Y cells were successfully constructed.

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