ORIGINAL ARTICLE

Effect of Nogo-A gene inhibition on dopamine release in PC12 cells

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Abstract

OBJECTIVES: Reticulon proteins, which are localized in the endoplasmic reticulum, have recently been shown to be involved in hormone secretion, in particular RTN1 and RTN3. The aim of the present study was to examine the effects of Nogo-A gene expression knockdown by RNA interference (RNAi) on dopamine release in PC12 cells.

METHODS: A small hairpin RNA (shRNA) eukaryotic expression vector, targeting the Nogo-A gene, was constructed and transfected into cultured PC12 cells by lipofecamine2000. Inhibition of Nogo-A gene expression was detected by semi-quantitative reverse transcription PCR and Western blot analysis. Following transfection, dopamine release was detected by high performance liquid chromatography.

RESULTS: The pGenesil-1-Nogo-A-2 plasmid was identified by gene sequencing. After transfection of the recombinant vector in PC12 cells, Nogo-A gene expression was significantly inhibited ($p<0.01$). Compared with the empty vector control group, dopamine release significantly decreased within 48 hours after transfection.

CONCLUSION: Results from this study suggest that Nogo-A might be involved in the mechanism of DA release in PC12 cells.

1. INTRODUCTION

Reticulon (RTN) is an endocytoplasmic reticulum membrane family member, which possesses many biological functions, including axonal inhibition and apoptosis induced by RTN4/Nogo expression. Studies have shown that many members of this family function as protein regulators or are involved in hormone secretion [Senden *et al.* 1997; Steiner *et al.* 2004, He *et al*. 2004, Wakana *et al.* 2004]. Steiner *et al*. demonstrated that RTN1C combines with the key molecular SNARE protein, which mediates fusion of the vesicle with the cell membrane of PC12 cells and other cell lines. Overexpression of RTN1C results in increased growth hormone secretion, which indicates that it plays a latent role in vesicle exocytosis [Steiner *et al.* 2004]. Nogo-A (also called Reticulon 4A) is the fourth member of RTN family [Chen *et al.* 2000]. Xiong *et al.* demonstrated that Nogo-A is strongly expressed in the hypothalamic supraoptic nucleus and paraventricular nucleus of rats. Furthermore, Nogo-A protein expression is greater in neuroendocrine cells of these regions than in cerebral cortical neurons, which suggests that Nogo-A may be involved in neuroendocrine activities [Hasegawa *et al.* 2005].

Vesicle exocytosis is the major route by which hormones and prohormones are secreted and neurotransmitters are released. Although the secretion mechanisms are different, the membrane fusion processes of the vesicles are similar. The PC12 cell line is a neuroendocrine cell line that secretes dopamine (DA), and has been used extensively in studies of dopaminergic neurotransmitters. Results from Northern blots, RT-PCR, and Western blots have demonstrated that Nogo-A is highly expressed in PC12 cells [Senden *et al.* 1997, Oertle *et al.* 2003]; however, to date, there are no studies that have shown that Nogo-A is involved in the regulation of DA release. RNA interference is a powerful tool to study gene function, and the cellular effects are similar to gene knock-out. The present study constructed a shRNA eukaryotic expression vector for the target gene, Nogo-A, and transfected it in PC12 cells. High-performance liquid chromatography (HPLC) was utilized to detect changes in dopamine release following knockdown of Nogo-A expression. The aim was to better understand the function of Nogo-A in dopamine release and the significance of Nogo-A expression in neuroendocrine cells.

2. MATERIALS AND METHODS

2.1 Designation of Nogo-A siRNA and cloning of siRNA hairpin loops

Nogo-A siRNA was designed according to recommendations from the Qiagen website (http://sirna.qiagen.com). The sequence was linked to the following pair of oligonucleotides through the use of restriction enzymes (produced by Wuhan GenSil Biotechnology, China): Nogo-A-2: sense 5'-GATCCGTTTGCAGTGTTGATGTGGTTCAAGACGCCACATCAACACTGCAAACTTTTTTGTCGACA-3' and anti-sense 3'-GCAAACGTCACAACTACACC AAGTTCTGCGGTGTAGTTGTGACGTTTGAAAAAACAGCTGTTCGA-5', where the target sequence was exhibited as antisense followed by sense orientations, separated by a 9-nucleotide spacer sequence and flanked at either end by *Hind*III or *Bam*H1 restriction enzyme sites. Annealed oligonucleotides were cloned into pGenSil-l (Wuhan GenSil Biotechnology, China), according to manufacturer's recommendations.

2.2 Cell culture, transfection, and selection of stably transfected cell clones

PC12 cells (Institute of Biochemistry and Cell Biology, SIBS) were cultured in DMEM (Hyclone), containing 10% heat-inactivated fetal calf serum (Gibco BRL, Gaithersburg, Md. USA). Cells were incubated at 37°C, in a 50 mL/L CO_2 air incubator with saturated humidity. Cells were seeded in 6-well plates, 2×10^6 cells/well, and allowed to grow overnight to 70% confluency. The cells were transfected with 10 μL liposome, in addition to either 4 μg pGenSil-1/Nogo-A-2, empty vector pGenSil-1, or a blank control. Because the plasmid pGenSil-1/Nogo-A-2 shRNA co-expressed Nogo-A shRNA and EGFP, the stably transfected clones could be selected by fluorescent microscopy. Clones containing empty vector did not co-express EGFP; therefore, these clones did not emit fluorescence. After transfection (24 or 48 hours), cells were washed and resuspended in PBS. The cells were subsequently observed under fluorescence microscopy.

2.3 Detection of Nogo-A mRNA expression by RT-PCR

Cells (1×10^6) were harvested 48 h after transfection. Total RNA was isolated with TRIzol (Gibco) and reverse transcribed into cDNA with human specific primers for Nogo-A and β-actin. Primer sequences for Nogo-A were as follows: sense 5'-GTC CTG CTT GAA ACT GCT-3', anti-sense 5'-CTT TCG GTT GCT GAG GTA-3', length 713 bp; β-actin: sense 5'-ATC ATG TTT GAG ACC TTC A-3', anti-sense 5'-CAT CTC TTG CTC GAA GTC-3', length 318 bp. Briefly, 35 PCR amplification cycles were performed: 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, in a total of 25 μL. PCR products were visualized by UV illumination. mRNA gene expression was corrected and compared to the housekeeping gene, β-actin, which served as an internal control.

2.4 Detection of Nogo-A protein expression by Western blot

Attached PC12 cells were washed twice with PBS, harvested in PBS, and pelleted by centrifugation. The pellet was weighed and suspended in 2× Laemmli buffer. Proteins were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking, membranes were incubated for 1 h with anti-Nogo-A antibody (Santa-Cruz), followed by a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Zhongshan). Specific bands were detected by an enhanced chemiluminescence system (Pierce, Rockford, IL).

2.5 Detection of the amount of DA release by HPLC

Supernatants (200 μL) were collected into centrifuge tubes after 24 and 48 h transfection, respectively. PC12 cells were washed 3 times, lysed with 0.2% Triton at 37°C for 15 min, and collected for the detection of DA. Samples were centrifuged at 1000 r/min for 30 min at 4°C. HClO (1 mol/L, 0.5 mL) was added to each sample (0.2 mL) to separate DA, followed by centrifugation. Supernatants were adjusted to pH 3.4 with phosphoric acid. The amount of DA in each sample (200 μL) was analyzed by HPLC (VARIAN Prosta, USA), using ECL detection (VARIAN, USA) at +0.65 V. The HPLC column was ODS C18 (46 mm × 250 mm, 5 μm), with a flow rate of 1 mL/min. The total amount of DA was calculated as follows: DA release + cell DA; the percentage of DA release was calculated as follows: release of DA/total DA × 100%.

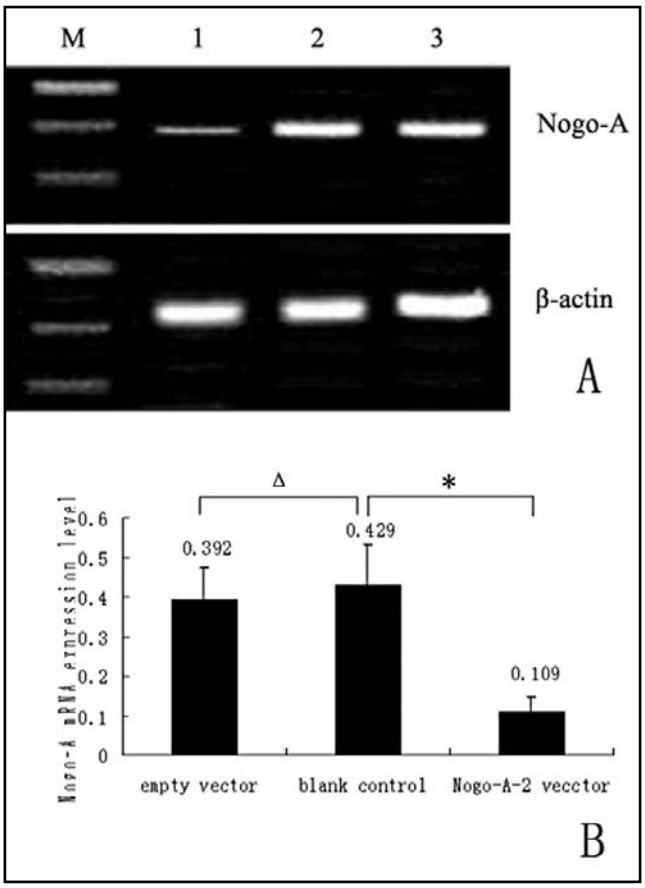

Fig. 1. RT-PCR detection of Nogo-A mRNA expression levels in cultured PC12 cells of different groups, including standard error bars. M: marker; 1: pGensil-1/Nogo-A group; 2: empty pGensil-1 vector group; 3: blank control group. B showed there was no significant difference between the blank control and empty vector group (Δ implies $p>0.05$), whereas band density in pGenesil-1/Nogo-A-2 group decreased (* implies $p<0.05$) compared to the blank control. Data are based on 12 repetitive experiments for each group.

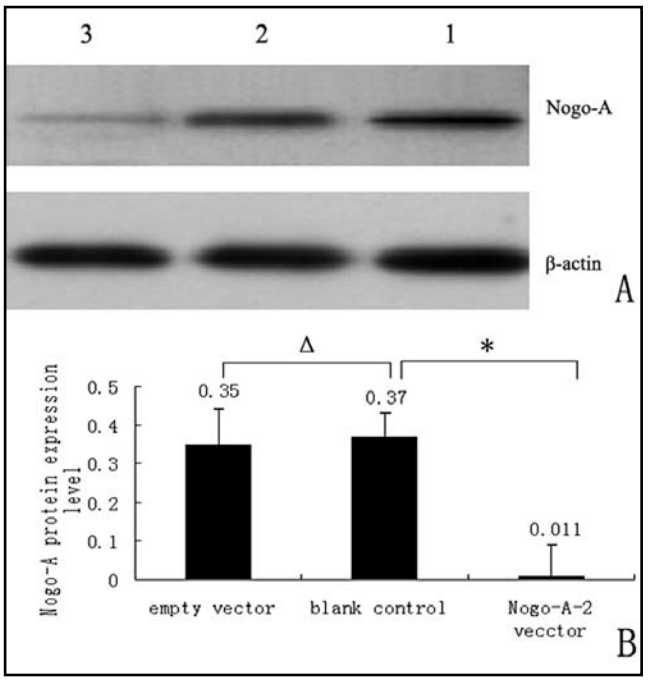

Fig. 2. Western blot detection of Nogo-A protein expression levels in cultured PC12 cells of different groups, including standard error bars. 1: pGensil-1/Nogo-A group; 2: empty pGensil-1 vector group; 3: blank control group. B showed there was no significant difference in Nogo-A gray scale between the blank control and empty vector group (Δ implies $p>0.05$), whereas the band density in pGenesil-1/Nogo-A-2 group dramatically decreased compared to the blank control group (* implies $p<0.05$). Data are based on 12 repetitive experiments for each group.

2.6 Statistical analysis

Data were expressed as means ± SD. Results were analyzed with one-way analysis of variance to determine statistical significance with SPSS13.0 statistic software. $p<0.05$ was considered statistically significant.

3. RESULTS

3.1 Nogo-A mRNA and protein expression changes after transfection

ANOVA results indicated that at least two groups demonstrated significant differences among all three groups ($F=7.34$, $p<0.05$). SNK post-hoc test revealed a significant difference between the pGenesil-1/Nogo-A-2 and blank control groups.

In all transfection groups, a 713-bp band was detected (Fig. 1), with a relative density of 0.429±0.082 (blank control), 0.392±0.103 (empty vector), and 0.109±0.038 (pGenesil-1/Nogo-A-2 vector). There was no significant difference between the blank control and empty vector group ($p>0.05$), whereas band density in pGenesil-1/Nogo-A-2 group was decreased ($p<0.05$) compared to the blank control, which indicates that the pGensil-1/Nogo-A-2 vector was successful at inhibiting Nogo-A mRNA expression following transfection in PC12 cells.

The relative density of Western blot bands (Fig. 2) in the blank control, empty vector, and pGensil-1/Nogo-A-2 groups was 0.37±0.06, 0.35±0.09, and 0.011±0.08, respectively. There was no significant difference in Nogo-A gray scale between the blank control and empty vector group ($p>0.05$), whereas the band density in pGenesil-1/Nogo-A-2 group decreased dramatically compared to the blank control group ($p<0.05$). These results suggest that the pGensil-1/Nogo-A-2 vector inhibited Nogo-A protein expression following transfection in PC12 cells.

3.2 Changes of DA release in PC12 cells after Nogo-A interference

HPLC detection of DA is shown in Fig. 3. Compared with the empty control group, there were no changes in DA release 24 and 48 h after transfection with the empty pGensil-1 vector ($p>0.05$). Following transfec-

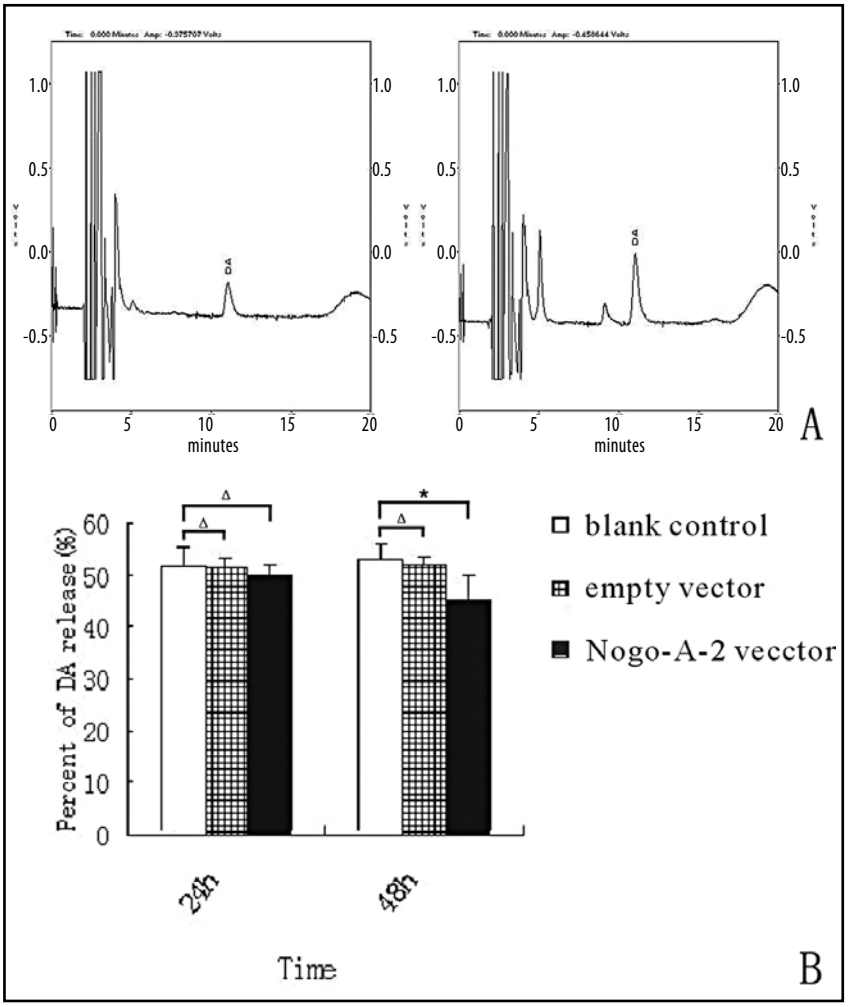

Fig. 3. HPLC results of DA. A depicts HPLC results of DA from a standard sample, whereas B shows the HPLC results of DA in PC12 cells. C illustrates the effects of Nogo-A shRNA on DA release in PC12 cells. DA release remained unchanged for 24 and 48 h after transfection in the empty pGensil-1 vector group compared to the blank control group (Δ implies $p>0.05$). DA release also remained unchanged 24 h after transfection (Δ implies $p>0.05$), but was significantly decreased 48 h after transfection in the pGenesil-1/Nogo-A-2 group (* implies $p<0.05$). Data are based on 9 repetitive experiments.

tion of the pGenesil-1/Nogo-A-2 vector, there were no changes in DA release by 24 hr; however, DA release significantly decreased by 48 h. There was no significant difference in total amount of DA among groups (data not shown).

These results demonstrated that the amount of DA release decreased after the down-regulation of Nogo-A gene in PC12 cells; however, the total amount of DA remained unchanged.

4. DISCUSSION

RTN family member proteins are localized in the endoplasmic reticulum and are involved in several biology functions. At least four different RTN genes have been identified in mammals: RTN1, RTN2, RTN3, and RTN4. RTN protein is ubiquitously expressed in vertebrates; all RTN proteins share a carboxylterminal 150-201 aa reticulon homology domain, comprising two large hydrophobic regions with a 66-aa loop in between. RTN1A and RTN1C are expressed in some neuroendocrine tumors, such as small cell lung cancer, and their functions have been shown to be involved in hormone secretion [Senden *et al.* 1997]. Steiner *et al.* demonstrated that a number of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, i.e., syntaxin 1, syntaxin 7, syntaxin 13, and VAMP2, co-immunoprecipitate with RTN1C. Moreover, overexpression of a RTN1C fragment in PC12 cells, which binds to SNAREs, significantly enhances human growth hormone secretion. These results suggest that RTN1C is involved in vesicle trafficking events, including the regulation of exocytosis. Wakana *et al.* (2004) showed that cells expressing RTN3 exhibit a filamentous/reticular distribution, blocked protein transport between the ER and Golgi, and dispersed Golgi proteins, which suggests that RTN3 plays a role in membrane trafficking in early secretory pathways. In addition, He *et al.* (2004) demonstrated that decreased RTN3 expression by RNA interference increases Aβ secretion, which suggests that reticulon proteins are negative modulators of BACE1 in cells. These results support a mechanism by which reticulon proteins block access of BACE1 to amyloid precursor protein and reduce cleavage of this protein.

RTN4-A/Nogo-A has been described as a potent neurite outgrowth inhibitor in the CNS [Chen *et al.* 2000]. Since the cloning of Nogo-A, much research has focused on Nogo-A and CNS regeneration. Moreover, several recent studies [Mingorance *et al.* 2004, Dodd *et al.* 2005, Jin *et al.* 2003] demonstrate that the func-

tion of Nogo-A is not limited to neural regeneration, but rather that it is a multifunctional protein. Although Nogo-A also belongs to RTN family, to date there have been no reports regarding the function of Nogo-A secreted proteins. Hasegawa *et al.* [Hasegawa *et al.* 2005] found that Nogo-A mRNA was strongly expressed in the hypothalamic nucleus, supraotic nucleus, and periventricular nucleus. Xiong *et al.* [Xiong *et al.* 2005] also showed that Nogo-A protein is expressed in the supraoptic nucleus and paraventricular nucleus, and these regions secrete higher hormone levels than cerebral cortical neurons. These results suggest that Nogo-A protein may participate in neuroendocrine activities.

The present study demonstrated that DA release significantly decreased after inhibition of Nogo-A gene expression by RNAi. These results suggest that Nogo-A might be involved in some mechanism of DA release in PC12 cells. However, the mechanisms involved remain unclear. It is well known that neuroendocrine cells contain neurotransmitters or neuropeptide hormones; these cells are functionally different from other neurons and endocrine cells. Vesicular transportation is not only a fundamental process in hormone secretion, but also a key method of neurotransmitter release. Neurotransmitters, hormones, and proteins are packaged in different types of vesicles, respectively; the formation of these vesicles and cycle processes are comparatively different, yet membrane fusion of vesicles is similar. PC12 cells originate from a pheochromocytoma cell line of the rat adrenal gland; these cells secrete DA and are able to reuptake DA. Therefore, PC12 cells are appropriate cells to study DA release, and the study of Nogo-A gene expression effects on DA release in these cells is relevant.

It is well accepted that synthesized DA is stored in mainly in the vesicle and released to synaptic cleft by vesicular release or exocytosis. RTN takes part in functions that relate to the endoplasmic reticulum, such as vesiculation and packaging of secretory products. It has been suggested that Nogo-A functions in neuroendocrine cells may be similar to other RTN family members, such as release of DA by regulating vesicular release or exocytosis.

This study provides evidence for the relationship between Nogo-A and neuroendocrine activity. Nevertheless, at present, the functional study of RTN is still very basic. To understand RTN functions and mechanisms during emiocytosis, further research is required. Vesicular transportation of eukaryotic cells is involved in the modulation of hundreds of proteins and regulatory factors. However, neurotransmitter release is different from hormone release. Thus, the effects and mechanisms of Nogo-A in neuroendocrine cells require further studies.

Acknowledgments

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