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Original article

Improved neurite outgrowth on central nervous system myelin substrate by siRNA-mediated knockdown of Nogo receptor

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ABSTRACT

Purpose: To investigate the *in vitro* effect of short interfering RNAs (siRNAs) against Nogo receptor (NgR) on neurite outgrowth under an inhibitory substrate of central nervous system (CNS) myelin.
Methods: Three siRNA sequences against NgR were designed and transfected into cerebellar granule cells (CGCs) to screen for the most efficient sequence of NgR siRNA by using reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence staining. NgR siRNA sequence 1 was found the most efficient which was then transfected into the CGCs grown on CNS myelin substrate to observe its disinhibition for neurite outgrowth.
Results: Compared with the scrambled control sequence of siRNA, the NgR siRNA sequence 1 significantly decreased NgR mRNA level at 24 h and 48 h ($p < 0.05$), which was recovered by 96 h after transfection. NgR immunoreactivity was also markedly reduced at 24 and 48 h after the transfection of siRNA sequence 1 compared with that before transfection ($p < 0.05$). The NgR immunoreactivity was recovered after 72 h post-transfection. Moreover, the neurite outgrowth on the myelin substrate was greatly improved within 72 h after the transfection with siRNA sequence 1 compared with the scrambled sequence-transfected group or non-transfected group ($p < 0.05$).
Conclusion: : siRNA-mediated knockdown of NgR expression contributes to neurite outgrowth *in vitro*.

Introduction

It is well known that the central nervous system (CNS) of adult mammalian fails to regenerate after injury, resulting in persistent neurological deficits. Although adult CNS neurons generally survive axotomy, axonal regeneration is transient and occurs only over a confined area, which inhibits the reformation of functionally-relevant synaptic contacts. As yet, there is a lack of an effective therapy to treat the patients with CNS injuries such as traumatic brain injury, spinal cord injury (SCI), and stroke. The reasons for the failure of the CNS to regenerate has been extensively investigated,[1–4] but the mechanism is still unclear.

It has been reported that the mechanism may be related with the inability of the neuron to regenerate, the limited availability of neurotrophins required for neuronal survival and axonal growth,[5,6] the generation of the intrinsic axonal outgrowth inhibitors,[1,7–9] and the formation of a glial/collagen scar, a biochemical barrier hindering axon advancement.[5,6,10] Among them, the axon growth inhibitors, mainly from the CNS myelin, may play a key role in the failure of the regeneration.[4,7] To date, several such inhibitors have been identified, including myelin-associated glycoprotein (MAG),[7,8,11] oligodendrocyte myelin glycoprotein (OMgp) and Nogo.[12–15] All these proteins bind to the same neuronal glycosylphosphatidylinositol (GPI)-anchored receptors—Nogo receptors (NgRs) and transduce inhibitory signals to cells by interacting with p75 neurotrophin receptor as a co-receptor. The Co-receptor p75-NgR induces growth cone collapse by triggering an inhibitory signaling pathway, thereby inhibiting axonal

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regeneration.[4,11,16,17] Thus, inhibiting or blocking NgR may improve axon outgrowth. Focusing on this target, several reagents have been developed to overcome the inhibition of myelin on axonal regeneration, including antibody or antagonist,[7,18–20] downstream signaling molecule and vaccine.[3,21–26]

Gene knockout or knockdown can effectively block the expression and/or function of target genes. Recently, the transfection of short interfering RNA (siRNA), a powerful tool to knockdown specific gene expression, has been intensively studied and used to block gene expression and/or function.[27,28] Because NgR plays an important role in the inhibition of axon outgrowth, we hypothesize that a siRNA against NgR is able to promote the axon outgrowth of the neuron. To test this hypothesis, we designed NgR-specific siRNA sequences. After screening for the most efficient sequence of siRNA against NgR, we conducted NgR blocking experiments and assessed the effect of NgR-specific siRNA on NgR expression and neurite outgrowth *in vitro*.

Materials and methods

Cerebellar granule cell culture

Cerebellar granule cells (CGCs) were cultured as previously described.[29,30] Briefly, cerebellar tissue was collected from rats (postnatal day 7). After meninges were removed, the tissue was digested with trypsin (0.3 mg/ml) for 12 min and then mixed with an equal volume of dissociation buffer (80% Dulbecco modified Eagle medium, 10% Ham's F-12 medium, 10% fetal bovine serum, and 0.1 mg/ml DNase). The tissue was centrifuged at 1000 × *g* for 5 min, and the pellet was resuspended with 1 ml of the dissociation buffer in a 12 mm × 75 mm tissue culture tube. Dissociated cells were collected in the supernatant after the tissue settled by gravity in the tube for 10 min. The dissociation process was repeated once. The dissociated cells were finally resuspended in 1.5 ml of Hank's Balanced Salt Solution (HBSS). Cell viability was determined using trypan blue dye exclusion and cell counts.

NgR-specific siRNA preparation and transfection

NgR siRNA sequences of the rat, designed with the criteria described by Elbashir et al[31] were shown in Table 1. All the sequences were subjected to a BLAST program to make sure there was no significant homology with other genes prior to the process of the synthesis by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

For siRNA transfection, we used TransMessenger transfection reagent (Qiagen, Hilden, Germany) in 24-well tissue culture plates following the manufactory's protocol. Briefly, 1.2 μg of siRNA and 1.6 μl of enhancer R, a specific RNA-condensing reagent, were mixed in a final volume of 100 μl of an RNA-condensing buffer (Buffer EC-R) at room temperature for 5 min, which was further incubated with 2 μl of TransMessenger transfection reagent for an additional 10 min to form a transfection-complex. After being mixed with 300 μl of Opti-MEM I reduced serum medium (Life Technologies, Carlsbad, CA, USA), the complex was added to the cultured cells. Following a 4-h incubation, the old culture medium was replaced with neurobasal medium, and the cells were incubated until they were ready for the following analyses.

Table 1
Seven siRNA sequences of the rat NgR.

Items	Sequences
Sequence 1 (sense)	5′-GCAGUACCUGCGACUCAAUTT-3;
Sequence 1 (antisense)	5′-AUUGAGUCGCAGGUACUGCAG-3′
Sequence 2 (sense)	5′-GGCCAGGUUGUUCCAGAAATT-3′
Sequence 2 (antisense)	5′-UUUCUGGAACAACCUGGCCTC-3′
Sequence 3 (sense)	5′-GCUUCCAGCCAUGCCGGAATT-3′
Sequence 3 (antisense)	5′-UUCCGGCAUGACUGGAAGCTG-3′
Scrambled control sequence (sense)	5′-UUCUCCGAACGUGUCACGUTT-3′
Scrambled control sequence (antisense)	5′-ACGUGACACGUUCGGAGAATT-3′

To calculate transfection efficiency, the scrambled siRNA labeled with Carboxy fluorescein (FAM) was transfected into the CGCs, too. At 6 h after transfection, the number of FAM-labeled CGCs, emitting green fluorescence, was counted. The transfection efficiency was calculated by the ratio between the number of cells with green fluorescence and the total cultured cells.

For the CGC transfection, a preliminary study was first conducted to screen for the most efficient siRNA sequence. Briefly, three sequences of NgR siRNA and one scrambled control sequence were transfected into the CGCs as mentioned above. The most efficient sequence of siRNA NgR, identified by reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence staining as described below, was used for further assessment of neurite outgrowth.

Preparation of the CNS myelin extract

The CNS myelin extract was prepared according to the method of Cuzner et al.[32] Briefly, adult Sprague–Dawley rat brains were homogenized in 0.32 mol sucrose with 1 mmol EDTA (pH 7.0). The suspension was centrifuged at 800 × *g* for 10 min and supernatant was collected. The cell pellet was resuspended in the original volume of 0.32 mol sucrose with 1 mmol EDTA (pH 7.0), and recentrifuged at the above speed. The second supernatant was collected and pooled with the first one, which was then centrifuged at 13,000 × *g* for 20 min. After the removal of the supernatant, the pellet was suspended in 0.9 mol/L sucrose, followed by carefully overlaid with 1–2 ml of 0.32 mol/L sucrose. The sucrose solutions were then centrifuged at 20,000 × *g* for 60 min. The white material at the interface of the two sucrose layers was collected in the minimum volume possible, dispersed in 20 volumes of 0.32 mol/L sucrose, and centrifuged at 13,000 × *g* for 25 min. The white pellet was then collected, diluted in 25 volumes of pure water, left on ice for 30 min before centrifuging at 20,000 × *g* for 25 min. Then the final white pellet was resuspended in a small volume of water and freeze-dried overnight. The protein content of the myelin extract was determined using nucleic acid/protein analyzer (DU640-type, Beckman, Brea, CA, USA).

RT-PCR

To quantify NgR expression in the CGCs before and at 24, 48, 72, and 96 h after transfection, we determined the mRNA levels of the NgR and the internal control hypoxantine phosphoribosyltransferase (HRPT) using RT-PCR. Total RNA was extracted with Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription of mRNA to cDNA was performed with reverse transcriptase as usual. PCR primer sequences are shown as follows: NgR sense, 5′-CTG CTG GCA TGG GTG TTA TGG-3′; NgR antisense, 5′-TCT GGC TGG AGG CTG GGA T-3′; HRPT sense, 5′-AAA GCC AAG TAC AAA GCC TAA A-3′; HRPT antisense, 5′-CTG TCT GTC TCA CAA GGG AAG T-3′. PCR amplification was carried out using Taq DNA polymerase in a 25-μl of PCR reaction mixture containing 3 μg cDNA. For NgR, the amplification protocol consisted of initial denaturation (94°C for 4 min), 35 cycles of reaction (denaturation at 94°C for 30 s, annealing at 61°C for 60 s, extension at 72°C for 45 s), and final extension at 72°C for 10 min. The method of HRPT amplification was similar to the one for NgR except that there were 32 cycles of reaction and annealing was carried out at 58°C for 50 s. The