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Functional expression of rat neuroligin-1 extracellular fragment by a bi-cistronic baculovirus expression vector

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ABSTRACT

The interaction between the synaptic adhesion molecules neuroligins and neurexins is essential for connecting the pre- and post-synaptic neurons, modulating neuronal signal transmission, and facilitating neuronal axogenesis. Here, we describe the simultaneous expression of the extracellular domain of rat neuroligin-1 (NL1) proteins along with the enhanced green fluorescent protein (EGFP) using the bi-cistronic baculovirus expression vector system (bi-BEVS). Recombinant rat NL1 protein, fused with signal sequence derived from human Azurocidin gene (AzSP), was secreted into the culture medium and the optimum harvest time for NL1 protein before the lysis of infected cells was determined through the release of cytosolic EGFP. The NL1 protein (0.129 ± 0.013 mg/8×10^7 High Five cells; ~96% purity by metal affinity chromatography) was obtained from the supernatant of the recombinant virus-infected insect cells. A novel chip was employed to address whether the recombinant NL1 is functional in axogenesis. The purified rat NL1 promoted and enhanced the growth rate (137.07 ± 9.74 μm/day) of the axon on NL1/PLL (poly-L-lysine)-coated fine lines on the chip compared to those lines that were coated with PLL alone (105.53 ± 4.53 μm/day). These results were confirmed by fluorescence immunocytochemistry and demonstrated that the recombinant protein can be purified by a one-step process using IMAC combined with monitoring of cell lysis by bi-BEVS. This technique along with our novel chip offers a simple, cost-effective and useful platform for understanding the roles of NL1 protein in neuronal regeneration and synaptic formation studies.

Introduction

Synapses are asymmetric cell–cell junctions that mediate information processing between neurons in the brain. Transmission of information in the synapse is fast, dynamic, efficient, and tightly regulated. The plasticity of synapse, like long-term potentiation or depression dictates the basic mechanism of memory formation. Thus, the synaptic formation is the most fascinating phenomenon and subject in neuroscience. At present, there are two different types of synapses: excitatory (E) and the inhibitory (I) synapse. The balance between E and I synapses is critical for the cognitive function of the brain and the alteration of normal E/I ratios may trigger psychiatric disorders, especially autism spectrum disorders (ASDs)[2] and mental retardation [1–3]. It has been well established that synapse formation and the pattern of synaptic diversity depend on the actions of synaptic cell-adhesion molecules [4]. Neuroligin (NL) and neurexin (Nrxn) are two of the most investigated synaptic cell-adhesion molecules that affect synapse specification through differential effects on E/I synapse development [5–8]. In addition, NLs and Nrxns found on the post- and pre-synaptic cell surfaces are involved in facilitating neuronal axogenesis [9] and the development of a mature synapse [10].

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[2] *Abbreviations used*: AChE, acetylcholinesterase; AcMNPV, *Autographa californica* multicapsid polyhedrosis virus; ASDs, autism spectrum disorders; AzSP, signal peptide sequence of human Azurocidin gene; bi-BEVS, bi-cistronic baculovirus expression vector system; DIV, Days *in vitro*; EGFP, enhanced green fluorescent protein; Hi5, High Five cells; hpi, hour post-infection; IMAC, immobilized metal affinity chromatography; MOI, multiplicity of infection; Ni-NTA, nickel (II) nitrilotriacetic acid; NL1, neuroligin 1; Nrxn, Neurexin; PDZ, post-synaptic density 95/Discs large/Zona occludens-1; PLL, poly-L-lysine; P_{PH}, *polyhedrin* promoter; PS, penicillin/streptomycin; RhPV, *Rhopalosiphum padi* virus; Sf21, *Spodoptera frugiferda* 21 cells; $TCID_{50}$, 50% tissue culture infectious dose.

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Currently, four NL genes have been identified in rodents and five in the human genome [11–14]. NLs are type-I transmembrane proteins consisting of the following parts: a signal peptide, an extracellular AChE-like domain, EF-hand binding motif, an O-linked carbohydrate binding region, a transmembrane region, and a short cytoplasmic tail containing a PDZ (post-synaptic density 95/Discs large/Zona occludens-1) binding domain [15]. Although the extracellular region exhibits 34% amino acid sequence identical to AChE [16], it still lacks the active site residue required for catalytic activity of AChE. Moreover, some functional redundancy exists in the non-catalytic region of NLs, including a neuritogenesis capacity shared with AChE that have been well-reported [8,17,18]. The cytoplasmic region of NLs interacts with a number of PDZ domain-containing scaffold proteins, which in turn couple to ion channels, transmitter receptors, and signaling molecules [15,19]. However, the precise mechanism of the neuroligin–neurexin interaction remains unclear. In order to explore the interactions between NLs and Nrxns related to neuronal axogenesis and the medical applications of NLs for neuronal diseases, it is important to produce large quantities of recombinant NL proteins.

In this study, the extracellular esterase domain of rat NL1 (residues 46–638) was produced in insect cells using a novel bicistronic baculovirus expression vector system. The recombinant baculovirus, vAcAzSNL1-Rhir-E, can express NL1 and green fluorescent protein, EGFP, simultaneously in High Five and Sf21 insect cells through the internal ribosome entry site (IRES) sequence derived from *Rhopalosiphum padi* virus (RhPV). A signal peptide sequence was incorporated into the N-terminal of NL1 gene and the recombinant NL1 protein was secreted into and harvested from the culture medium to be further purified. Our results indicated that the average yield of the NL1 protein produced from 8×10^7 High Five cells was approximately 116–142 μg. A fabricated glass chip [20] stamped and coated with rat NL1 was utilized in order to explore the function of the recombinant NL1 on axogenesis. Finally, immunocytochemical analysis also revealed that the recombinant rat NL1 expressed by insect cells increased the rate of axogenesis in rat hippocampal neurons.

Materials and methods

Insect cell culture

The *Spodoptera frugiperda* IPBL-Sf21 (Sf21) cell line was routinely maintained at 27 °C in TNM-FH insect medium (Sigma, USA) supplemented with 8% heat-inactivated fetal bovine serum for the generation of recombinant baculovirus. For protein production, Sf21 cells were maintained at 27 °C in Sf-900II Serum-Free Medium (Invitrogen, USA). High Five™ cells (*Trichopulsia ni*) were maintained at 27 °C in Express Five® Serum-Free Medium (Invitrogen) supplemented with 90 μL/mL of 200 mM L-glutamine (Invitrogen).

Construction of the recombinant transfer vector

In the construction of a baculovirus transfer vector, pBacAzSNL1-Rhir-E, the *NL1* gene from nucleotides 977–2756 (GenBank Accession No. U22952) was PCR-amplified from the gene containing plasmid provided by Dr. Thomas C. Südhof (U. Texas Southwestern Medical Center, USA). The sequence of the primers used in the PCR were as follows: P1 (Forward, 5′-ATAGCTAGCATGACCCGGCTGACAGTCCTGGCCCTGCTGGCTGGTCTGCTGGCGTCCTCGAGGGCCGGCTCCAGCCCCCTTTTGGACCAAAAGTTGGATGTAGAC-3′, the sequence of *Nhe*I site and Azurocidin signal peptide are underlined, respectively) and P2 (Reverse, 5′-GCGGCGCGGAATTCTTAGTGATGGTGATGGTGATGAATGTCATTGAGATTATG-3′, the sequence of *Eco*RI site and hexa-histidine tag are underlined, respectively). The PCR product was cloned into *Nhe*I and *Eco*RI sites of pBacIR-GFP vector [21] to the generate transfer vector pBacAzSNL1-Rhir-E.

Generation of the recombinant virus

A recombinant virus, vAcAzSNL1-Rhir-E, was generated from Sf21 co-transfected with pBacAzSNL1-Rhir-E plasmid DNA (1.0 μg) and Bac-N-Blue linear AcMNPV viral DNA (0.25 μg, Invitrogen) by cellfectin. The recombinant virus formed green fluorescent plaques as identified under an inverted fluorescence microscope (Nikon, Japan) at day 6 after co-transfection. A virus stock of vAcAzSNL1-Rhir-E was selected by three round end-point dilutions. The titer of the generated virus was determined by end-point dilution and calculated via the green fluorescence in a 96-well plate according to the 50% tissue culture infectious dose ($TCID_{50}$) method [22].

Expression of the recombinant rat NL1 in insect cells

To investigate the NL1 protein expression as well as EGFP generation by the baculovirus, Sf21 cells (2×10^5 cells) were seeded on 24-well plates, infected with three different vAcAzSNL1-Rhir-E virus colonies (a, b and c) at multiplicity of infection, MOI 1.0, and were incubated for 96 h. The harvested culture supernatants (300 μL) were centrifuged (3000*g* for 15 min) to remove cell debris, treated with protease inhibitor cocktail (PI, Sigma), and were immediately frozen at −20 °C. The cells were lysed for 10 min in 300 μL culture cell lysis reagent containing 100 mM potassium phosphate (pH 7.8), 1 mM EDTA, 1.0% Triton X-100, and 7 mM β-mercaptoethanol. After centrifugation at 12,800 rpm for 30 min, the lysate supernatants (100 μL) were treated with PI and stored at −20 °C.

To obtain an efficient NL1 protein production in insect cells, Sf21 and High Five cells were seeded on 24-well plates (2×10^5 cells/well), infected with vAcAzSNL1-Rhir-E virus-b and incubated for 72 h at an MOI 1.0. The harvested culture supernatants (300 μL) were centrifuged (3000*g* for 15 min) to remove cell debris, treated with PI, and were immediately frozen at −20 °C.

SDS-PAGE and Western blot analysis

The protein concentration of samples was determined by the protein assay kit (Bio-Rad Laboratories, USA). About 30 μL of the supernatant and 30 μg of lysate were loaded and separated by SDS-PAGE on 10% polyacrylamide gels under reducing conditions and then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, USA) at 4 °C for 3 h at 70 V. The resulting membranes were blocked and incubated with anti-neuroligin-1 (1:2000 dilution; Santa Cruz Biotechnology, USA) and anti-EGFP antibodies (1:2000 dilution; Millipore, USA) at 4 °C overnight. This was followed by an incubation with horseradish peroxidase (HRP) conjugated secondary antibody (1:5000 dilution; Molecular probe, USA) for 1 h at room temperature. The HRP on the membrane was detected by an enhanced chemiluminescence kit (Pierce, USA) following the manufacturer's instructions.

Neuroligin-1 protein purification

High Five cells were seeded in T-75 flasks (1×10^7 cells/flask) containing 10 mL of Express Five™ SFM. Incubation was done at 27 °C in a non-humidified incubator and the insect cells were infected with vAcAzSNL1-Rhir-E at MOI = 5. The supernatant was harvested 72 h post-infection (hpi) and loaded onto Proteus Midi immobilized metal affinity chromatography, IMAC (Pro-Chem, USA). The column was washed three times with 10 mL for each wash with a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl,

30 mM imidazole, pH 7.5. Bound proteins were eluted with 10 mL (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 7.5). The eluates were concentrated to 250 μL by a 10 kDa cut-off concentrator (Millipore) and dialyzed three times with phosphate-buffered saline (pH 7.5) with protease inhibitor cocktail (Sigma). Samples recovered from the purification process including the supernatant, flow through, wash buffer, and the eluted protein were loaded onto 10% polyacrylamide gels for SDS-PAGE separation and Coomassie brilliant blue G (Merck, Germany) staining. The purity of the protein was determined by quantitative densitometry using UN-ISCAN IT Software (Silk Scientific, Inc., Utah, USA).

Chip fabrication and neuronal culture

The procedures for fabricating the chip are described previously [20]. Briefly, the glass chips, stamps and stencils were prepared as follows: round glass coverslips were cut into small squares and linear grooves were formed on two opposite surfaces. After, either the poly-L-lysine (PLL) or recombinant NL1 with PLL (NL1/PLL) was stamped onto the glass chip as fine lines (yellow color in Fig. 4A). On the other hand, polydimethylsiloxane stencil in which the opening is coated with PLL was placed on the chip (red color in Fig. 4A). Primary hippocampal neurons were dissociated from the rat fetuses at embryonic day 18 (E18) according to the procedures previously described [23,24]. Dissociated hippocampal neurons were first plated at a density of 2080 cells/mm^2 into the stencil openings in minimum essential medium (Invitrogen) supplemented with 5% horse serum, 5% fetal bovine serum, 0.5 mM glutamine and penicillin/streptomycin (PS, all from Invitrogen). After 72 h, the stencil was removed from the glass chip to make the axons capable of growing along the PLL-coated or NL1/PLL-coated fine lines. The glass chips were maintained in serum-free culture medium (NB with 2% B27, glutamine and PS, Invitrogen). The chips were treated with 5 mM cytosine-β-D-arabinofuranoside (Sigma) at 3 days *in vitro* (DIV3) for 24 h to reduce the growth of glial cells.

Axonal growth rate assay

Only one axon growing along the coated lines of either PLL or rat NL1/PLL was chosen and its length was monitored daily through the labeled grids on the chip. Axonal outgrowth was observed under a bright-field optical microscope (Nikon, Japan) and images were recorded from DIV3 to DIV8. The length of the distal end (growth cone) of the axons was measured everyday from DIV3 to DIV8. The daily growth rate (μm/day) equals axon's length of the current day minus the axon's length of the previous day. Axon length from three independent experiments for NL1/PLL group ($n = 247$) and PLL group ($n = 359$) were measured. Data were analyzed and statistical analysis was performed by Student's *t*-test.

Fluorescence immunocytochemistry for axonal outgrowth

Fluorescence immunocytochemistry was performed according to procedures previously reported [25]. Glass coverslips were coated with rat NL1 (50 μg/mL) combined with PLL (100 μg/mL) or with PLL (150 μg/mL) alone. Cultured hippocampal neurons (2080 cells/mm^2) on the coverslips were incubated for 72 h. Cells were fixed with 4% paraformaldehyde (PFA, Merck) for 25 min, permeabilized with Triton X-100 for 15 min, and blocked with 3% goat serum in phosphate-buffered saline (PBS) for 1 h. The cells were then incubated overnight at 4 °C with rabbit anti-MAP2 antibody (1:500 dilution; Chemicon, USA) and mouse anti-SMI-312 (1:1000 dilution; Covance, USA). The same cells were incubated with secondary antibodies, including FITC-conjugated goat anti-rabbit IgG (1:200 dilution; Santa Cruz Biotechnology) and Cy5-conjugated goat anti-mouse IgG (1:250 dilution; Jackson ImmunoResearch, USA) antibodies for 1.5 h at 25 °C. They were then incubated with Alexa Fluor 546 phalloidin (1:20 dilution; Molecular probe, USA) for 70 min to detect F-actin while the nuclei were detected subsequently with DAPI (Sigma) for 15 min at 25 °C. Fluorescence images of selected fields of the resultant cells were acquired using a confocal laser scanning microscope (LSM 510, Carl Zeiss MicroImaging, Inc., USA) and were analyzed using LSM 510 software.

The axon's length of neurons at DIV3 was measured from the cell bodies (DAPI) to the phalloidin-labeled growth cone while the single major axonal branch was seen by staining with SMI312. Axonal outgrowth data were expressed as mean ± standard error of the mean (SEM) from three independent experiments and statistical analysis was performed using Student's *t*-test.

Results and discussion

Expression of recombinant NL1 in insect cells

The gene encoding the truncated transmembrane region of NL1 (46–638 amino acid residues) was amplified by PCR and the N-terminal of NL1 was inframe-fused with a signal peptide sequence derived from the human Azurocidin gene (AzSP) that can increase the secreted proteins produced by insect cells up to 3-folds [26]. Meanwhile, a hexa-histidine tag (His-tag) was attached to the C-terminal of NL1 to facilitate the purification of NL1 protein. The amplified PCR product was cloned into the bi-cistronic transfer vector developed with six restriction sites flanked between *polyhedrin* promoter and the RhPV–IRES–EGFP sequence capable of facilitating the recombinant virus isolation and titer determination [21]. The resultant transfer vector was named pBacAzSNL1-Rhir-E (Fig. 1A). After co-transfection of pBacAzSNL1-Rhir-E with the viral DNA into Sf21 cells, green fluorescent plaques (Fig. 1B) were observed and the recombinant viral progenies were allowed to propagate in Sf21 cells (Fig. 1C). The green fluorescence, in turn, was used to guide facile isolation of recombinant virus. The resultant recombinant viruses were named vAcAzSNL1-Rhir-E.

To isolate the recombinant virus that co-expressed the EGFP and NL1 proteins, three vAcAzSNL1-Rhir-E viral colonies (a, b and c) were randomly selected under fluorescence microscope to infect Sf21 cells, then, the cell medium and lysate were collected and subjected to Western blot. The 80-kDa NL1 protein was found in the culture medium of virus-infected Sf21cells (Fig. 1D upper panel; lanes 4, 6 and 8). In contrast, the 27-kDa cytosolic EGFP protein was detected mainly in the cell lysates seen as a strong band at the cell lysate lane (Fig. 1D lower panel; lanes 3, 5 and 7) of virus-infected Sf21 cells. In addition, vAcAzSNL1-Rhir-E-a-infected Sf21 cell lysates and supernatants yielded visibly weaker NL 1 signals by Western blot as compared to that expressed in the vAcAzSNL1-Rhir-E-b or -c-infected Sf21 cells (Fig. 1D, upper panel). Thus, vAcAzSNL1-Rhir-E-b was chosen for the production of a functional recombinant NL1 protein.

To test which insect cell line was more feasible for NL1 protein production, High Five and Sf21 cells were infected with vAcAzSNL1-Rhir-E-b (MOI = 1) for three days and the culture medium was collected, and subjected to Western blot. The NL1 protein expression in the High Five cells was significantly higher than that of Sf21 cells grown in either TNM-FH supplemented with serum or Sf-900II Serum-Free Medium (Fig. 1E). This was consistent with the previous study [27], indicating that the yield of secreted proteins was higher in High Five than in Sf21 cells. Taken altogether, these findings indicated that vAcAzSNL1-Rhir-E-b can generate efficiently the NL1 protein as well as the cytosolic EGFP simultaneously in insect cells. Moreover, efficient NL1 production was obtained in High Five cells.

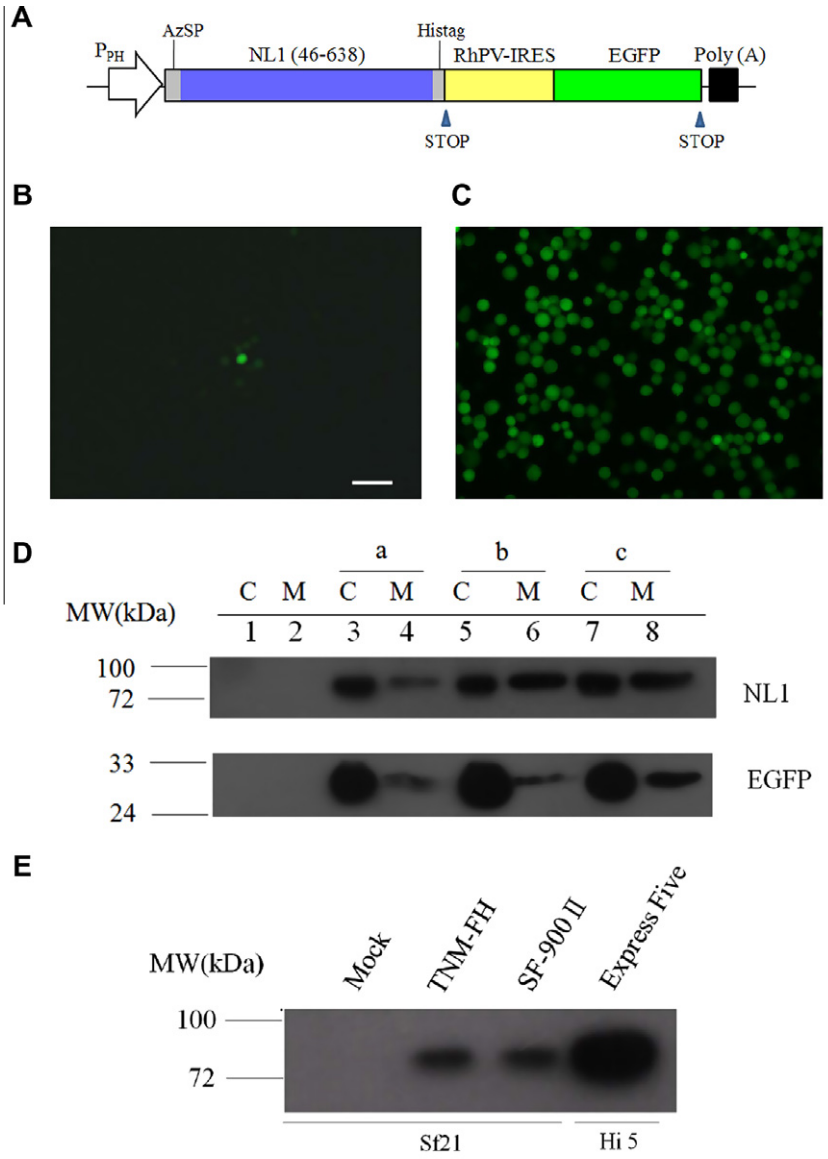

Fig. 1. Expression of neuroligin-1 in insect cells. (A) Construct of the recombinant virus, vAcAzSNL1-Rhir-E showing: P_{PH}, polyhedrin promoter; NL1, rat neuroligin-1 gene; RhPV–IRES, element of RhPV 5′ IRES; EGFP, enhance green fluorescence protein gene; AzSP, Azurocidin signal peptide gene; His-tag, hexa-histidine tag sequence. (B) Recombinant transfer vector, pBacAzSNL1-Rhir-E (1 μg) was co-transfected into Sf21 cells (2×10^5 cells seeded in a 24-well plate) with linear viral DNA Bac-N-Blue (0.25 μg) by cellfectin. Successful recombination formed green fluorescent plaques as revealed under fluorescence microscopy. Photographs were taken via conventional FITC channel with 450/490 filter set and at an exposure time of 264 ms, scale bar = 30 μm. (C) The cells were infected with vAcAzSNL1-Rhir-E (MOI = 1); the progeny of the virus can be easily observed under fluorescence microscope. (D) Western blot analysis of the vAcAzSNL1-Rhir-E-infected Sf21 cell with anti-NL1 and anti-EGFP antibodies. Sf21 cells (2×10^5 cells seeded in a 24-well plate) were infected at an MOI of 1 with three different recombinant viruses, vAcAzSNL1-Rhir-E-a (lanes 3 and 4), vAcAzSNL1-Rhir-E-b (lanes 5 and 6) and vAcAzSNL1-Rhir-E-c (lanes 7 and 8), respectively. Cells were then harvested 4 days, post-infection (dpi). Lanes 1 and 2: mock infection; C, cell lysates; M, medium. Molecular mass marker is indicated on the left. (E) Evaluation of *Spodoptera frugiferda* (Sf21) and High Five (Hi5) cell lines grown in serum-free or serum-supplemented medium and infected with vAcAzSNL1-Rhir-E for the production of NL1 protein.

Time-course study on EGFP and NL1 expression in High Five cells for the determination of harvest time

High Five cells were chosen to generate recombinant NL1 and optimum harvest time for NL1 production was determined. High Five cells were infected with vAcAzSNL1-Rhir-E-b at an MOI of 5, and the culture medium were harvested at different time post-infection and subjected to Western blot analysis. The NL1 protein was visible from 48 h post-infection (hpi) and last to 144 hpi (Fig 2, up panel) to determine the optimal harvest time for recombinant NL1 production.

Through the RhPV–IRES–EGFP module in the bi-BEVS expression vector, the cytosolic EGFP was used to monitor the cell lysis after vAcAzSNL1-Rhir-E-b infection and to signal the release of intracellular proteins which may interfere in the purification of the NL1 protein. Western blot analysis of the EGFP was initially found in the culture medium at 96–120 hpi as demonstrated by a faint band and a darker band at 144 hpi (Fig. 2 low panel) indicating cell lysis caused by the virus and leaking of the intracellular proteins including cytosolic EGFP into the culture medium. Since the cells began to lyse at 96 hpi (MOI = 5) as demonstrated by the detection of EGFP in the medium and upon considering the contamination of other intracellular proteins in the culture medium at 96 hpi, the culture medium therefore was harvested at 72 hpi avoiding interference from other intracellular proteins in the succeeding purification.

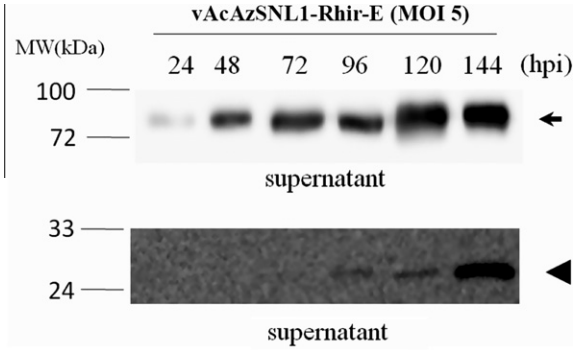

Fig. 2. Time-course analysis of the secreted NL1 protein and EGFP in the culture medium of the vAcAzSNL1-Rhir-E-infected High Five cells. Cell lysis was monitored by the release of EGFP. High Five cells were infected with vAcAzSNL1-Rhir-E at an MOI of 5. After infection, the culture supernatants were collected at the indicated time and subjected for Western blot analysis with anti-NL1 (up panel) and anti-EGFP (low panel) polyclonal antibody.

Purification of recombinant NL1 protein one step by IMAC

Immobilized metal affinity chromatography (IMAC) was used to purify the recombinant NL1 proteins from culture medium of vAcAzSNL1-Rhir-E-b-infected High Five cells. The final purification products were identified by using Coomassie brilliant blue-staining on SDS-PAGE, revealing a single band corresponding to the recombinant NL1 protein approximately at 80 kDa with 96% purity (Fig. 3, lane 5) determined through quantitative densitometry. Western blot analysis of this purified proteins probed with anti-NL1 antibodies also confirmed the purified protein band is the recombinant NL1 proteins (Fig. 3, lane 6). The yield of NL1 protein was 0.129 ± 0.013 mg per 8×10^7 High Five cells (Table 1). In connection with this, a recent study indicated that with the use of the baculovirus expression system, the complete extracellular domain of a similar cell-adhesion molecule, i.e. L1 protein, can be obtained at a production yield of 0.250 mg/8×10^7 High Five cells [28]. Thus, these results demonstrated by using this cell lysis monitoring bi-BEVS combined with IMAC and serum-free medium the recombinant secreted proteins can be purified by one-step purification. We also tried to purify the recombinant NL1 proteins from the vAcAzSNL1-Rhir-E-b-infected High Five cells harvested at 96 hpi by IMAC. The Coomassie brilliant blue-staining on SDS-PAGE revealed three or four visible protein bands other than the NL1 protein and with only 76% purity as determined through quantitative densitometry (data not show). According our knowledge, this is the first

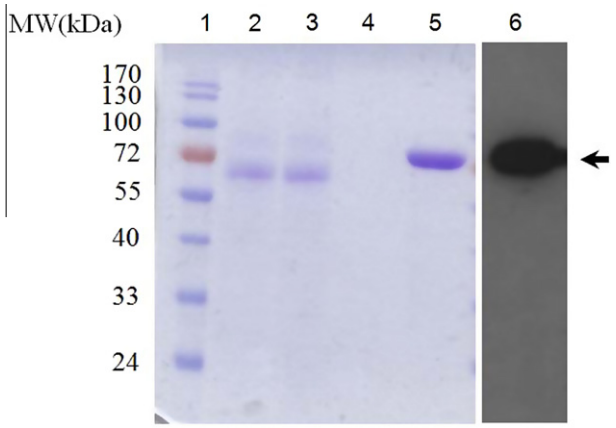

Fig. 3. Purification of secreted recombinant NL1 protein expressed in the supernatant of vAcAzSNL1-Rhir-E-infected High Five cells. Hi5 (1×10^7 cells seeded in a 75 cm^2 tissue culture flasks) were infected with vAcAzSNL1-Rhir-E-b at an MOI of 5 and harvested at 72 hpi. Proteins were separated using SDS-PAGE and Coomassie brilliant blue staining. Lane 1: molecular weight marker; lane 2: the culture supernatants; lane 3: flow through; lane 4: 10 mM imidazole buffer wash; lane 5: eluate was obtained by passing with 300 mM imidazole buffer and dialyzed and concentrated by 10 kDa molecular sieve; lane 6: eluted samples were diluted 40-fold and subjected to Western blot analysis with the specific antibody against NL1. Lane 2–5: 10 μL for each lane.

Table 1
Purification of secreted rat NL1 protein expressed in BEVS[a,b].

Sample	Total protein (mg)	Purity[d] (%)	NL1 (mg)
Collected culture medium[c]	2.0 ± 0.18	N.D.	N.D.
IMAC purification sample	0.133 ± 0.013	96 ± 2	0.129 ± 0.013

[a] Data are expressed as mean ± SD values of three experiments.
[b] 8×10^7 Hi5 cells were used for the purification of NL1.
[c] 80 mL of culture medium was used for the purification of NL1.
[d] Purity was determined and analyzed by quantitative densitometry of proteins stained with Coomassie brilliant blue.

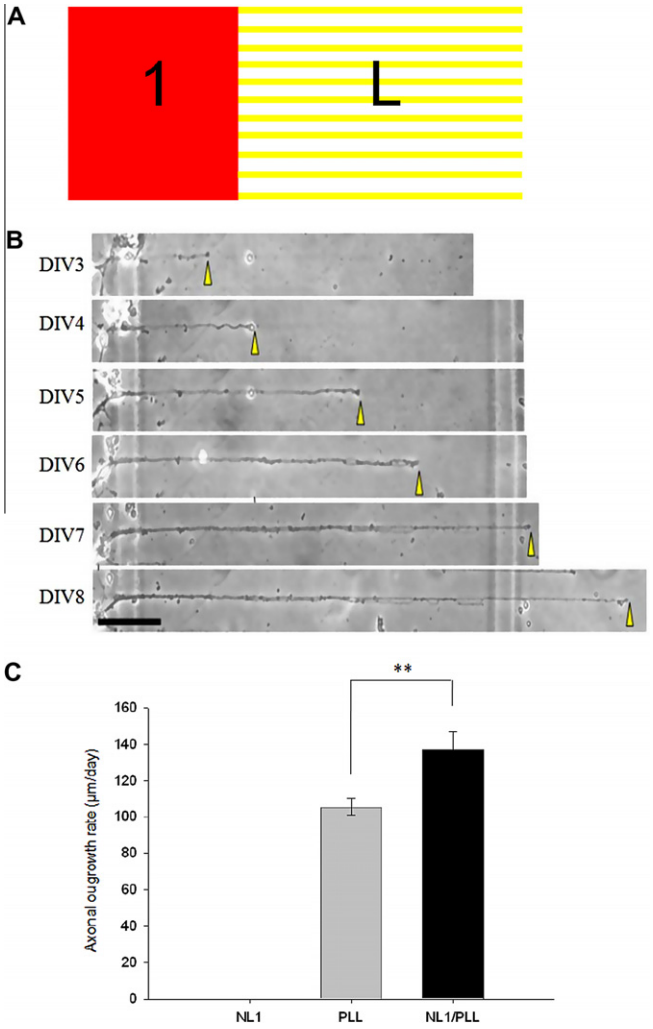

Fig. 4. Recombinant NL1 proteins produced in Hi5 cells increased the daily growth rate of the axons of cultured rat hippocampal neurons. (A) Schematic representation of neurons growing on glass surface containing a rectangularly patterned PLL-coated (region 1 indicated as red) and NL1/PLL-coated fine lines (region L indicated as yellow). The cell bodies are confined to grow along the PLL-coated area while some axons grow along the NL1/PLL-coated fine lines. (B) Photographs of an axon growing along NL1/PLL-coated fine line taken at different days *in vitro* (DIV). Arrowheads indicate the positions of the growth cone of the axon at different time points. Scale bar: 100 μm. (C) Growth rate (μm/day) histograms of axons growing along fine lines coated with NL1/PLL (black bars, $n = 247$) and with PLL (gray bars, $n = 359$). $^{**}P$ value < 0.001 by Student *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

report on the use of EGFP and bi-BEVS in the straightforward expression of target protein(s) and offsets the laborious and tedious multiple chromatography purification steps. This may reflect that the monitoring of EGFP is useful in tracing the lysis of the infected cells and avoiding the interference of other cytosolic proteins during purification. However, for the polyhedrin promoter is a very late promoter and the expression of EGFP began at about 1 dpi, this result suggests that a copy of *egfp* gene present in the host genome or controlled by an earlier promoter may make the EGFP as a more reliable indicator of cell lysis during recombinant viruses infection.

Effect of the baculovirus-expressed recombinant NL1 protein on axogenesis

Neuroligins are cell-adhesion molecules that bind to a specific subset of neurexins and play a role in neuronal differentiation and neurite extension [9,29,30]. The function of the purified recombinant NL1 protein from the supernatant of vAcAzSNL1-Rhir-E-b-infected High Five cells on the axons of rat hippocampal neurons was ascertained on its effect on axogenesis. The neurons were cultured on one area of the glass coverslip (as described in materials and methods) with parallel fine lines labeled accordingly. The location of the neuron's axon on either NL1/poly-L-lysine (PLL) or PLL-coated fine lines (Fig. 4A, letter L) on the surface of the chips were traced so as to investigate the effects of extrinsic molecules specifically on the axons instead of the neuronal somatodendrites [20]. It was observed that axons did not grow along NL1-coated fine lines (data not shown). On the other hand, it was seen that axons were capable of extending along PLL or NL1/PLL-coated fine lines as seen after day 3 *in vitro* (DIV3) until day 8 *in vitro* DIV8 (Fig. 4B). The rates of axonal outgrowth (μm per day) were obtained after DIV3 up to DIV8 by calculating the increase in the axonal length (Fig. 4B) as described in Materials and Methods. It was found that the neurons grown on NL1/PLL (137.07 ± 9.74 μm/day) coated lines gave a faster rate of axonal elongation than those grown on PLL (105.53 ± 4.53 μm/day) by up to 1.29-fold (Fig. 4C). This result indicates that the baculovirus-expressed NL1 protein in combination with PLL augments the rate of neuron axonal outgrowth.

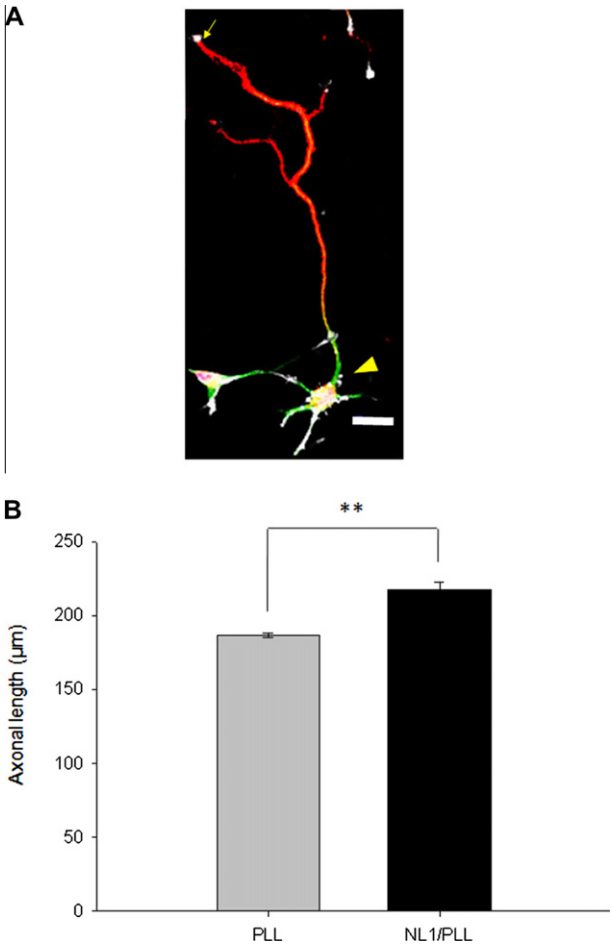

Fig. 5. Exposure of neurons to NL1 resulted in an increased axonal length. (A) Rat hippocampal neurons growing on a NL1-coated glass coverslip at DIV 3 were observed under double fluorescence microscopy after immunostaining with antibodies to SMI312 (an axon marker, red) and MAP2 (a dendrite marker, green) and labeled with Alexa Fluor 546 phalloidin (an F-actin marker, white) and DAPI (a nucleus marker, pink). The length of the longest axon was measured between the grow cone at the end of the axon (indicated by arrow) and where axon emerges from the cell body (arrowhead). Scale bar, 20 μm. (B) The lengths of the axons of rat hippocampal neurons grown on glass coverslips coated with NL1 (50 μg/mL) plus PLL (100 μg/mL) (black bar, $n = 210$) and on glass coverslips coated with PLL (150 μg/mL) (gray bar, $n = 159$) at DIV3. $^{**}P$ value < 0.005 by Student t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fluorescence immunocytochemical analysis was performed on rat hippocampal neurons seeded for three days onto PLL- or NL1/PLL-coated glass chips to confirm the effect of the recombinant NL1 protein on the promotion of axonal growth. The axon's length was measured from the growth cone (as indicated by an arrow in Fig. 5A, stained with phalloidin and anti-SMI312 antibody) down to proximal end (as indicated by the arrowhead in Fig. 5A, stained with anti-SMI312 and anti-MAP2 antibody) that was attached to DAPI-stained cell body. Fig. 5B shows axons longer by 1.6-fold in hippocampal neurons cultured on NL1/PLL (217.29 ± 5.46 μm)-coated coverslips than in those grown on PLL (186.45 ± 1.39 μm). These results indicated that the recombinant NL1 produced in BEVS was able to promote axonal outgrowth in rat hippocampal neurons and confirmed the earlier results obtained using the fabricated chip device. The advantages of using this chip device included simplicity, high efficiency in quantifying the length of neuronal axons, and ease in defining the function of extrinsic molecules specifically on neuronal axons.

Conclusion

This study reported the expression of a functional 80 kDa extracellular fragment of NL1 in the bi-cistronic baculovirus/insect cell system. Recombinant NL1 was successfully expressed and secreted as a soluble protein into the culture medium and the optimum harvest time was determined by the cell lysis indicator, EGFP. The one-step purified recombinant NL1 proteins were able to promote the rate of neuron axonal outgrowth as demonstrated using the fabricated chip. These studies will facilitate to investigate the applications of NLs either alone or coupled with other cell-adhesion molecules in promoting axonal regeneration and inducing presynaptic formation.

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