

NCAM-140 Translocation into Lipid Rafts Mediates the Neuroprotective Effects of GDNF

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Abstract Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor for substantia nigra dopaminergic (DA) neuronal cells. Recent studies have demonstrated that neural cell adhesion molecule functions as a signal transduction receptor for GDNF. The purpose of this study is to reveal whether neural cell adhesion molecule (NCAM) mediates the protective effects of GDNF on DA neuronal cells and further explore the mechanisms involved. We utilized SH-SY5Y cell line to establish a model of 6-hydroxydopamine (6-OHDA)-injured DA neuronal cells. Lentiviral vectors were constructed to knockdown or overexpress NCAM-140, and a density gradient centrifugation method was employed to separate membrane lipid rafts. 3-(4,5-Dimethylthiazol-

2-yl)-2,5-diphenyl tetrazolium bromide (MTT), flow cytometric analysis, and western blotting were used to evaluate the protective effects of GDNF. The results showed that GDNF could protect 6-OHDA-injured SH-SY5Y cells via improving cell viability and decreasing the cell death rate and cleaved caspase-3 expression. NCAM-140 knockdown decreased cell viability and increased the cell death rate and cleaved caspase-3 expression, while its overexpression had the opposite effects. Notably, the amount of NCAM-140 located in lipid rafts increased after GDNF treatment. Pretreatment with 2-bromopalmitate, a specific inhibitor of protein palmitoylation, suppressed NCAM-140 translocation to lipid rafts and reduced the NCAM-mediated protective effects of GDNF on injured DA neuronal cells. Our results suggest that GDNF have the protective effects on injured DA cells by influencing NCAM-140 translocation into lipid rafts.

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Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disease that affects millions of people worldwide. Glial cell line-derived neurotrophic factor (GDNF) exerts specific protective effects on the dopaminergic (DA) neurons that are damaged in PD pathogenesis; however, the signaling mechanisms underlying these effects remain obscure.

Many previous reports have shown that GDNF plays important biological roles, mainly via the RET-GFR α 1-dependent signaling pathway [1–3]. However, GFR α s are much more widely distributed than RET in the central nervous system,

which suggests the existence of RET-independent signaling pathways [4–6]. In 2003, Paratcha et al. reported that GDNF could promote axonal growth in hippocampal and cortical neurons through the neural cell adhesion molecule (NCAM) signaling pathway and proposed that NCAM was an alternative signaling receptor for GDNF [7].

NCAM was originally characterized as a homophilic cell adhesion molecule, but recent studies have shown that NCAM can function as a signaling receptor, as well as a cell–cell adhesion molecule [8]. NCAM is encoded by a single gene that undergoes alternative splicing to generate three major isoforms of different molecular weights, NCAM-180, NCAM-140, and NCAM-120. The extracellular domains of these three NCAM subtypes are completely conserved. NCAM-120 lacks transmembrane and cytoplasmic domains and is attached to plasma membrane with glycosylphosphatidylinositol (GPI) membrane anchor, while NCAM-180 and NCAM-140 are both transmembrane proteins and differ in the size of their cytoplasmic domain. There is increasing evidence indicating that NCAM could mediate multiple biological processes induced by GDNF including cell viability, differentiation, migration, and neurite outgrowth [9–11]. However, few investigations have been carried out to examine the potential roles of NCAM in mediating the protective effect of GDNF on DA neurons.

Lipid rafts are highly dynamic plasma membrane microdomains enriched in cholesterol and sphingolipids [12]. A growing body of evidence suggests that lipid rafts play an important role in compartmentalizing the components involved in NCAM signal transduction pathways. The various signaling molecules are differently distributed inside or outside lipid rafts, and NCAM in and out of rafts will activate different signaling pathways and induce diverse biological functions [13]. However, whether the raft distribution of NCAM is involved in the GDNF-NCAM-mediated protective effects on DA neurons is not clear.

The present study aimed to explore whether and how NCAM could mediate the protective effects of GDNF on DA neural cells. We utilized the human SH-SY5Y neuroblastoma cell line to establish 6-hydroxydopamine (6-OHDA)-injured DA neuronal cells. A short hairpin RNA (shRNA) lentiviral vector targeting NCAM-140 and a lentiviral vector expressing NCAM were used to knockdown or overexpress NCAM, respectively, and then tested the influences of NCAM knockdown or overexpression on the protective effects of GDNF. Finally, we employed a density gradient centrifugation to separate lipid rafts and explored whether lipid rafts were involved in the neuroprotective effects of GDNF-NCAM signaling pathway.

We found that GDNF could protect 6-OHDA-injured SH-SY5Y cells via improving cell viability, decreasing the apoptotic rate, and inhibiting cleaved caspase-3 expression. NCAM-140 knockdown weakened the protective effects of

GDNF, while NCAM overexpression was accompanied by enhanced protective effects of GDNF on 6-OHDA-injured SH-SY5Y cells. In addition, our results showed that GDNF could induce NCAM translocation into lipid rafts, and inhibition of NCAM-140 translocation induced by 2-bromopalmitate (2-BP) could depress NCAM-mediated protective effect of GDNF. Collectively, these findings suggest that NCAM-140 could mediate the protective effects of GDNF on injured DA cells via its translocation into lipid rafts.

Materials and Methods

Cell Culture

The dopaminergic neuroblastoma SH-SY5Y cell line was obtained from the Shanghai Institutes for Biological Sciences (SIBS) of Chinese Academy of Sciences (CAS). The cells were plated at a density of 5×10^5 cells on 25-cm² dishes (Corning, Corning, NY, USA). The cells were cultured in Dulbecco's modified Eagle media, nutrient mixture F-12 (DMEM/F12; Sigma, St. Louis, MO, USA) supplemented with 15 % fetal bovine serum (Cambrex, East Rutherford, NJ, USA) in a water-jacketed incubator at 37 °C in a humidified incubator with 5 % CO₂. The differentiation of SH-SY5Y cells was induced using 10 μM all-trans-retinoic acid (ATRA) for 6 days before cells were used for experiments.

6-OHDA and GDNF Treatment

The neurotoxin 6-OHDA·HBr (Sigma, St. Louis, MO) was made fresh for each experiment in a non-oxidizing vehicle. Cultures were exposed to 40 μM 6-OHDA for 24 h (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability assay) or 10 h (western blot and flow cytometric), followed by two washes of serum-free media, and then were prepared for further testing. GDNF (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS). GDNF (50 ng/ml) was co-incubated with 6-OHDA in every experiments.

MTT Cell Viability Assay

Vybrant[®] MTT Cell Viability Assay Kit was used to measure cell viability. SH-SY5Y cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100-μl medium. The cultures were grown for 48 h, and then, 10 μM ATRA was added to differentiate cells for 6 days before cells were used for experiments (changed the medium every 3 days). After co-incubation with 6-OHDA (40 μM) and GDNF (50 ng/ml) for 24 h, the medium was removed and replaced with 100 μl of fresh culture medium. Of the 12 mM MTT reagent

(0.5 mg/ml MTT in PBS containing 10 mM HEPES), 10 μ l was added to each well and incubated in the CO₂ incubator for 4 h. Of the sodium dodecyl sulfate (SDS)-HCl solution (add 10 ml of 0.01 M HCl to 1 g of SDS), 100 μ l was added to each well and mixed thoroughly using the pipette. The microplate was incubated at 37 °C for 4 h in a humidified chamber. Each sample was mixed again using a pipette, and absorbance of the solution was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad, USA). Wells were added with 100 μ l of medium, and 10 μ l of the MTT reagent alone was used as a negative control.

Flow Cytometric Analysis of Cell Death Rate

The SH-SY5Y cells were plated at a density of 5×10^5 cells on 25-cm² dishes. The cultures were grown for 48 h, and then, 10 μ M ATRA was added to differentiate cells for 6 days before cells were used for experiments. After incubation with 6-OHDA (40 μ M) and GDNF (50 ng/ml) for 10 h, death cells were quantified by cytofluorometric analysis using a FACSCalibur (Becton–Dickinson, Mountain View, CA). In order to quantify cell death rate induced by 6-OHDA, using Annexin V-APC Apoptosis Detection Kit (BD Pharmingen, USA) according to the manufacturer's instructions, briefly, cells were incubated with 5- μ l APC-conjugated Annexin-V and 10 μ l PI staining solution for 15 min in the dark at room temperature and further screened by flow cytometry. Under these conditions, Annexin V+/PI+ cells correspond to either secondary necrotic or late apoptotic cell and Annexin V+/PI- cells correspond to early apoptotic cell. The cell death rate was estimated by the relative amount of Annexin V+/PI- and Annexin V+/PI+ cell populations.

Real-Time Polymerase Chain Reaction

Total RNA was extracted from cells with TRIzol Reagent (Invitrogen, USA). Then, 2 μ g was used to synthesize complementary DNA (cDNA) with the Superscript First-Strand Synthesis Kit (Promega, USA) following the manufacturer's protocols. The real-time polymerase chain reaction (PCR) reaction kit contained 0.2- μ M sense primer, 0.2- μ M anti-sense primer, 12.5- μ l SYBR Green I (Toyobo, Osaka, Japan), and 5 μ l of previously synthesized cDNA in a total volume of 25 μ l. A pair of GAPDH primers in identical reactions was used to control the starting template. The sense and anti-sense primers were 5'-TTGGCTACAGCAACAGGGTG-3' and 5'-TCTACATGGCAACTGTGAGGAG-3', respectively. The target genes detected were human NCAM (forward 5'-CCGATTCATAGTCCTGTCCAA-3 and reverse 5'-ATAAGTGCCTCATCTGTTTTCT-3'.

Western Blotting

The cells were lysed in a lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Triton X-100, 2 mM EDTA, 1 % deoxycholic acid (DOC), 0.1 % SDS, 1 mM NaVO₃, 10 mM NaF, and 1 mM DTT) and centrifuged to yield whole-cell lysates. Bradford protein concentration assay was used to measure total protein concentration. Aliquots of the lysates (20 μ g of protein) were separated on a 5–10 % SDS-polyacrylamide gel with a SDS-running buffer (25 mM Tris (pH 8.3), 250 mM glycolol, and 0.1 % SDS) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA) with a transfer buffer (25 mM Tris-HCl (pH 8.3), 192 mM glycolol, and 20 % methyl alcohol). After blocking the non-specific site with 5 % non-fat dry milk, the membrane was then incubated with specific primary antibody (in 3 % BSA at 4 °C for overnight). The membrane was washed with 1 \times PBS prior to incubating with IR Dye[®] 800 anti-rabbit or anti-mouse secondary antibodies (Li-Cor, Lincoln, NE), at room temperature. Immunoactive proteins were detected using Odyssey[®] Imaging Systems (Li-Cor, Lincoln, NE). Anti-NCAM antibody, anti-flotillin-1 antibody, and anti-CD71 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-cleaved caspase 3 antibody was obtained from Millipore Corporation (Bedford, MA, USA). Band intensities were quantified using Image J software.

Isolation of Detergent-Resistant and Detergent-Sensitive Fractions

For isolation of detergent-resistant (DR) and detergent-sensitive (DS) fractions, the cell sample was first incubated for 30 min on ice in TNET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and protease inhibitor cocktail) containing 1 % Triton X-100 [14]. Following centrifugation at 100,000g at 4 °C for 1 h, the DR (pellet) and DS (supernatant) fractions were collected and analyzed for western blots.

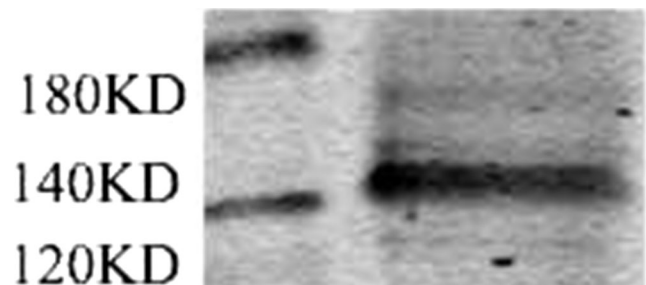


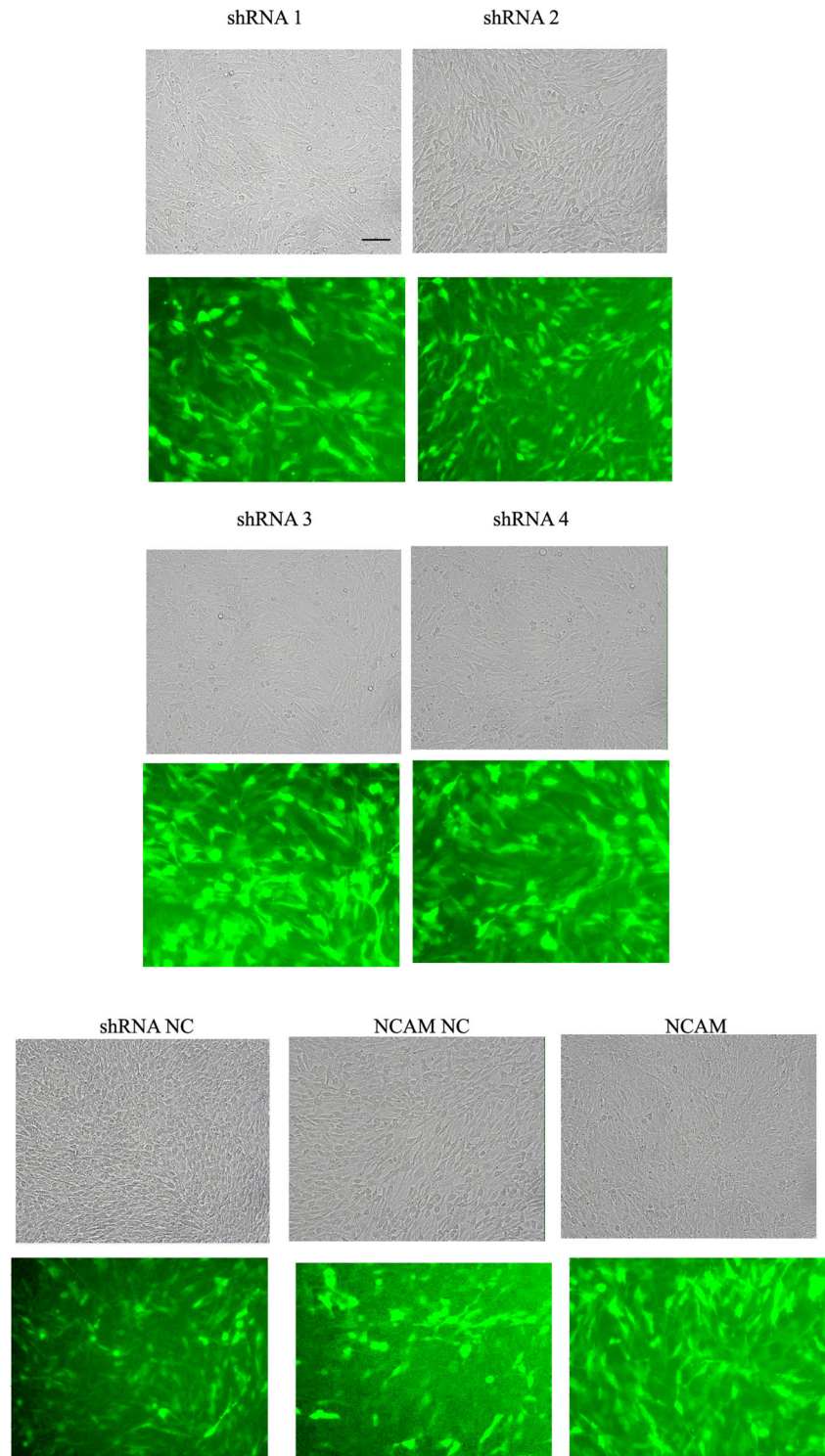
Fig. 1 NCAM isoform expressed in differentiated SH-SY5Y cells. Lysates were analyzed by western blot with the anti-NCAM rabbit antibody (H-300) against amino acids 1–300 at the N-terminus which recognizes all three NCAM isoforms

Isolation of Lipid Raft Fractions by Non-Detergent Density Gradient Ultracentrifugation Using OptiPrep™

Isolation of lipid raft fractions by non-detergent density gradient ultracentrifugation using OptiPrep™ was prepared as previously described [15]. Briefly, cells were washed and scraped into 2-ml

base buffer (20 mM Tris-HCl, pH 7.8, 250 mM, 1 mM CaCl₂, 1 mM MgCl₂, and protease inhibitor cocktail). After homogenizing the cells by 20 passages through a 22-g syringe needle, centrifuge the homogenate at 1000g for 10 min. The supernatant was adjusted to 50 % OptiPrep™ in base buffer (2 ml) and laid at the bottom of a 12-ml centrifuge tube. After which, 4 ml each of

Fig. 2 Transfection efficiency detected by fluorescent microscopy. Differentiated SH-SY5Y cells were observed by a fluorescent microscope 72 h after lentiviral vectors transfection. The percentage of GFP-positive cells in 10 fields was counted to evaluate the transfection efficiency (*bar* = 100 μm)



20 and 0 % OptiPrep™ in base buffer were overlaid. Following centrifugation at 52,000×*g* at 4 °C for 90 min, a density gradient was formed. Of the fractions, ×12 1000 μl were collected from the top to the bottom of the gradient and analyzed for western blots for NCAM, flotillin, and CD71.

Plasmid and Transfection

According to full sequence of human NCAM1 (NM_000615.6), the NCAM expression vector LV5- green fluorescent protein (GFP)-NCAM lentivirus; control lentivirus LV5-GFP (named as NCAM and NCAM NC, respectively); and NCAM shRNA expression vector LV3-GFP-shRNA1, LV3-GFP-shRNA2, LV3-GFP-shRNA3, LV3-GFP-shRNA4 lentivirus, and control lentivirus LV3-GFP control shRNA (named as shRNA1, shRNA2, shRNA3, shRNA4, and shRNA NC, respectively) were designed and constructed by Genepharma Corporation (Shanghai, China). The target sequences of shRNAs were GCAGGAGATGCCAAAGATAAA (shRNA1), GGACTTCTACCCGGAACATCA (shRNA2), GCTGCTGCCAAGCTCCAATTA (shRNA3), GCAGTTGGTGAAGAAGTATGG (shRNA4), and TTCTCCGAACGTGTACAGT (shRNA NC), respectively. Sequencing analysis was performed to confirm the recombinant plasmid. SH-SY5Y cells were transfected with the lentiviral vectors for 72 h, using the Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's recommendations. To assess the efficiency of lentiviral transduction, SH-SY5Y cells were plated at a density of 5×10^5 cells/well into 24-well plates and infected with lent-GFP at a dose of multiplicity of infection (MOI) 10. After 72 h, GFP-expressing cells were detected by fluorescence microscopy (Olympus, Japan). The percentage of GFP-positive cells in 10 fields was counted to evaluate the transfection efficiency. Western blot was performed to detect NCAM-140 protein, and RT-PCR was performed to detect the level of NCAM messenger RNA (mRNA).

Statistical Analysis

All data are reported as mean ± SEM. Statistical significance between multiple groups was performed using one-way analysis of variance (ANOVA), and differences between groups were determined with Scheffe's *t* test. A value of $P < 0.05$ was considered significant.

Results

NCAM Expression in Human Neuroblastoma SH-SY5Y Cells

The NCAM H-300 antibody detects against amino acids 1–300 at the N-terminus of human NCAM and can detect

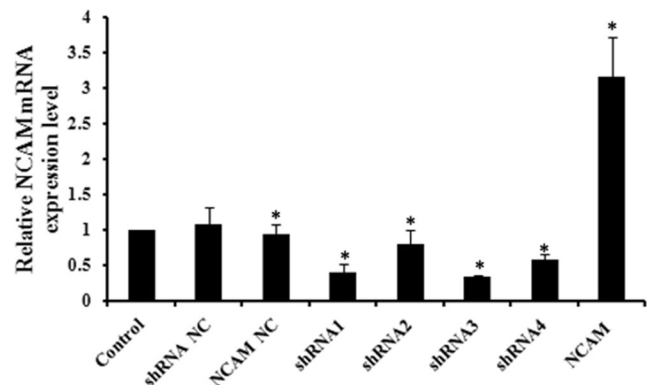


Fig. 3 The effects of lentiviral vectors (shRNA1, shRNA2, shRNA3, shRNA4, shRNA NC, NCAM NC, and NCAM) on NCAM mRNA levels in differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were transfected with lentiviral vectors for 72 h, and NCAM mRNA levels were evaluated using real-time PCR. The data represent the means ± SEM of three independent experiments. * $P < 0.05$ vs. control

all three NCAM isoforms. Western blotting revealed that the expression of NCAM-140 was much higher than that of NCAM-180 and NCAM-120 in SH-SY5Y cells (Fig. 1).

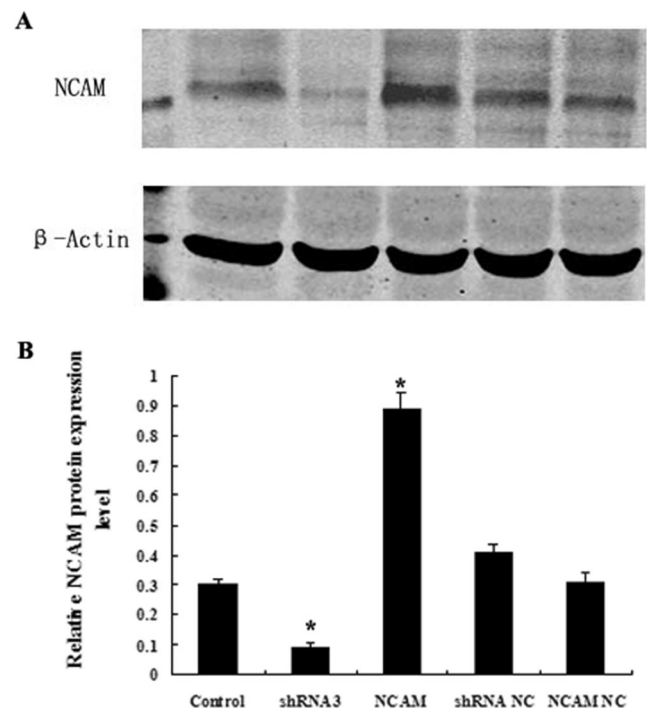


Fig. 4 The effects of lentiviral vectors (shRNA3, NCAM, shRNA NC, and NCAM NC) on NCAM protein expression in differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were infected with lentiviral vectors 72 h, and NCAM protein levels were evaluated by western blot. The protein levels were quantified by densitometry and normalized to the amount of β-actin. The bar chart depicts the densitometric results. The data represent the means ± SEM of three independent experiments. * $P < 0.05$ vs. control

Identification of shRNA Lentiviral Vectors and Lentiviral Expression Vectors of NCAM-140

The sequencing results confirmed that the oligo DNA sequence of shRNA was correctly inserted into the former vector (Supplement 1), while the sequences of the NCAM gene carried in NCAM lentiviral vector were consistent with the NCAM gene sequences in the Genbank database (Supplement 2). Lentiviral vectors were used to transfect SH-SY5Y cells for 3 days. Then, the transfection efficiency of shRNA targeting NCAM and lentiviral vector overexpressing NCAM-140 were assessed by examining the expression of the GFP gene tag. More than 70 % cells were infected with lent-GFP at a dose of MOI 10 after 72-h treatment (Fig. 2).

After transfection, the mRNA and protein expression of NCAM-140 were examined by real-time PCR and western blot analysis, respectively, to assess the knockdown and overexpression effect of lentiviral vectors. Results showed that NCAM-140 mRNA levels were decreased to 41, 81, 34, and 58 % with shRNAs 1, 2, 3, and 4, respectively, and shRNA3 had the most prominent inhibitory effect on NCAM-140 mRNA expression (Fig. 3). Therefore, shRNA3 was used in the subsequent experiments and suppressed the expression of NCAM. Conversely, the lentiviral vector overexpressing NCAM-140 significantly increased NCAM-140 mRNA and protein levels (Figs. 3 and 4).

Effects of NCAM-140 Knockdown or Overexpression on Protective Effects of GDNF

To determine whether NCAM-140 mediated the protective effects of GDNF on 6-OHDA-injured SH-SY5Y cells, we performed MTT assays to assess cell viability, flow cytometry to examine cell death rate, and western blotting to measure cleaved caspase-3 expression.

The cells were divided into the following nine groups: control group, the cells were cultured under normal condition; 6-OHDA group, treated with 40 μ M 6-OHDA for 24 h (MTT assays) or 10 h (flow cytometry and western blotting); NCAM + 6-OHDA group, after transfection with lentiviral vector overexpressing NCAM-140, treated with 40 μ M 6-OHDA for 24 or 10 h; shRNA + 6-OHDA group, after transfection with shRNA 3, treated with 40 μ M 6-OHDA for 24 or 10 h; 6-OHDA + GDNF group, treated with 40 μ M 6-OHDA and 50 ng/ml GDNF for 24 or 10 h; shRNA + 6-OHDA + GDNF group, after transfection with shRNA 3, treated with 40 μ M 6-OHDA and 50 ng/ml GDNF for 24 or 10 h; NCAM + 6-OHDA + GDNF group, after transfection with lentiviral vector overexpressing NCAM-140, treated with 40 μ M 6-OHDA and 50 ng/ml GDNF for 24 or 10 h; shRNA NC + 6-OHDA + GDNF group, after transfection with shRNA NC, treated with 40 μ M 6-OHDA and 50 ng/ml GDNF for 24 or 10 h; and NCAM NC + 6-OHDA + GDNF group, after transfection with NCAM NC, treated with 40 μ M 6-OHDA and 50 ng/ml GDNF for 24 or 10 h.

MTT assays results showed that the cell viability in the 6-OHDA group was less than that in the group without 6-OHDA treatment (Fig. 5). Similarly, the cell death rate, and expression of cleaved caspase-3 with 6-OHDA treatment were increased in 6-OHDA group (Figs. 6 and 7). These results indicated that the 6-OHDA treatment significantly affected cell viability, cell death rate and expression of cleaved caspase-3. In 6-OHDA + GDNF group, cell viability was more while the cell death rate and expression of cleaved caspase-3 were less than that in 6-OHDA group. These results showed that GDNF could protect 6-OHDA-injured SH-SY5Y cells via improving cell viability and decreasing the cell death rate and cleaved caspase-3 expression.

Based on this experimental model describing the protective effects of GDNF on 6-OHDA-injured SH-SY5Y cells,

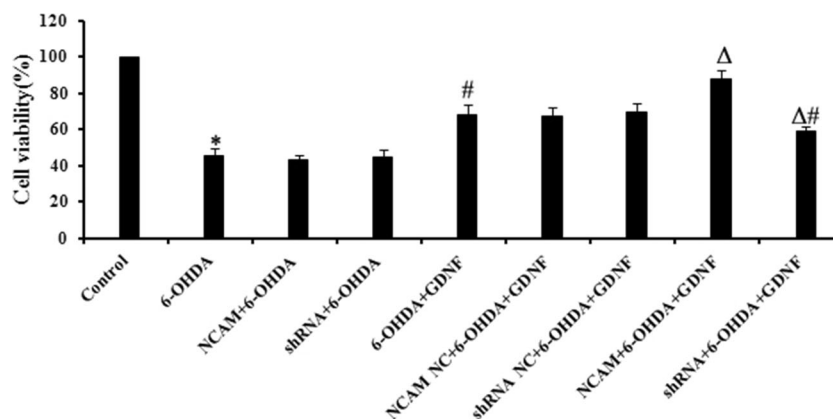
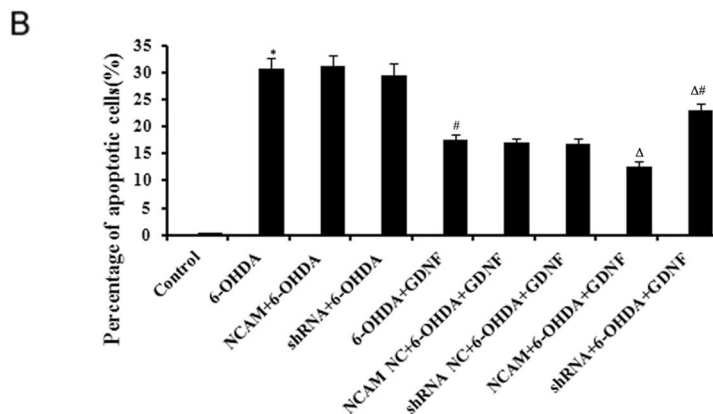
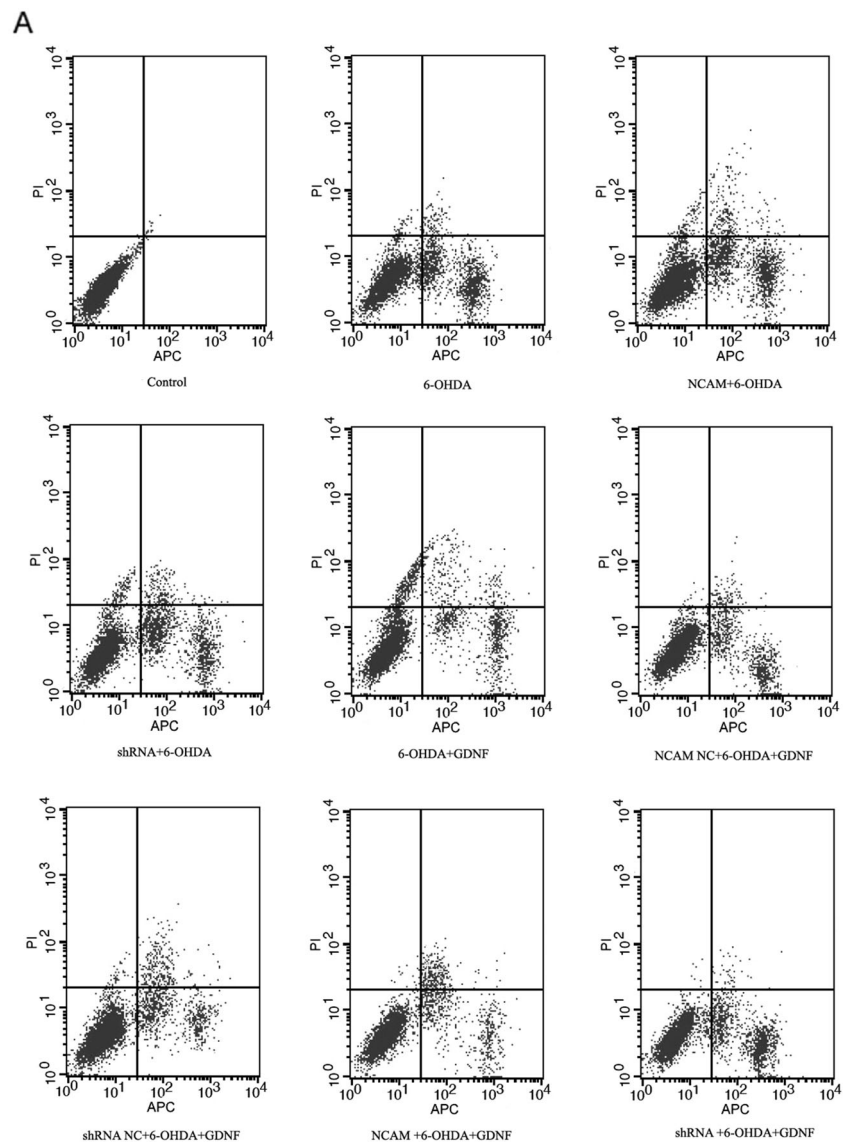


Fig. 5 The effect of NCAM overexpression or inhibition on cell viability. Differentiated SH-SY5Y cells were divided into the following nine groups: control, 6-OHDA, NCAM + 6-OHDA, shRNA + 6-OHDA, 6-OHDA + GDNF, shRNA + 6-OHDA + GDNF, NCAM + 6-OHDA + GDNF, shRNA NC + 6-OHDA + GDNF, and NCAM NC + 6-OHDA +

GDNF and treated as described in the text. SH-SY5Y cell viability was detected with MTT assays. The data represent the means \pm SEM of three independent experiments. * P < 0.05 vs. control, # P < 0.05 vs. 6-OHDA, Δ P < 0.05 vs. 6-OHDA + GDNF

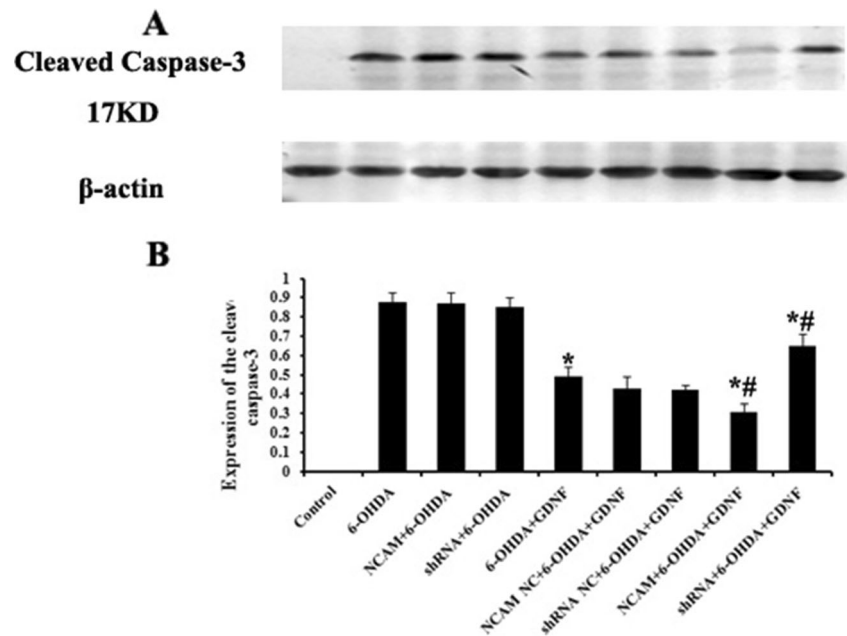
Fig. 6 The effect of NCAM overexpression or inhibition on cell death rate. Differentiated SH-SY5Y cells were divided and treated as mentioned in the text, then viable cell (annexin V⁻/PI⁻), early apoptotic cell (annexin V⁺/PI⁻), secondary necrotic or late apoptotic cell (annexin V⁺/PI⁺), and the residual damaged cell (annexin V⁻/PI⁺) were quantified by flow cytometric analysis. The cell death rate was estimated by the relative amount of annexin V⁺/PI⁻ and annexin V⁺/PI⁺ cell populations. The data represent the means ± SEM of three independent experiments. **P* < 0.05 vs. control, #*P* < 0.05 vs. 6-OHDA, Δ*P* < 0.05 vs. 6-OHDA + GDNF



shRNA 3 targeting NCAM-140 or lentiviral vector overexpressing NCAM-140 were transfected into cells to knock-down or overexpress NCAM-140, respectively. When shRNA was transfected into cells, the effects of GDNF to

stimulate cell viability and to inhibit cell death rate and cleaved caspase-3 expression were antagonized. And, the overexpression of NCAM-140 enhanced the influences of GDNF on cell viability, cell death rate, and cleaved

Fig. 7 The effects of NCAM overexpression or inhibition on cleaved caspase-3 expression. Differentiated SH-SY5Y cells were divided into nine groups, SH-SY5Y cells were divided and treated as mentioned in the text, and then, cleaved caspase-3 and β -actin levels were evaluated by western blotting. The protein levels were quantified by densitometry and normalized to the amount of β -actin. The bar chart depicts the densitometric results. The data represent the means \pm SEM of three independent experiments. * $P < 0.05$ vs. 6-OHDA, # $P < 0.05$ vs. 6-OHDA + GDNF



caspase-3 expression. These results demonstrated that NCAM-140 could mediate, at least partially, the protective effect of GDNF.

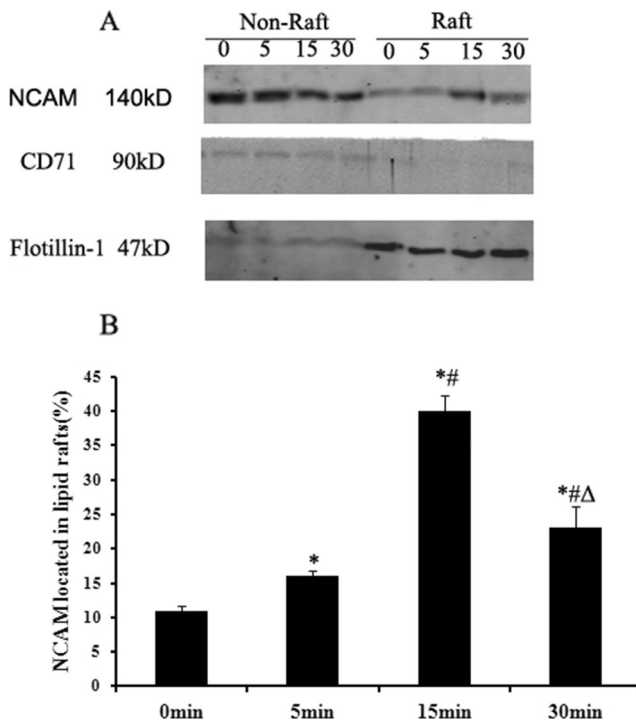


Fig. 8 GDNF induced NCAM translocation into lipid rafts isolated by non-ionic detergents. Differentiated SH-SY5Y cells were stimulated with medium alone or GDNF (50 ng/ml) for 5, 15, or 30 min, and then, lipid rafts were extracted with Triton X-100. NCAM, flotillin-1 (marker of lipid raft), and CD71 (marker of non-raft fractions) in lipid rafts and non-raft membrane fractions were detected by western blotting. This experiment was performed three times with similar results. * $P < 0.05$ vs. 0 min, # $P < 0.05$ vs. 5 min, $\Delta P < 0.05$ vs. 15 min

GDNF-Induced NCAM-140 Translocation into Lipid Rafts

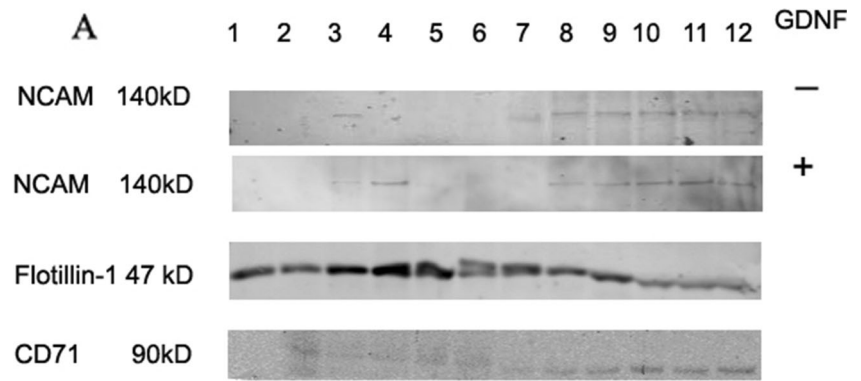
Insolubility in cold non-ionic detergents and flotation on OptiPrep™ density gradients are well-established techniques for separating lipid rafts, and we employed these two methods to isolate lipid rafts. Our initial aim was to determine whether GDNF treatment could result in the NCAM-140 translocation to or from the lipid rafts. Using non-ionic detergents, we found that only a small number of NCAM-140 was identified in lipid raft microdomains without GDNF. After GDNF treatment, NCAM-140 located in lipid rafts gradually increased and reached a maximum at 15 min (Fig. 8).

To further confirm NCAM-140 translocation into lipid rafts after GDNF treatment, OptiPrep™ density gradient centrifugation was used to separate lipid rafts, and similar results were obtained (Fig. 9). As mentioned above, we observed that a small fraction of NCAM-140 localized to lipid raft microdomains in the absence of GDNF, confirming an association of NCAM-140 with rafts under basal conditions, and that GDNF treatment could further induce NCAM-140 translocation into lipid rafts. These results suggested that lipid rafts might be involved in GDNF-NCAM-140 signaling events.

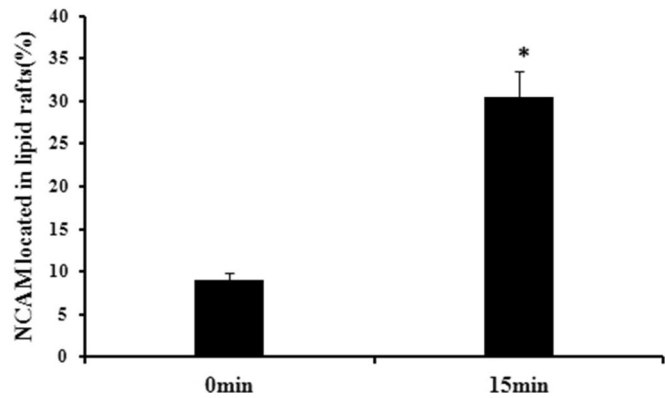
Inhibition of NCAM-140 Translocation into Lipid Rafts Suppressed GDNF-NCAM-140-Mediated Protective Effects

To further investigate whether lipid rafts were involved in the protective effects of GDNF-NCAM-140 on injured DA neuronal cells, 2-BP pretreatment was administered to block NCAM-140 translocation into lipid rafts. NCAM

Fig. 9 GDNF induced NCAM translocation into lipid rafts isolated by OptiPrep™ density gradient centrifugation. Differentiated SH-SY5Y cells were treated with medium alone or with GDNF (50 ng/ml) for 15 min. The cells were then lysed and subjected to OptiPrep™ density centrifugation as described in the methods. Twelve equal fractions were taken from the top of the tubes and labeled from 1 to 12, with fraction 12 being the densest. These fractions were analyzed by western blotting for NCAM, flotillin-1, and CD71. This experiment was performed three times with similar results. * $P < 0.05$ vs. 0 min



B



palmitoylation is required for NCAM displacement to lipid rafts [13, 16]. 2-BP inhibits the activity of protein

acyltransferase and protein palmitoylation modification, thus reducing NCAM in lipid rafts [17]. Our results also showed that the amount of NCAM located in lipid raft microdomains was significantly reduced after 2-BP pretreatment, confirming that 2-BP could inhibit GDNF-induced NCAM translocation into lipid rafts (Fig. 10). Then, we assessed cell viability, cell

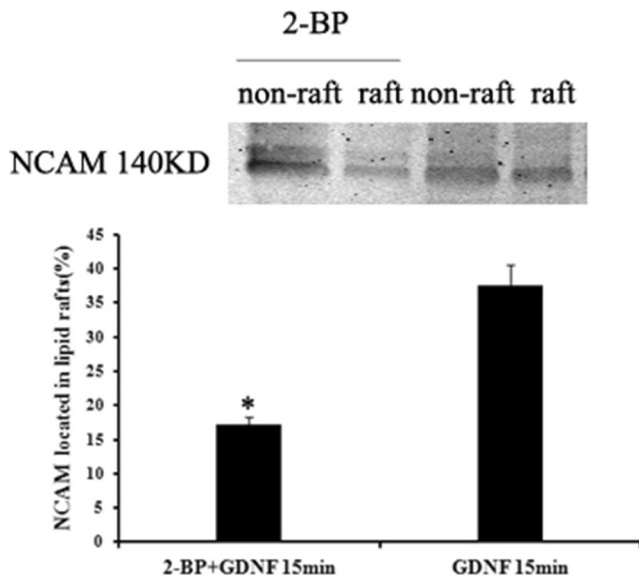


Fig. 10 2-BP inhibited NCAM translocation into lipid rafts induced by GDNF. Differentiated SH-SY5Y cells were pretreated with or without 20 μ M 2-BP for 15 min followed by treatment with GDNF (50 ng/ml) for 15 min. Lipid rafts were isolated by non-ionic detergents, and then, NCAM in lipid rafts and non-raft membrane fractions were detected by western blotting. This experiment was performed three times with similar results. * $P < 0.05$ vs. GDNF 15 min

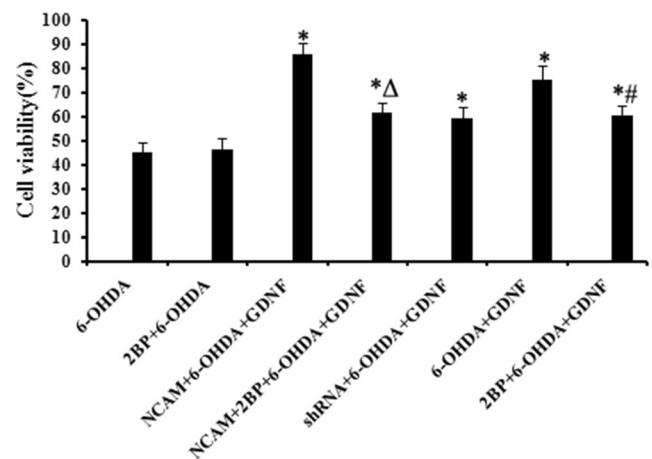


Fig. 11 The effect of 2-BP pretreatment on cell viability. Differentiated SH-SY5Y cells were divided into seven groups as mentioned in text, and cell viability was detected with MTT assays. The bar chart depicts SH-SY5Y cell viability as the means \pm SEM of three independent experiments. * $P < 0.05$ vs. 6-OHDA, # $P < 0.05$ vs. 6-OHDA + GDNF, $\Delta P < 0.05$ vs. NCAM + 6-OHDA + GDNF

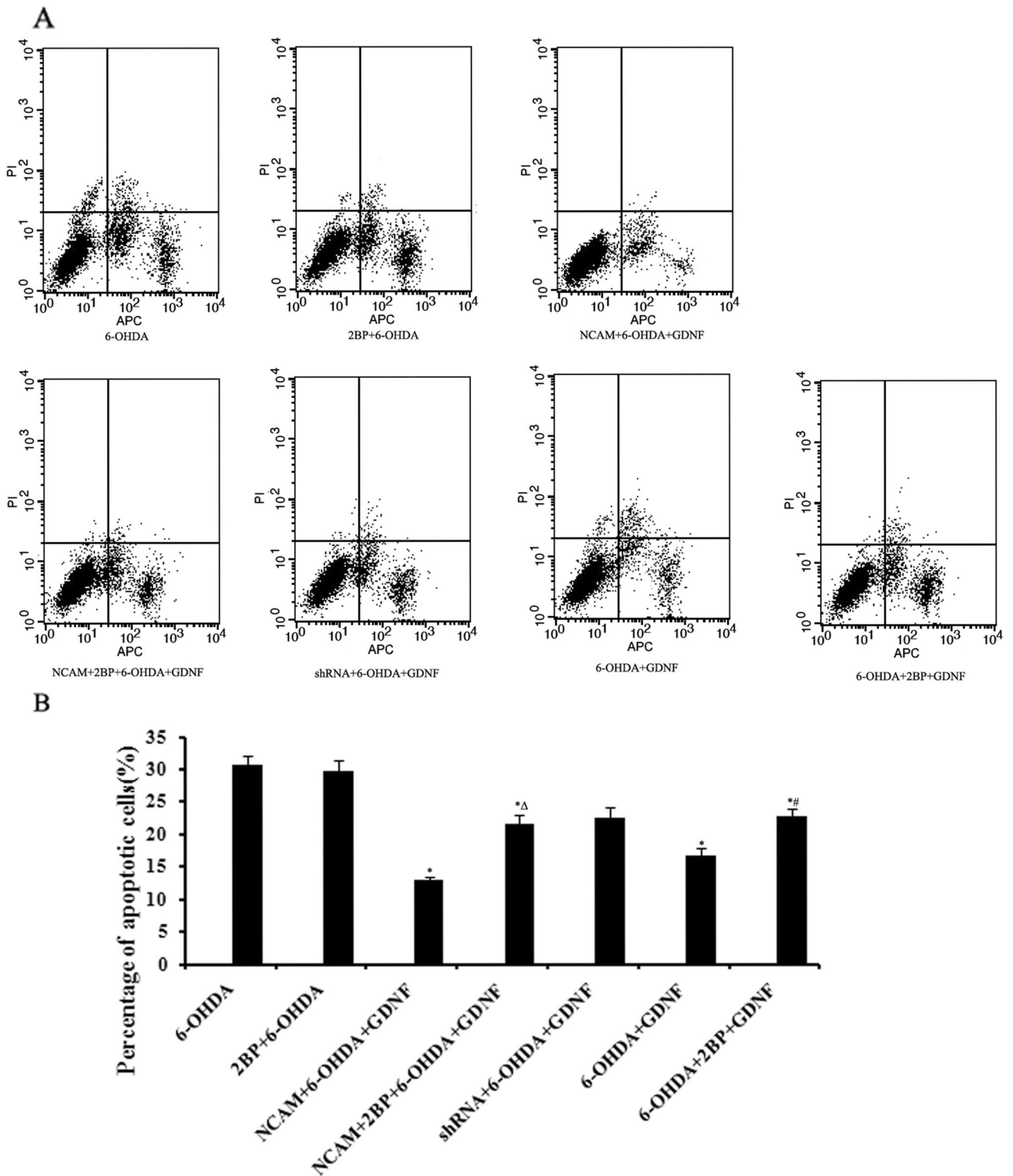


Fig. 12 The effect of 2-BP pretreatment on cell death rate. Differentiated SH-SY5Y cells were divided and treated as mentioned in the text; then, viable cell (annexin V⁻/PI⁻), early apoptotic cell (annexin V⁺/PI⁻), secondary necrotic or late apoptotic cell (annexin V⁺/PI⁺), and the residual damaged cell (annexin V⁻/PI⁺) were quantified by flow

cytometric analysis. The cell death rate was estimated by the relative amount of annexin V⁺/PI⁻ and annexin V⁺/PI⁺ cell populations. The data represent the means \pm SEM of three independent experiments. * P < 0.05 vs. 6-OHDA, # P < 0.05 vs. 6-OHDA + GDNF, ΔP < 0.05 vs. NCAM + 6-OHDA + GDNF

apoptosis rates, and the expression of cleaved caspase-3 to explore the influences of NCAM translocation into lipid rafts on the protective effects of GDNF-NCAM-140 pathway.

The cells were divided into the following seven groups: 6-OHDA group, treated with 40 μ M 6-OHDA for 24 (MTT assays) or 10 h (flow cytometry and western blotting); 2BP + 6-OHDA group, pretreated with 20 μ M 2-BP for 15 min followed by 6-OHDA and GDNF treatment for 24 or 10 h; NCAM + 6-OHDA + GDNF group, after transfection with lentiviral vector overexpressing NCAM-140, 6-OHDA, and GDNF treatment for 24 or 10 h; NCAM + 2-BP + 6-OHDA + GDNF group, after transfection with lentiviral vector overexpressing NCAM-140, pretreated with 20 μ M 2-BP for 15 min followed by 6-OHDA and GDNF treatment for 24 or 10 h; shRNA + 6-OHDA + GDNF group, after transfection with shRNA 3, treated with 6-OHDA and GDNF for 24 or 10 h; 6-OHDA + GDNF group, treated with 6-OHDA and GDNF for 24 or 10 h; and 2-BP + 6-OHDA + GDNF group, pretreated with 20 μ M 2-BP for 15 min followed by treatment with 6-OHDA and GDNF for 24 or 10 h.

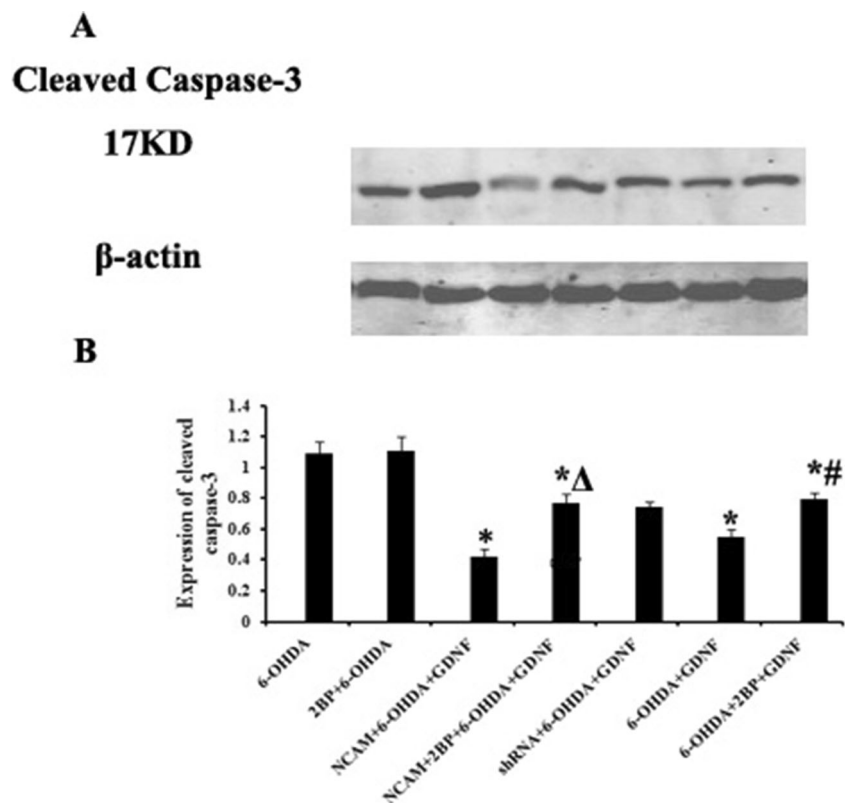
In 2-BP + 6-OHDA + GDNF group, pretreatment with 2-BP counteracted GDNF-induced effects on cell viability, cell death rate, and expression of cleaved caspase-3 when compared with 6-OHDA + GDNF group. In contrast to NCAM + 6-OHDA + GDNF group, results in NCAM + 2-BP + 6-OHDA + GDNF group showed decreased cell viability and increased cell death rate and cleaved caspase-3

expression, and the results are similar to that in shRNA + 6-OHDA + GDNF group (Figs. 11, 12, and 13). These above results showed that 2-BP pretreatment inhibited the protective effects of GDNF-NCAM-140 on 6-OHDA-injured SH-SY5Y cells, indicating that NCAM displacement to lipid rafts played a key role in GDNF's beneficial effect.

Discussion

The present study was conducted to determine whether NCAM could mediate the protective effects of GDNF and, if so, whether lipid raft translocation of NCAM was involved. To determine whether NCAM could mediate the protective effects of GDNF on 6-OHDA-injured SH-SY5Y cells, we constructed shRNA lentiviral vectors targeting NCAM-140 and expressing NCAM-140 to inhibit and increase the protein's expression, respectively. The results showed that NCAM knockdown suppressed the protective effect of GDNF, while overexpression promoted protection of 6-OHDA-injured cells. In accordance with our findings, many other studies have shown that NCAM can mediate neuronal survival. For example, Ditlevsen et al. found that stimulation of NCAM with the synthetic NCAM mimetic peptide C3d could promote the survival of cerebellar and dopaminergic neurons induced to undergo apoptosis [18, 19]. In addition, Chao et al. reported that function blocking anti-NCAM

Fig. 13 The effect of 2-BP pretreatment on cleaved caspase-3 protein levels. Differentiated SH-SY5Y cells were divided into seven groups as mentioned in text, and cleaved caspase-3 and β -actin levels were detected by western blotting. The protein levels were quantified by densitometry and normalized to the amount of β -actin. The *bar chart* depicts the densitometric results. The data represent the means \pm SEM of three independent experiments. * $P < 0.05$ vs. 6-OHDA, # $P < 0.05$ vs. 6-OHDA + GDNF, $\Delta P < 0.05$ vs. NCAM + 6-OHDA + GDNF



antibodies antagonized the survival-promoting effect of GDNF in tyrosine hydroxylase-positive DA neurons [20].

NCAM can interact with several different transmembrane and intracellular signaling molecules to activate a complex network of signaling pathways. NCAM signaling is markedly affected by the protein's compartmentalization into lipid rafts. These highly dynamic membrane microdomains enriched in cholesterol and sphingolipids are reported to be involved in important intracellular signaling events [12, 21, 22]. Among several signal molecules linked to NCAM, the Src kinase family, heteromeric G proteins, and the neurite outgrowth-associated protein growth-associated protein-43 (GAP-43) are enriched in lipid rafts; nevertheless, the fibroblast growth factor (FGF) receptor is out of lipid rafts [23–25]. NCAM-mediated signaling events via lipid rafts are distinct from its signaling outside of lipid rafts. Within lipid rafts, NCAM-140 associates with Fyn, leading to the recruitment of focal adhesion kinase and subsequently activating the Ras–Raf extracellular signal-related kinase ERK1/2 pathway [13, 26]. Outside of lipid rafts, NCAM-140 signaling is dependent on the FGF receptor to increase intracellular Ca^{2+} and activate phospholipase C, protein kinase C, and Ca^{2+} /calmodulin-dependent protein kinase II [13, 27].

Because NCAM can activate different signaling molecule depending on its subcellular location, we first investigated the distributions of NCAM within cytoplasmic membranes. We separated lipid rafts and found that NCAM-140 was the main isoform expressed in differentiated SH-SY5Y cells and was found in both raft and non-raft fractions in unstimulated cells. This is consistent with previous researches [13, 28, 29]. In addition, our results showed a gradual increase in the amount of NCAM-140 in lipid rafts after GDNF treatment, with a peak at 15 min. This finding is in accordance with previous studies showing that dynamic regulation of NCAM-140 raft association with specific redistribution upon NCAM clustering [28, 29]. There is growing evidence that movement of cellular receptors into rafts can enhance downstream signaling pathways [30, 31]. Therefore, we asked whether NCAM raft localization was essential for the protective effect of GDNF. We employed the palmitoylation inhibitor 2-BP to inhibit NCAM translocation into lipid rafts. It has been suggested that NCAM-140 is targeted to lipid rafts by the palmitoylation of four residues in the NCAM intracellular juxtamembrane region and that 2-BP pretreatment could inhibit its translocation into lipid rafts [16, 17]. Our results showed that 2-BP pretreatment indeed attenuated GDNF-induced NCAM redistribution to lipid rafts. The protective effect of GDNF-NCAM-140 was also inhibited after 2-BP pretreatment. Collectively, these results suggest that NCAM translocation into lipid rafts is necessary for GDNF-NCAM-140-mediated protective effects on injured DA cells. Considering that a major downstream mediator of NCAM located in lipid rafts is Fyn, we hypothesize that the interplay among these proteins is

important. However, elucidation of the exact signaling pathways activated downstream of NCAM translocation into lipid rafts will require further investigation.

In conclusion, the present results suggest that NCAM could mediate the protective effect of GDNF on DA neurons. Lipid rafts are involved in this process, and subcellular compartmentalization of NCAM into lipid raft microdomains is necessary for GDNF-NCAM-140-mediated protective effects.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Maeda K, Murakami H, Yoshida R, Ichihara M, Abe A, Hirai M, Murohara T, Takahashi M (2004) Biochemical and biological responses induced by coupling of Gab1 to phosphatidylinositol 3-kinase in RET-expressing cells. *Biochem Biophys Res Commun* 323:345–354
2. He Z, Jiang J, Kokkinaki M, Golestaneh N, Hofmann MC, Dym M (2008) Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. *Stem Cells* 26:266–278
3. Paveliev M, Lume M, Velthut A, Phillips M, Arumäe U, Saarma M (2007) Neurotrophic factors switch between two signaling pathways that trigger axonal growth. *J Cell Sci* 120:2507–2516
4. Trupp M, Arenas E, Fainzilber M, Nilsson AS, Sieber BA, Grigoriou M, Kilkenny C, Salazar-Grueso E et al (1996) Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature* 381:785–789
5. Poteryaev D, Titievsky A, Sun YF, Thomas-Crusells J, Lindahl M, Billaud M, Arumäe U, Saarma M (1999) GDNF triggers a novel ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor alpha1. *FEBS Lett* 463:63–66
6. Trupp M, Scott R, Whittemore SR, Ibáñez CF (1999) Ret-dependent and -independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells. *J Biol Chem* 274: 20885–20894
7. Paratcha G, Ledda F, Ibáñez CF (2003) The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell* 113:867–879
8. Kiss JZ, Muller D (2001) Contribution of the neural cell adhesion molecule to neuronal and synaptic plasticity. *Rev Neurosci* 12:297–310
9. Euteneuer S, Yang KH, Chavez E, Leichtle A, Loers G, Olshansky A, Pak K, Schachner M et al (2013) Glial cell line-derived neurotrophic factor (GDNF) induces neurogenesis in the cochlear spiral ganglion via neural cell adhesion molecule (NCAM). *Mol Cell Neurosci* 54:30–43
10. Charoy C, Nawabi H, Reynaud F, Derrington E, Bozon M, Wright K, Falk J, Helmbacher F et al (2012) GDNF activates midline repulsion by semaphorin3B via NCAM during commissural axon guidance. *Neuron* 75:1051–1066

11. Ledda F, Paratcha G, Sandoval-Guzmán T, Ibáñez CF (2007) GDNF and GFR α 1 promote formation of neuronal synapses by ligand-induced cell adhesion. *Nat Neurosci* 10:293–300
12. Pike LJ (2009) The challenge of lipid rafts. *J Lipid Res Suppl*: S323–328
13. Niethammer P, Delling M, Sytnyk V, Dityatev A, Fukami K, Schachner M (2002) Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neurogenesis. *J Cell Biol* 157:521–532
14. Janich P, Corbeil D (2007) GM1 and GM3 gangliosides highlight distinct lipid microdomains within the apical domain of epithelial cells. *FEBS Lett* 581:1783–1787
15. Macdonald JL, Pike LJ (2005) A simplified method for the preparation of detergent-free lipid rafts. *J Lipid Res* 46:1061–1067
16. Little EB, Edelman GM, Cunningham BA (1998) Palmitoylation of the cytoplasmic domain of the neural cell adhesion molecule NCAM serves as an anchor to cellular membranes. *Cell Adhes Commun* 6:415–430
17. Webb Y, Hermida-Matsumoto L, Resh MD (2000) Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *J Biol Chem* 275: 261–270
18. Ditlevsen DK, Köhler LB, Pedersen MV, Risell M, Kolkova K, Meyer M, Berezin V, Bock E (2003) The role of phosphatidylinositol 3-kinase in neural cell adhesion molecule-mediated neuronal differentiation and survival. *J Neurochem* 84: 546–556
19. Ditlevsen DK, Berezin V, Bock E (2007) Signalling pathways underlying neural cell adhesion molecule-mediated survival of dopaminergic neurons. *Eur J Neurosci* 25:1678–1684
20. Chao CC, Ma YL, Chu KY, Lee EH (2003) Integrin α v and NCAM mediate the effects of GDNF on DA neuron survival, outgrowth, DA turnover and motor activity in rats. *Neurobiol Aging* 24:105–116
21. Schley PD, Brindley DN, Field CJ (2007) (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. *J Nutr* 137:548–553
22. Lucero HA, Robbins PW (2004) Lipid rafts-protein association and the regulation of protein activity. *Arch Biochem Biophys* 426:208–224
23. Simons K, Toomre D (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 1:31–39
24. Arni S, Keilbaugh SA, Ostermeyer AG, Brown DA (1998) Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues. *J Biol Chem* 273: 28478–28478
25. Aarts LH, Verkade P, Schrama LH, Oestreicher AB, Gispen WH, Schotman P (1999) Local accumulations of B-50/GAP-43 evoke excessive bleb formation in PC12 cells. *Mol Neurobiol* 20:17–28
26. Beggs HE, Baragona SC, Hemperly JJ, Maness PF (1997) NCAM140 interacts with the focal adhesion kinase p125 (fak) and the SRC-related tyrosine kinase p59 (fyn). *J Biol Chem* 272: 8310–8319
27. Povlsen GK, Ditlevsen DK, Berezin V, Bock E (2003) Intracellular signaling by the neural cell adhesion molecule. *Neurochem Res* 28: 127–141
28. Bodrikov V, Leshchyns'ka I, Sytnyk V, Overvoorde J, den Hertog J, Schachner M (2005) RPTP α is essential for NCAM-mediated p59fyn activation and neurite elongation. *J Cell Biol* 168:127–139
29. Leshchyns'ka I, Sytnyk V, Morrow JS, Schachner M (2003) Neural cell adhesion molecule (NCAM) association with PKC β 2 via beta1 spectrin is implicated in NCAM-mediated neurite outgrowth. *J Cell Biol* 161:625–639
30. Cinek T, Horejsi V (1992) The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. *J Immunol* 149: 2262–2270
31. Tansey MG, Baloh RH, Milbrandt J, Johnson EM Jr (2000) GFR α -mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. *Neuron* 25:611–623