Overexpression of nucleoside diphosphate kinases induces neurite outgrowth and their substitution to inactive forms leads to suppression of nerve growth factor- and dibutyryl cyclic AMP-induced effects in PC12D cells

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Abstract Whether nucleoside diphosphate kinase (NDPK) is involved in neuronal differentiation was investigated with special reference to its enzyme activity. Neurite outgrowth of PC12D cells induced by nerve growth factor or a cyclic AMP analog was suppressed to some extent when inactive NDPKs (the active site histidine 118 was replaced with alanine), not active forms, were transiently overexpressed. This suppression was more definite in their stably expressed clones. NDPKβ-transfected clones and, to a lesser extent, NDPKα-transfected clones, but not inactive NDPK-transfected clones, extended neurites without differentiation inducers. These results imply that NDPKs may play a role by exerting their enzyme activity during differentiation of PC12 cells.

Key words: Nucleoside diphosphate kinase; Enzyme activity; Neurite outgrowth; NGF; Cyclic AMP; PC12 cell

1. Introduction

Nucleoside diphosphate kinase (NDPK, EC 2.7.4.6) is a ubiquitous enzyme catalyzing a transfer of the terminal phosphate from nucleoside triphosphates ((d)NTPs) to nucleoside diphosphates ((d)NDPs) by forming a high energy phosphorylated enzyme intermediate [1]. NDPK plays a key role in maintaining intracellular pools of (d)NTPs utilized for syntheses of cellular compounds such as nucleic acids, proteins, polysaccharides and lipids. Also, this enzyme is suggested to play roles as a GTP-supplying machinery in the vicinity of heterotrimeric GTP-binding proteins in signal transduction [2] and tubulins in microtubule polymerization [3].

In recent years, it has been suggested that NDPK may have multi-functions as shown by its identity to nm23 [4–7], Awd [8,9], Puf [10,11] and I-factor [12,13]. NDPK enzyme activity is not required for the latter two functions [11,13]. Whether NDPK/nm23 requires the enzyme activity for suppression of metastasis still remains ambiguous. Since NDPK is presumed to be involved in the regulation of wide variety of cellular functions, including cell proliferation [14,15], differentiation [16–18], development [9,19] and apoptosis [16], it is essential to know whether its enzyme activity is required for each of these cases for total understanding of NDPK in the cell. In the present study, we investigated the possible role of NDPK in the neuronal differentiation of PC12D cells, a sub-cell line of rat pheochromocytoma PC12 cells, which can be induced to differentiate very quickly by stimulation of nerve growth factor (NGF) or elevation of intracellular cyclic AMP (cAMP) levels [20]. When PC12D cells were transfected with expression vectors harboring cDNAs of hemagglutinin (HA)-tagged active NDPK isoforms and their mutant NDPKs defective in their enzyme activity, those cell clones transfected with inactive NDPKs showed reduced neurite outgrowth in response to NGF or dibutyryl cAMP (dbcAMP). Furthermore, active NDPK-transfected clones, not inactive NDPK-transfected clones, became capable of extending neurites with no stimulation by differentiation inducers. Therefore, the enzyme activity of NDPK was found to be crucial for the neurite outgrowth of PC12D cells.

2. Materials and methods

2.1. Materials

PC12D cells were kindly provided by Dr. M. Sano (Institute for Developmental Research, Aichi Human Service Center). Monoclonal antibodies for rat NDPK isoforms were previously described [7,21]. An anti-HA mouse monoclonal antibody was kindly donated from Dr. N. Hanai (Tokyo Research Laboratories, Kyowa Hakko Kogyo Co.). All other materials were obtained from commercial sources, unless described otherwise.

2.2. Construction of expression vectors

The cDNAs coding for active NDPK and their mutant proteins in which the active site histidine 118 in rat NDPKs was replaced with alanine (NDPKα^{H118A} and NDPKβ^{H118A}), were constructed using PCR-based technique from the pBluescript KS(+) plasmid harboring NDPKα or NDPKβ cDNA cloned previously [22,23]. In another set of cDNAs the sequence coding for the HA epitope YPYDVPDYA was inserted in frame between alanine 2 and asparagine 3 by PCR using primers containing HA sequence. These cDNAs were inserted in *Eco*RI site of the pBluescriptII SK(+) plasmid (Stratagene). These

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Abbreviations: NDPK, nucleoside diphosphate kinase; NGF, nerve growth factor; dbcAMP, dibutyryl cyclic AMP; HA, hemagglutinin; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; PBS, Dulbecco's phosphate-buffered saline

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cDNAs were subcloned in pET-17b vector (Novagen) for production of recombinant NDPKs in *E. coli* and in pcDNA3.1(+) vector (Invitrogen) for expression in PC12D cells. The sequence of all constructs was confirmed using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham) and an automated DNA sequencing system (Shimazu, model 1000L).

2.3. Expression and purification of recombinant NDPKs

E. coli BL21 (DE3) pLysS competent cells were transformed with the pET-17b vectors harboring active and inactive NDPK cDNAs. The bacterial culture was grown at 37°C and the expression was induced by the addition of 1 mM IPTG. After centrifugation the resulting cell pellet was suspended in a lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF) and treated with freezing and thawing, followed by sonication on ice until it lost viscosity. The recombinant NDPKs were recovered from high-speed supernatant fraction and purified to almost homogeneity by one-step affinity chromatography using an ATP-Sepharose column as previously described [24].

2.4. Assay of NDPK activity

NDPK activity was measured by the pyruvate kinase-lactate dehydrogenase coupled enzyme reaction essentially as described previously [25]. Protein concentration was measured using a BCA Protein Assay Kit (Pearce) with BSA as a standard.

2.5. Cell culture

PC12D cells were cultured at 37°C under a humidified 5% CO_2 atmosphere in a growth medium, Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l glucose supplemented with 10% horse serum and 5% fetal calf serum in non-coated tissue culture dishes until 60–70% confluence. The cells were passaged by incubating for 10 min with 1 mM EDTA in Dulbecco's phosphate-buffered saline (PBS).

2.6. Transient transfection and immunostaining

PC12D cells were transfected with pcDNA3.1(+) vectors harboring HA-NDPK cDNAs by lipofection using a Lipofect AMINE PLUS Reagent (Gibco BRL) according to the manufacturer's instructions. After 24 h, the cells were transferred to a chamber slide (Nunc) coated with poly-L-lysine at a density of 1.0×10^4 cells/cm^2 and cultured for another 24 h. For analyses of neurite outgrowth, the medium was replaced with the fresh one containing 50 ng/ml NGF (Takara) or 1 mM dbcAMP (Sigma) and the cells were cultured further for 48 h. The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min, washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 3 min. After blocking with 1% BSA in PBS at room temperature, samples were treated with an anti-HA mouse monoclonal antibody in PBS containing 1% BSA at 4°C overnight, followed by incubation with a second antibody, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody in PBS containing 1% BSA at room temperature for 1 h. For quantification of neurite outgrowth, the cells expressing exogenous HA-NDPK were identified (at least more than 150, up to 300, in number) and those forming neurites longer than 2- or 5-fold the diameter of the cell body were counted.

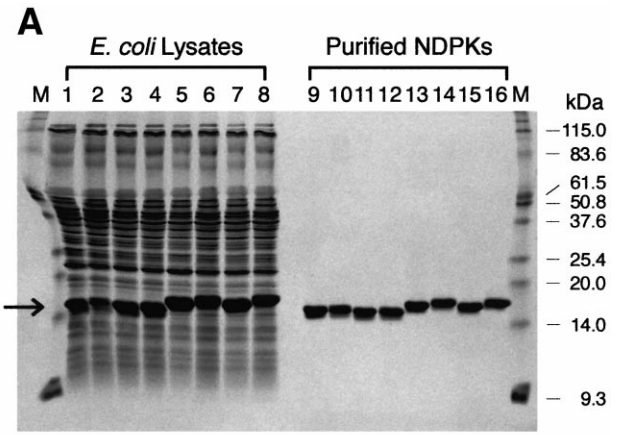
2.7. Stable transfection

Stable transfection was performed by electroporation as follows. Cells (4.0×10^6) in 0.4 ml of PBS with 15 μg of pcDNA3.1(+) vector harboring HA-NDPK cDNAs were treated using a Bio-Rad gene pulser (setting: 0.3 kV, 250 microfarad) as described [7]. Selection was done with the growth medium containing 0.7 mg/ml G418 for 3 weeks. G418-resistant colonies were isolated using the cloning cylinders and expanded in the growth medium containing 0.5 mg/ml G418. Two clones of each group showing the highest expression were selected by Western blotting with an anti-HA antibody. Control clones transfected with the vector alone were selected randomly from those that integrated the vector into their chromosomal DNA. The selected clones were plated on poly-L-lysine-coated dishes at a density of 1.0×10^4 cells/cm^2 for differentiation experiments. After 24 h, medium was replaced with the fresh one containing 50 ng/ml NGF or 1 mM dbcAMP. The morphological change was observed every day, and the relative ratio of cells forming neurites longer than 2-fold the diameter of the cell body out of total cells counted under a phase-contrast microscope (from 120 to 330 in number) was calculated.

3. Results

3.1. Purification and enzyme activity of recombinant NDPKs

For the purpose of examining whether the replacement of amino acid in the active site and the insertion of HA epitope tag influence the NDPK enzyme activity, we prepared recombinant proteins using each cDNA, purified them and examined their enzyme activity (Fig. 1). The recombinant NDPKs expressed in *E. coli* were purified to almost homogeneity by one-step affinity chromatography on the ATP-Sepharose column. The HA-tagged NDPKs were shown to have larger molecular mass than NDPKs without HA epitope tag by SDS-PAGE. When the enzyme activity was measured by the enzyme-coupled assay method, the replacement of the active site histidine 118 with alanine (NDPKα^{H118A} and NDPKβ^{H118A}) resulted in the loss of enzyme activity. On the other hand, there was no difference in the specific activity between NDPKs with and without HA epitope tag. From these results we concluded that HA-NDPK cDNAs and their protein products can be used for further experiments to dis-



B

NDPK Activity (μmol/min/mg)		
	Lysate	Purified NDPK
NDPKα	283	1100
NDPKβ	203	554
NDPKα^{H118A}	4.5	12
NDPKβ^{H118A}	4.5	8.6
HA-NDPKα	293	1002
HA-NDPKβ	235	501
HA-NDPKα^{H118A}	3.4	4.5
HA-NDPKβ^{H118A}	3.6	3.8

Fig. 1. Purification and enzyme activity of recombinant NDPKs. The recombinant NDPKs expressed in *E. coli* were purified by one-step affinity chromatography on an ATP-Sepharose column. A: The *E. coli* lysates and purified NDPKs were subjected to SDS-PAGE, followed by staining with coomassie brilliant blue. Lanes 1 and 9, NDPKα; lanes 2 and 10, NDPKβ; lanes 3 and 11, NDPKα^{H118A}; lanes 4 and 12, NDPKβ^{H118A}; lanes 5 and 13, HA-NDPKα; lanes 6 and 14, HA-NDPKβ; lanes 7 and 15, HA-NDPKα^{H118A}; lanes 8 and 16, HA-NDPKβ^{H118A}. Arrow shows the position of recombinant proteins. B: The enzyme activity of the *E. coli* lysates and purified recombinant NDPKs.

criminate exogenous NDPK from the endogenous NDPK in the cells.

3.2. Transient expression of NDPKs in PC12D cells

PC12D cells were transfected with the pcDNA3.1(+) vector containing cDNA of HA-NDPKα, HA-NDPKβ, HA-NDPKα^{H118A} or HA-NDPKβ^{H118A}. After 48 h cultivation, the cells were treated with 50 ng/ml NGF or 1 mM dbcAMP and incubated for additional 48 h, followed by fixation and staining with an anti-HA antibody. Then, the cells expressing exogenous HA-NDPK were identified under a fluorescence microscope (Fig. 2A), and among those the cells forming neurites longer than 2- or 5-fold the diameter of the cell body were counted. The control cells treated with vector alone were examined under a phase-contrast microscope because of the absence of fluorescence signal. The neurite outgrowth stimulated by NGF or dbcAMP showed a tendency to be suppressed in PC12D cells expressing inactive HA-NDPK (Fig. 2B). This led to the speculation that the enzyme activity of NDPK may take no small part in neurite outgrowth in PC12D cells. This was more extensively investigated by stable transfection experiments as described below.

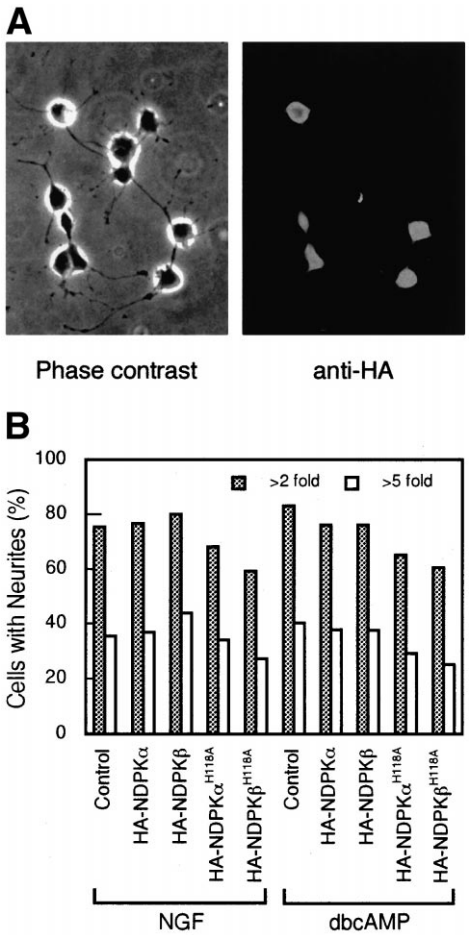


Fig. 2. Neurite outgrowth of PC12D cells transiently transfected with various HA-NDPK cDNAs. A: After 48 h of transfection, the cells were treated with 50 ng/ml NGF or 1 mM dbcAMP for additional 48 h to induce neurite outgrowth. Then the cells were fixed, stained with an anti-HA antibody, and the cells expressing exogenous HA-NDPK were identified under a fluorescence microscope (right). Phase-contrast photograph is shown on the left side. B: The percentage of the cells having neurites with length exceeding 2- or 5-fold the diameter of cell body among those expressing HA-NDPK is shown.

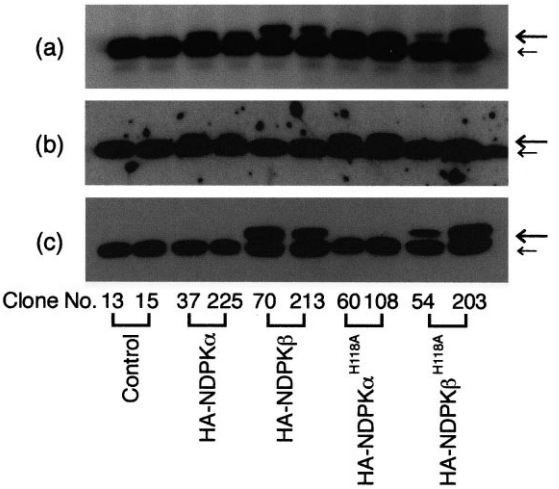


Fig. 3. Western blotting of the cell lysates extracted from PC12D transfectants. The stable transfectants were established as described in Section 2. Their lysates were subjected to Western blotting with an anti-NDPK antibody cross-reactive to both isoforms (a), an anti-NDPKα-specific antibody (b) or an anti-NDPKβ-specific antibody (c), and the expression of exogenous HA-NDPKs was confirmed by their mobility and indicated by large arrows. Endogenous NDPKs are indicated by small arrows.

3.3. Stable expression of NDPKs in PC12D cells

The stable transfectants were prepared as described in Section 2 and clones expressing exogenous HA-NDPKs were selected by Western blotting. Two representative clones selected from each group are shown in Fig. 3. Morphological difference of these transfectants was examined after 2 days incubation in the absence and the presence of NGF or dbcAMP (Fig. 4A). Surprisingly, cell clones expressing HA-NDPKβ were capable of extending neurites without any inducers of differentiation. HA-NDPKα-transfected clones showed only marginal neurite outgrowth under the condition. The clones expressing inactive NDPKs were indistinguishable from the control clones transfected with the vector alone. On the other hand, when the differentiation of each transfectant was examined in the presence of NGF or dbcAMP, cell clones overexpressing HA-NDPKα or HA-NDPKβ extended neurites to an extent similar to the control clones. In contrast, neurite outgrowth of transfectants expressing inactive NDPK, HA-NDPKα^{H118A} or HA-NDPKβ^{H118A} in response to the inducers was remarkably reduced. In this case, inactive forms of both NDPK isoforms exhibited almost similar potency to inhibit the morphological change as shown in Fig. 4B. It is important to note that NDPK enzyme activity in these cell extracts was not largely altered by the overexpression of mutant inactive NDPKs (data not shown).

We examined possible relationship between differentiation potential and proliferative potential in the transfected PC12D cells. Interestingly, doubling time of these transfectants was approximately 30 h except HA-NDPKβ transfectants in which doubling time was prolonged to over 40 h.

4. Discussion

The distribution of rat two NDPK isoforms differs from

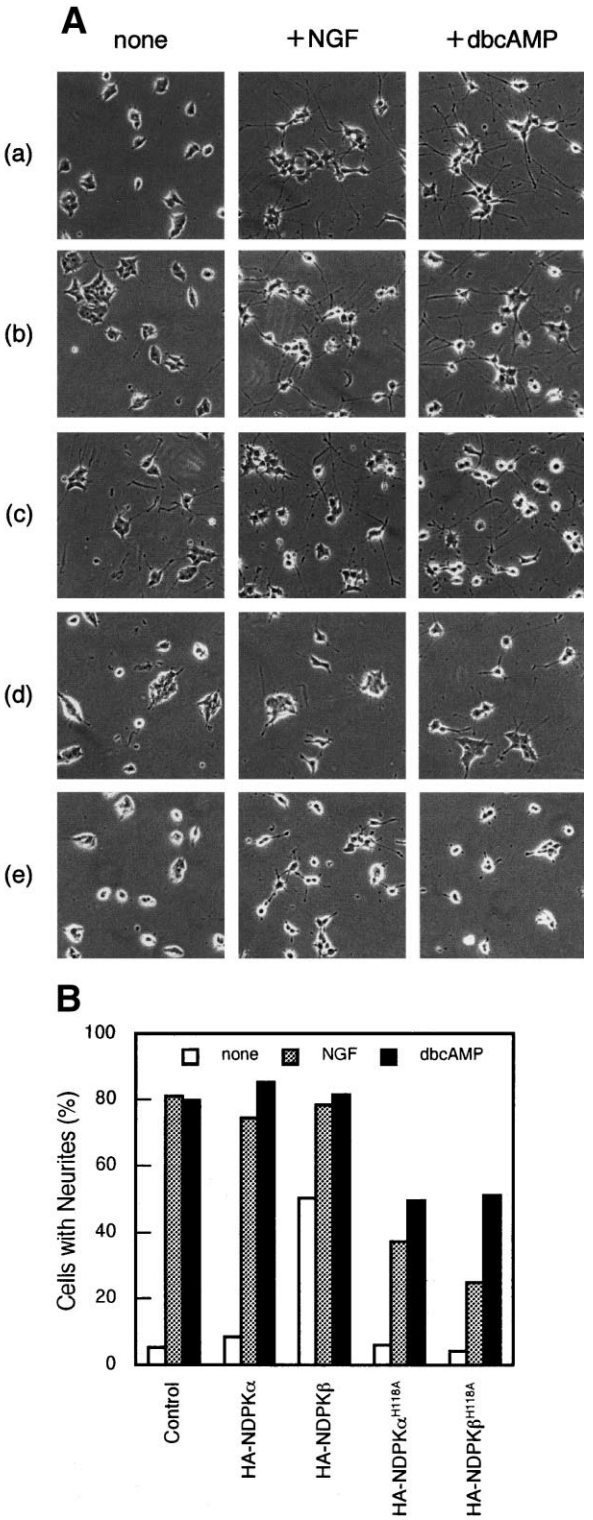


Fig. 4. Neurite outgrowth in stable PC12D transfectants. A: PC12D cell clones stably transfected with the vector alone (clone #15) (a), HA-NDPKα (clone # 225) (b), HA-NDPKβ (clone #70) (c), HA-NDPKα^{H118A} (clone #108) (d), or HA-NDPKβ^{H118A} (clone #203) (e). Neurite outgrowth of these PC12D transfectants was examined after 48 h treatment with or without 50 ng/ml NGF or 1 mM dbcAMP under a phase-contrast microscope. B: Quantification of neurite outgrowth in stable transfectants. Each transfectant was treated as in A. The relative number (in percentage) of cells having neurites with length exceeding 2-fold the diameter of cell body is shown.

tissue to tissue [23]; NDPKα is the major form in most tissues, whereas NDPKβ is highly expressed in a limited number of tissues such as cerebrum and testis. These observations led us to speculate that NDPKβ may play some specific roles in neuronal cells and to investigate the role of NDPK in neuronal differentiation of PC12 cells. To our surprise was the finding that PC12D cell clones overexpressing NDPKβ obviously became to be capable of extending neurites without any differentiation inducers and showed decreased proliferative activity. This effect was dependent on its enzyme activity because NDPKβ^{H118A} of which enzyme activity was nullified could not reproduce this phenotypic change. Since overexpression of NDPKα showed only marginal effect, specific role of NDPKβ in the neuronal differentiation is strongly suggested. However, the role of NDPK isoforms in the differentiation processes seems to be more complicated. This is exemplified in the experiments in which stable transfectants were examined regarding their response to differentiation inducers, NGF and dbcAMP. In this case, not only NDPKβ^{H118A} but also NDPKα^{H118A} acted inhibitory for morphological change induced by these reagents. These differences may be partly due to the possible distinct potencies of NDPK isoforms and/or to the differential expression levels of exogenous proteins employed in this study, and remain to be studied further.

It has been believed that NDPKs mostly exist in the cell cytosol as a soluble free form [1]. However, we previously revealed membrane-associated form of NDPK [25] and postulated importance of its physical interaction with G protein-coupled signal transduction systems [26–28]. Some recent literatures describe another possible target proteins which NDPK interacts with [29,30]. To extend this idea, it is essential to demonstrate convincing data that suggest protein-protein interaction in any NDPK-related phenomena in vivo. In this respect, it should be emphasized that the overexpression of mutant inactive NDPKs suppressed NGF- or dbcAMP-induced neurite outgrowth of PC12D cells, since under these conditions NDPK enzyme activity in these cell extracts was not largely affected. Therefore, suppression by these inactive enzymes is not likely due to the altered NDPK enzyme activity in the cell but rather to their dominant negative effects. In other words, it is conceivable that the enzyme activity of endogenous NDPKs is required at a specific site where they should be for extending neurites. When inactive NDPKs are present, the endogenous enzymes may be displaced from their binding site, as a result, leading to incomplete neurite outgrowth of PC12D cells. Recent studies revealed that NGF signaling is linked to mitogen-activated protein kinase (MAPK) cascades [31] and that elevation of intracellular cAMP levels leads to activation of MAPK cascade via activation of cAMP-dependent protein kinase (PKA) and Rap1, a member of the Ras superfamily [32] in PC12 cells. Thus, both differentiation inducers seem to share in part their signaling pathway. Taking into account the fact that mutant inactive NDPKs can interfere with both signaling pathways, the interaction site of NDPKs may be located in the common part of these two signaling pathways. On the other hand, it cannot be ruled out that NDPK may influence neurite outgrowth by altering microtubule dynamics as shown by its colocalization with microtubules [3,33]. However, whether NDPK has an ability to influence tubulin polymerization has been argued [34] and remains to be studied further. In PC12 cells overexpression of NDPK/nm23 increases expression of β-tubulin

as well as neurofilament, but no description is provided for the interaction between β-tubulin and NDPK/nm23 [35]. Identification of the target protein(s) which NDPKs interact with for the neuronal differentiation of PC12D cells is under current investigation.

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