Chapter 3

Cultured Cell Line Models of Neuronal Differentiation: NT2, PC12

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Abstract

The lack of a convenient, easily maintained and inexpensive in vitro human neuronal model to study neurodegenerative diseases, prompted us to develop a rapid, 1-h differentiated neuronal cell model based on human NT2 cells and C3 transferase. Here, we describe the rapid differentiation of human neuronal NT2 cells, and the differentiation, transduction and transfection of rat PC12 cells to obtain cells with the morphology of differentiated neurons that can express exogenous genes of interest at high level.

Key words Human neuronal cells, NT2, PC12, Differentiation, NGF, Rho kinase inhibitor, C3 transferase

1 Introduction

Developing of suitable and affordable neuronal models was always an important issue in neuroscience research. Although, several in vitro neuronal models have been proposed previously [1–3], all these techniques are time-consuming and require long-term (up to 4–6 weeks) incubations and expensive reagents. Differentiated human NT2 and rat PC12 cells may represent an excellent neuronal source for in vitro studies of neurodegenerative diseases, investigating pro-survival pathways and agents protecting human neuronal cells from cell death-mediating stresses.

The most ideal cell culture for analysis of the processes of neuronal differentiation would be one that can be differentiated rapidly so that it could be maintained easily and, at the same time, could be transfected with high efficiency to produce a stable or transient population of cells expressing exogenous gene products. These cells would promote extensive neuronal processes similar to that of primary neurons in culture. NTera (NT2), a human teratocarcinoma cells are capable of differentiating in response to retinoic acid, RA. To obtain pure neuronal cultures from RA-treated NT2 cells, further treatment with mitotic inhibitors should be

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performed for at least 6 weeks [1]. These neurons (NT2-N cells) express all ubiquitous neuronal markers that can be identified in axons or dendrites using molecular and functional criteria. The aim of this study was to develop a neuronal culture system that can be easily maintained and can be rapidly differentiated. Thus, we further developed an in vitro neuronal model to obtain rapidly differentiated NT2 and PC12 cells.

2 Materials

1. NTera 2/cl.D1 (NT2), a human teratocarcinoma cell line. NT2 cells are obtained from American Type Culture Collection (ATCC, Manassas, VA).
2. PC12 rat pheochromocytoma cells (ATCC No. CRL-1721) cells are from American Type Culture Collection.
3. Retinoic acid, RA.
4. F-12 medium supplemented with 15 % horse serum and 2.5 % fetal bovine serum.
5. Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 U/ml penicillin and 10 μg/ml streptomycin).
6. Dulbecco's modified Eagle's medium high glucose (DMEM HG) supplemented with 10 % fetal bovine serum and penicillin/streptomycin.
7. DMEM for neurite regeneration with 10 % FBS, penicillin/streptomycin, supplemented with mitotic inhibitors (1 μM cytosine arabinoside and 10 μM uridine).
8. DMEM/F12 medium for PC12 cells containing 10 % fetal bovine serum (FBS) and 5 % horse serum (HS).
9. 37 °C humidified Incubator containing 7 % CO_2.
10. Collagen-coated 60 mm tissue culture dishes.
11. Serum-free F-12 medium.
12. NGF solution (100 ng/ml).
13. Cold phosphate-buffered saline (PBS).
14. pLECFP-C1 (Clontech, Mountain View, CA).
15. Commercially purchased synthetic peptide containing the arginine rich protein transduction domain (PTD) of HIV-1 Tat (amino-acids 47–57) fused to an N-terminal 6 histidine epitope tag.
16. Cover Glass (finest grade, Premium Hard, Glass/Clear/Pre-Cleared/Non-Corrosive/Non-Fogging, Thickness No 1 Size: 24 mm × 40 mm.

17. Chamber Slide Lab-Tec System Well Permanox Slide 4 chambers mounted on Permanox slide with cover.
18. Chamber Slide System and Chambered Coverglass Lab-TekII Chamber Slide.
19. 2 well Permanox slide.
20. Tissue Culture Dish Polystyrene 60 × 15 mm.
21. Tissue Culture Dish 100 × 20 mm Style.
22. Tryple Express enzyme (Invitrogen, Carlsbad, CA).
23. RhoA inhibitor, C3 transferase.

3 Methods

3.1 Cell Culture

3.1.1 Human Neurons

Ntera (NT2) human embryonic teratocarcinoma cells are maintained in Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C in a humidified atmosphere containing 7 % CO_2. Cells are plated in T75 flasks at a density of one million cells, and 12× well dishes at a density of 3×10^4/well and cultured in 3 ml medium.

3.1.2 Postmitotic Terminally Differentiated Polarized Human Neurons

Postmitotic Terminally Differentiated Polarized Human Neurons are derived from NT2 according to published protocols [1–3] with some modifications. Cells are cultured in Dulbecco's modified Eagle's medium high glucose (DMEM HG). Cells are induced to differentiation upon incubation with RA 10 μM RA twice a week for 4 weeks. For neurite regeneration following RA treatment, cells are incubated in DMEM with mitotic inhibitors for 2–4 weeks. Then DMEM is replaced with Neurobasal medium. Differentiated neurons establish polarized neurites with the characteristics of axons and dendrites after 4 weeks of RA treatment and 2–4 weeks of incubation with mitotic inhibitors. Cells are maintained at 37 °C in a humidified incubator containing 7 % CO_2 (*see* **Note 1**).

3.1.3 PC12

PC12 rat pheochromocytoma cells are cultured on 60 mm poly-D-lysine-coated dishes at a density of 1×10^5 cells per 60 mm dishes in DMEM/F12 medium containing 10 % fetal bovine serum (FBS) and 5 % horse serum (HS). To induce neuronal differentiation, PC12 cells are plated on collagen IV-coated dishes and treated with 20 ng/ml nerve growth factor (NGF) in serum-free medium (SFM). Neuronal processes begin to form within first 24 h following the treatment and are preserved as differentiated neuronal cultures for 5 days (*see* **Note 2**).

3.2 Treatment

3.2.1 PC12 Cells

PC12 Cells are differentiated in the presence of NGF (20 ng/ml) for 5 days. The images of neurite-like processes were taken from series of photographs from selected microscopic fields and reflect an average length of neurite-like processes in a particular microscopic field.

3.2.2 NT2 Cell Treatment

NT2 cells are plated onto 12× well tissue culture plates at a density of 3×10^4 and cultured for 1 day. Cells are starved in serum free DMEM for 2 h prior to C3 transferase stimulation (1 μg/ml) for 1 h or 16 h. Each culture is also incubated with C3 transferase in the presence of serum [4].

3.3 Transfection

PC12 cells are transfected with pLEGFP-C1 plasmid (2 μg) using a high efficiency transfection reagent such a Lipofectamine 2000 (3 μl). Human neuronal cells are also transfected with pLEGFP-C1 expression plasmid (3 μg each) using a high efficiency transfection reagent (*see* **Note 3**).

3.4 Microscopy

Contrast and brightness were adjusted equally for all images using Adobe Photoshop version 5.5. Original magnification was 200×.

3.5 Antibodies

Expression of the GFP protein was examined by immunocytochemistry, using Living Colors full-length monoclonal antibody (BD Biosciences/Clontech). The presence of Protein Transduction Domain (PTD) from HIV-1 Tat protein fused to 6 Histidine amino acid sequence was examined by immunocytochemistry using anti-His(C-term)-tag Antibody (Invitrogen).

3.6 Protein Transduction and Fluorescence Staining

Approximately 1×10^5 PC12 cells are seeded in 60 mm dishes in medium containing fetal bovine and horse serum. After 8 h, medium is replaced with DMEM/F12 without serum for 16 h, after which the cells were treated with NGF for 48 h in 1 ml DMEM/F12 plus fetal bovine and horse serum. For staining, approximately 1,000 cells were seeded in poly L-lysine coated slide chambers and after 0, 2, and 24 h treatment with 25 μM of synthetic Tat PTD peptide tagged with six histidine amino acids, cells were fixed with cold acetone for 3 min and washed with PBS. Immunocytochemistry was performed by blocking with normal horse serum and incubating with anti-histidine antibody (Invitrogen) at 1:200 dilution for 16 h at room temperature. Cells were incubated with a fluoroscein labeled secondary antibody for 1 h. For nuclear counter staining, propidium iodide was included in the mounting medium. Protein Transduction Domain (PTD) was visualized in fluorescence green.

3.7 Expected Results and Conclusions

The lack of an easy maintained in vitro neuronal models to study human neurodegenerative diseases and signaling pathways that control neuronal differentiation, prompted us to develop a rapid, 1-h differentiated neuronal cell model based on NT2 human neuronal cells. Our aim was to develop an advanced in vitro model, generating sustainable cells with morphology of human, mature neurons during short period of time. First, we have developed a modified protocol to obtain a terminally differentiated NT2 cells that show the typical neuronal morphology with neuronal features,

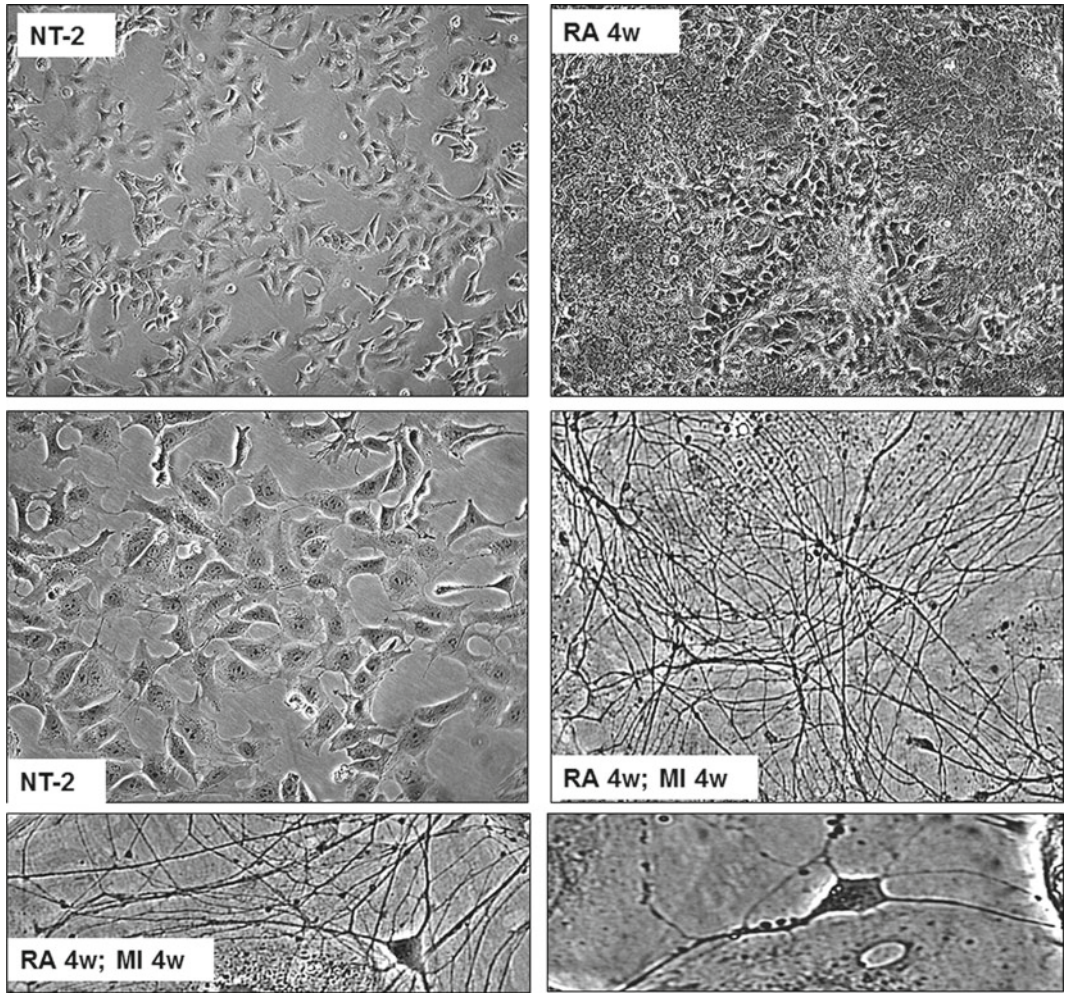

Fig. 1 Differentiation of neuronal NT2 cells. Phase images of undifferentiated and differentiated (8-week) human NT2 cells showing the morphologic changes during the terminal differentiation of NT2 cells. (*Top left panel*) Untreated NT2 cells (×1,000); (*second left panel*) untreated NT2 cells at higher magnification (×200); (*top right panel*) NT2 cells following 4-weeks of RA treatment; (*second right panel*) NT2 cells following replate and 4-weeks of treatment with mitotic inhibitors (note that most cells exhibit the morphology of neurons and extensive process outgrowth has occurred); (*bottom left panel*) terminally differentiated NT2 cells showing the typical neuronal morphology of these cells at higher magnification ×200 (note cell body and defined neuronal processes); (*bottom right panel*) single differentiated cell with neuronal features, the long process resembling an axon and the three major processes that resemble dendrites

the long process resembling an axon and the three major processes that resemble dendrites (Fig. 1). Inhibition of RhoA activation by a RhoA inhibitor, C3 transferase, also promotes massive long-lasting neurite outgrowth during short period of treatment of NT2 cells. Treatment of cells with C3 transferase produced extensive neurite outgrowth after as early as 1 h of incubation. Massive neurite outgrowth after 3 h of C-3 transferase treatment in

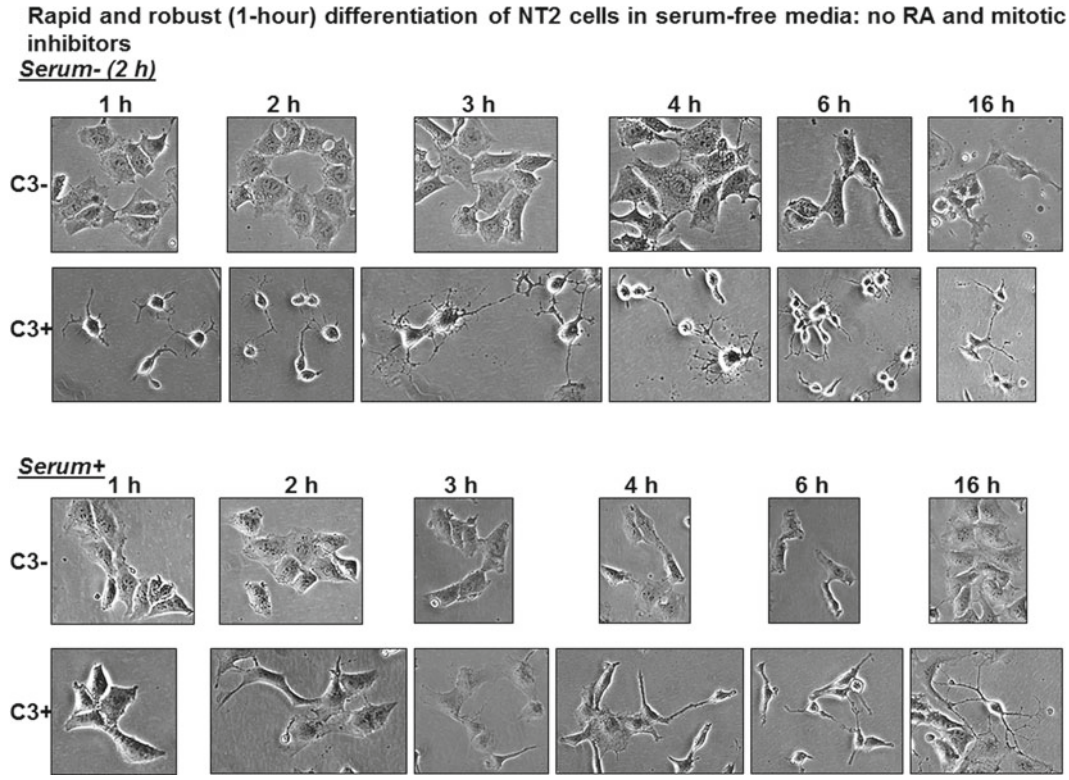

Fig. 2 Time-dependent effect of C3 transferase on neurite outgrowth in human NT2 neuronal cells. (*Top*) The effect of C3 transferase on the formation of neuronal processes in NT2 cells after 1 h incubation in serum-free medium. (*Bottom*) Neuronal outgrowth in NT2 cells, mediated by C3 transfeease in the presence of serum

GFP-expressing cells (Fig. 2). Interestingly, removal of C3 transferase does not lead to neurite retraction indicating that the C3 transferase in neuronal cells is critical for neuronal differentiation at early stages of treatment (Fig. 3). This model is useful to study signaling pathways resulting in neuronal cell injury. We also improved transfection protocols, specifically using Lipofectamine 2000, to efficiently transfect differentiated or undifferentiated neurons with various plasmids (Fig. 4). Immunocytochemical analysis of NT2 neuronal cells overexpressing GFP revealed cytoplasmic immunoreactivity for GFP and massive neuronal outgrowth (Fig. 5). Thus, it is possible transfect undifferentiated NT2 cells with expression plasmids allowing the introduction of expressed proteins into cells that can subsequently undergo induction to develop into stable, postmitotic, human neurons.

Another neuronal model, actively used by our group, is a PC12 rat neuronal cell line [5–7]. PC12 cells are differentiated upon NGF treatment (20 ng/ml) and can be used for rapid protein transduction assays. We have developed differentiation/protein transduction assay followed by immunocytochemistry and microscopy (Fig. 6). Results from PC12 differentiation and protein

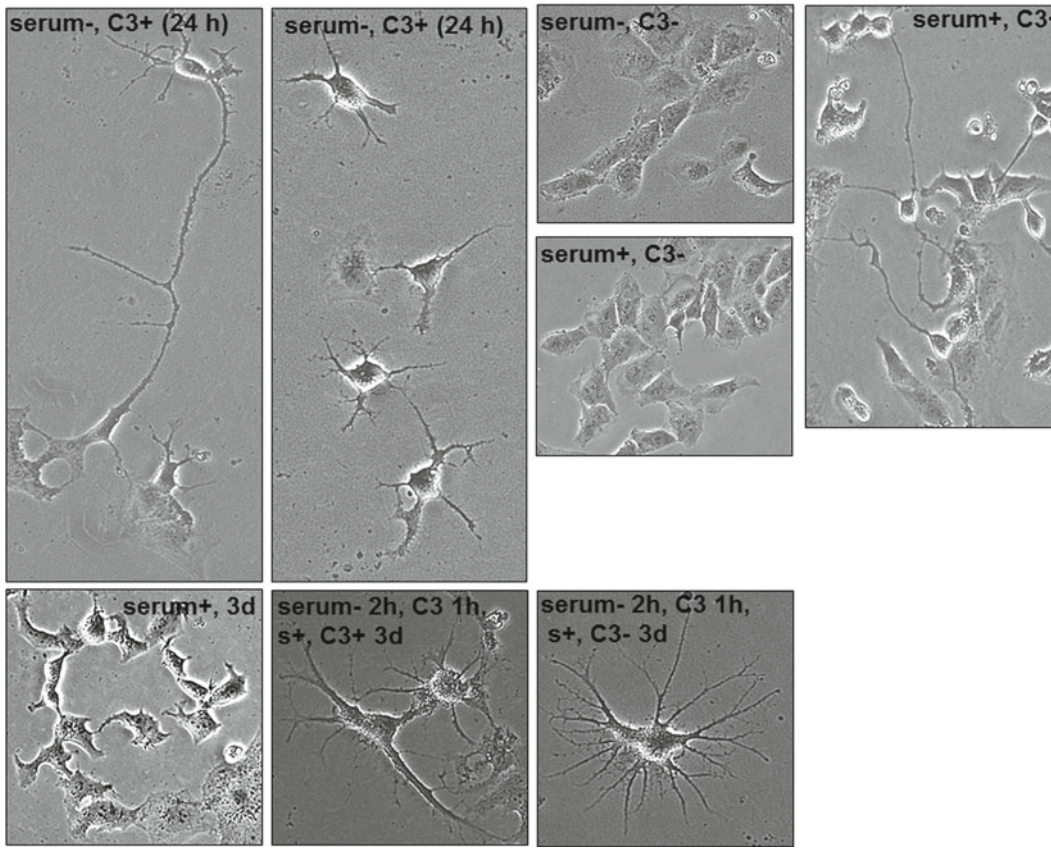

Fig. 3 Differentiation and rescue assays. Incubation of neuronal cells with C3 transferase longer than 1 h effects on. (*Top*) Neurite outgrowth in NT2 cells treated with the RhoA inhibitor, C3 transferase for 24 h. (*Bottom panels*) Removal of C3 transferase does not lead to neurite retraction, indicating that the C3 transferase in neuronal cells is critical for neuronal differentiation at early stages of treatment

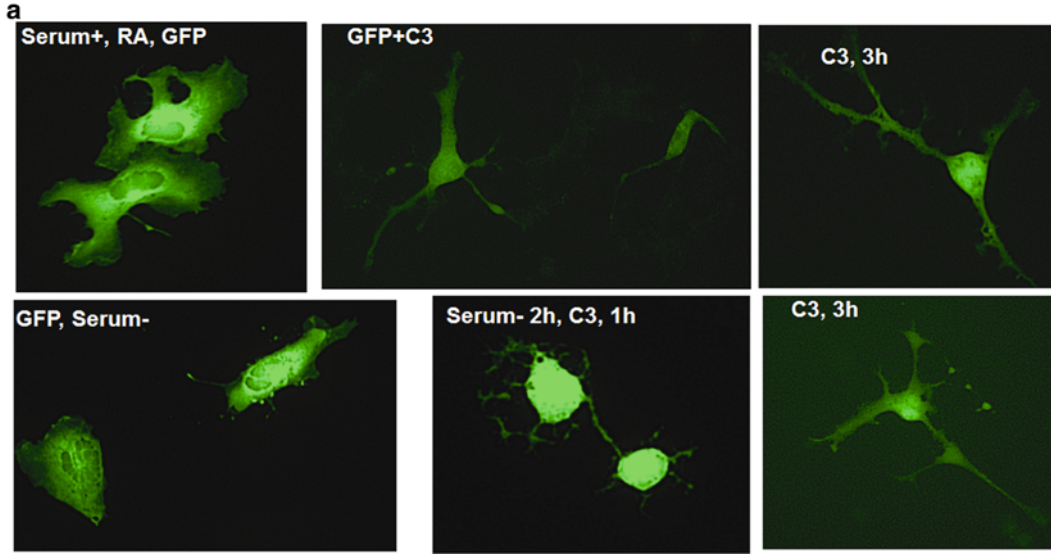

Fig. 4 NT2: Rapid differentiation, transfection, and neurite outgrowth. NT2 neuronal cells were transiently transfected with pLEGFP-C1 plasmid for 16 h and cells, after 2 h of serum starvation, were treated with C3

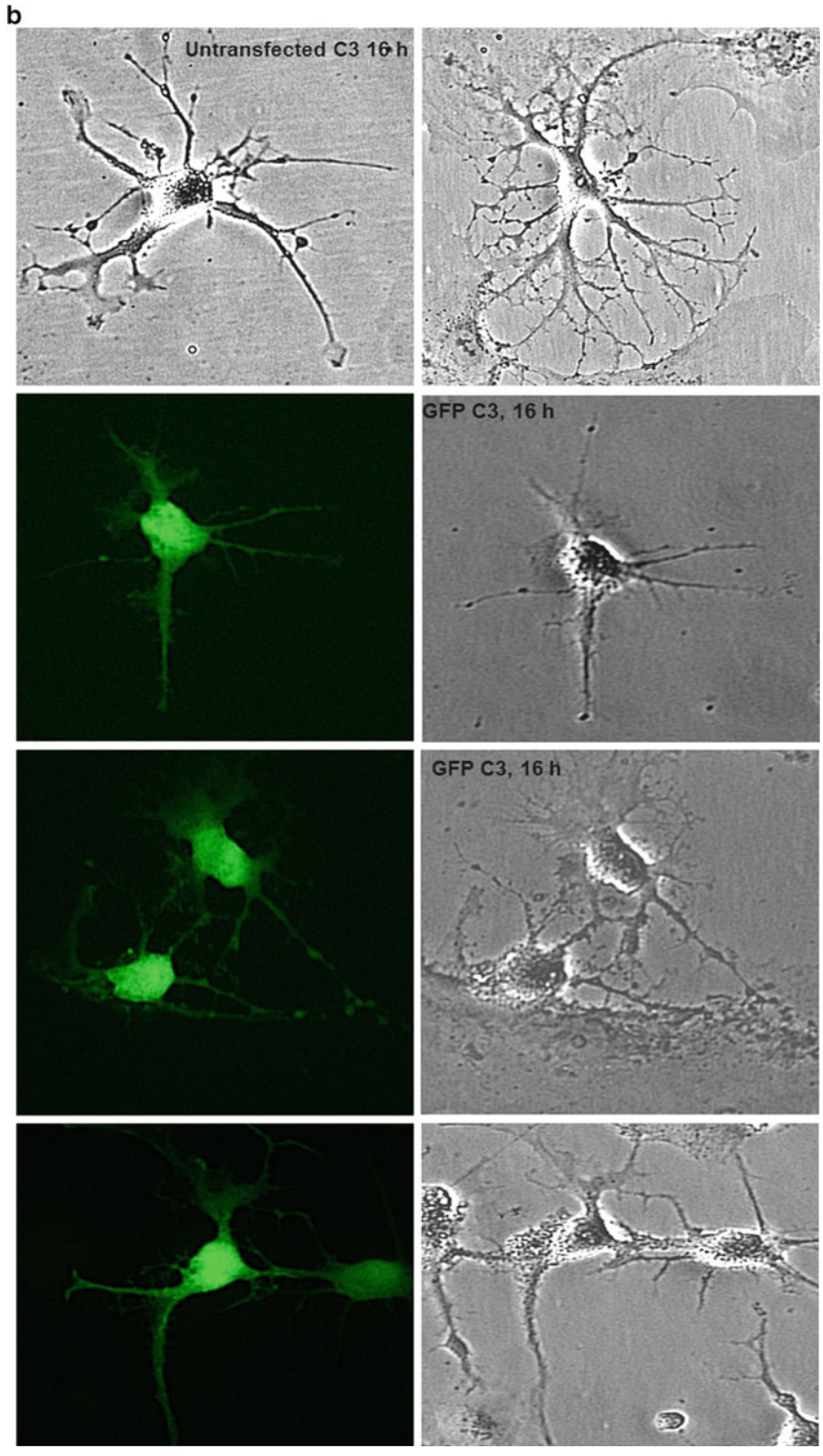

Fig. 4 (continued) transferase for 3 or 16 h to induce differentiation via RhoA inactivation. (**a**) Fluorescence images (magnification ×200) of neuronal cells transfected with GFP and incubated with C-3 transferase for 3 (**b**). Phase and fluorescence images of transfected and differentiated cells. Cells were kept in serum-free medium for 2 h and then incubated with C3 transferase for 16-h. C-3 transferase causes massive outgrowth in GFP-expressing cells

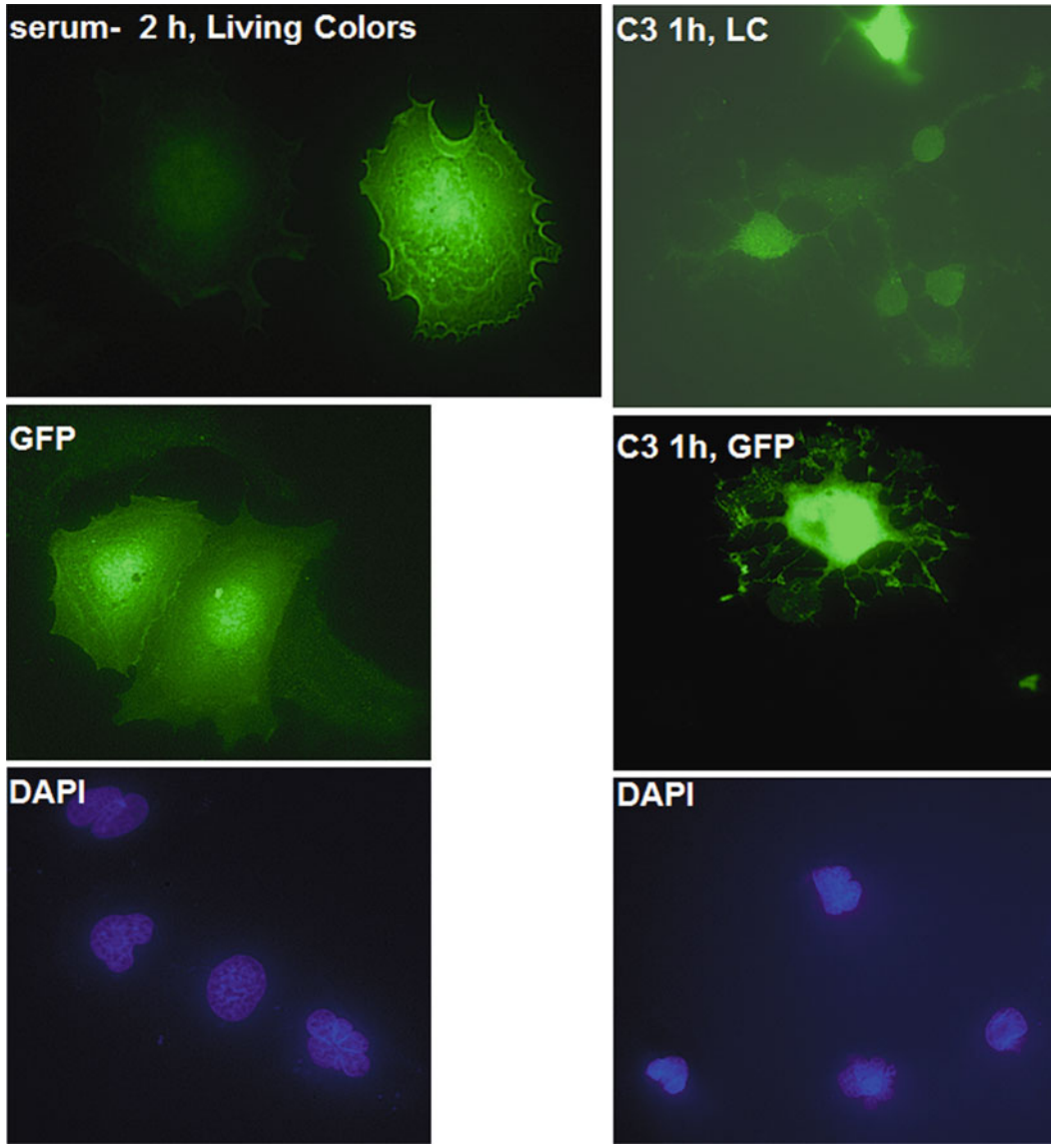

Fig. 5 NT2: Rapid differentiation, transfection, and immunostaining. Immunocytochemical analysis of NT2 neuronal cells overexpressing GFP and incubated with C3 transferase for 1 h. (*Top panels*) Immunostaining was performed with Living colors (for GFP protein) on cells with C3 transferase treatment (*right panels*) or without C3 incubation (*left panels*). Cytoplasmic immunoreactivity is seen for GFP. GFP-expressing cells demonstrate massive neuronal outgrowth (*right panels*). (*Bottom panels*) DAPI nuclear staining was performed in cells without or with C3 transferase

transduction experiments using a synthetic peptide containing the arginine rich Protein Transduction Domain (PTD) of HIV-1 Tat (aa 47–57) and a His Tag revealed cellular internalization and nuclear appearance of the PTD peptide after 2 h and its detection in nuclei up to 24 h after treatment. PC12 cells were also transfected with a plasmid expressing a gene of interest prior to differentiation (Fig. 7).

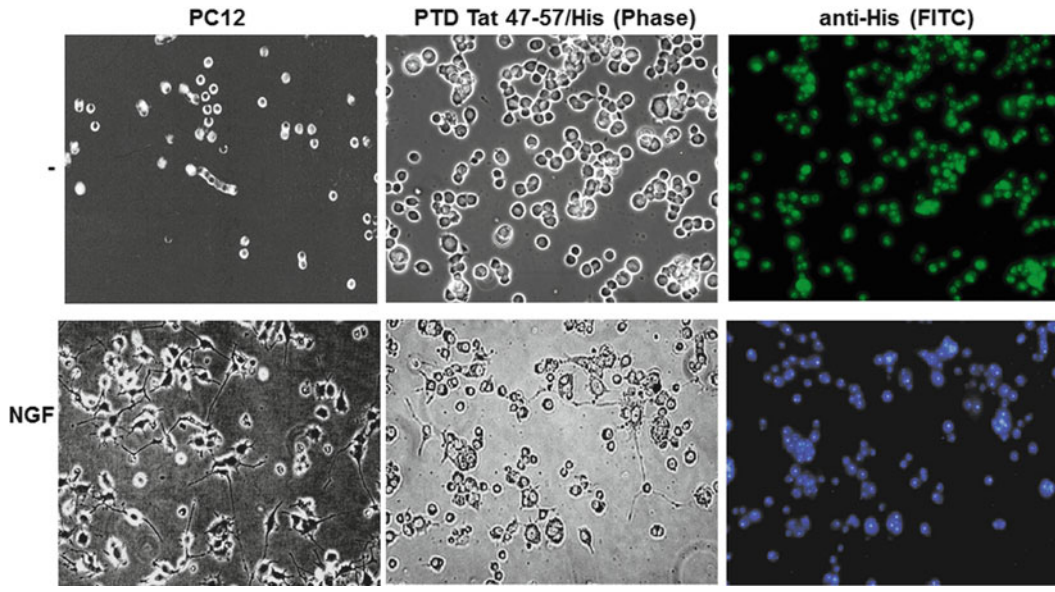

Fig. 6 PC12: Differentiation, protein transduction and detection. Undifferentiated (*top panels*) or differentiated PC12 cells (*bottom panels*) upon NGF treatment (20 ng/ml) were used for rapid protein transduction assays. We have developed differentiation/protein transduction assay followed by immunocytochemistry and microscopy. Results from PC12 differentiation and protein transduction experiments using the synthetic peptide representing the arginine rich Protein Transduction Domain (PTD) of HIV-1 Tat revealed cellular internalization and nuclear appearance of the PTD peptide visualized with anti-histidine antibody after 2 h and its detection in nuclei up to 24 h after treatment

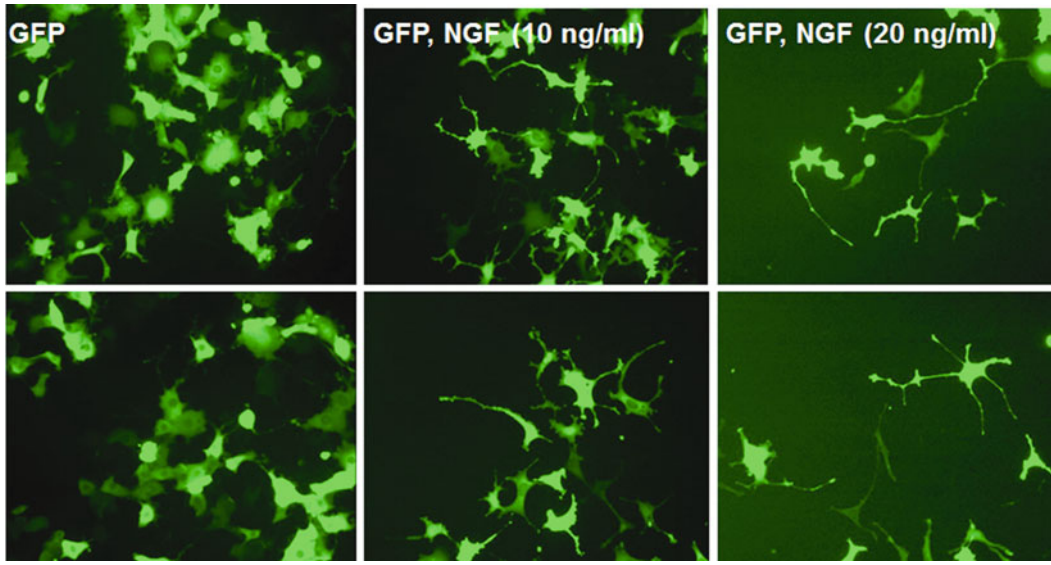

Fig. 7 PC12: differentiation and transient transfection. GFP-expressing PC12 cells were grown on 12× well plates (Falcon) for 1 day, then treated with increased concentrations of NGF (10–20 ng/ml). Results from transfection/differentiation experiments using two concentrations of NGF revealed robust cellular internalization of GFP and massive neuronal outgrowth after 48 h after treatment

Thus, NT2 cells and PC12 cells represent a unique model system for studies of human and rat neurons, and signaling pathways that control neuronal differentiation and represent novel models for the expression of diverse gene products in terminally differentiated polarized neurons, or robustly differentiated neuronal cells.

4 Notes

1. Make sure to add antibiotics to media for long incubation of NT2 cells before cells start to differentiate.
2. It is important to use fresh poly-D-lysine-coated dishes or collagen IV-coated dishes for efficient attachment and differentiation of PC12 cells.
3. The presence of antibiotics or serum in the media does not inhibit the transfection efficiency, if highly purified DNA used in transfection reactions.

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