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ABBREVIATIONS: NP25, Neuronal protein 25; NSC(s), neural stem cell(s); FGF-2, fibroblast growth factor 2; EGF, epidermal growth factor; HGF, hepatocyte growth factor; FBS, fetal bovine serum; TBST, Tris-buffered saline containing Tween-20; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; GST, glutathione S-transferase; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR. reverse transcription-PCR.

ABSTRACT

Neuronal protein NP25 is a neuron-specific protein present in highly differentiated neural cells, but its functional properties has not been well characterized. NP25 shows high amino acid sequence homology with the smooth muscle cell cytoskeleton-associated proteins, SM22 α, mp20, and calponin. To gain an insight into the biological functions of NP25, we examined changes in NP25 mRNA and protein levels during neural differentiation. When PC12 cells were stimulated with NGF, NP25 mRNA and protein levels increased. Similar increases in NP25 protein was also observed during HGF-or FBS-induced differentiation of mouse neural stem cells. These results suggest that NP25 is involved in neural cell differentiation.

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Key words : Neuronal protein 25; Neural differentiation; PC12; Neural stem cells

INTRODUCTION

The neuron-specific protein, NP25, is only found in highly differentiated neural cells. In rats, NP25 mRNA is detected in the brain and spinal cord, but not in the liver, kidney, testis, ovary, intestine, uterus, heart, or skeletal muscle[1]. This distribution pattern suggests that NP25 is expressed solely in the central nervous system. When different regions of the rat brain were examined, NP25 mRNA was distributed widely, but not uniformly, in the brain, with strong signals in the hippocampus, frontal cortex, cerebellum, and midbrain[1]. This wide and differential distribution of NP25 in the brain suggests that it may play an important role in the functioning of specific neuronal systems. However, its functional properties remain to be clarified.

Brain development and neuronal cell differentiation are controlled by a complex program. Rat PC12 cells have been widely used as a model system to study neuronal differentiation[2)~4)]. Neural stem cells (NSCs) can self-renew and give rise to various types of neurons, astrocytes, and oligodendrocytes *in vitro*[5)~8)], and are considered the best model for studying neural differentiation. Nerve growth factor (NGF) is a well known neurotrophic factor and hepatocyte growth factor (HGF), originally cloned as a hepatocyte mitogen, has recently been shown to promote the proliferation and neuronal differentiation of NSCs from mouse embryos[9].

In the present study, we used these experimental models to examine NP25 expression during differentiation of PC12 cells and NSCs induced by NGF and HGF, respectively. Our data support the idea that NP25 is involved in the neural cell differentiation.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and rabbit antibody against actin were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and horse serum were from Themo-Trace (Melbourne, Australia). Penicillin and streptomycin were from ICN Pharmaceuticals (Costa Mesa, CA), B27 supplement and DMEM/F-12 medium (1 : 1)

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were from Invitrogen (Grand Island, NY), and human EGF, FGF-2, and HGF were from R&D Systems (Minneapolis, MN). Isogen and ImmunoStar Reagents were from WAKO Pure Chemicals (Osaka, Japan). PowerScript reverse transcriptase and oligo d(T) were from Clonetech (Palo Alto, CA). Pyrobest DNA polymerase was from TAKARA Biomedicals (Tokyo, Japan). "Complete", a mixture of protease inhibitors, was from Roche (Indianapolis, IN). The Immobilon-P membrane filters were from Millipore (Bedfold, MA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody were from Amersham Biosciences (Buckinghamshire, England). The prestained protein markers, phenylmethylsulfonyl fluoride (PMSF), and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Nacalai Tesque (Kyoto, Japan). Poly-D-lysine hydrobromide was from BD Biosciences (Bedford, MA). The 100 bp DNA Ladder Plus was from COSMOBIO (Tokyo, Japan). Rat β-NGF was from Genzyme TECHNE (Minneapolis, MN).

PC12 cells cultures

The rat pheochromocytoma cell line, PC12, a generous gift from Dr Y. Sugimoto (Shirakawa Institute of Animal Genetics, Fukushima, Japan), was grown in 100mm culture dishes in DMEM supplemented with 10% (v/v) FBS, 5% (v/v) horse serum, penicillin (100μ g/m ℓ), and streptomycin (100μ g/m ℓ) in a humidified atmosphere containing 5% CO_2 at 37℃. For neuronal differentiation, 2×10^5 cells were plated on 100 mm dishes coated with poly-D-lysine for 24 h and differentiation was initiated by replacing the medium with DMEM containing 10% FBS, 5% horse serum, and 50 ng/m ℓ of NGF for 48 and 96 h. Control cultures were carried out in the absence of NGF in DMEM with serum for 48 and 96 h.

Primary culture of neonatal mouse striatal cells and neurosphere passage

Striatal cells were removed from14-day-old mouse embryos (C57 BL/6, plug day=1.0) in phosphate-buffered saline, pH7.4, (PBS) containing penicillin (50μ g/m ℓ) and streptomycin (50μ g/m ℓ). The tissue was mechanically dissociated by repeated aspiration through a fire-polished pipette in serum-free medium consisting of DMEM and F-12 nutrient (1 : 1). The cells were plated at a concentration of 1.5×10^5cells/m ℓ in growth medium [DMEM and F-12 nutrient (1 : 1), 0.6% glucose, 2 mM glutamine, 2% B27 supplement, and 20ng/m ℓ each of EGF and FGF-2] in culture flasks, 6-well dishes, or 24-well dishes (all from Falcon). Half of the medium was replaced every 4 days with fresh growth medium. After 7 days, primary neurospheres were collected by centrifugation (2,300× g for 2 min at 4℃), resuspended in fresh growth medium, and dissociated with a fire-polished pipette. The dissociated NSCs were plated onto poly-D-lysine-coated 100 mm culture dishes. To examine the effects of differentiation, the NSCs were cultured for 2 - 5 days in DMEM and F-12 (1 : 1) containing either 1% FBS plus HGF (20ng/m ℓ) or 10% FBS.

Expression of GST-fusion protein

During the course of our study, we cloned and sequenced human NP25 cDNA by screening a brain two-hybrid library. Homology analysis was performed using BLAST algorism at NCBI, NLM, NIH (Bethesda, MD), and the obtained sequence was the same as that for human NP25 cDNA (accession number XP002852). Glutathione S-transferase (GST) - NP25 was constructed by inserting the human NP25 cDNA, amplified by PCR using Pyrobest DNA polymerase (TAKARA Biomedicals), into the Bam H 1 site of the pGEX-3X vector(Amersham Biosciences). The construct obtained was confirmed by sequencing. GST-NP25 fusion protein and GST were produced in *E. coli* and purified on glutathione beads. The GST-NP25 fusion protein was used in absorption experiments with anti-NP25 antibody.

Anti-NP25 antibody

A rabbit was immunized with the synthetic peptide, GEPSWFHRKAQQ, corresponding to amino acids 146-157 of rat, human, and mouse NP25[10] and the antibodies purified from the sera by affinity chromatography on immobilized peptide. For control experiments, during incubation of the membrane with primary antibody, the anti-NP25 antibody was preabsorbed with GST-NP25 protein produced in *E. coli.*

RT-PCR

Total RNA was isolated from PC12 cells at various stages of differentiation using Isogen according to the manufacturer's instructions, then 1 mg was reverse-transcribed using oligo d(T). The PCR primers, designed using the sequence for rat NP25 cDNA (accession number AF459788), were 5'-AAAGGATCCATGGCTAACAGGGGTCCGAGC-3'(sense) and 5'-AAAGAATTCATTACATGATCTGCCTGGGCATCCC- 3 '(antisense); these were used to amplify a 619 bp fragment of rat NP25 cDNA. The primers for the control, rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH), were 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3'(sense)and 5'-CATGTAGGCCATGAGGTCCACCAC-3'(antisense); these were used to amplify a 983 bp rat G3PDH cDNA. The PCR conditions used were 26 cycles of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min, and extension at 72℃ for 1 min. The amplified DNA fragments were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The intensity of the bands was quantified using a densitometer

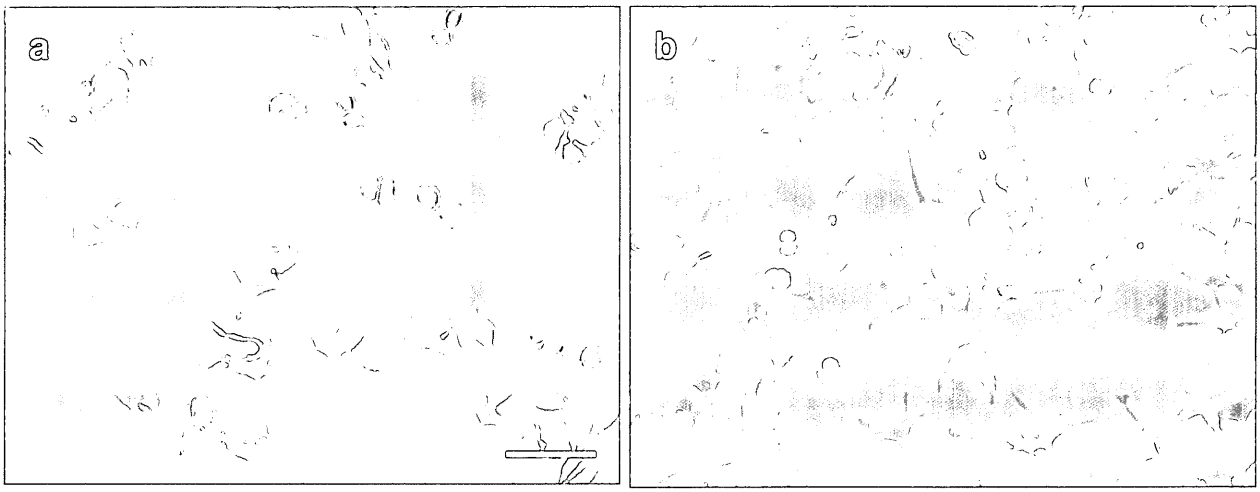

Fig. 1 Phase contrast micrographs of PC12 cells. (a) Untreated PC12 cells ; (b) PC12 cells after differentiation by NGF for 96 h. Scale bar, 50μm.

(ATTO Densitograph, Tokyo, Japan).

Western blot analysis

PC12 cells and NSCs at various differentiation stages were suspended in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and "Complete"). Adult female mouse (C57BL/6) brain tissue was suspended in ice-cold lysis buffer and sonicated for approximately 30 second. Insoluble material was removed by centrifugation at 13,000× g for 20 min at 4℃. The protein concentration of the supernatant was determined using the Bio-Rad protein assay, then the proteins (30μg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels, and electrophoretically transferred to polyvinylidene difluoride membranes. Blocking was performed for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skimmed milk. The membranes were incubated for 1 h at room temperature with rabbit antibodies recognizing either NP25 (1:2,000) or actin (Sigma; 1:5,000) in blocking buffer, then, after washing in TBST, for 1 h at room temperature with horseradish peroxidase-coupled anti-rabbit IgG antibody at a dilution of 1:5,000 in TBST. Labeled bands were detected using the ImmunoStar system and the intensity of the bands quantified using a densitometer.

Statistical analysis

Three independent experiments were performed and the data expressed as the mean±S.D. Student's t test was used to assess the significance of the differences, a p value less than 0.05 being considered statistically significant.

RESULTS

NP25 mRNA and protein levels increase during neuronal differentiation of PC12 cells

PC12 cells are often used neuronal cell differentiation model. We treated PC12 cells with NGF and observed neurite outgrowth as shown in Fig. 1.

Using these cells, we first examined changes in NP25 mRNA during NGF-induced differentiation by RT-PCR. As shown in Fig. 2, a significant increase to 150% of the zero hour levels was seen after 48 h of NGF treatment, but, at 96 h after NGF addition, the levels were below the basal value. In undifferentiated cultures, NP25 mRNA levels gradually decreased with time. No significant changes were observed in G 3 PDH mRNA levels during the time-course examined.

We then examined changes in NP25 protein using Western blotting. Our affinity-purified antibody raised against peptide 146-157 of rat NP25 recognized a band of 22.4 kDa in a PC12 cell lysate, and binding was inhibited by the preincubation of the antibody with GST-NP25. In addition, the antibody recognized a 22.4 kDa band in lysates of NSCs and adult mouse brain (data not shown). As shown by the Western blots in Fig. 3, the amount of NP25 protein showed a slight, but not significant increase, after 48 h of NGF treatment, and a significant increase to 150% of the zero hour value after 96 h of NGF treatment. In non-NGF-treated cultures, NP25 protein levels were unchanged at 48 h and slightly decreased at 96 h. No significant changes were observed in the levels of the reference protein, actin.

The results show that, during neuronal differentiation of PC12 cells, there was a significant increase in NP25 mRNA,

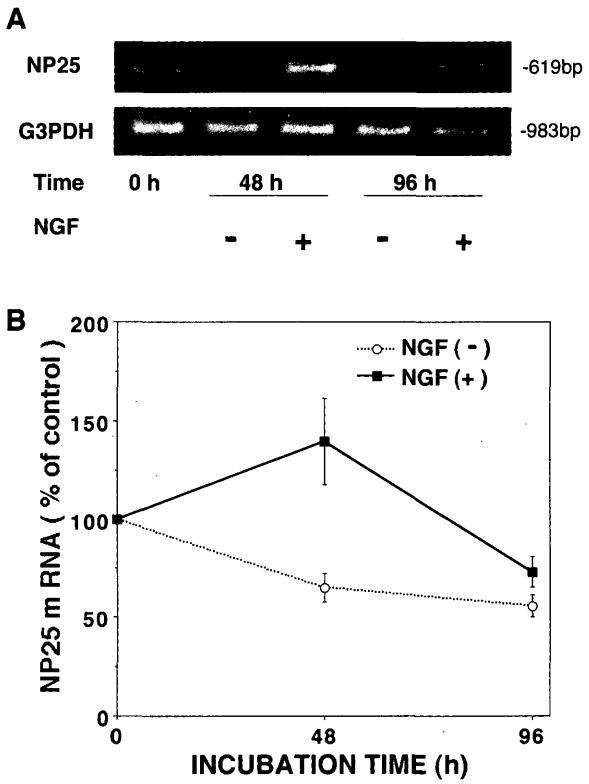

Fig. 2 NGF-induced increases in NP25 mRNA levels in PC12 cells. PC12 cells were incubated for 48 or 96 h in the presence or absence of NGF, then NP25 and G3PDH mRNA levels were analyzed by RT-PCR. (A) : Gels showing that bands of the expected sizes. (B) : Densitometric analysis of the NP25 band expressed as a percentage of that in untreated cells. The values are the mean±S. D. of 3 independent experiments.

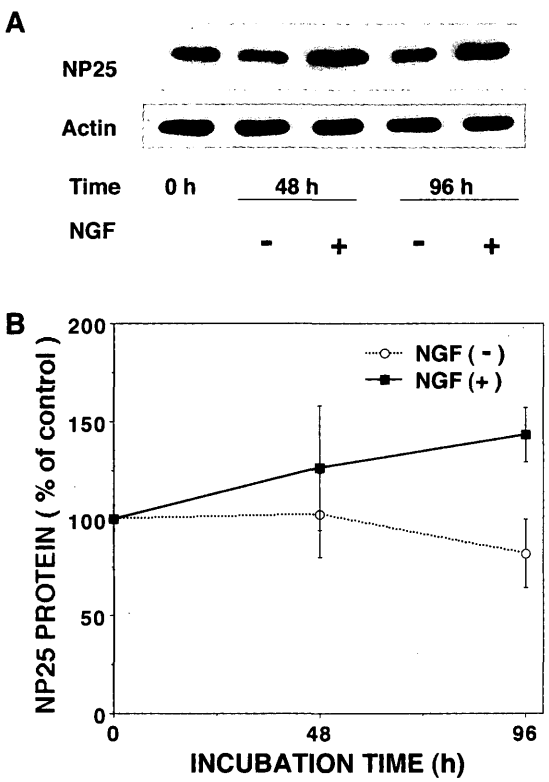

Fig. 3 NGF-induced increases in NP25 protein levels in PC12 cells. PC12 cells were treated as in Fig. 2, and the levels of NP25 protein analyzed by Western blotting. (A) : Changes in NP25 protein levels during PC12 cell differentiation. (B) : The intensity of each band was analyzed by densitometry and expressed as a percentage of that in untreated cells. The values are the means±S.D. of the results of 3 independent experiments.

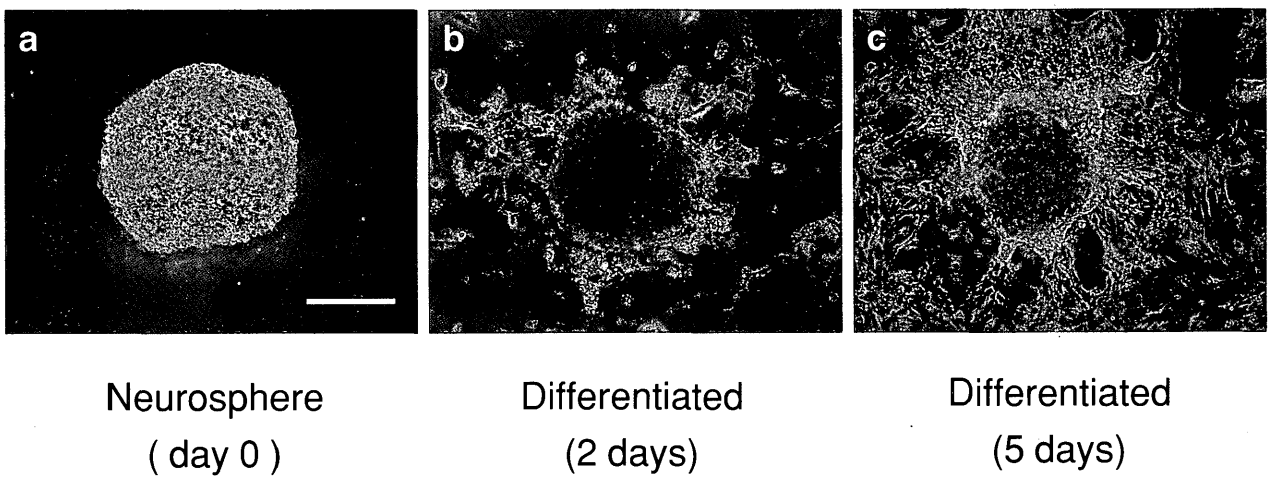

Fig. 4 Phase contrast micrographs of primary neurospheres and differentiated neural stem cells. (a) Untreated primary neurospheres (day 0) ; (b and c) Neural stem cells after differentiation in 10% FBS for 2 days (b) or 5 days (c). Scale bar, 50μm.

followed by an increase in NP25 protein.

NP25 protein levels increase during neural differentiation of NSCs

To examine changes in NP25 protein during NSC differentiation, primary mouse striatal neurospheres were treated for 2-5 days with either 1% FBS plus HGF (20ng/m ℓ) or 10% FBS alone. Adult mouse brain was also examined as a control. As shown in Fig. 4, NSCs grown in 10% FBS-containing medium underwent drastic morphological changes.

As shown by the Western blots in Fig. 5, NP25 protein levels in untreated neurospheres were approximately 10% of those in the adult mouse brain, and increased significantly during differentiation in a time-dependent manner, irrespec-

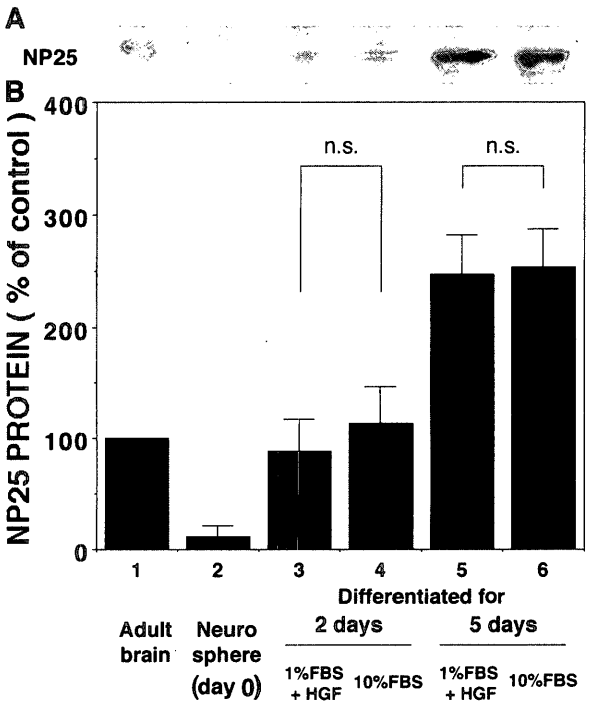

Fig. 5 Differentiation-induced increases in NP25 protein levels in NSCs. Primary neurospheres were either untreated (day 0 ; lane /column 2) or treated for 2 days (lanes/columns 3 and 4) or 5 days (lanes/columns 5 and 6) with 1% FBS plus 20 ng/m ℓ of HGF (lanes/columns 3 and 5) or 10% FBS (lanes/columns 4 and 6). The levels of NP25 protein were analyzed by Western blotting. (A) : Changes in NP25 protein levels in neural stem cells. (B) : The intensity of each band was analyzed by densitometry and plotted as a percentage of that of the same band in adult brain (lane/column 1). The values shown are the mean±S.D. for 3 independent experiments.

tive of whether differentiation was induced by 10% FBS or 1% FBS plus HGF. At day 5, the levels of NP25 protein were significantly increased 2.5-fold compared to adult brain and 25-fold compared to untreated neurospheres. It has been reported that, in 10%FBS, most neurospheres differentiate into astrocytes, whereas, in 1% FBS plus HGF, about 50% differentiate into neurons, 45% into astrocytes, and 5% into oligodendrocytes[9]; these results were confirmed in our experiments (data not shown). No significant differences were seen between cells grown in 1% FBS plus HGF and those grown in 10% FBS.

These results showed that NSCs expressed a relatively low amount of NP25 protein compared to differentiated brain tissues, and that the amount of NP25 protein increased greatly during neural differentiation from NSCs to astrocytes and neurons.

DISCUSSION

It is well documented that neural differentiation processes, such as neurite extension, involve the dynamic reorganization of actin filaments[11]. Regulation of actin dynamics is therefore crucial for precise neuronal development.

NP25 shows high homology with members of the calponin family of actin-binding proteins, such as SM22 and calponin (63% and 45% identity of amino acids, respectively). These proteins are major components of differentiated smooth muscle cells, showing actin-binding properties. Increased SM 22 expression has been reported to correlate with muscle cell differentiation[12)~14)]. In addition, it was recently reported that SM22 and calponin regulate actin filament stability[15)].

The present experiments showed that levels of NP25 mRNA and protein increased during the NGF-induced neuronal differentiation of PC12 cells. NP25 protein also increased during the neural differentiation of NSCs. Ren et al[1)] reported that no significant change was observed during PC12 differentiation induced with NGF. We do not have good explanation for this discrepancy, though there are many types of PC12 cells and some of them might not be good for differentiation. SM22 and calponin are only present in smooth muscle cells, whereas NP25 is expressed only in neural system. It is reasonable to consider that NP25 might play a similar role (s) in neural cells to those in smooth muscle cells by SM22 or calponin. In this context, it is interesting to note that we also observed that NP25 interacts with filamentous actin in SK-N-SH cells[16)]. Thus, it is possible that NP25, which binds to actin filaments, might regulate actin dynamics during neurite extension in neural differentiation. Although we have demonstrated that NP25 increases during neural differentiation, further investigations are needed to clarify the biological significance of this. Experiments with neuronal model cells overexpressing or lacking NP25 would be very helpful in future investigations and are currently being undertaken in our laboratory.

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Neuronal Protein 25は神経細胞の分化により増加する

中枢神経組織にのみ発現するタンパク質 Neuronal Protein 25 (NP25) はアクチン結合タンパク質の Calponin ファミリーと高い相同性を示すが、その機能に関する報告はほとんどない。本研究では NP25の機能を明らかにするため、神経細胞モデルである PC12及び神経幹細胞 neural stem cells (NSCs) を分化誘導し、その発現量を解析した。PC12細胞は NGF を添加し分化誘導した。また NSCs は mouse embryos から得た neurosphere を HGF あるいは FBS を用いて neuron と astrocyte に分化誘導した。NP25の発現量は RT-PCR 法と Western blot 法により解析した。その結果、PC12細胞に NGF を添加し分化誘導すると、NP25の mRNA は約1.5倍に増加した。タンパク質レベルでも NP25は約1.5倍に増加した。neuroshere では NP25の発現は低値であったが、分化が進むに従い著明に増加した。neuron あるいは astrocyte への分化では発現量に有意差を認めなかった。さらに我々は別の実験において NP25がアクチン結合タンパク質であることを証明している。以上の結果から NP25は神経細胞の分化に伴い著明に増加し、神経細胞におけるアクチンの機能制御に重要な役割をもつ可能性が示唆された。