

Developmental Expression of Neurofilament 3 (NF-M) in the Cultured Rat Cortical Neurons

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Neurofilament (NF) proteins constitute the major intermediate filament type in adult neurons. They are made up by the copolymerization of the neurofilament light (NF-L, 61 kDa), medium (NF-M, 90 kDa), and heavy (NF-H, 115 kDa) proteins. Although neurofilaments play a crucial role in neuronal growth, organization, shape, and plasticity, their expression pattern and cellular distribution in the developing neurons remain unknown. In this study, we have produced a rabbit polyclonal antibody specific to NF-M and investigated expression of NF-M in cultured cortical neurons. Immunostaining of 12 and 24 h cultures revealed strong expression of NF-M in axonal growth cone and in the region of a soma toward the axon. Doublestaining of 4 and 14 DIV cortical neurons with NF-M and PSD95 antibodies revealed that both axon and dendrites were stained intensely with NF-M antibody, and that NF-M immunostaining along dendrites is often punctate and colocalize with PSD95 puncta, indicating that the puncta represent postsynaptic spines. Presence of NF-M in the postsynaptic spine was also indicated by immunoblot analysis of the postsynaptic density fraction. Taken together, our results show intensive targeting of NF-M into axons in the early axonal development, and into spines in mature neurons, indicating its important functions in axon and spine development.

Key words – cortical culture, growth cone, immunocytochemistry, neurofilament, spine,

The neuronal cytoskeleton is composed of three interconnected filaments: the actin microfilaments, microtubules, and intermediate filaments (IFs). Whereas microtubules and actin filaments are dynamic structures known to play key roles in neuronal development, the functions of IF proteins in neurons have remained more elusive. IF proteins constitute a large gene family that confers intracellular scaffold and mechanical stability to eukaryotic cells.

IFs constitute a family of proteins expressed in different cell types[7,8]. Five major types of IF proteins are expressed in adult neurons: the three neurofilament proteins, peripherin, and α -internexin. Neurofilament proteins constitute the major IF type in adult neurons. They are made up by the copolymerization of the neurofilament light (NF-L, 61 kDa), medium (NF-M, 90 kDa), and heavy (NF-H, 115 kDa) proteins[11]. Neurofilaments are obligate heteropolymers requiring NF-L with either NF-M or NF-H for proper polymer formation into 10 nm-thick filaments[3,13].

Although neurofilaments play a crucial role in neuronal growth, organization, shape, and plasticity, their expression pattern and cellular distribution in the developing neurons remain unknown. Following their synthesis in the perikaryon, NF proteins are rapidly assembled into filamentous structures and then translocated into the axons. Many neurons extend their axon over great distances, up to 1 m in human, to form synapses with appropriate target cells. It is well established that actin filaments and microtubules play key roles in axonal outgrowth and stability. In a recent report, Lopez-Picon *et al*[15] found that, during the first postnatal week, NF-M was the most abundantly expressed NF, followed by NF-L, whereas the expression of NF-H was very low. Through P7-P14, the expression of NF-H increased dramatically and later began to plateau, as also occurred in the expression of NF-M and NF-L.

In this work, we investigated the expression of NF-M during morphological development of cortical neurons. We found that NF-M is highly expressed in the growth cone and perikaryon toward axon in the early stage of development, and then distributed into dendrites in later stage, often colocalizing with PSD95, a postsynaptic marker.

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Materials and Methods

Generation of antibody JS-1Ba

A DNA fragment corresponding to the N-terminal region (aa 173-230) of eukaryotic elongation factor 1Ba (eEF1Ba) was amplified by PCR (92°C, 2 min; 50°C, 1 min; 72°C, 2 min; 35 cycles) from a rat brain lambda ZAPII cDNA library (Stratagene) with sense (5'-GGATCCACAGCTGCGG-GAGGAGC-3') and antisense (5'-GAATCTTGTTGAAAG-CTGCGAT-3') primers, and cloned in-frame into BamHI/EcoRI sites of the pGEX1 vector (Pharmacia Biotech, Piscataway, NJ) to create a glutathione sulfotransferase (GST) fusion construct. The recombinant plasmids were transformed into *Escherichia coli* BL21 and cultured at 30°C, induced with 0.1 mM isopropyl- β -thiogalactopyranoside (IPTG) for 5 h at 30°C, and the cells pelleted at 5,000 \times g for 10 min. Inclusion bodies were prepared from the pellet and fractionated by sodium dodecyl sulfate (SDS)-8% polyacrylamide gel electrophoresis (PAGE). The GST-eEF1Da fusion protein band was identified by staining with Coomassie dye, electroeluted, mixed with Freund's adjuvant and used for immunization. Antibodies, named as JS-1Ba (see Results and Discussion) were affinity-purified with GST-eEF1Da blotted onto nitrocellulose membrane. In brief, GST-eEF1Da (~20 μ g) was transferred to a nitrocellulose membrane, and this was cut into small pieces (4 \times 4 mm) and blocked overnight at 4°C with TTBS [0.2% Tween-20, 10 mM Tris-HCl (pH 7.5), and 0.2 M NaCl] in a 1.5 ml microtube. After washing the membrane with TTBS (4 \times 10 min) at room temperature (RT), 1.0 ml of antiserum (diluted 5 \times in 10 mM Tris-HCl, pH 7.5 plus 1.0 mM PMSF) was added and incubated for 2 h at RT. Following several more washes, the antibody was eluted for 5 min with 500 μ l of 100 mM glycine-HCl (pH 2.5) and the pH immediately adjusted to 8.0 with 1.0 M Tris-HCl (pH 8.5).

PSD preparation and detergent extraction

'One-Triton' postsynaptic density (PSD) fractions were prepared from rat forebrain (FB) and cerebellum (CBL) by washing synaptosomes with 0.5% Triton X-100 (Triton) for 15 min at 4°C, as described previously[16]. In brief, synaptosomes were isolated from homogenates by sucrose step-gradient centrifugation using 0.85, 1.0 and 1.2 M sucrose layers, and then extracted with 0.5% Triton X-100 for 15 min at 4°C. The resulting 'One-Triton' PSD fraction was pelleted by centrifugation at 36,800 \times g for 45 min. In the

detergent extraction experiments, the fraction was extracted again at 4°C, either with 1.0% Triton or 1.0% n-octyl glucoside (OG) for 15 min or with 3.0% N-lauroyl sarcosine for 10 min. Thereafter, the pellet and supernatant were separated by centrifugation at 201,800 \times g for 1 h at 4°C.

Immunoblot

After SDS-PAGE, proteins were transferred to a nitrocellulose membrane. After incubation of the blots overnight at 4°C in TTBS [0.2% Tween-20, 10 mM Tris-HCl (pH 7.5) and 0.2 M NaCl], a primary antibody was added and further incubated for 2 h at RT. Blots were rinsed in TTBS (4 \times 20 min), and the antigen/antibody complex was visualized with alkaline phosphatase-conjugated secondary antibodies, according to the manufacturer's instructions (Roche, Germany).

Immunoprecipitation

OG-P fractions (600 μ g) were brought to 2.5% SDS and boiled for 3 min. The volume was adjusted to 1.0 ml with RIPA buffer [10 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 150 mM NaCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.1% SDS (added after boiling step)]. Then, the affinity-purified rabbit JS-1Ba antibody (1:50) was added. After incubation of the mixture for 2 h at 4°C, protein A-agarose slurry (1:1) was added and further incubated for 2 h. The agarose beads were washed with RIPA buffer (5 \times 5 min, 1.0 ml each). After a brief spin, supernatant was discarded and SDS-gel sample buffer was added to the mixture. After the mixture was boiled for 3 min and centrifuged briefly, the supernatant was applied to SDS-PAGE.

Enzymatic digestion of protein in-gel

Protein spots were enzymatically digested in-gel in a manner similar to that previously described by Shevchenko *et al*[21] and using modified porcine trypsin. Gel pieces were washed with 50% acetonitrile to remove SDS, salt and stain, dried to remove solvent and then rehydrated with trypsin (8~10 ng/ μ l) and incubated 8~10 h at 37°C. The proteolytic reaction was terminated by addition of 5 μ l 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extraction of gel pieces with 50% aqueous acetonitrile. After concentration the peptide mixture was desalted using C₁₈ZipTips (Millipore), and peptides eluted in 1~5 μ l of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α -cyano-4-hydroxycinnamic acid

in 50% aqueous acetonitrile, and 1 μ l of mixture spotted onto a target plate.

Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry and database search

Protein analysis were performed using a Ettan MALDI-TOF (Amersham Biosciences). Peptides were evaporated with a N₂ laser at 337 nm, and using a delayed extraction approach. They were accelerated with 20 Kv injection pulse for time of flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak mass/charge (*m/z*) (842.510, 2211.1046) as internal standards.

Neuronal culture and immunocytochemistry

Embryonic day 18 (E18) cortical cells were dissociated from E18 rat cerebra by triturating trypsin-treated tissues, and were grown on poly-D-lysine-coated coverslips (1,000~1,500 cells/mm²) in the Neurobasal medium supplemented with B27 and 0.25 mM glutamax I (Invitrogen, Carlsbad, CA), as previously described [1,4,16,17,18]. Cells were fixed with methanol for 20 min at -20°C and then doublestained with mouse monoclonal anti-PSD95 (Upstate Biotechnology) and rabbit polyclonal JS-1Ba antibodies, according to Moon *et al.* [18]. The primary antibodies were visualized with Alexa Fluor 568-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000, Molecular Probes; Leiden, The Netherlands). Images of the immunostained cells were obtained using a fluorescence microscope equipped with filter systems I3 and N2.1 (Leica Microsystems, Wetzlar, Germany). There was no bleed-through between the two channels. Fluorescence images were processed with Adobe Photoshop 5.0 (Adobe Systems Inc.).

Results and Discussion

Generation of a rabbit polyclonal antibody JS-1Ba against GST-eEF1Ba.

Generation of antibody against NF-M was fortuitous. We amplified a DNA fragment corresponding to amino acid 173-230 of eEF1Ba by PCR (Fig. 1A, lane 1). The PCR fragment (Fig. 1A, arrowhead) was inserted into pGEX1 vector

(Fig. 1A, lane 2). The in-frame insertion was confirmed by DNA sequencing. The resulting GST-eEF1Ba protein was expressed in *E. coli* (Fig. 1B, arrowhead) as described in detail in Materials and Methods. SDS-PAGE analysis showed the expected fusion protein of ~45 kDa. The fusion protein band was cut out of the gel and used to generate an antiserum. Antibody was affinity-purified using the GST-eEF1Ba fusion protein and named as JS-1Ba. Immunoblotting showed that the antibody recognizes two protein bands of ~25 and ~40 kDa, respectively, in uninduced bacterial lysate (Fig. 1C, lane 1). The protein with a smaller molecular size was not recognized in the induced lane (Fig. 1C, lane 2). The larger one was still recognized in the induced lane, together with a new band with molecular size of ~45 kDa (Fig. 1C, lane 2, arrowhead), which matched well to the expected fusion protein.

JS-1Ba recognizes NF-M

Immunoblotting was carried out to test specificity of the antibody using brain homogenates and the postsynaptic density (PSD) fraction. Unexpectedly, the JS-1Ba recognized a ~130 kDa protein band, instead of eEF1Ba (~34 kDa), in both rat forebrain homogenate and PSD fraction (Fig. 2A, lane 1 and 2, respectively). The recognition was very specific

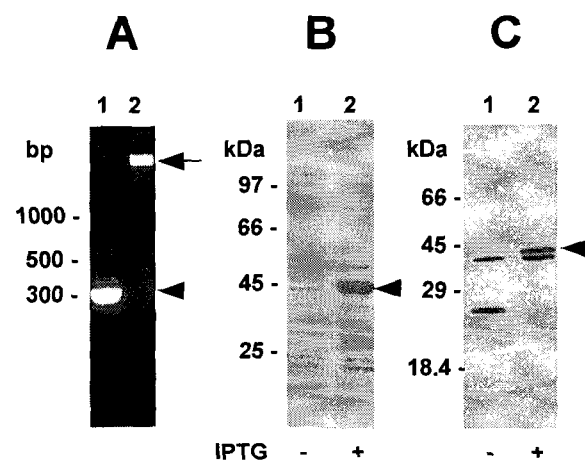


Fig. 1. Generation of a rabbit polyclonal antibody against GST-eEF1Ba. A. Agarose gel electrophoresis. Lane 1, PCR product. Lane 2, pGEX-eEF1Ba digested with BamHI and EcoRI. Arrow, vector. Arrowhead, insert. B. SDS-PAGE (10%). Lane 1, IPTG-uninduced bacterial lysate. Lane 2, IPTG-induced bacterial lysate. Arrowhead, the position of GST fusion protein. C. Western blot. Uninduced and induced bacterial lysates were electrophoresed in 12% SDS-gel, transferred to a nitrocellulose membrane and immunoblotted with JS-1Ba. Arrowhead, the position of the fusion protein.

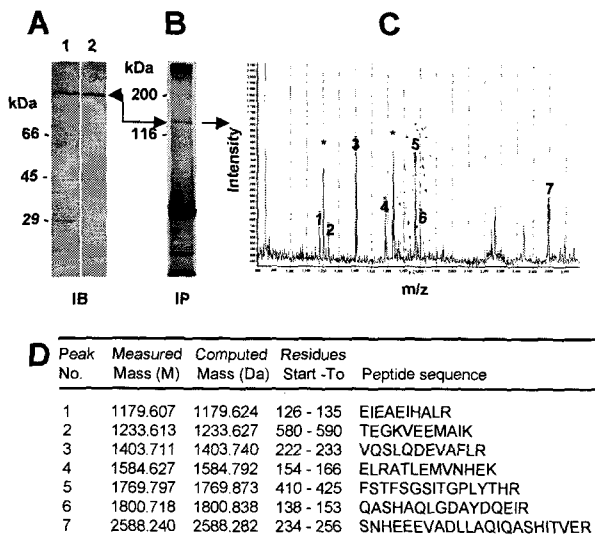


Fig. 2. Identification of the JS-1Ba-recognized 130 kDa protein as NF-M. A, Immunoblots. Brain homogenate (lane 1) and PSD fraction were immunoblotted with JS-1Ba. B, Immunoprecipitation. The 130 kDa protein was immunoprecipitated by JS-1Ba. C, MALDI-TOF. The immunoprecipitated band was subjected to mass spectrometry (MALDI-TOF). The masses of numbered peaks matched with the fragments in NF-M. The peaks with asterisks indicate no matches with NF-M. D, The peak information. Peak numbers correspond to those in C.

because there were virtually no other bands recognized. Since this result was completely unexpected, we identified the 130 kDa protein. For identification, the ~130 kDa protein was immunoprecipitated (Fig. 2B) and subjected to MALDI-TOF analysis (Fig. 2C). Database search using ProFound [The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe)] identified the 7 out of 9 peptide masses in NF-M (NCBI ACCESSION NP_058725)[23] (Fig. 2D). Since the apparent molecular size of NF-M in SDS-PAGE is ~130 kDa[22], this result confirms that the 130 kDa protein recognized by the JS-1Ba is indeed NF-M. The reason why an antibody raised by eEF1Ba antigen recognizes NF-M instead of its orthogonal protein is not known. There was no similarity in both DNA and protein sequences between the two proteins. Variation of three reading frames of the eEF1Ba DNA sequence did not result in any sequence similarity to NF-M. However, it is possible that the GST-fusion protein might form 3-dimensional configuration which is very similar to that of NF-M, and this configuration worked as a strong antigenic epitope.

Association of NF-M with the PSD fraction

Since immunoblotting of the PSD fraction with JS-1Ba

antibody recognized a strong and specific band at 130 kDa, which is identified as NF-M, we carried out detergent extraction experiments to find its association characteristics with the PSD. Non-ionic detergents Triton X-100 (1.0%) and *n*-octylglucoside OG (1.0%) were not efficient in dissociating NF-M from the PSD core (Fig. 3, Triton and OG). Very harsh detergent *N*-lauroyl sarcosinate (Sarc) dissociated most NF-M from the PSD core (Fig. 3, Sarc). Interestingly, salt (1.0 M) was also very efficient in dissociating NF-M from the PSD core (Fig. 3, NaCl). These data indicate that NF-M is associated with the core of the PSD through ionic interactions.

Expression of NF-M in the early stage of morphological development

The subcellular distribution of NF-M in the early stage of morphological development of neurons was investigated. For this purpose we cultured rat cortical cells that were dissociated from E18 brains. Neurons begin to extend neurites from ~2 h after plating and most neurons have growth cones at 12~24 h *in vitro*. Immunostaining of ~12 h cultures revealed strong expression of NF-M in the processes with growth cones (Fig. 4A, block arrows). Interestingly, strong immunoreactivity was also associated with the region of soma toward the axon (Fig. 4A, arrows). The opposite side to the axon was not stained strongly. At 24 h, the immunoreactivity was still stronger in the axon (Fig. 4B, a series of small arrows) and growth cones (block arrows) than in dendrites. Strong immunostaining in the soma region toward axon was also seen (arrows). In an enlarged image of a growth cone, string-like arrays could be seen radiating from center of the growth cones, suggesting that NF-M is expressed along filopodia of the growth cone (Fig. 4B, box).

Expression of NF-M in the maturing and mature neuron

To investigate the expression of NF-M in morphologically

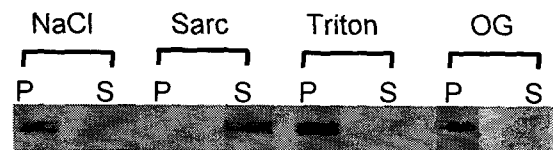


Fig. 3. Association of NF-M with the PSD fraction. The PSD fraction was extracted with salt (NaCl, 1.0 M), *N*-lauroyl sarcosinate (Sarc), Triton X-100 (1.0%, Triton), and *n*-octyl glucoside (OG, 1.0%). Pellet (P) and supernatant (S) fractions were subjected to immunoblot analysis with JS-1Ba.

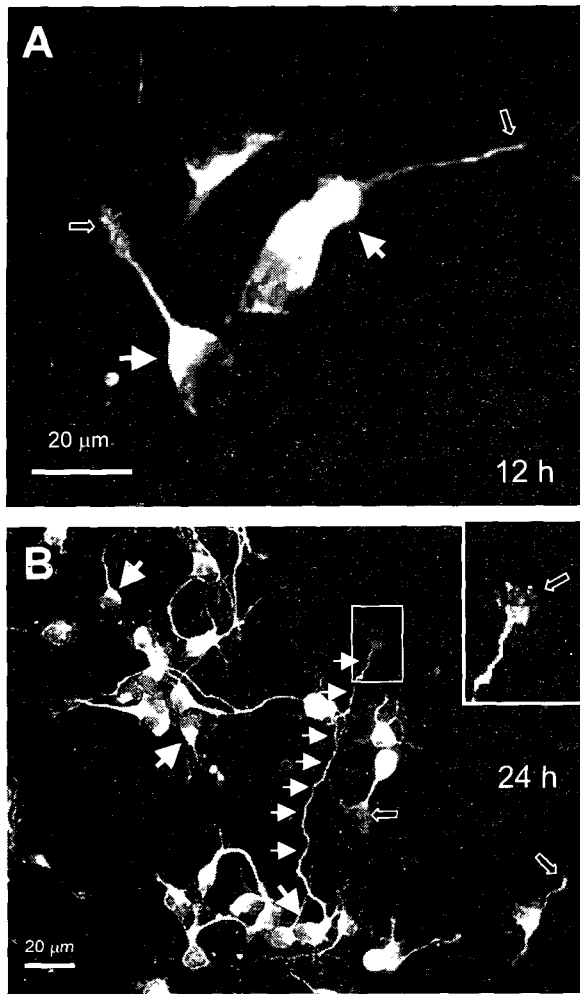


Fig. 4. Immunocytochemistry showing expression of NF-M in early stage of neuronal development. E18 rat cortical cells were grown in Neurobasal media and immunostained with affinity-purified JS-1Ba at 12 h (A) or 24 h (B) *in vitro*. Block arrow, growth cone. Large arrow, perikarya close to axon. A series of small arrows in B indicate an axon, of which growth cone is shown enlarged in the inset. Bar, 20 μ m.

maturing neurons, 4 DIV cortical cultures were immunostained with affinity-purified JS-1Ba. At this stage of maturation, intense immunostaining was still associated with the soma region toward the axon (Fig. 5A, big arrows). Axons were stained intensely (Fig. 5A, a series of small arrows). Dendrites were stained almost as intensely as axons. Since spines begin to appear at this stage of development, we investigated localization of NF-M in spines by double-staining the culture with NF-M and PSD95 antibodies. The latter is a marker for PSD. Although the density of PSD95 puncta is low at this developmental stage, the positions of the most PSD95 puncta overlapped with those of NF-M (Fig.

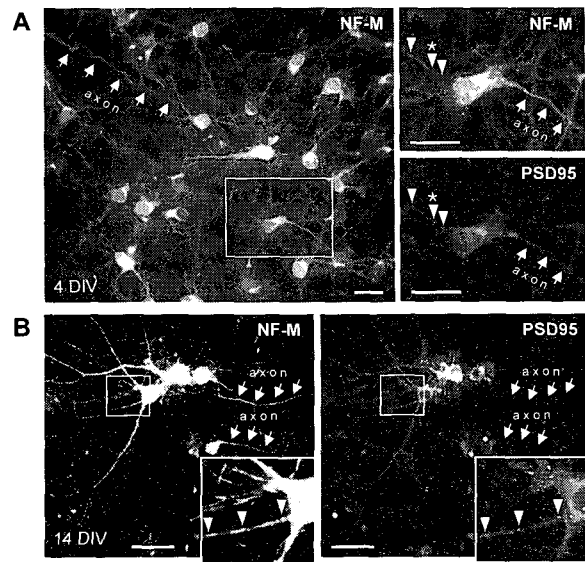


Fig. 5. Immunocytochemistry showing expression of NF-M in maturing stage of neuronal development. E18 rat cortical cells were grown in Neurobasal media and double-stained with affinity-purified JS-1Ba and PSD95 antibodies on 4 DIV h (A) or 14 DIV (B). Axons are indicated by a series of arrows. NF-M and PSD95 punctae were marked by arrowhead. An example of punctae which does not colocalize each other is marked by an arrowhead with asterisk. Insets are enlarged images of the corresponding boxed areas of each panel. Bar, 20 μ m.

5A, arrowheads in insets), indicating that two proteins are colocalized. Where NF-M colocalizes with PSD95 puncta, NF-M immunostain is also punctate. This fact, together with the fact that NF-M colocalize with PSD95, strongly suggests that NF-M puncta represents spines. However, some of the two kinds of punctae did not colocalize each other (Fig. 5A, arrowhead with an asterisk in insets).

Next, the expression of NF-M in the mature neurons were investigated using 14 DIV cortical cultures. The cells were double-stained with NF-M and PSD95 antibodies. In this mature neuron, both axon (Fig. 5B, a series of arrows) and dendrites were stained intensely. The density of PSD puncta is much higher than that of 4 DIV, and most of them colocalize with NF-M (arrows in the inset of Fig. 5B). Again, where FM-M colocalizes with PSD95, they are punctate, indicating that the puncta represent spines. Taken together, these immunocytochemical staining results strongly indicate that NF-M is expressed in spines of mature neurons.

In this work, we have found that NF-M is highly targeted to axon growth cone in the early stage of morphological development of neurons. This result strongly suggests NF-M's function in the development of axons. Indeed, the gene tar-

getting approach yielded results regarding the specific roles of NF-M and NF-H subunits in IF assembly and function. The carboxy terminals of the NF-M and NF-H subunits form sidearm projections at the periphery of neurofilaments [2,10]. It is believed that phosphorylation of multiple Lys-Ser-Pro (KSP) repeats in the carboxy-terminal region, increases neurofilament spacing. The complete loss of NF-H in mice with targeted disruption of the gene had little effect on radial growth of motor axons during development [5, 20,24]. Also, the loss of NF-H tail and all of its phosphorylation sites did not affect axonal diameter of motor and sensory axons [19]. Interestingly, it turns out that the NF-M protein is more important than NF-H as mediator of axonal growth. The targeted disruption of NF-M gene or NF-M together with NF-H genes resulted in a two- to threefold reduction in the caliber of large myelinated axons [5,9]. The absence of NF-M caused decreases in NF-L levels and in axonal neurofilament content resulting in axonal atrophy [6]. Mice with null mutations in both NF-M and NF-H genes develop atrophy in ventral and dorsal roots as well as a hind limb paralysis with aging. So far, there is no indication that the other neuronal IF proteins, peripherin and α -internexin, are modulators of axon caliber. Disrupting the peripherin or α -internexin genes in mice had no effect on the caliber of axons [12,14]. Since this study showed that NF-M is highly targeted to axons in early morphological development, our result confirm the crucial role of NF-M in the initial development of axons.

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초록 : 배양한 흰쥐 대뇌신경세포에서 신경미세섬유 3(NF-M)의 발생학적 표현

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신경미세섬유(neurofilament, NF) 단백질은 신경세포의 주된 중간세사로서, NF-L (61 kDa), NF-M (90 kDa) 및 NF-H (115 kDa) 단백질의 공동중합체로 구성된다. 신경세사섬유는 신경세포의 성장, 구성, 형태 및 가소성에 중요한 역할을 하지만 발생학적 표현에 대하여는 아직 잘 알려지지 않았다. 본 연구에서는 NF-M에 특이한 항체를 제조하여 배양한 대뇌신경세포에서 NF-M의 표현을 조사하였다. 배양 12 및 24 시간 세포에서 NF-M은 축삭과 그 성장추 그리고 축삭에 가까운 세포체에 강하게 표현하였다. 배양 4 및 14일 신경세포를 NF-M과 PSD95 항체로 이중염색한 결과 NF-M은 축삭과 가지돌기에 공히 강하게 표현되었으며, PSD95와 같이 위치할 경우에는 점박이로 나타났다. 면역염색에서도 NF-M이 PSD 분획에서 검출되었는데, 따라서 이 점박이는 가지돌기가시임을 시사한다. 본 연구의 결과는 NF-M이 신경세포의 초기 형태발달과정에서 축삭으로 강하게 물러가며, 성숙한 신경세포에서는 가지돌기 및 가지돌기가시에도 위치하여 특정기능을 수행함을 시사한다.