Molecular characterization of a transport vesicle protein
Neurensin-2, a homologue of Neurensin-1,
expressed in neural cells

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Abbreviations:
FCS, fetal calf serum
SDS, sodium dodecylsulfate
DMEM, Dulbecco’s modified Eagle’s medium
PBS, phosphate-buffered saline
GST, glutathione S-transferase
NGF, nerve growth factor
DRG, dorsal root ganglion
CNS, central nervous system

ABSTRACT
We have isolated and characterized a novel cDNA encoding a small neuronal membrane protein showing high sequence homology to Neuro-p24/Neurensin-1, a protein containing a microtubule-associated domain at the carboxyl-terminus and exclusively localized to small vesicles of neurons. The newly identified Neurensin-2 constitutes two-membrane spanning domains but not the microtubule-binding domain, with a molecular mass of 28 kDa. Neurensin-2 mRNA is expressed only in brain, whereas the protein expressed in various neurons including those of the thalamus/hypothalamus and hippocampus, of postnatally developing mice. While the levels of Neurensin-1 mRNA and protein in retinoic acid-exposed mouse neuroblastoma Neuro2a cells increased, those of Neurensin-2 mRNA and protein remained unchanged. When the Neurensin-2 cDNA was transfected into Neuro2a cells, Neurensin-2 was expressed in small vesicles including lysosomes in the perinuclear region. On the cotransfection of Neurensin-1 and -2 cDNA into Neuro2a cells, Neurensin-2 was mainly found in small vesicles of the cell body and Neurensin-1 in those of growth cones. In nerve growth factor-stimulated PC12 cells, the intracellular localization of these proteins also differed. Furthermore, immunohistochemical staining of mouse brain revealed that Neurensin-1 and -2 had a similar distribution in many regions such as the Diagonal band, hippocampus, amygdaloid nucleus, and habenula nucleus, but differed in the intracellular localization as follows: Neurensin-1 was found mainly in neuritic processes, while Neurensin-2 was found in small vesicles in neural cells, but their localizations of the vesicles are not always the same by each other, suggesting that they are under separate regulation.

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1. Introduction

Neurons are signaling cells with a unique polarized composition, which is paralleled by a highly specific microtubule network. Neurons vigorously extend neurites during the development of nervous tissue, and this process is a characteristic neuronal feature that is also observed in axonal regeneration and dendritic plasticity during adulthood. Since the distance between a cell body and the neuronal periphery can be quite extensive, the localization and transport of membrane-bound organelles over microtubule tracks are interacted and can be tightly organized (Baas, 1997; Rogers and Gelfand, 2000). Since protein synthesis takes place in the cell body, the neuron must move vital materials made in this region to the most distal parts of axons using elaborate transport systems (Dent and Gertler, 2003; Greka et al., 2003; Mori and Morii, 2002). Although there is complex machinery involved in vesicle transport, and membrane fusion to expand the plasma membrane, the precise mechanism for nerve fiber extension needs to be explored in more detail (Garcia et al., 1995; Martinez-Arca et al., 2000; Scott and Luo, 2001; Wong and Ghosh, 2002).

Neurensin-1/Neuro-p24 encodes a membranous protein with a molecular mass of 24 kDa (Kadota et al., 1997). We previously showed its molecular structure and regional distribution in the brain and cultured neurons. Namely, Neurensin-1 is specifically localized to growing neurites and not present in synaptic vesicles. We also showed that Neurensin-1 plays certain roles in the transport of small vesicles to the growing distal end of neurites in association with microtubules, thereby contributing to neurite extension (Araki et al., 2002; Ida et al., 2004). The extension of long branching processes in epithelial COS-7 and neuroblastoma Neuro2a cells was dependent on the transient expression of Neurensin-1, and small vesicles containing Neurensin-1 were often fused with the plasma membrane at the growth cones (Araki et al., 2002). In cultured neuroblastoma Neuro2a cells, the expression of Neurensin-1 was up-regulated when cells were induced to differentiate to neurons with retinoic acid, and immunoreactive vesicles were concentrated at the growth cones. Furthermore, in vivo and in vitro pull-down assays confirmed that Neurensin-1 binds to tubulin, indicating that Neurensin-1 is associated with microtubules (Ida et al., 2004), and that Neurensin-1 plays an essential role in neurite extension. In a computer search of TBLASTIN, we found a novel protein, named Neurensin-2, whose amino acid sequence has similarity to that of Neurensin-1. We report here the neural expression of Neurensin-2 and analyze the different localization and regulation between Neurensin-1 and -2.

2. Results

2.1. Identification, cloning, and expression of analysis of Neurensin-2

A systematic search of the EST databases (Boguski et al., 1993) using mouse Neurensin-1 (Kadota et al., 1997) as a query resulted in several partial Neurensin-like cDNA sequences from human and mouse that did not correspond to the vertebrate Neurensin-1. Specific primers were used to amplify the mouse Neurensin-2 cDNA from the mouse brain cDNA library. They each code for a novel protein of 202 amino acids 28 kDa (Fig. 1). After confirmation of the cDNA, 5’-RACE of Neurensin-2 with the mouse brain Marathon cDNA library (BD Bioscience-Clontech) was carried out. Several different cDNAs were isolated, and the sequence of 5’-upstream of the putative translation initiation site was examined. The cDNAs encode a protein that fits well into a Neurensin alignment based on the conserved two membrane-spanning domains. The protein sequence of the novel Neurensin is clearly longer than that of Neurensin-1, which comprises about 190 amino acids. The overall identity in amino acid sequence with mouse Neurensin-1 was 31% for mouse Neurensin-2 and 41% for human Neurensin-2, respectively. The difference is because of the long C-terminus which did not consist of a domain similar to the microtubule associated proteins-like region (Kadota et

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**Fig. 1** – Alignment of the amino acid sequences of mouse and human Neurensin-1 and -2. The two membrane-spanning domains are underlined. The dotted line indicates the microtubule-associated domain of Neurensin-1.
al., 1997). Computer predictions using the PSORT II program (Nakai and Horton, 1999) indicate that the novel Neurensin does not contain any signal peptide.

The expression of the newly identified Neurensin-2 gene was analyzed by Northern blotting to a filter containing mRNA from different mouse tissues (Fig. 3A). We observed a specific expression in the brain (~1.7 kb), similar to that of Neurensin-1. When Neuro2a cells were induced to differentiate by treatment with 20 μM retinoic acid, the increased expressions of Neurensin-1 mRNA and protein, but not Neurensin-2 mRNA and protein, were observed (Figs. 2A, B). Immunoblot analysis using the developing cerebral cortex revealed that Neurensin-2 was expressed at E15.5, and the high levels at postnatal stages were similar to those seen for Neurensin-1 (Fig. 3B). When their distribution in various tissues of the brain was examined, Neurensin-1/2 was found to be broadly expressed in the cerebrum, and Neurensin-2 was found to be highly expressed in the thalamus/hypothalamus (Fig. 3C).

2.2. Expression of Neurensin-2 in cultured cells

To examine the intracellular localization of Neurensin-2, a plasmid carrying the HA-tagged cDNA was constructed and transfected into COS-7 cells. Immunoblotting was done with anti-HA monoclonal antibody. A band corresponding to a molecular mass of 28 kDa was detected in cells expressing HA-tagged Neurensin-2 (data not shown). The molecular mass of Neurensin-2 was greater than that of HA-tagged Neurensin-1 (24 kDa). Indirect immunofluorescence microscopy with the transfected COS-7 cells revealed that the staining of Neurensin-2 mainly occurred in lysosomes, similar to that of a lysosomal protein, Lamp-2 (Fig. 2C). When transfected Neuro2a cells were stained for Neurensin-2, immunoreactive small vesicles were found in the perinuclear region and also in the processes. Transfected cells were often embellished with numerous fine spikes, whose cell membrane appeared positively stained. On the cotransfection of pcDNA3-Neurensin-2 and pCG-HA-Neurensin-1 into Neuro2a cells, Neurensin-2 was expressed in intracellular organelles, and the organelles of the cell body were densely stained, and the staining of Neurensin-1 was found in intracellular organelles near growth cone (Fig. 3C).

Fig. 2 – Expression of Neurensin-2 in cultured cells. (A) RNA blots of Neurensin-1 and -2 in Neuro2a cells. RNA was isolated from Neuro2a cells treated with 20 μM retinoic acid for 48 h, and Northern blotting was performed with 32P-DNA fragment of Neurensin-1 and -2. (B) Expression of Neurensin-1, and -2 in Neuro2a cells. Cellular proteins from the cells treated as above were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto a membrane. Immunoblotting was performed with anti-Neurensin-1, -2 and actin. (C) Immunofluorescent staining of COS-7 cells expressing Neurensin-2. Cells transfected with pCG-HA-Neurensin-2 were plated on glass coverslips and fixed. After blocking, the fixed cells under permeabilized conditions were incubated with anti-Neurensin-2 antibody (red), followed by a monoclonal antibody for a lysosomal protein, Lamp-2 (green). The merged image confirmed that the dots colocalized. (D) The localization of Neurensin-1 and -2 in Neuro2a cells. The cells were cotransfected with pcDNA3-Neurensin-2 and pCG-HA-Neurensin-1 and incubated for 20 h. They were fixed, permeabilized, and reacted simultaneously with anti-HA (green) and anti-Neurensin-2 (red) to show the localization. (E) Intracellular localization of Neurensin-1, and -2 in NGF-stimulated PC12 cells. The cells co-transfected with pcDNA3-Neurensin-2 and pCG-HA-Neurensin-1 were cultured in the absence or presence of NGF (50 ng/ml). Immunostaining of permeabilized cells was conducted as above. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Thus, these locations were different. Furthermore, intracellular localization of Neurensin-1 and -2 was also examined by transfection of these cDNAs into NGF-stimulated PC12 cells. Neurensin-1 was distributed mainly in the peripheral region of neurites, while Neurensin-2 was expressed in the perinuclear region (Fig. 2E). These results indicated that small vesicles containing Neurensin-1 and -2 were different.

2.3. Localization of Neurensin-2 in the mouse brain

Immunocytochemical staining of mouse brains with anti-Neurensin-2 revealed that the overall distribution of Neurensin-2 was approximately consistent with that of Neurensin-1 (Fig. 4). Neurensin-2 was always found in neural cell bodies and no other types of cells were stained. Neurensin-2-immunoreactive cells were detected in many regions of the limbic system, such as the septum nucleus, horizontal and vertical limbs of the Diagonal band, hippocampus, amygdaloid nucleus, and habenula nucleus. Many immunoreactive neural cells were also found in the cerebral cortex, particularly in the pyriform cortex. Prominent immunostaining was found in the cell bodies of cerebellar Purkinje cells. Although Neurensin-1 and Neurensin-2 showed a similar distribution in the central nervous system, they differed in their intracellular localization. Neurensin-1 was mostly found in neuritic processes (Kadota et al., 1997), while Neurensin-2 was normally found in the somatic regions (Figs. 4C, D) Both of them appear to be expressed in the intracellular vesicles.

3. Discussion

In the present study, we report a novel type of Neurensin specifically expressed in neural cells. Named Neurensin-2, it is a membrane protein of vertebrate brain with an undefined role. To investigate the role of Neurensin-1, we searched the database with an EST entry showing significant sequence homology to Neurensin-1 and identified one cDNA coding a protein homologous to Neurensin-1. The predicted primary structure of this protein shows very high sequence identity in the amino-terminal region and two membrane-spanning domains. However, Neurensin-1 contains an obvious microbody-targeting signal (SRV) near the end of the carboxy-terminus, whereas Neurensin-2 does not. The most striking
Neurites (Ida et al., 2004; Kadota et al., 1997). Our previous study showed that Neurensin-1 is expressed in the mouse cerebral cortex. (A, B) Neurensin-1-immunoreactive granules are found in apical dendrites in addition to faintly positive reaction product in cell bodies. (C, D) Neurensin-2-immunoreactive granules are mostly found in cell bodies and occasionally some immunoreactive materials are found at the beginning of apical dendrites. Scale bars = 100 μm (A, C). Scale bars = 10 μm (B, D).

The overexpression of several neuronal growth-associated proteins such as Tau, GAP-43, Syntaxin1A/HPC-1, SNAP-25 induces a neuron-like appearance in neuronal and non-neuronal cells (Knops et al., 1991; Osen-Sand et al., 1993; Piontek et al., 2002; Saunders et al., 1995; Yamaguchi et al., 1996). Furthermore, amphiphysin I seems to serve as a physiological binding partner for dynamin I in the endocytosis of synaptic vesicles (Takei et al., 1999), and antisense oligonucleotides for amphiphysin I mRNA inhibited neurite outgrowth in cultured hippocampal neurons (Mundigl et al., 1998). Similarly, we previously showed that the transfection of antisense oligonucleotide for Neurensin-1 mRNA resulted in the transient retraction and re-extension of neuritic processes. A unique form of neurons that were intensely stained with anti-SNAP-25 and showed thick processes (Kosik, 1993; Osen-Sand et al., 1993) was also observed when the cultured DRG neurons were treated with antisense oligonucleotide for Neurensin-1 (Ida et al., 2004). Immunocytochemistry of Neurensin-2 revealed that this protein was stained intensely at cell bodies of CNS neurons. These vesicles are probably different from those containing Neurensin-1. Since membrane turnover and the continuous transport of membranous vesicles must occur in neurites to maintain neuronal fibers, particularly in the areas where active synaptic remodeling.
continues, Neurensin-2 also has a role of the maintenance and/or transport of vesicles. Further studies are required to demonstrate the precise roles of vesicles containing Neurensin-1 or -2, which can be transported to growing neurites.

4. Experimental procedures

4.1. Materials

[$\alpha$-$^{32}$P] dCTP (6000 Ci/mmole) was obtained from Amersham Biosciences (Buckinghamshire, UK). Marathon mouse brain cDNA library was a product of BD Biosciences-Clontech (Alto, CA). Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Co. (Tokyo, Japan) and Toyobo Co. (Tokyo, Japan). Anti-HA (clone: 12CA5) monoclonal antibody was purchased from Roche Molecular Biochemicals (Mannheim, Germany). FluoroLink-Cy2-labeled anti-mouse IgG was from Amersham Biosciences Co. Other reagents used were of analytical grade.

4.2. Cloning and sequencing of mouse Neurensin-2

Mouse and human expressed sequence tag (EST) databases were searched using the TBLASTN algorithm (Altschul et al., 1990) with the amino acid sequence of the mouse Neurensin-1 (Kadota et al., 1997) as a query. Several partial EST matches for mouse (accession number BC088982, XM.130718.5 and AL928568) and human (BC001963, NM.024958 and HSM801881) were extracted from the databases and aligned. Specific oligonucleotide primers were designed according to the aligned EST sequences (mouse p28N: 5′-AATCTAGAAGCTGCACTTCCT-GCCCGGCTGGGGC′-3′ and p28C: 5′-AAAAGCTTAAGTCCGCTGGGGG-3′) and used to amplify mouse Neurensin-2 cDNA making up the complete coding region from the mouse brain cDNA library. The PCR product was cloned into the vector pGEM-T easy (Promega, Madison, WI) and sequenced on both strands.

4.3. Cell culture and DNA transfection

COS-7 cells and mouse Neuro2a cells obtained from the Japan Cell Bank (Tsukuba, Japan) were grown in DMEM supplemented with 10% FCS and antibiotics (Mizutani et al., 2002). PC12 cells were grown in DMEM containing 5% FCS, 5% horse serum, and antibiotics. The cells were transfected using Lipofectamine (Invitrogen Co. San Jose, CA) or calcium phosphate. The cells were grown in DMEM containing 5% FCS, 5% horse serum, and antibiotics. The cells were transfected using Lipofectamine (Invitrogen Co. San Jose, CA) or calcium phosphate.

4.4. Plasmids

The full-length cDNA of mouse Neurensin-2 was isolated by PCR using a Mouse Brain Marathon-ready cDNA (BD Biosciences-Clontech). The primers used were AATCTAGAAGCTGCACTTCCT-GCCCGGCTGGGGC′-3′ and AAAAGCTTAAGTCCGCTGGGGG-3′. The DNA fragment was ligated into pGEM-T easy (Promega Co.). The resulting plasmid (pGEM-Neurensin-2) was digested with Xbal and HindIII and ligated into Xbal-HindIII-digested hemagglutinin (HA) epitope-tagged expression plasmid pCG-N-Bl (pCG-HA-Neurensin-2) (Kadota et al., 1997). To construct pCG-HA-Neurensin-1 or -2, the Neurensin-2 cDNA was amplified by PCR with AAGAATTCATGTGCCAGTCGCCCTGCTGTGT and GTAATAGACTCACATATAGGCC (T7 primer) from pGEM-Neurensin-2. The fragment was digested with EcoRI and HindIII and ligated into EcoRI-HindIII-digested pcDNA3.1 (Invitrogen Co.) (pcDNA-Neurensin-2).

4.5. Isolation of anti-Neurensin-2

To construct expression plasmids for GST-fusion proteins, a portion of mouse Neurensin-2 cDNA was amplified by PCR, and the resulting fragments were ligated into vector pGEX-4T (Amersham Biosciences). The primers used were AAGGATCC-CAGGACATCAAGGCCAGA and GTAATAGACTCACATATAGGCC (T7 primer). The plasmid thus constructed was pGEX-Neurensin-2C, which encoded the C-terminal region of Neurensin-2 (amino acids 143–202) fused with GST (GST-Neurensin-2C). The protein was expressed in E. coli (strain: DH-5α) with 0.3 mM isopropyl-1-thio-β-D-galactoside at 37 °C for 2 h and affinity-purified using Glutathione-Sepharose CL-4B (Amersham Biosciences). Antibodies against mouse Neurensin-2 were prepared by injecting a rabbit with 0.5 mg of GST-Neurensin-2C fusion protein in Freund’s complete adjuvant. After three subsequent injections at 2-week intervals, antiserum was collected. The resulting antiserum was affinity purified (Mizutani et al., 2002). Antibodies against the synthetic polypeptide of Neurensin-2 (amino acid position 154 to 168) were also raised in rabbits and antiserum was affinity purified. Fig. 5 shows immunoblot analysis of Neurensin-2 in Neurensin-1 or -2 cDNAs transfected Cos7 cells. The obtained anti-Neurensin-2 reacted with a single protein corresponding to the molecular mass of 28 kDa in Neurensin-2-expressing cells and did not cross-react with Neurensin-1.

4.6. RNA blots

Total RNA was isolated from Neuro2a cells using the guanidium isothiocyanate method as described previously (Kadota et al., 1997). The RNA was loaded on a 1% agarose/formaldehyde gel, electrophoresed, and transferred onto a nylon membrane (Amersham Biosciences) for hybridization with $^{32}$P-labeled DNA fragments of mouse Neurensin-2 and Neurensin-1 cDNA (Kadota et al., 1997; Mizutani et al., 2002), and then the filter was hybridized and washed as described.

Fig. 5 – Immunoblot analysis of Neurensin-1 and -2. COS-7 cells transfected with pCG-HA control vector (lane 1), pCG-HA-Neurensin-1 (lane 2) or pCG-HA-Neurensin-2 (lane 3) and incubated for 20 h. Immunoblotting was performed with anti-HA, anti-Neurensin-1, and anti-Neurensin-2, as the primary antibodies.
previously. Mouse adult multiple tissues RNA blot membrane was purchased from BD Biosciences-Clontech.

4.7. Immunofluorescence microscopy

For immunostaining, the cells were fixed with 4% paraformaldehyde in PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂ (PBS (+)) for 20 min and permeabilized with 0.1% Triton-X100 in PBS (+) for 30 min. The cells were incubated with anti-HA or anti-Neurensin-2, as described (Kadota et al., 1997; Mizutani et al., 2002). After a wash with PBS (+), they were incubated with a fluorolink Cy2-labeled anti-mouse IgG (Amersham Biosciences). To stain the cell surface, the treatment of the cells with Triton-X-100 was omitted. Fluorescence microscopy was performed with a Zeiss microscope.

4.8. Immunocytochemistry of the mouse brain

Adult mice were examined for Neurensin-2 in the brain by the avidin-biotin method as previously described (Araki et al., 2002; Kadota et al., 1997). Briefly, adult male mice were sacrificed with an intraperitoneal injection of excess pentobarbital sodium (20 mg/kg) (Nembutal, Boehringer Ingelheim, Australia). Brains were perfused with a mixture of 4% paraformaldehyde and 0.3% glutaraldehyde in 0.1 M phosphate buffer at 4 °C for 10 min and were then immersed in 4% paraformaldehyde at 4 °C overnight. Frozen sections 20 μm thick were cut on a cryostat and were processed for immuno-chemistry, the primary antibody (48 h, 4 °C), a biotinylated anti-IgG (Amersham Biosciences). To stain the cell surface, the treatment of the cells with Triton-X-100 was omitted. Fluorescence microscopy was performed with a Zeiss microscope.

4.9. Immunoblotting

The lysates from Neuro2a cells transfected with the empty vector pCG-HAor pCG-HA-Neurensin-2 were also subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Homogenates (10 μg) from various tissues of mouse brain were also used. Immunoblotting was done with anti-Neurensin-1 or anti-Neurensin-2. The protein concentration was estimated by the method of Bradford (1976).

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