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Distinct neuronal localization of microtubule-associated protein 4 in the mammalian brain

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ABSTRACT

Although recent studies have suggested the role of microtubule-associated protein (MAP) 4 in some neuron-specific events, there are no reports that directly observed its neuronal localization. Here we show the detailed expression of MAP4 in the mammalian brain. Immunoblotting revealed the presence of MAP4 in all neuronal tissues. The site-specific localization of MAP4 was observed in sagittal brain sections: MAP4 was rich in brain-specific cells, cerebellum Purkinje cells and hippocampus pyramidal cells. When primary cultures of cortical neurons were immunostained, MAP4 was detected in the cell bodies and processes with patchy staining pattern. These results suggested that MAP4 play some roles in the central nervous system, such as the dynamic cytoskeletal reorganization and regulation of the microtubule-dependent long-range transport.

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Microtubule-associated proteins (MAPs) belong to a family of proteins that bind to microtubules and regulate their function. Among the major mammalian MAPs, MAP1, MAP2 and tau are expressed predominantly in the brain, while MAP4 is widely present in various types of cells and tissues [12]. Although a small amount of MAP4 is also expressed in the brain, its importance in the central nervous system has been overlooked partly because of its ubiquitous nature.

We recently identified a novel isoform of MAP4 which contains an extensive deletion in the Pro-rich region (hereby referred to as the short Pro-rich region variant) (Fig. 1(5)), and revealed that the isoform was expressed in the brain and adrenal medulla, but was absent from adrenal cortex, heart, kidney, liver, skeletal muscle, and lung [8]. The expression of the variant in PC12 cells was augmented by the addition of nerve growth factor (NGF). Consequently, this isoform was concluded to be neural cell specific [8], and its rapid turnover on cytoplasmic microtubules [5] suggested the involvement of the variant in dynamic cytoskeletal reorganization that occurs within developing neurons. Meanwhile, the longer Pro-rich region variants (Fig. 1(1–4)) reportedly altered the microtubule surface properties [15] and effected the kinesin-driven microtubule movement *in vitro* [16]. The long Pro-rich region variants possibly regulate the microtubule-dependent long-range transport in neurons. These findings elucidate some roles of MAP4 in neuron-specific events, and have prompted us to reveal its neuronal localizations.

Although we have already investigated the intracellular behavior of MAP4 variants in neuroblastoma cells using GFP-fused proteins [6], it is essential to clarify the localization of intrinsic MAP4 in neurons of the brain to reveal the role of MAP4 in the central nervous system. In this study, we examined the cellular and subcellular localization of the neuronal MAP4, and successfully observed detailed localization of MAP4 in neurons of the mammalian brain for the first time.

Antibodies used in this study were: a rabbit polyclonal antibody against the N-terminal region (amino acid residues 1–300, Fig. 1, Epitope) of mouse MAP4 which can detect all MAP4 isoforms (sc-67153, Santa Cruz); mouse anti-MAP2 monoclonal antibody (MAB3418, Chemicon); mouse anti-β-tubulin monoclonal antibody (G712A, Promega); rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (Z0334, Dako); Alexa488-conjugated goat anti-rabbit antibody (A11008, Invitrogen); Alexa568-cojugated goat anti-mouse antibody (A11031, Invitrogen); and anti-rabbit IgG-peroxidase antibody (A-6154, Sigma).

Abbreviations: MAP, microtubule-associated protein; NGF, nerve growth factor; GFAP, glial fibrillary acidic protein; CA, cornu ammonis; DG, dentate gyrus.

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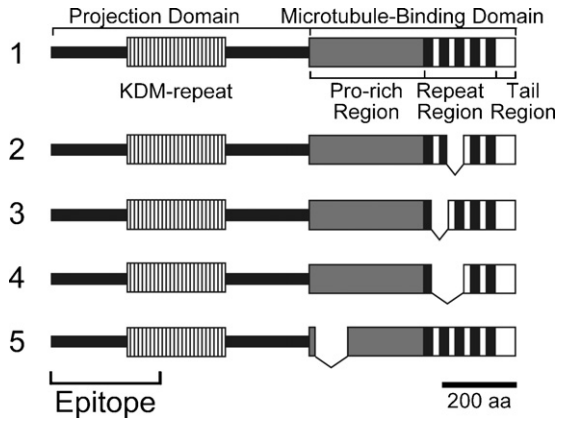

Fig. 1. Schematic structures of MAP4 isoforms. The longest version (1) and four spliced isoforms (2–5) are shown. The shortest (5) variant lacks 72 consecutive amino acid residues within the Pro-rich region, while the others (2–4) are devoid of one or two repeat sequences, which are indicated by filled thick boxes in the Repeat region. "Epitope" denotes the antigenic site of the anti-MAP4 polyclonal antibody.

Western blotting using commercially available polyvinylidene difluoride membrane containing extracts from 15 different mice neuronal tissues (MW – 200, ZYAGEN) was performed according to the manufacturer's instructions. Immunohistochemistry of the mammalian brain was performed using adult wild type mice (B6129) as described previously [11,19]. Cerebral cortical neurons were prepared from B6129 embryos (embryonic day 16) for immunocytochemical analysis as described elsewhere [7,19]. The neurons were plated onto poly-D-lysine-coated round coverslips (BD Bioscience), and were primary cultured in 24-well tissue culture plates (Fisher Scientific) for 10 days before observations. The cells were fixed, permeabilized, and stained with antibodies. Glial contamination was checked using anti-GFAP antibody, anti-MAP2 antibody, and Hoechst 33342 (Molecular Probes).

The samples were observed using a wide-field fluorescence microscope (TE-300, Nikon) equipped with a CCD camera (DP71, Olympus). Alexa488, Alexa568, and Hoechst33342 were imaged using an FITC filter set (ex 480/40 and em 535/50, Chroma/Nikon), red filter set (ex 560/55 and em 645/75, Chroma/Nikon), and blue filter set (ex 390/22 and em 460/50, Chroma/Nikon), respectively.

First, antibody specificity was assayed by immunoblotting, using the commercially available neuronal tissue extracts (Fig. 2). Since the antibody used to detect MAP4 recognizes an epitope region common to all MAP4 isoforms (Fig. 1), we can expect the antibody to recognize MAP4 variants with deletions as well. In all samples, prominent bands were observed in the molecular mass range of around 200,000. As the isoforms of MAP4 are approximately 200,000 Da in size, the bands were most likely the MAP4 isoforms, since massive degradation was unlikely to have occurred. Consequently, it can be concluded that all the tested samples contained MAP4 variants. The varied banding patterns (Fig. 2) may be

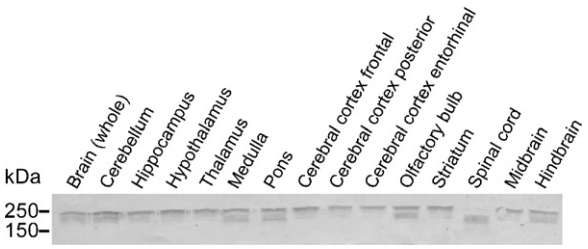

Fig. 2. Expression of MAP4 in various neuronal tissues. Extracts of various neuronal tissues of adult mice were stained with the anti-MAP4 antibody (1/10,000 dilution). Positions of molecular markers are indicated on the left.

due to the differential expression of MAP4 isoforms in the different tissues [2]. The highest and lowest molecular mass bands probably represent the long and short Pro-rich isoforms containing five repeats (Fig. 1(1 and 5)), respectively, judging from their molecular masses. The intermediate molecular mass bands may represent the other long Pro-rich region variants (Fig. 1(2–4)). No bands were detected beyond this molecular mass range, including those corresponding to MAP1, MAP2, and tau, indicating that the antibody is specific to MAP4 (Supplementary Fig. 1).

Next, we examined the localization of MAP4 in the brain by immunohistochemistry. Distinct cell-specific expression patterns of MAP4 were observed in the cerebellum (Fig. 3a and b) and hippocampus (Fig. 3c–e). In the cerebellum, MAP4 antibody stained well in the molecular but not the granular cell layer (Fig. 3a and b, brown staining). Under a higher magnification, Purkinje cell bodies (arrow heads) and their dendrites (arrows) were predominantly stained, while the Purkinje cell axons in the granular cell layer were not (Fig. 3b). In the hippocampus, MAP4 staining was seen in the pyramidal cell layer in the cornu ammonis (CA) and granule cells in the dentate gyrus (DG) (Fig. 3c). A high magnification micrograph shows that all pyramidal cell bodies in the pyramidal cell layer and their apical dendrites in the radiatum layer were well stained, while basal dendrites in the oriens layer were not stained (Fig. 3d). The brown-stained cells in the radiatum layer were likely to be astrocytes, judging from their shapes (Fig. 3d). In the DG, approximately 70% of the granule cells were counted to be stained positive for the presence of MAP4 (Fig. 3e).

Lastly, the subcellular localization of MAP4 in mouse cortical neurons was examined in relation to MAP2 and microtubules (Fig. 4). Fig. 4a shows staining for MAP2, MAP4, and Hoeschst, which marks the nuclei of the cells. While the majority of the neurons are stained positive for both MAP2 and MAP4, about 1/10th of them were stained positive for MAP4 only (indicated in Fig. 4a by an arrow). Since this neuron culture contained approximately 10% contamination of astrocytes, which were stained by a GFAP marker (data not shown), the non-MAP2 cells were likely to be astrocytes (as MAP2 is a neuronal only protein). In contrast to the neuron-specific nature of MAP2, MAP4 was found in both neurons and astrocytes. The merged image shows the colocalization of MAP2 and MAP4 (Fig. 4a), indicating a simultaneous expression of both MAPs in a single cell. The colocalization is not surprising since we have reported that MAP2 can bind to MAP4-bound microtubules using its uncompetitive biding site *in vitro* [13]. The green signal, as indicated by the arrow, in the merged image (Fig. 4a) suggests the exclusive presence of MAP4 in astrocytes. The double-staining with anti-MAP4 and anti-tubulin antibodies (Fig. 4b and Supplementary Fig. 2a) revealed colocalization of MAP4 and tubulin in the processes. Although individual microtubules in a process cannot be visualized using this technique, the colocalization can most reasonably be interpreted as MAP4 binding to microtubules in the neuronal processes. An interesting characteristic of neuronal MAP4 was its patchy staining patterns (Fig. 4b and Supplementary Fig. 2b), which may be the signs of MAP4 clusters on microtubules. A high-power magnification micrograph (Fig. 4c) shows the colocalization of MAP4 and microtubules in the branched processes. The MAP4 staining in branched processes was punctate, and approximately half of the processes stained green in their tips (Fig. 4c and Supplementary Fig. 3a and b), suggesting the presence of microtubule-free MAP4 in this region (unbound MAP4). The green region was also observed when the MAP4 image was merged with intensified tubulin image (Supplementary Fig. 3c), indicating that the green staining is not an artifact of fluorescence intensity.

Western blotting revealed the presence of MAP4 in all neuronal tissues tested (Fig. 2). The apparent MAP4 isoforms were expressed differentially. The abundance of the lowest molecular mass versions (short Pro-rich variant, Fig. 1(5)) in some samples (such as

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