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Expression of Septin 3 Isoforms in Human Brain

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Septin 3 is a novel member of the septin subfamily of GTPase domain proteins. Human *septin 3* was originally cloned during a screening of genes expressed in human teratocarcinoma cells induced to differentiate with retinoic acid. Alternative splicing of the *septin 3* gene transcript produces two isoforms, A and B, in the human brain, though their regional expression and physiological function remain to be determined. The purpose of the present study was to identify the expression patterns of human septin 3 isoforms in normal human brain and a human neuroblastoma cell line, SH-SY5Y, after retinoic acid-induced differentiation. The expression and distribution patterns of septin 3 isoforms A and B were similar and resembled that of another septin, CDCrel-1. Septin 3A and 3B were expressed in normal human brain in a region-specific manner, with the highest level in the temporal cortex and hippocampus and the lowest level in the brainstem regions. Prominent immunoreactivity was observed diffusely in the neocortices in association with neuropils and punctate structures suggestive of synaptic junctions. Immunoprecipitation studies revealed that septin 3A, 3B, and CDCrel-1 form a complex in the frontal cortex of human brain. These findings, taken together, suggest that septin 3A and 3B, along with CDCrel-1, play some fundamental role(s) in synaptogenesis and neuronal development.

Key words: CNS; Neurons; Septins; Differentiation

SEPTINS are a family of GTPase domain proteins that are present in most cell types of multicellular organisms. Several members of the septin family form complexes that are involved in various activities within different cell types of the central nervous system (CNS), including: vesicular trafficking at neuronal synapses, neurite outgrowth, and transporter-mediated glutamate uptake by astrocytes (5,13,24). These findings clearly indicate a functional relevance of mammal septins within the CNS. To be noteworthy are the reports suggesting the involvement of septins in neurodegenerative conditions. In postmortem brains of patients with Alzheimer's disease (AD), septin members, such as Nedd5, H5, and Diff6, were identified in neurofibrillary tangles, neuropil threads, and dystrophic neurites, which indicates their participation in the formation of tau-based neurotoxic filaments (9). More recently, we found a genetic association of *septin 3* polymorphisms with AD, but not the Lewy body variant of AD or Parkinson's disease (PD) (23). Moreover, septin H5 has been consistently found in α-synuclein-positive cytoplasmic inclusions, a pathological hallmark of several neurodegenerative disorders including PD, dementia with Lewy bodies, and multiple system atrophy (6). Interestingly, another neural septin, CDCrel-1, has been identified as a substrate of parkin, which is a ubiquitin-protein ligase encoded by the *PARK2* gene and lacking in individ-

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uals with autosomal recessive juvenile parkinsonism (28).

Septin 3 is a novel member of the septin family. Mouse *septin 3* expression is increased in neuronal tissues during development (26), while rat septin 3 is a specific substrate for type I cGMP-dependent protein kinase and is present as a phosphoprotein in nerve termini (27). Human *septin 3* is upregulated upon neuronal differentiation of a human teratocarcinoma cell line, NT2, and is known to have at least two isoforms, A and B, produced by the alternative splicing of transcript (16). The *septin 3* gene has been mapped to chromosome 22q13.2 in the vicinity of *CYP2D6* gene and contains a microsatellite of dinucleotide repeats in exon 11.

The purpose of the present study was, in an attempt to approach the functional role of septin isoforms in neural tissues, to determine their expression and distribution in adult human brain and a human neuroblastoma cell line, SH-SY5Y, induced to differentiate by all-*trans* retinoic acid (RA), with special reference to possible interactions with CDCrel-1.

MATERIALS AND METHODS

Plasmid Construction

The human cDNA fragments of septin 3A, 3B, and Nedd5 were produced by polymerase chain reaction (PCR) from the Human Fetal Brain Large-Insert cDNA library (BD Biosciences Clontech, Palo Alto, CA). Oligonucleotide primers were designed after the published sequence in GeneBank (Bethesda, MD) and supplemented with an additional *Xba*I restriction site plus a Myc-tag sequence in 5′-sense primers or *Afl*II site in 3′-antisense ones. The PCR products were digested with *Xba*I and *Afl*II and then ligated into the pcDNA3.1(−) vector (Invitrogen Corp., CA). All constructs were confirmed by DNA sequencing.

Cell Culture

HeLa cells, used for septin 3A and 3B preparation, were cultured in α-MEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum. The undifferentiated SH-SY5Y human neuroblastoma cells were cultured in D-MEM/F-2 (Invitrogen) supplemented with 10% fetal bovine serum. Both cells were grown in a humidified incubator with 5% CO_2 at 37°C. For neuronal differentiation of SH-SY5Y cells, RA (Sigma-Aldrich) dissolved at 10 mM in dimethyl sulfoxide was added to the culture medium to a final concentration of 2.5 μM.

Preparation and Characterization of Anti-Septin 3 Antibodies

The following peptides derived from septin 3A and 3B were synthesized: GEGLLGTVLPPVPATPC (residues 325–341 of septin 3A) and CVSVDTEESHDSN (cysteine plus residues 325–336 of septin 3B). These peptides were conjugated to bovine thyroglobulin for immunization. Anti-septin 3A and 3B polyclonal antibodies were raised in rabbits and affinity purified with antigen peptides. HeLa cells were transfected with Myc-tagged expression vectors using FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN). After 24 h of transfection, the lysates of HeLa cells were subjected to Western blotting (as described below), using anti-Myc (Clone 9E10; BD Biosciences Clontech), anti-septin 3A, or anti-septin 3B antibody.

Reverse Transcription-Coupled Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from SH-SY5Y cells treated with or without RA by using RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). First-strand cDNA was synthesized from 3 μg total RNA using oligo$(dT)_{20}$ primer (Toyobo Co., Ltd., Osaka, Japan) and 100 units of Moloney murine leukemia virus reverse transcriptase, ReverTra Ace (Toyobo), in a total volume of 20 μl. The cDNA thus formed served as a template for PCR. The following primers were used to detect mRNAs of septin 3A (GenBank; Accession No. AF285107), 3B (AF285109), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; BC026907): 5′-GAT TCA TGT CCA AAG GGC TC-3′ (septin 3A and 3B; forward), 5′-ACA GCA CTA AAA AGG GCT TGG-3′ (3A; reverse), 5′-CTT ACA AGG GAC TCT CCA GG-3′ (3B; reverse), 5′-CCA GGG CTG CTT TTA ACT CT-3′ (GAPDH; forward), and 5′-CTC TCT CTT CCT CTT GTG CT-3′ (GAPDH; reverse). PCR was performed with 0.65 unit of Pyrobest DNA polymerase (Takara Bio, Inc., Otsu, Japan) in 20 μl of 1× PCR buffer containing 0.4 μl cDNA prepared as above, 0.2 mM each 2′-deoxyribonucleoside-5′-triphosphates mixture, 1.0 mM $MgCl_2$, and 0.2 μM each of the primers. The mixture was subjected to 35 cycles (for septin 3A and 3B) or 25 cycles (for GAPDH) of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 2 min. The PCR products were electrophoresed on 1% agarose gel containing ethidium bromide.

Western Blotting

Multiple tissue blots containing proteins from various tissues in normal human subjects (Oncogene Re-

search Products, San Diego, CA) were probed with anti-septin 3A and 3B antibodies. Protein extracts from various regions in normal human brain (Cosmo Bio Co., Ltd., Tokyo, Japan) were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting on polyvinylidene difluoride membrane (Hybond-P; Amersham Bioscience Corp., Piscataway, NJ) and then probed with anti-septin 3A, anti-septin 3B, anti-CDCrel-1 rabbit polyclonal (IBL Co., Ltd., Fujioka, Japan), anti-hCDC10 rabbit polyclonal (sc-20620; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-β-actin mouse monoclonal (Clone AC-15; Sigma-Aldrich) antibody. Immunoreactive bands were visualized with the ECL Western blotting analysis system (Amersham Bioscience).

Human Brain Tissues

Three autopsied human brains were obtained from Kyoto University-affiliated hospitals. One of the brains came from a case without neurological disorders (male; 76 years old) and two came from sporadic AD cases (females; 58 and 82 years old). Informed consent was obtained for all samples.

Immunoprecipitation

Frontal cortex of normal human brain was homogenized in 10 volumes of ice-cold lysis buffer [20 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA 2 Na, 10 μg/ml aprotinin, 3 μg/ml leupeptin, 1 μg/ml pepstatin A, and 100 μM 4-amidinophenylmethanesulfonyl fluoride hydrochloride]. Homogenized samples were centrifuged at $40{,}000 \times g$ for 30 min at 4°C. The supernatant was precleared by incubation with protein A-sepharose (Sigma-Aldrich) and then incubated with unimmunized rabbit IgG (Sigma-Aldrich), or anti-septin 3A, anti-septin 3B, or anti-CDCrel-1 antibody, overnight at 4°C. After the addition of protein A-sepharose, the mixture was incubated for 1 h at 4°C. The sepharose beads were washed five times with lysis buffer and eluted in SDS sample buffer by boiling. Immunoprecipitates were subjected to Western blotting with anti-septin 3A, anti-septin 3B, or anti-CDCrel-1 antibody.

Immunohistochemistry

Immunohistochemical procedures were performed as described previously (7). Briefly, the cortex of human mid-frontal gyrus was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 days at 4°C. After cryoprotection in 15% sucrose in 0.1 M phosphate-buffered saline (pH 7.4), the tissue was sliced into 30-μm sections. The sections were pretreated with 0.3% H_2O_2 for 30 min to eliminate endogenous peroxidase and then blocked with 5% bovine serum albumin overnight at 4°C, followed by a 72-h incubation with anti-septin 3A or 3B antibody at 4°C. Immunoreactive signals were visualized by using Vectastain® ABC kit (Vector Laboratories, Inc., Burlingame, CA). The specific immune complex was detected with a solution containing 0.01% 3,3′-diaminobenzidine, 0.6% nickel ammonium sulfate, 50 mM imidazole, and 0.00015% H_2O_2. Sections were mounted on glass slides, dehydrated, and coverslipped with Entellan (Merck Ltd., Tokyo, Japan).

RESULTS

Characterization of Antibodies Against Septin 3 Isoforms

To explore the properties of *septin 3* gene products, polyclonal antibodies were generated against the septin 3 isoforms, A and B. Peptides with sequences specific for each of these isoforms were used to immunize rabbits. The specificity of the antibodies was confirmed with Myc-tagged septin proteins overexpressed in HeLa cells. As shown in Figure 1, anti-septin 3A antibody specifically recognized Myc-septin 3A, and not Myc-septin 3B or Myc-Nedd5, by Western blot analyses. Similarly, anti-septin 3B antibody recognized only Myc-septin 3B. Preincubation of the antibodies with the peptides used for immunization selectively inhibited the immunoreactivity (data not shown).

Upregulation of Septin 3 During Neuronal Differentiation of SH-SY5Y Cells

The expression of septin 3A and 3B in human, fetal and adult, brains has been reported by the use of RT-PCR (23). To further clarify the role of these proteins during development, we examined their expression in SH-SY5Y cells after neuronal differentiation with RA. On the fourth day of RA treatment, the levels of both septin 3A and 3B mRNAs were increased compared with dimethyl sulfoxide (vehicle)-treated controls (Fig. 2a). Septin 3A and 3B mRNAs were detected as the bands of expected sizes by RT-PCR (Fig. 2a). The protein levels of septin 3 isoforms showed a comparable increase to those of mRNA during RA-induced neuronal differentiation (Fig. 2b).

Expression of Septin 3 Isoforms in Multiple Human Tissues and Brain Regions

To determine the tissue distribution of septin 3 isoforms, the antibodies specific for isoforms A and B were used for Western blotting of various tissues de-

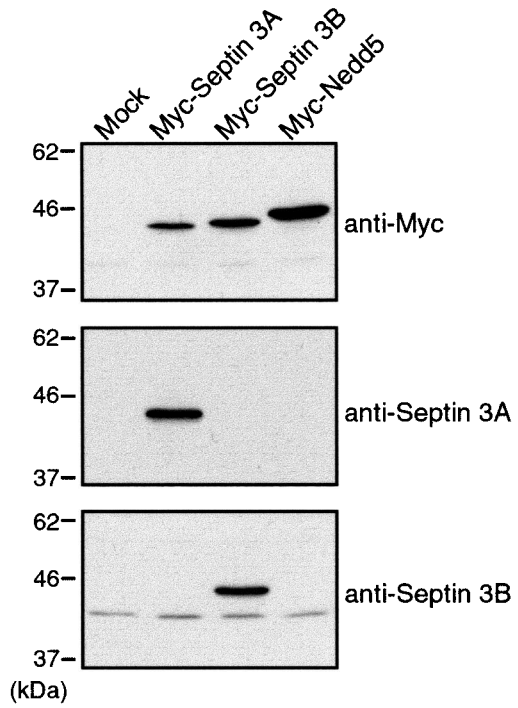

Figure 1. Specificity of antibodies for septin 3 isoforms. Lysates from HeLa cells transiently expressing Myc-tagged septin 3A, 3B, or Nedd5 were separated by 10% SDS-PAGE and then subjected to Western blotting with antibodies indicated on the right. Using antibody against Myc-tag (top panel), the bands of Myc-tagged septin 3A, 3B, and Nedd5 were detected with estimated molecular weights of 43, 44, and 46 kDa, respectively. Antibodies against septin 3A (middle) and 3B (bottom) specifically recognized Myc-septin 3A and 3B, respectively. Mock (transfection of the empty vector) served as a negative control.

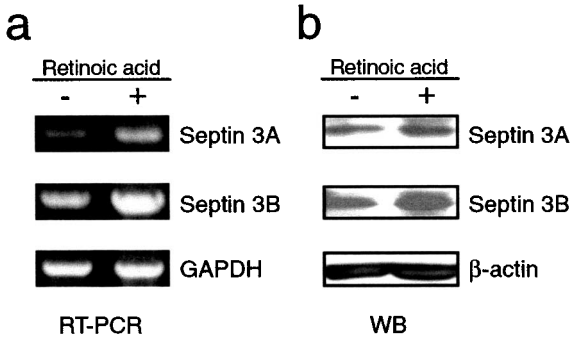

Figure 2. Upregulation of septin 3 isoforms during RA-induced neural differentiation of SH-SY5Y cells. SH-SY5Y cells were treated for 4 days with vehicle (−) or 2.5 μM RA (+). (a) cDNA synthesized from mRNA of treated cells was used as template for PCR. Three sets of primers were used to detect mRNAs for septin 3A, 3B, and GAPDH as an internal control. The expected sizes of septin 3A (top), 3B (middle), and GAPDH (bottom) mRNAs were 1160, 1308, and 1000 bp, respectively. (b) Lysates from treated cells were separated by 10% SDS-PAGE and then subjected to Western blotting (WB) with antibodies against septin 3A (top), 3B (middle), or β-actin as an internal loading control (bottom).

rived from adult human subjects (Fig. 3a). Based on the estimated molecular weights of Myc-tagged septin 3 isoforms (Fig. 1), the highest level of septin 3A expression was found in the brain, and the lowest was found in the kidney. The antibody against septin 3A revealed an additional band with an estimated molecular weight of 47 kDa in the liver, kidney, and heart. Whether this band represents a cross-reacting protein or an unknown, alternatively spliced, variant of septin 3 remains to be determined. Septin 3B was highly expressed in the brain and heart. To determine whether the expression of the isoforms occurs throughout the human brain, Western blotting of tissue extracts from various brain regions was performed (Fig. 3b). Septin 3A and 3B expression was detected in all brain regions examined, with the highest level in the temporal cortex and hippocampus and the lowest level in the brainstem regions. A notable difference in reactions with septin 3A and 3B antibodies was observed in the cerebellum. Expression of other members of

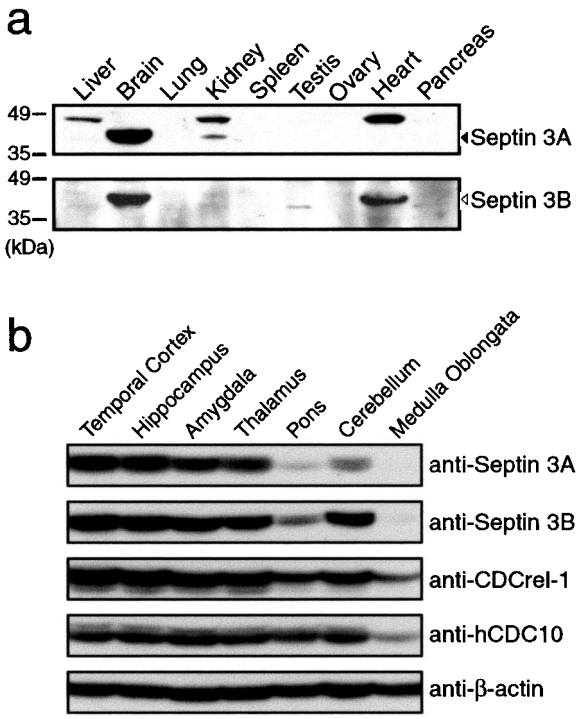

Figure 3. Expression of septin 3 isoforms in multiple human adult tissues and brain regions. (a) Each lane of the membrane was a blot with 75 μg proteins isolated from specified human tissues. The blots were probed with anti-septin 3A (upper) or 3B (lower). Septin 3A (42 kDa) and septin 3B (43 kDa) are indicated by closed and open arrowheads, respectively, on the right. (b) Each lane of the membrane was a blot with 20 μg proteins isolated from various regions of human brain. The blots were probed with antibodies indicated on the right. Septin 3A and 3B proteins in medulla oblongata were not detectable until a longer exposure.

the septin family, CDCrel-1 and hCDC10, was also examined (Fig. 3b). The expression of CDCrel-1 was variable among brain regions, closely resembling that of septin 3B, whereas the hCDC10 expression was almost constant in all brain regions except medulla oblongata.

Formation of a Complex From Septin 3A, 3B, and CDCrel-1 in Human Brain

To determine if septin 3 is involved in hetero-oligomeric complex formation like other septins (5,12), proteins were extracted from the frontal cortex of normal human brain and immunoprecipitated with antibody against septin 3A, 3B, or CDCre-1. As shown in Figure 4, the antibody specific for septin 3A precipitated not only septin 3A but also septin 3B and CDCrel-1. Similarly, the antibody against septin 3B precipitated septin 3A and CDCrel-1, and the antibody against CDCrel-1 precipitated both septin 3A and 3B. These results of coprecipitation of septin 3A, 3B, and CDCrel-1 with either antibody indicate that they make a hetero-oligomeric protein complex in the human brain.

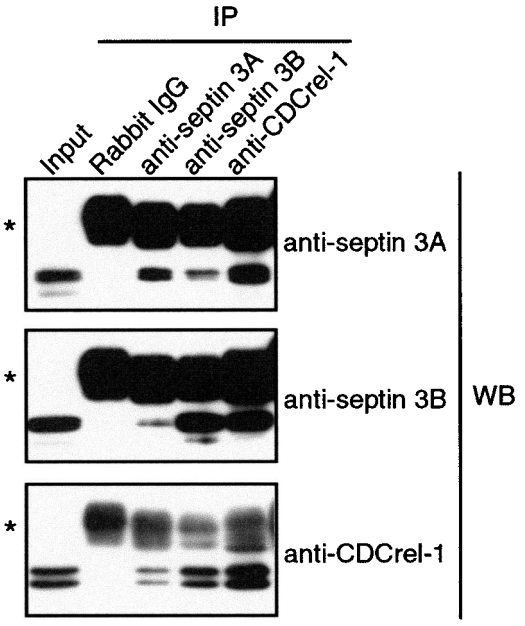

Figure 4. Coimmunoprecipitation of septin 3A, 3B, and CDCrel-1 in the human brain. Homogenates of human brain frontal cortex were subjected to immunoprecipitation (IP) with the antibodies indicated at the top. Unimmunized rabbit IgG was used as a negative control. Immunoprecipitates were subjected to Western blotting (WB) with antibodies indicated on the right. Initial brain homogenates were also analyzed (Input). The asterisk indicates the position of the rabbit IgG heavy chain.

Immunohistochemistry of Septin 3A and 3B in the Cortex of Human Mid-Frontal Gyrus

Immunoreactivity for septin 3A and 3B exhibited a similar distribution pattern in human brain tissues (data not shown). A large number of dotty structures were immunostained in the neocortices. Many of them were scattered as fine granules like synapses in the cortical layers 1, 2, 3, 5, and 6 (Fig. 5A and B). Some punctuates were clustered and appeared in the cells with astrocytic profiles (Fig. 5C). Septins were also immunolocalized in a few axons with dystrophic contours (Fig. 5B).

DISCUSSION

In mammalian cells, there are 12 *septin* genes so far identified, and several of the transcripts have been shown to undergo the alternative splicing to generate multiple protein products (11,15). These many septins are present in different combinations in various tissues (1,25) and also in different cell types within a particular tissue (8). The expression of a variety of septins within particular cells or tissues might govern the formation of septin complexes of distinct compositions and thus distinct functions. This notion is apparently evidenced in *Saccharomyces cerevisiae* where the septins Cdc3p, Cdc10p, Cdc11p, and Cdc12p are involved in cytokinesis, while only Cdc3p and Cdc11p, in conjunction with Spr3p and Spr28p, are expressed during sporulation (3,4).

In the present study, septins 3A and 3B proved to be expressed predominantly in the human brain (Fig. 3a). This brain-specific expression confirmed previous results of Northern blot analysis on mouse and rat orthologs (26,27). Our analysis (Fig. 3b) further indicated that septin 3 is distributed in a similar fashion to CDCrel-1 throughout the human brain. CDCrel-1 is another septin almost exclusively expressed in the brain (1,2), unlike other septins that exhibit a broader tissue distribution (17,18,21,25). CDCrel-1 is reportedly associated with membranes including synaptic vesicles (2) and binds directly to syntaxin, a protein that mediates the fusion of synaptic vesicles with calcium channels at the presynaptic active zone (1). CDCrel-1 is also known to associate with at least two other septins, Nedd5 and CDC10 (19). Overexpression of wild-type CDCrel-1 in HIT-T15 cells inhibited evoked secretion, whereas a predicted dominant-negative mutation in the GTPase domain enhanced secretion (1). Deletion of CDCrel-1 does not apparently affect synaptic properties or neuronal development, suggesting that CDCrel-1 is functionally

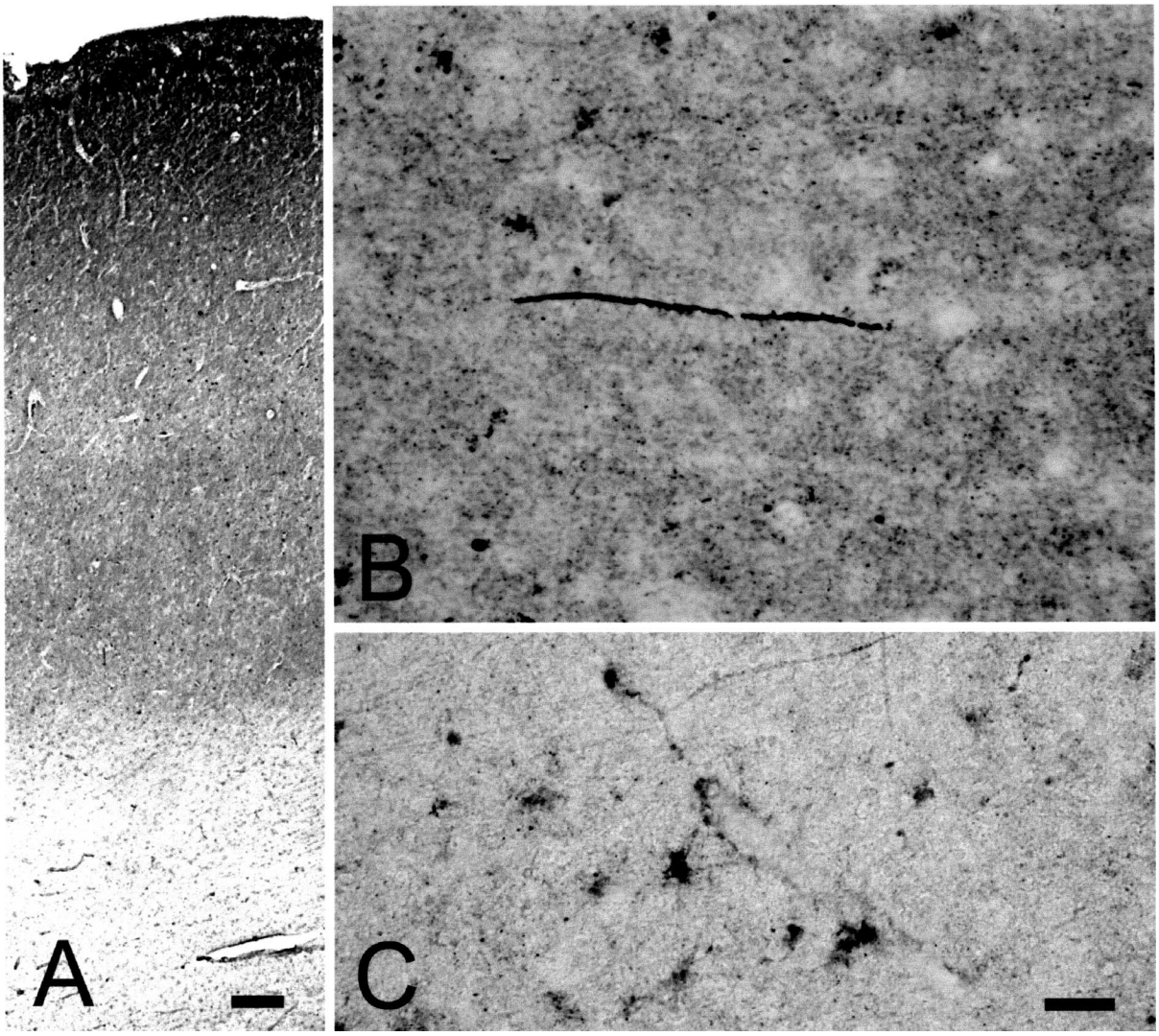

Figure 5. Immunohistochemistry of septin 3A and 3B in the cortex of human mid-frontal gyrus. Dotty structures were densely immunolabeled for septin 3A in the gray matter of the cortex (A and B). In the cortex, layers 1, 2, 3, 5, and 6 were strongly immunopositive compared with layer 4 or subcortical white matter (A). Many dots were scattered around neuropils, and some were clustered in layer 6, where a few axons were strongly stained (B). Clusters of the punctuates were immunoreactive for septin 3B in the cells with astrocytic profiles surrounding a vessel in the white matter (C). Scale bars: (A) 1 mm; (C) 100 μm. (B) and (C) are presented at the same magnification.

redundant with other septins (19). In the present study, we obtained evidence for a complex formation from septin 3A, 3B, and CDCrel-1 in the frontal cortex of human brain (Fig. 4). The pattern of immunoreactivity of septin 3 in the cortex of human mid-frontal gyrus (Fig. 5) supports the view of its functional role at synaptic junctions.

The majority of septins are predicted to have one or more coiled-coil regions, mostly near at the carboxyl termini (10), and some of them are necessary for formation of hetero-oligomeric septin complexes (20). Septin 3 has no predictable coiled-coil domain, which suggests the existence of other mechanisms of inter-septin interactions, similar to that of septin MSF (22). The similar distribution and expression patterns of septin 3 and CDCrel-1 may indicate a redundancy in their functions in synaptogenesis and neuronal development.

Several isoforms of mammalian septins have been shown to be distributed in different compartments of the cell. For example, septin H5 is distributed in the cytosol, while its splicing variant, ARTS, which mediates TGF-β-induced apoptosis (14), is localized in mitochondria and translocated into the nucleus when apoptosis is to be primed. Septin 3A and 3B differ only in 21 and 13 C-terminal amino acids,

keeping the central regions with a GTPase domain conserved. Our preliminary analysis, by using confocal microscopy, has revealed that both isoforms are distributed in the cytoplasm of HeLa cells forming filament-like structures and that the filaments of septin 3A are slightly longer than those of septin 3B (unpublished data). Structural and functional differences of these isoforms in neuronal cells are under investigation.

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