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Isolation and characterization of synaphins, novel cytosolic proteins associated with the docking/fusion complex for neurotransmitter release

Toru Ishizuka

Doctoral Program in Biosystem Science
Graduate School of Science and Technology
Niigata University

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General introduction

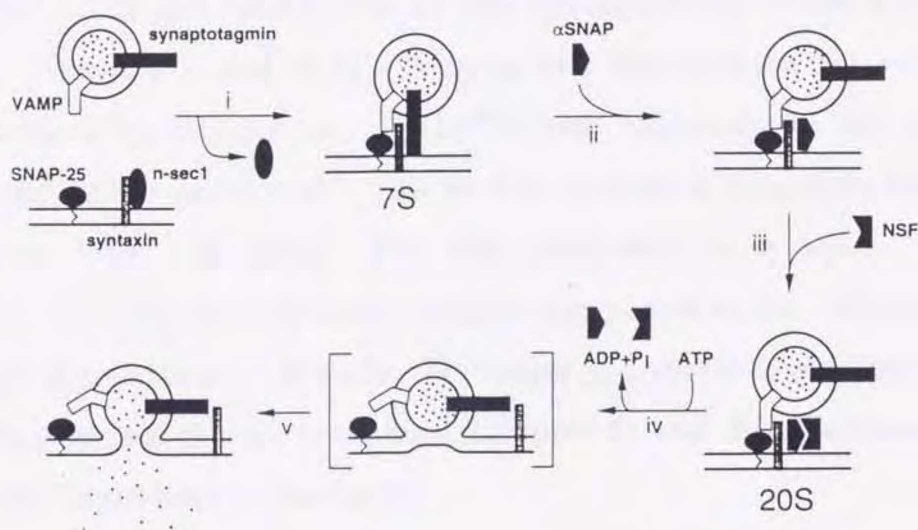
Release of neurotransmitters from synaptic vesicles is the result of a highly orchestrated process of membrane trafficking in the presynaptic nerve terminal. At the chemical synapse, synaptic transmission is accomplished by exocytotic release of neurotransmitters stored in synaptic vesicles that fuse with the presynaptic plasma membrane in response to Ca^{2+} influx within 200 μs of activation of voltage-gated calcium channels (Almers, 1990). This rapid signaling suggests that a subset of synaptic vesicles, likely those clustered at the active zone of presynaptic terminals, are primed and held poised for Ca^{2+}-signaled exocytosis (Burgoyne and Margan, 1995).

As a first step for elucidating the molecular mechanisms of neurotransmitter release, efforts have focused on characterizing molecules unique to synaptic vesicles and have identified many proteins of nerve terminals that are possibly involved in neurotransmitter release. Among these are synaptotagmin, synapsin, synaptophysin, VAMPs/synaptobrevins and rab3A, all in synaptic vesicles, and syntaxins and SNAP-25 in the presynaptic plasma membrane (Südhof, 1995).

Recent insights into the mechanisms of synaptic vesicle docking and fusion have come from several independent approaches described below. First, we (Saisu et al., 1991; Morita et al., 1992; Horikawa et al., 1993) and others (Bennett et al., 1992; Yoshida et al., 1992) found the a protein complex associated with N-type Ca^{2+} channels using monoclonal and polyclonal antibodies that immunoprecipitate N-type Ca^{2+} channels. The complex contains syntaxins, SNAP-25, rab3A, VAMP/synaptobrevin, and synaptotagmin. The presence of this complex indicates that some synaptic vesicles are docked to the presynaptic plasma membrane. Second, investigations on intracellular membrane transports identified *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs), which are cytoplasmic proteins required for the vectorial

movement of vesicles from the endoplasmic reticulum (ER) to the Golgi complex and from Golgi complex to the plasma membrane (Beckers et al., 1989; Orci et al., 1989; Graham and Emr, 1991). Docking and fusion of vesicles was shown to involve the formation of a complex in which NSF and SNAPs bring together a vesicle protein and a target membrane protein. An assay looking for membrane components of this complex in brain identified syntaxins, SNAP-25, and VAMP/synaptobrevin (Söllner et al., 1993). Third, studies on clostridial neurotoxins (tetanus toxin and botulinum neurotoxins) that block synaptic vesicle membrane fusion revealed that these constitute a family of metalloproteases which specifically cleave either VAMP/synaptobrevin, syntaxin, or SNAP-25 (Montecucco and Schiavo, 1993; Niemann et al., 1994). These findings have emphasized the importance of syntaxin, SNAP-25, and VAMP/synaptobrevin in synaptic vesicle exocytosis.

Originally, synaptic vesicle exocytosis was thought to be mechanistically unique. However, the recent convergence of these approaches strongly suggests neurotransmitter release takes places by similar mechanisms to those of intracellular membrane transports. One working hypothesis (Pevsner et al., 1994b, *see page 7*) proposes that two proteins on the synaptic vesicle, VAMP/synaptobrevin and synaptotagmin, interact with two molecules on the plasma membrane, SNAP-25 and syntaxin, to form a 7S complex. Syntaxin is proposed to be associated with a cytosolic protein, Munc-18/n-sec1, prior to and perhaps during formation of the 7S complex. Dissociation of synaptotagmin from the 7S complex is proposed to allow the addition of NSF and SNAPs, forming a 20S complex. This 20S complex dissociates upon ATP hydrolysis by NSF, a process that may lead to synaptic vesicle fusion.



Working hypothesis of the biochemical events involved in synaptic vesicle docking, activation, and fusion.

A pool of syntaxin initially binds Munc-18/n-sec, which dissociates (step i), leading to the docked synaptic vesicle (7S complex). Then synaptotagmin is released, allowing soluble α–SNAP binding (step ii), which leads to the association of NSF (step iii). This forms the 20S complex, which dissociates upon ATP hydrolysis (step iv) and may lead to membrane fusion via unknown intermediates (brackets). Finally, the neurotransmitter contents of the vesicle are released (step v).

In this thesis, I first describe the characterization of a 19 kDa protein that copurified with syntaxin, SNAP-25 and rab3A on SPM-1- and SPM-2-Sepharose. Two monoclonal antibodies, SPM-1 and SPM-2, are immunoprecipitate brain N-type Ca2+ channels. On immunoaffinity chromatography of a digitonin extract of bovine brain membranes on SPM-1- and SPM-2-Sepharose, proteins of molecular masses of 36 kDa (syntaxins), 28 kDa (SNAP-25 and rab3A), and 19 kDa are specifically retained by both columns. The 36 kDa and 28 kDa proteins have already been characterized and identified by Morita et al. (1992) and Horikawa et al. (1993), respectively, in our laboratory. The 19 kDa protein is separable into two components (19a and 19b). The 19a component is a novel protein associated with the docking/fusion complex unreported so far. Thus in the latter part of this thesis, I describe the cloning and sequence analyses of the novel 19a proteins, named synaphins (Chapter 2), and distributions of its mRNAs in the rat brain (Chapter 3).

Abbreviations

BCIP: 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt

bp(s): base pair(s)

cDNA: complementary deoxyribonucleic acid

CNBr: cyanogen bromide

DTT: dithiothreitol

EDTA: ethylenediamine-*N,N,N',N'*-tetraacetic acid

EGTA: *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid

GVIA: ω-conotoxin GVIA

HEPES: 2-[4-(2-hydroxyethyl)-1-piper azinyl]ethanesulfonic acid

HPLC: high-performance liquid chromatography

kb(s): kilobase(s)

kDa: kilodalton

NBT: nitro blue tetrazolium

NSF: *N*-ethylmaleimide-sensitive fusion protein

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PVDF:polyvinylidene difluoride

SDS: sodium dodecyl sulfate

SNAP(s): soluble NSF attachment protein(s)

SNAP-25: synaptosomal-associated protein of 25 kDa

SNARE(s): SNAP receptor(s)

SSC: standatd saline citrate

TFA: trifluoroacetic acid

Tris: tris(hydroxymethyl)aminomethane

VAMP(s): vesicle-associated membrane protein(s)

Part 1.

Syntaxins, SNAP-25, rab3A, VAMP/synaptobrevin and the 19a protein form a complex for neurotransmitter release in brain presynaptic terminals

Introduction

Neurotransmitter release at the presynaptic terminal of neurons is initiated primarily by the entry of Ca^{2+} through voltage-gated calcium channels (Smith and Augustine, 1988; Robitaille et al., 1990). The increase in cytosolic Ca^{2+} concentration triggers the fusion of a portion of synaptic vesicles with the presynaptic plasma membrane, resulting in neurotransmitter release. Exocytosis of synaptic vesicles occurs at specialized regions of the nerve terminal called active zones. These zones may contain clusters of presynaptic calcium channels that supply calcium for neurotransmitter release (Pumplin et al., 1981; Pumplin, 1983; Zucker, 1993). The release of neurotransmitter from the presynaptic nerve terminal occurs very rapidly (within 0.2 ms) following the influx of Ca^{2+}. The brief rise in calcium concentration to the level necessary for exocytosis likely occurs only in proximity to the calcium channels (Llinás et al., 1981). Synaptic vesicles available for rapid transmitter release must presumably be predocked in the vicinity of calcium channels. Thus, it is likely that proteins involved in vesicle docking and fusion associate directly with calcium channels.

A combination of electrophysiological and pharmacological criteria have defined four main types of high voltage-activated calcium channels that are widely distributed in mammalian neurons. These are ω-conotoxin GVIA-sensitive N-type calcium channels, ω-agatoxin IVA- and ω-conotoxin MVIIC-sensitive P-type and Q-type calcium channels, and dihydropyridine-sensitive L-type calcium channels (Bean, 1989; Hess, 1990; Tsien et al., 1991; Miller, 1992; Zhang et al., 1993). GVIA blocks synaptic transmission in some synapses in the nervous system, suggesting that GVIA-sensitive calcium channels are directly involved in neurotransmitter release from presynaptic

terminals (Gray et al., 1988). Thus it is essential to characterize GVIA-sensitive N-type calcium channel molecules and their regulations for elucidating the mechanisms underlying neurotransmitter release and their plasticity.

Saisu et al. (1991) attempted to generate monoclonal antibodies against the brain N-type calcium channel using synaptic plasma membranes (SPMs) as antigen and obtained two monoclonal antibodies (SPM-1 and SPM-2) that immunoprecipitated significant portions of the N-type calcium channel solubilized from brain membranes. In immunoblot analysis of lysed P2 fractions from bovine and rat brains, proteins with molecular masses of 36 kDa and 28 kDa were detected by SPM-1 and SPM-2, respectively. When a digitonin extract of bovine or rat brain lysed P2 fraction was applied to a column of SPM-1- and SPM-2-Sepharose, three major proteins with molecular masses of 36 kDa, 28 kDa, and 19 kDa were retained by both columns. The 36 kDa and 28 kDa proteins reacted with SPM-1 and SPM-2, respectively. However, the 19 kDa protein did not react with either SPM-1 and SPM-2. The 36 k and 28 k proteins might be subunits of the N-type calcium channel. However, these proteins were mostly separated from the N-type calcium channel by sucrose density gradient centrifugation. Therefore, the two proteins are very unlikely to be unique subunits of the N-type calcium channel, but are probably bound to a fraction of N-type calcium channels directly or indirectly. SPM-1 and SPM-2 immunoprecipitated solubilized N-type calcium channels even in a solution containing 0.5% Triton X-100 and 0.5 M NaCl, suggesting strong binding between the two proteins and N-type calcium channels. Thus, it is likely that some N-type calcium channels in the brain form a firm complex with these two proteins. The conservation of the 36 k and 28 k proteins during evolution, at least from amphibian to mammals, and their

localization in the nervous system and paraneurons strongly suggest these proteins play fundamental roles in the nervous system. An immunohistochemical study indicated that these proteins are abundant in synapses in the brain, suggesting their involvement in synaptic functions (Bennett et al., 1992; Ishikawa et al., 1993).

Morita et al. (1992) have demonstrated the presence of several isoforms of the 36 k protein in the brain on the basis of closely-spaced bands that react with SPM-1 in immunoblots and partial amino acid sequence, and have isolated a cDNA clone encoding the 36 kDa protein (named synaptocanalin I). Synaptocanalin I is a novel presynaptic plasma membrane protein consists of 288 amino acid residues with a calculated molecular mass of 33,274. Virtually identical and highly homologous proteins to synaptocanalin I have been reported independently: a rat brain protein recognized by a monoclonal antibody HPC-1 that stains amacrine cells in the retina (Inoue et al., 1992) and two rat brain proteins (syntaxins) associated with the synaptic vesicle membrane protein, synaptotagmin (Bennett et al., 1992). Horikawa et al. (1993) have shown that the 28 k protein contains a synaptic plasma membrane protein, SNAP-25 (synaptosomal-associated protein of 25 kDa) (Oyler et al., 1989) and a synaptic vesicle protein, rab3A (Touchot et al., 1987; Matsui et al., 1988).

In this chapter, I describe the characterization of the 19 kDa protein, and discuss possible functions of the complex that consists of syntaxin, SNAP-25, rab3A, and the 19 kDa protein.

Chapter 1. Characterization of the 19 kDa protein copurified with syntaxins, SNAP-25 and rab3A

1.1 Experimental procedures

1.1.1 Materials

Iodoacetamide and phenylmethylsulfonyl fluoride were obtained from Sigma. Pepstatin A and leupeptin were from Peptide Institute (Osaka, Japan). Cyanogen bromide and lysyl endopeptidase from *Achromobacter* sp. was a product of Wako Pure Chemicals. Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore. Keyhole limpet hemocyanin was purchased from Calbiochem. Complete and incomplete Freund's adjuvant were from DIFCO Labs. Nitrocellulose sheet was from Schleicher and Shuell. Alkaline phosphatase-labeled anti-rabbit IgG was from Kirkegaard & Perry. Other reagents were of analytical grade.

1.1.2 Preparation of lysed P2 fraction from bovine brain

Fresh bovine brains were obtained from the local slaughterhouse, and cerebral cortices were immediately removed. The following procedure was carried out at 0-4 °C. All of the solutions used in preparation of membranes contained four protease inhibitors (1 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 2 μM leupeptin). Cortices (approximately 150 g) were chopped up with scissors, and homogenized in a Waring blender in 9 volumes of a solution (0.32 M sucrose/ 20 mM Tris-HCl pH 7.5). The homogenate was centrifuged at 700 x g for 10 min. The resultant supernatant was sedimented at 10,000 x g for 60 min to obtain P2 fraction. The P2 fraction was suspended in 8 volumes of a solution (20 mM Tris-HCl pH 8.1) and was subjected to 10 up-and-down

strokes in a glass-Teflon homogenizer by hand. The suspension was then stirred gently for 30 min and resedimented at 10,000 x g for 60 min to yield lysed P2 fraction. The lysed P2 fraction was suspended in a solution (20 mM Tris-HCl pH 7.5) to a final volume of 100 ml (approximately 15 mg protein/ml).

1.1.3 Affinity chromatography on SPM-1-Sepharose

The lysed P2 fraction prepared as described above was incubated with an equal volume of solution A (0.15 M NaCl/5 mM EDTA/20 mM Tris-HCl pH 7.5) containing the protease inhibitors and 1% (w/v) Triton-X100 for 40 min at 4 °C. The mixture was centrifuged at 100,000 x g for 60 min and the resultant supernatant was then diluted with an equal volume of solution A. The diluted supernatant was filtrated through a 0.2 μm filter, and the filtrate was applied to a column of SPM-1-Sepharose 4B equilibrated with solution A. The resin was washed first with solution A containing 0.1% Triton X-100, second with solution A, and finally with solution B (5 mM EDTA/20 mM Tris-HCl pH 7.5). The bound material was eluted with solution B containing 0.1% SDS at room temperature.

1.1.4 SDS-polyacrylamide gel electrophoresis and electroelution

The SDS-eluate from SPM-1-Sepharose (10 ml) was concentrated 10-fold by ultrafiltration. The concentrate was mixed with an equal volume of 2x SDS sample buffer (20% (w/v) glycerol/0,125 M Tris-HCl pH 6.8/4% SDS/10% (v/v) 2-mercaptoethanol/0.01% bromophenol blue). The mixture was incubated at 95 °C for 5 min and then electrophoresed in an 11-cm-long 15% SDS-polyacrylamide gel according to Laemmli (1970). After electrophoresis, the gel was stained with 0.2% Coomassie Brilliant Blue R-250/25% 2-propanol/10% acetic acid for 60 min, and destained with several

changes of 10% 2-propanol/10% acetic acid.

The 19a and 19b components were electroeluted with 50 mM NH_4HCO_3, 0.1% SDS using Model 422 Electro-Eluter (Bio-Rad). After electroelution, the eluate was recovered and lyophilized. To remove the SDS and Coomassie blue, lyophilized samples of protein-SDS complexes were dissolved directly in the extraction solvent, which was a freshly prepared mixture of acetone : triethylamine : acetic acid (90 : 5 : 5), and incubated at -20 °C for 3 h. The protein precipitate was collected by centrifugation at 1,600 x g for 20 min, washed twice with cold acetone to remove traces of the extraction solvent, and the residual acetone was removed in a vacuum desiccator.

1.1.5 Partial amino acid sequence analyses

The acetone-dried preparations of the 19a and 19b protein (~10 μg) were treated with 0.15 M CNBr/70% formic acid for 24 h in the dark at room temperature, then recovered by lyophilization after diluting 10-fold with distilled water. The CNBr peptides were electrophoresed in an 11-cm-long 10%-20% polyacrylamide gradient gel according to Horigome et al. (1980). and electroblotted onto polyvinylidene difluoride (PVDF) membrane. The CNBr peptides were visualized by staining with 0.2% Amido Black 10B/50% methanol for 10 min, and the band was applied to a protein sequencer (Model 470A, Applied Biosystems).

The acetone-dried preparation of the 19a protein (~10 μg) was denatured in 50 μl of 6 M guanidine hydrochloride/20 mM sodium phosphate pH 7.2 at 50 °C for 15 min. After dilution with 100 μl of distilled water, the protein was digested with 2 μg of lysyl endopeptidase for 16 h at 37 °C. The resultant peptides were fractionated by reversed-phase high-performance liquid chromatography (HPLC) on an octadecylsilane column (TSKgel ODS-

120T, 4.6 mm x 250 mm, Tosoh) with a linear gradient of 0.062% (v/v) TFA/1% (v/v)CH_3CN-0.05% (v/v) TFA/75% (v/v) CH_3CN at a flow rate of 1 ml/min for 60 min. Major peaks of absorbance at 215 nm were subjected to sequence analysis.

1.1.6 Generation of polyclonal antibodies

Antibodies were raised against synthetic peptides coupled to keyhole limpet hemocyanin. Three peptides were used for antibody production (peptide sequences are shown in single-letter amino acid code) : Peptide I: MSATAATAPPAAPAGEGG. This peptide corresponds to the N-terminus of bovine VAMP/Synaptobrevin-2. Peptide II: VDKVLERDQKLSELDD RADA. This sequence corresponds to amino acid residues 50-69 of bovine VAMP/synaptobrevin-2 and contains the sequence obtained from the purified CNBr peptide of the 19b protein. Peptide III: EKVRQQIRDKYGL KK. This peptide was from the sequence obtained from the purified CNBr peptide of the 19a protein. These three peptides were synthesized using a Milligen peptide synthesizer (9050 PepSynthesizer) and purified by HPLC on a Waters μBondapak C_{18} column (19 mm x 150 mm). Each purified peptide (2 mg) was conjugated overnight with 12 mg of keyhole limpet hemocyanin in 0.1 M sodium phosphate buffer pH 7.4 containing 0.06% (v/v) glutaraldehyde, and dialyzed against 20 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl. The conjugate (corresponding to 0.3 mg of the original peptide) was emulsified with an equal volume of Freund's complete (1st injection) or incomplete adjuvant (subsequent injections) and injected subcutaneously into a rabbit at two-week intervals. Ten days after the 4th or 6th injection, the animal was bled. The blood was kept at room temperature for 2 hours, and then incubated overnight at 4 °C. The serum was prepared by centrifugation at 10,000 x g for 10 min, and sodium azide

was added to a final concentration of 0.05%. This serum was directly used in immunoblot analysis.

1.1.7 Immunoblotting

Electrophoresis was performed on SDS-polyacrylamide gels and the proteins were electrophoretically transferred to nitrocellulose sheet in transfer buffer (25 mM Tris/192 mM glycine/20% (v/v) methanol), blocked by a 60-min incubation in a blocking solution (3% nonfat dried milk/10 mM Tris-HCl pH 8.0/0.15 M NaCl/0.05% (v/v) Tween-20/0.05% NaN_3), and probed with antisera described above at a 1/500 dilution in the blocking solution containing 5% goat serum for 90 min at room temperature. The blots were washed five times (5 min each) in TTBS (10 mM Tris-HCl pH 8.0/0.15 M NaCl/0.05% (v/v) Tween-20/0.05% NaN_3) and then incubated with alkaline phosphatase-labeled anti-rabbit IgG at a 1/1000 dilution in the blocking solution containing 5% goat serum for 90 min at room temperature. After washing five times, immunoreactive bands were visualized by color development in 0.1 M Tris-HCl pH 9.6/0.1 M NaCl/5 mM $MgCl_2$/0.03% NBT/0.015% BCIP.

1.2 Results

1.2.1 Separation of the 19 kDa protein into two components

The monoclonal antibodies SPM-1 and SPM-2, which recognize proteins of 36 kDa (syntaxins) and 28 kDa (SNAP-25 and rab3A), respectively, immunoprecipitate significant fractions of N-type calcium channels solubilized from bovine brain (Saisu et al., 1991). The proteins of 36 kDa, 28 kDa and 19 kDa are major brain membrane components and specifically bound to SPM-1- and SPM-2-Sepharose. Since only the 36 kDa and 28 kDa proteins are recognized by SPM-1 and SPM-2, respectively, all these proteins must be associated with each other. On SDS-PAGE in the presence of urea, the 36 kDa and 28 kDa proteins are separated into two major bands (36a, 36b and 28a, 28b), corresponding to syntaxins 1B and 1A, SNAP-25 and rab3A, respectively (Morita et al., 1992; Horikawa et al., 1993). Material in the 19 kDa band is also separable into two components (19a and 19b). Even in the absence of urea, the 19 kDa protein was easily separated by SDS-PAGE using ≥12.5% uniform gels (Fig. 1-1).

1.2.2 Partial amino acid sequences of the 19a and 19b component

To obtain the amino acid sequence information, the 19a and 19b proteins from SPM-1-Sepharose were blotted onto PVDF membranes and directly applied to a protein sequencer. However, neither component gave any signal, indicating that the amino acid termini of both components were blocked. Therefore, the 19b component from SPM-1-Sepharose was treated with cyanogen bromide, and the resultant peptides were separated by SDS-PAGE. A few bands were detected, but only one fragment gave a partial sequence (Fig. 1-2), which exactly corresponds to amino acid residues 48-79 of bovine brain VAMP/synaptobrevin-2 (Fig. 1-3). The amino acid sequence

of the corresponding part of VAMP/synaptobrevin-1 is almost identical, but it does not contain methionine-46 and therefore should not be cleaved by cyanogen bromide at the position in VAMP/synaptobrevin-1.

The 19a component from SPM-1-Sepharose was also treated with cyanogen bromide and a few fragments were obtained. One CNBr peptide gave the partial sequence (Fig. 1-2). This peptide sequence did not show any significant homology to protein sequences contained in the protein data banks (PIR release 37.0, SWISS-PROT release 25.0). To obtain further sequence information, the 19a protein was digested with lysyl endopeptidase and resultant peptides were fractionated by reversed-phase HPLC. Some peaks were analyzed by gas-phase sequencer, and obtained six lysyl endopeptidase peptide sequences (Fig. 1-2). However, none of the partial sequences obtained from the lysyl endopeptidase peptides showed significant homology to any other protein reported so far.

1.2.3 Immunoblot analyses with antisera against synthetic peptides

In order to characterize the 19a and 19b components, we prepared two rabbit antisera against partial sequences of VAMP/synaptobrevin-2 and one rabbit antiserum against partial sequence of the 19a protein.

In immunoblot analysis of SDS-eluate from SPM-1-Sepharose, two antisera against partial sequence of VAMP/synaptobrevin-2 reacted only with the 19b component, but not with the 19a component. Conversely, antiserum against partial sequence of the 19a component reacted specifically with the 19a component, but not with the 19b or other components (Fig. 1-4A). Thus, the 19a component is probably not a breakdown product of a larger component. These results, together with the results from analyses of partial amino acid sequences, indicate that the 19b

component is probably identical to VAMP/synaptobrevin-2, a small synaptic vesicle membrane protein. On the other hand, the 19a component is not an isoform of the VAMP/synaptobrevin-2, but is a novel protein.

1.2.4 Tissue and subcellular distribution of the 19a component

The tissue distribution of the 19a component was examined by immunoblots of lysed P2 fractions prepared from various tissues of rat with the antibody described above. The protein was found only in the brain among the tissue examined (Fig. 1-5). The 19a component was not detected in the soluble fractions of these tissues other than the brain (data not shown). The size of rat 19a protein was identical to that of bovine 19a protein, indicating the similarity between these two species.

The 19a protein in the brain was much more concentrated in the soluble fraction than in the insoluble fraction (Fig. 1-6A). The protein in the insoluble fraction is not derived from the contamination of the soluble fraction, because similar amounts of the protein could be detected in the pellet even after extensive washing of the pellet (data not shown). Furthermore, the 19a protein copurified with syntaxin (36 kDa), SNAP-25 (28 kDa) and VAMP/synaptobrevin (19b) (all membrane proteins), and does not dissociate even in the presence of detergents and high salts as described above. Thus the 19a protein seems to exist both in the soluble and insoluble fractions in the brain. The 19a protein in both the soluble and insoluble fractions was separable into two components by SDS-PAGE in the presence of urea (Fig. 1-6B). They may indicate the presence of the 19a protein isoforms or homologous proteins.

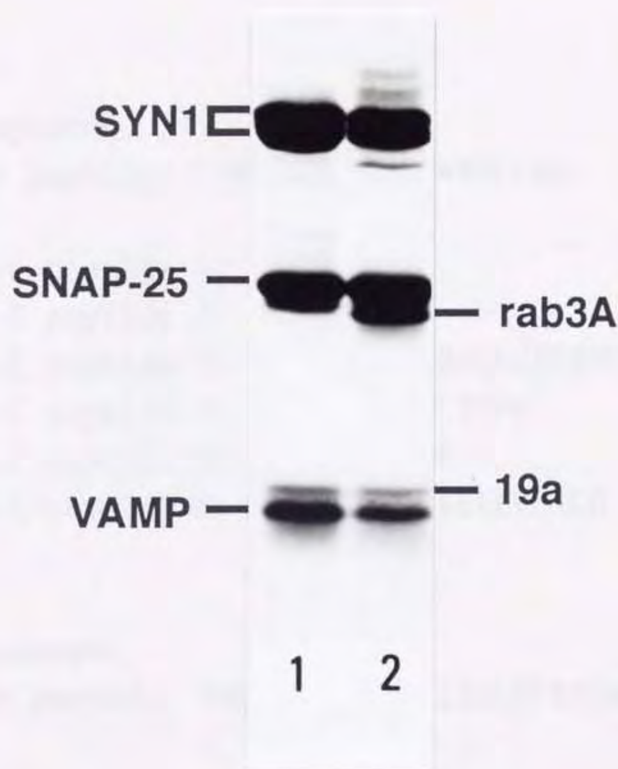


Figure 1-1. Fractionation by SDS-PAGE of proteins bound to SPM-1- and SPM-2-Sepharose.

A Triton X-100 extract of bovine brain membranes was applied to SPM-1- or SPM-2-Sepharose and eluted with SDS. The eluate from SPM-1- (lane 1) and SPM-2-Sepharose (lane 2) were stained with Coomassie blue. The positions of identified proteins are shown on both sides. SYN1, syntaxins 1A and 1B.

```
19a component
   CNBr peptide: EAEREKVRQQIRDKYGLKKXE

   Lys-C peptide 1: HARMEAEREK
   Lys-C peptide 2: QALGGATK
   Lys-C peptide 3: MLGGEEEKDPDAQKKEEERQ
   Lys-C peptide 4: AALEQPEEGSLTRPK
   Lys-C peptide 5: YLPGPLQDMFK
   Lys-C peptide 6: AIPAGEGDEEEEEEESILD


19b component
   CNBr peptide: XVNVDKVLERDQKLSELDDRADALQAGASQFET
```

Figure 1-2. Partial amino acid sequences of the 19a and 19b component.

The 19a component was treated with cyanogen bromide or with lysyl endopeptidase. One cyanogen bromide peptide (CNBr peptide) and six lysyl endopeptidase peptides (Lys-C peptide 1-6) could be sequenced. The 19b component was cleaved with cyanogen bromide and one CNBr peptide sequence was obtained. Amino acid residues were given with one-letter code. X denotes an unidentifiable residue.

```
 1  MSATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVD    40

           XVNVDKVLERDQKLSELDDRADALQAGASQFET
41  EVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETS   80

81  AAKLKRKYWWKNLKMMIILGVICAIILIIIIVYFSS      116
```

Figure 1-3. Identities of the 19b component with VAMP/ synaptobrevin-2.

The partial sequence obtained is shown above the corresponding sequence of bovine VAMP/synaptobrevin-2. X denotes an unidentifiable residue. Numbers of amino acid residues are given at both ends.

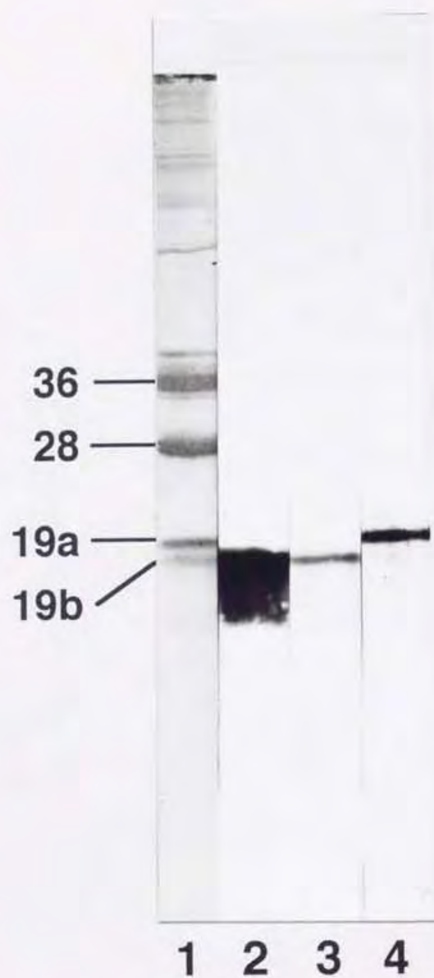


Figure 1-4. Immunoblotting with antibodies against synthetic peptides.

SDS-eluate from SPM-1-Sepharose was analyzed by SDS-PAGE. lane 1, Amidoblack 10B staining; lanes 2 and 3, immunoblots with antisera against residues 1-18 and 50-69 of VAMP/synaptobrevin-2, respectively; lane 4, immunoblot with an antiserum against a partial sequence of the 19a component. Positions of bands are shown on the left.

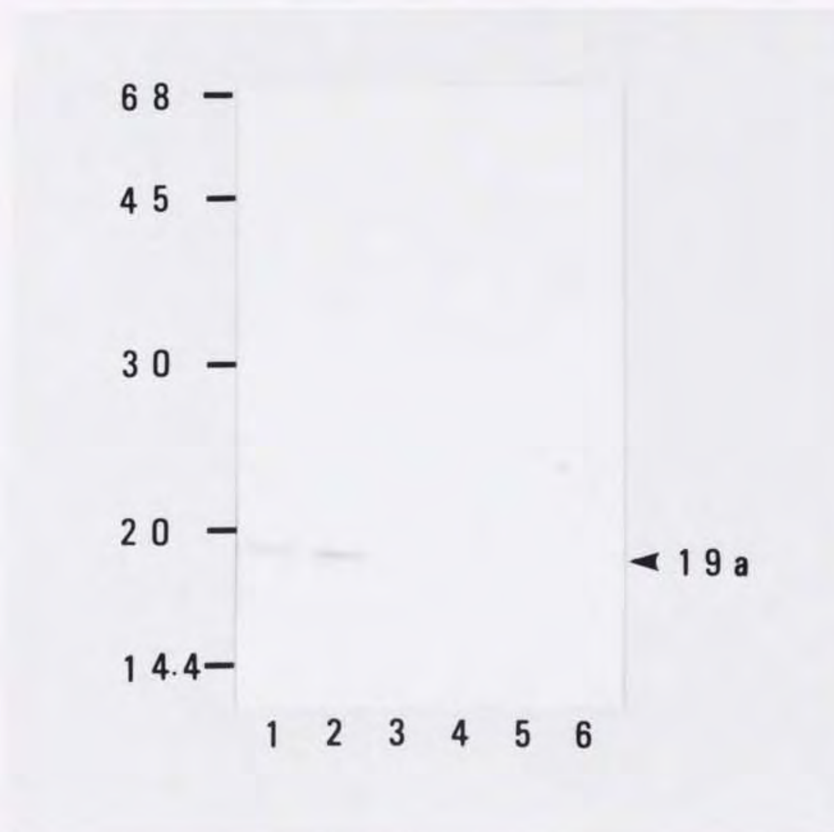


Figure 1-5. Tissue distribution of the 19a component.

Lysed P2 fractions (70 μg protein) prepared from various tissues were analyzed by immunoblotting. Lanes 1-5, rat cerebrum, cerebellum, kidney, liver, heart, respectively; lane 6, bovine adrenal medulla. Molecular masses (in kDa) of marker proteins and position of the 19a component are shown on the left and right, respectively.

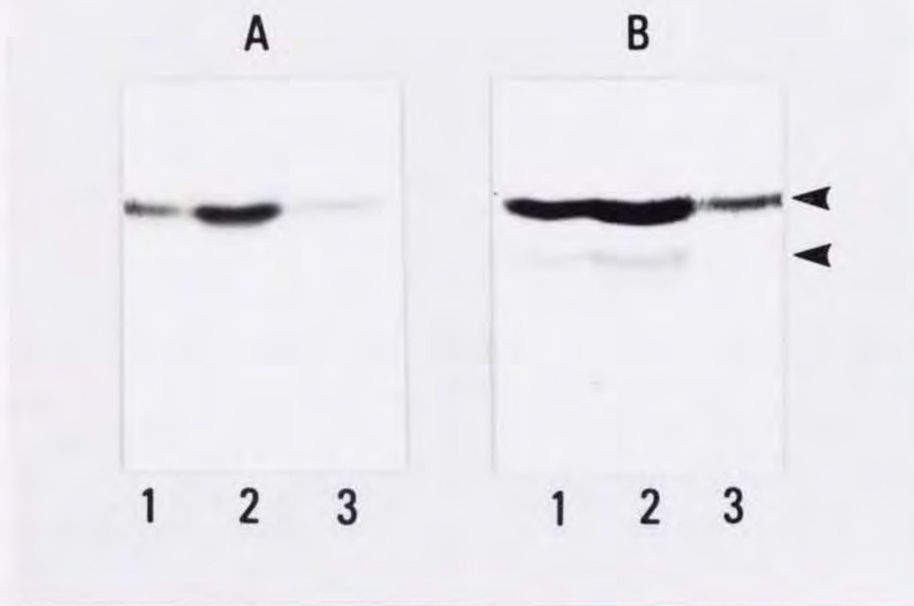


Figure 1-6. Presence of the 19a component in the soluble and insoluble fractions.

Rat cerebra were homogenized and centrifuged at 2 x 10^5 g for 1 h. The resultant supernatant and pellet together with the homogenate were subjected to immunoblotting. SDS-PAGE (100 μg protein/lane) was carried out in the absence (A) or presence (B) of urea. For both panels A and B, lane 1, homogenate; lane 2, soluble fraction; lane 3, insoluble fraction. Arrowheads in B indicate the two components. The 19a component tended to be stained with the antibodies more strongly when SDS-PAGE was carried out in urea.

Discussion

Two monoclonal antibodies (SPM-1 and SPM-2) immunoprecipitate brain N-type calcium channels. On immunoaffinity chromatography of digitonin or Triton X-100 extracts of bovine brain membranes on SPM-1- and SPM-2-Sepharose, proteins of 36 kDa (syntaxins), 28 kDa (SNAP-25 and rab3A) and 19 kDa are retained by both columns. In this chapter, I have characterized the 19 kDa protein. The 19 kDa protein is separable into two component (19a and 19b). Partial amino acid analysis of the 19b component demonstrated that one CNBr peptide gave the sequence that exactly corresponded to amino acid residues 48-79 of bovine brain VAMP/synaptobrevin-2. The amino acid sequence of the corresponding part of VAMP/synaptobrevin-1 is almost identical, but it does not contain methionine-46 and therefore should not be cleaved by cyanogen bromide at the position. Moreover, 19b, but not 19a, was recognized by antisera against two partial sequences of VAMP/synaptobrevin-2. Thus the 19b component is probably identical to VAMP/synaptobrevin-2. On the other hand, none of the partial amino acid sequences of several peptides derived from the 19a protein showed significant homology to any other protein reported so far. The 19a protein existed only in the brain among the tissues examined, and was much concentrated in the soluble fraction. Thus it is probable that the 19a component is a novel brain-specific cytosolic protein that has not yet been reported. Some of the 19a component definitely exists in the insoluble fraction. Thus our previous and present results indicate that syntaxins, SNAP-25, rab3A, VAMP/synaptobrevin-2 and the 19a protein form a complex in brain presynaptic terminals (Fig. 1-7).

Söllner et al. (1993) discovered that three membrane proteins present in synapses, VAMP/synaptobrevin-2, syntaxins and SNAP-25 are receptors

(SNAREs) for the general fusion proteins SNAPs (soluble NSF attachment proteins) and that they assemble with SNAPs and the *N*-ethylmaleimide-sensitive factor (NSF) fusion protein into 20S fusion particles. They proposed that this disassembly may be an intrinsic step in the fusion mechanism (SNARE hypothesis). In our experiments, no significant amounts of SNAPs (α, β and γ) (33-36 kDa) or NSF (76 kDa) were detected in SDS-eluates from either column, as judged from SDS-PAGE patterns. Our procedures for preparing the lysed P2 fraction and/or for extraction of membrane proteins may dissociate SNAPs and NSF from the complex, but our results indicate that syntaxins, SNAP-25, rab3A, VAMP/synaptobrevin-2 and the 19a component form a complex even in the apparent absence of SNAPs and NSF.

Rab3A and VAMP/synaptobrevin-2 exist in synaptic vesicles. In addition, syntaxins bind another synaptic vesicle protein, synaptotagmin. Thus synaptic vesicles located at the release site are probably associated with the presynaptic membrane by binding between these synaptic vesicle proteins and presynaptic membrane proteins such as syntaxins and SNAP-25. These synaptic vesicles should be situated very close to N-type calcium channels, as syntaxins and the 28 kDa protein (rab3A and/or SNAP-25) are associated with N-type calcium channels. Thus the present results suggest tight linkage of components probably participating in synaptic neurotransmitter release. This presynaptic complex may represent an essential machinery for the extremely rapid synaptic vesicle exocytosis. Studies on clostridial neurotoxins (tetanus toxin and botulinum neurotoxins) that block synaptic vesicle membrane fusion revealed that these constitute a family of metalloproteases which specifically cleave either VAMP/ synaptobrevin, syntaxin, or SNAP-25 (Montecucco and Schiavo, 1993; Niemann et al., 1994). These findings strongly support our idea.

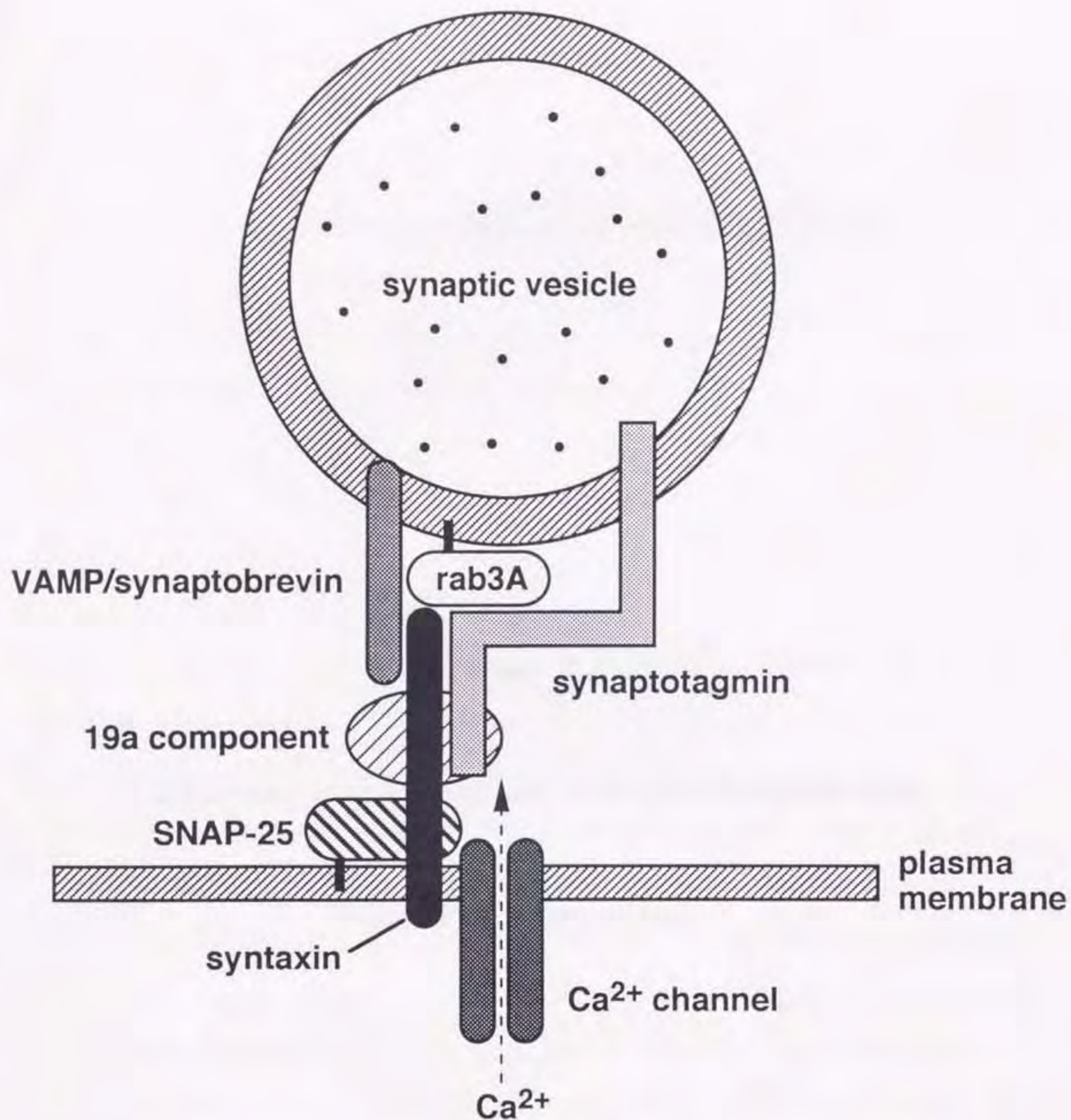


Figure 1-7. Proposed model for the molecular machinery involved in synaptic vesicle exocytosis.

Syntaxin, SNAP-25, rab3A, VAMP/synaptobrevin and the 19a component form a firm complex in brain presynaptic terminals, and this complex is situated close to N-type calcium channels. Synaptotagmin, a synaptic vesicle protein, has been shown to bind to syntaxins (Bennett et al., 1992).

Part 2.

Isolation, identification and characterization of the 19a component

Introduction

Various approaches contributed to the identification of several proteins critical to neurotransmitter release at presynaptic terminals. Firstly, using monoclonal and polyclonal antibodies that immunoprecipitate solubilized ω-conotoxin GVIA-sensitive (N-type) calcium channels we (Saisu et al., 1991; Morita et al., 1992; Horikawa et al., 1993) and others (Bennett et al., 1992; Yoshida et al., 1992) found a protein complex associated with N-type calcium channels. The complex (docking/fusion complex) contains syntaxins 1A and 1B, SNAP-25, VAMP/synaptobrevin-2, rab3A and synaptotagmin I. The presence of this complex indicates that some synaptic vesicles are docked to the presynaptic plasma membrane. Secondly, searches for the substrates for proteolytic activities of clostridial neurotoxins (tetanus toxins and botulinum neurotoxins) led to the identification of VAMP/synaptobrevin-2, SNAP-25 and syntaxins 1A and 1B as the targets of these toxins (Montecucco and Schiavo, 1993; Niemann et al., 1994). Since these neurotoxins specifically block neurotransmitter release, the identification provided convincing evidence for the involvement of these targets in neurotransmitter release. Thirdly, investigations on intracellular membrane transports in the brain have made a fundamental contribution to understanding the basic molecular mechanism underlying neurotransmitter release. NSF (*N*-ethylmaleimide-sensitive fusion protein) and SNAPs (soluble NSF attachment proteins) had been identified as ubiquitous agents for intracellular membrane fusions. Syntaxin 1A and 1B, SNAP-25 and VAMP/synaptobrevin-2 were found to be receptors for SNAPs (SNAREs) (Söllner et al., 1993). The finding strongly suggests that neurotransmitter release takes places by similar mechanisms to those of intracellular membrane transports. The SNARE hypothesis (Söllner et al,

1993; Söllner and Rothman, 1994) proposes that fusion in membrane transport occurs by combination between a SNARE in the transport vesicle (v-SNARE) and a SNARE in the target membrane (t-SNARE). VAMP/synaptobrevin-2 exists exclusively on the synaptic vesicle membrane and serve as a v-SNARE, while syntaxins 1A and 1B and SNAP-25 are mostly on the presynaptic plasma membrane and thus t-SNAREs. Fourthly, mutations of *Cenorhabditis elegans* and *Drosophila*, and gene knockout mice for the components of the docking/fusion complex indicate their importance for normal synaptic transmission (Südhof, 1995).

Very recent studies have shown that a cytoplasmic protein, Munc-18/n-sec1, interacts with syntaxin (Hata et al, 1993; Pevsner et al., 1994a; Garcia et al., 1994). It is a mammalian homolog of unc-18 of *C. elegans* whose mutants cause abnormal accumulation of acetylcholine (Gengyo-Ando et al., 1993), and seems inhibitory to neurotransmitter release (Schulzu et al., 1994). Besides the components described above, a protein of 19 kDa (19a component) constantly copurifies with the docking/fusion complex irrespective of detergent (digitonin, Triton-X100 or CHAPS) used for solubilization of the complex (Horikawa et al., 1993). The protein does not dissociate from the complex even in the presence of 0.5 M NaCl, indicating its tight binding to the complex. The 19a component is retained by Sepharoses conjugated with monoclonal antibodies SPM-1 (specifically recognizes both syntaxins 1A and 1B) and SPM-2 (specifically recognizes both SNAP-25 and rab3A) but not by Sepharose conjugated with normal mouse IgG (Saisu et al., 1991; Horikawa et al., 1993). Thus the protein binds to SPM-1 and SPM-2 specifically.

In the present study, we isolated and analyzed the sequences of cDNA clones for the 19a proteins and examined its subcellular distribution (Chapter 2). The 19a protein has two highly homologous isoforms (named

synaphins 1 and 2). We examined the tissue distribution and the localization in the rat brain of its mRNAs by Northern blotting and *in situ* hybridization, respectively (Chapter 3).

Chapter 2. Cloning and sequence analyses of cDNAs encoding the 19a component from bovine and rat brains

2.1 Experimental Procedures

2.1.1 Materials

Bovine and rat brain cDNA libraries constructed in phage λgt10 and bovine brain cDNA (Quick-clone cDNA) were obtained from Clontech. Plasmid vectors, pBluescript II SK(+) and pCR-Script SK(+), were from Stratagene. [α-^{32}P]dCTP was purchased from Amersham. The kit for DNA labeling was from TaKaRa Shuzo Ltd. A mouse monoclonal antibody against synaptophysin (SY38) was purchased from Progen Biotechnik. A mouse monoclonal antibody against synaptotagmin and a rabbit polyclonal antibody against VAMP were from Wako Pure Chemicals. Alkaline phosphatase-labeled anti-mouse IgG and alkaline phosphatase-labeled anti-rabbit IgG were from Kirkegaard & Perry. All other reagents used were of the highest quality commercially available.

2.1.2 Cloning of bovine synaphin

To generate a probe recognizing the above peptide sequence, a PCR was carried out (5 cycles of 95 °C, 30 s; 45 °C, 30 s; 70 °C, 1 min, followed by 35 cycles of 95 °C, 30 s; 55 °C, 30 s; 70 °C, 1 min) using 5 ng bovine brain cDNA and degenerate primers [5'-GA(AG)GC(AGCT)GA(AG)TC(AGCT)GA(AG)-AA(GA)GT-3' and 5'-TT(CT)TT(AGCT)AG(AGCT)CC(AG)TA(CT)TT(AG)TC-3'] for the CNBr peptide sequence of the 19a component. The product was cloned into pCR-Script SK(+) and sequenced by the method of Sanger et al. (1977). The nucleotide sequence corresponding to the above peptide sequence was obtained. This oligonucleotide was labeled by PCR using [α-

^{32}P]dCTP and used as a probe for screening a λgt10 bovine brain cDNA library. Filters were hybridized at 45 °C for 16 h in a solution containing 50 mM Tris-HCl pH 8.0/1 M NaCl/10 mM EDTA/0.1% SDS/100 μg /ml of heat-denatured salmon sperm DNA. The filters were washed with 6x SSC/0.1% SDS at 55 °C for 30 min and autoradiographed. Three clones were isolated from the library and sequenced after subcloning into pBluescript II SK(+). The nucleotide sequence of the cDNA clone was deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases (accession number D50807).

2.1.3 Cloning of rat synaphins

cDNA library from rat brain in λgt10 was screened with random-primed DNA probes from bovine synaphin. Hybridization was performed at 42 °C for 18 h in a hybridization solution containing 4x SSC/25% formamide/50 mM HEPES-NaOH pH 7.0/10x Denhardt's solution/100 μg/ml of heat-denatured salmon sperm DNA. Filters were washed with 0.5x SSC/0.1% SDS for 30 min at 50 °C. To obtain the inserts from λgt10 vectors, a PCR was performed. The oligonucleotide primers used flank the *Eco* RI cloning site of the λgt10 vector [forward primer: 5'-CTGCAGAGCA-AGTTCAGCCTGGTTAAGT-3' and reverse primer: 5'-GATATCTTATGAGT-ATTTCTTCCAGGG-3']. Reaction conditions consisted of 3 min at 94 °C followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C and 3 min extension at 72 °C, and a final incubation at 72 °C for 15 min. The amplified inserts were sequenced after subcloning into the *Srf* I site of pCR-Script SK(+). The nucleotides sequences of the cDNA clones were submitted to the DDBJ, EMBL and GenBank databases (accession number D70816 and D70817).

2.1.4 Preparation of subcellular fractions from rat brain

The preparation of subcellular fraction was performed according to the method of Huttner et al. (1983). The preparation procedure is outlined schematically in Fig. 2-1.

Fifteen rats were anesthetized with ether followed by decapitation. The brains were removed from the skull and placed into an ice-cold "buffered sucrose" (320 mM sucrose/4 mM HEPES-NaOH pH 7.3) containing protease inhibitors (1 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 2 μM leupeptin). From this point on, the material was kept at 4 °C throughout the preparation. Each cerebral cortex was dissected free of cerebellum, brain stem, and most of the midbrain. The cerebral cortices were homogenized in 10 volumes of buffered sucrose and centrifuged at 800 x g for 10 min. The resulting pellet (P1) was discarded, while the supernatant (S1) was collected and centrifuged at 9,200 x g for 15 min. The supernatant (S2) was removed, and the pellet (P2) was washed by resuspending in 120 ml of buffered sucrose and recentrifuged at 10,200 x g for 15 min to yield a supernatant, S2, and a pellet, P2'. The pellet (P2') was resuspended in 13 ml of buffered sucrose and transferred to a glass-Teflon homogenizer. Nine volumes of ice-cold water were added, and whole suspension was immediately homogenized. The resultant P2'-lysate (L) was poured into a beaker containing 1 ml of 1 M HEPES-NaOH pH 7.4, and the suspension was kept on ice for 30 min. It was then centrifuged at 25,000 x g for 20 min to yield a lysate pellet (LP1) and a lysate supernatant (LS1). The supernatant was centrifuged at 165,000 x g for 2 h. The supernatant (LS2) was removed, and the pellet (LP2) was resuspended in a total of 10 ml of 10 mM HEPES-NaOH pH 7.4.

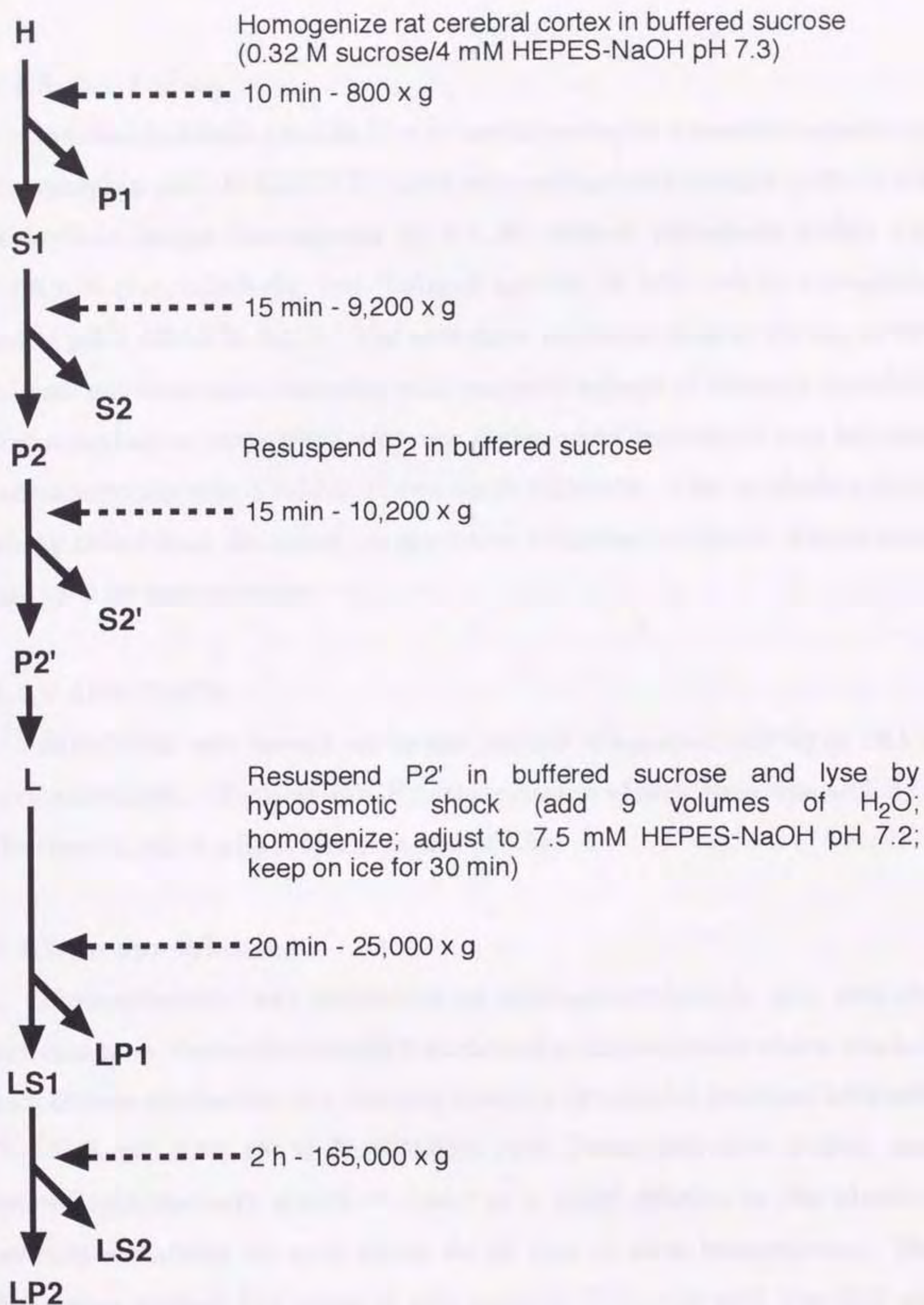


Figure 2-1. Scheme for preparation of subcellular fractions from rat brain.

2.1.5 Antibodies

Purified synthetic peptide (2 mg) corresponding to a partial sequence of the synaphin (EKVRQQIRDKYGLKK) was conjugated overnight with 12 mg of keyhole limpet hemocyanin in 0.1 M sodium phosphate buffer pH 7.4/0.06% glutaraldehyde, and dialyzed against 20 mM sodium phosphate buffer pH 7.4/0.15 M NaCl. The conjugate (corresponding to 0.3 mg of the original peptide) was emulsified with an equal volume of Freund's complete (1st injection) or incomplete adjuvant (subsequent injections) and injected subcutaneously into a rabbit at two week intervals. The antibodies (IgG) were purified from the serum using protein A-Sepharose CL-4B (Pharmacia) and used for immunoblots.

2.1.6 SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli (1970) in 12.5% acrylamide gels. For SDS-PAGE in the presence of urea, urea was added to the running gel at a final concentration of 5 M.

2.1.7 Immunoblotting

Electrophoresis was performed on SDS-polyacrylamide gels and the proteins were electrophoretically transferred to nitrocellulose sheet, blocked by a 60-min incubation in a blocking solution (3% nonfat dried milk/10 mM Tris-HCl pH 8.0/0.15 M NaCl/0.05% (v/v) Tween-20/0.05% NaN_3), and probed with antisera described above at a 1/500 dilution in the blocking solution containing 5% goat serum for 90 min at room temperature. The blots were washed five times (5 min each) in TTBS (10 mM Tris-HCl pH 8.0/0.15 M NaCl/0.05% (v/v) Tween-20/0.05% NaN_3) and then incubated with either alkaline phosphatase-labeled anti-rabbit IgG or alkaline phosphatase-labeled anti-mouse IgG at a 1/500 dilution in the blocking

solution containing 5% goat serum for 90 min at room temperature. After washing five times, immunoreactive bands were visualized by color development in 0.1 M Tris-HCl pH 9.6/0.1 M NaCl/5 mM $MgCl_2$/0.03% NBT/0.015% BCIP.

2.1.8 Glycerol gradient centrifugation and immunoprecipitation

The 7S particle was formed by solubilizing an LP2 fraction in HKA buffer (10 mM HEPES-KOH pH 7.5/140 mM potassium acetate/1 mM magnesium chloride/0.1 mM EGTA) supplemented with 2% Triton X-100 for 1 h at 4 °C. Insoluble material was removed by centrifugation at 100,000 x g for 1 h. The solubilized preparation was layered onto a linear 10%-35% glycerol gradient (in HKA buffer containing 0.5% Triton X-100) in an SW28 centrifuge tube (Beckman) and subjected to centrifugation at 27,000 rpm (100,000 x g) for 24 h. Sixteen 2 ml fractions were collected, and one-forth of each fraction was precipitated with 10% trichloroacetic acid (total sample) and the remainder was immunoprecipitated with anti-syntaxin antibody SPM-1, anti-SNAP-25 antibody SPM-2, and anti-synaphin antibody 12C5, respectively. The samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the following antibodies: anti-synaptotagmin monoclonal antibody, anti-syntaxin monoclonal antibody SPM-1, anti-SNAP-25 monoclonal antibody SPM-2, anti-synaphin monoclonal antibody 12C5 and anti-synaptobrevin-2 polyclonal antibody. Bovine serum albumin (4.6S), catalase (11.3S) and thyroglobulin (19.3S) were resolved on a parallel gradient and sedimented to fractions 13, 10 and 6, respectively.

2.2 Results

2.2.1 Structure of the bovine 19a component

The nucleotide sequence for the amino acid sequence of the cyanogen bromide peptide of the 19a component was obtained by PCR using degenerate primers for both ends of the peptide. The oligonucleotide (56 bps) was used as a probe for screening a bovine brain cDNA library. From 480,000 clones screened, three identical clones (about 1.1 kbps) were obtained. These clones contained an open reading frame of 402 nucleotides including a sequence corresponding to the partial amino acid sequence obtained by cyanogen bromide cleavage and lysyl endopeptidase digestion (Fig. 2-2). The most upstream ATG in the open reading frame was assumed to be the initiation site for translation, as the immediate upstream of this ATG is consistent with the Kozak criteria and an in-frame termination codon (TAG) exists upstream of the ATG.

The protein encoded by these clones contains 134 amino acid residues without a signal peptide. No strongly homologous proteins were found in the databases. Amino acids 15-68, 34-102 and 33-94 of the protein are weakly homologous (28-34% identical) to a protein of myosin heavy chain-B, troponin T and calreticulin, respectively. The protein is highly charged (48% of the total residues) and especially rich in glutamic acid and lysine residues (accounting for more than 1/3 of the total residues). The protein is acidic (calculated isoelectric point: 4.9) consistent with its behavior on 2-dimensional electrophoresis (data not shown). The region from amino acid 20 to 80 is especially hydrophilic as indicated by its hydropathy profile (Fig. 2-5). Lacking any putative transmembrane segments or strongly hydrophobic domain, the protein appears to be soluble by itself. A potential site (serine-115) for phosphorylation by casein kinase II exists.

Although the function of the 19a component is unknown at present, it is associated with the docking/fusion complex and presumably participates in or regulates the mechanisms of neurotransmitter release in harmony with the other components of the complex. We designate the protein **synaphin** from the Latin *synaphia*, meaning "maintenance of the same rhythm".

2.2.2 Isolation and sequence analyses of rat synaphin cDNAs

The coding region of the bovine synaphin cDNA was used to screen a rat brain cDNA library, and we have isolated two cDNAs encoding highly homologous proteins (named synaphin 1 and 2). Figures 2-3 and 2-4 show the nucleotide and deduced amino acid sequences of representative clones. Comparison of the rat synaphin nucleotide sequences with the cDNA sequence of bovine synaphin indicated that the only homology between these three sequences was in the coding region of the synaphin protein. The cDNA sequence for rat synaphin 1 is 912 nucleotides in length and the open reading frame encodes a predicted protein of 134 amino acid residues, which is completely identical to the bovine synaphin protein sequence. The cDNA sequence presented for rat synaphin 2 is 1212 nucleotides in length and the open reading frame encodes a protein containing 134 amino acid residues with a calculated molecular mass of 15,122. The protein sequence of rat synaphin 2 shares 84.3% homology with the rat and bovine synaphin 1 sequences (Fig. 2-6). Similar homologies are also observed at the nucleotide sequence level. A putative phosphorylation site (serine-115) by casein kinase II, which exists in the bovine and rat synaphin 1, is also conserved in the rat synaphin 2.

2.2.3 Subcellular distribution of synaphin

Since synaphins are highly charged and lacking any strong hydrophobic

domain, the protein appears to be soluble by itself. Indeed, synaphin in the brain was much more concentrated in the soluble fraction than in the insoluble fraction (Chapter 1). Synaphin in the insoluble fraction is not derived from the contamination of the soluble fraction, because similar amounts of synaphin could be detected in the pellet even after extensive washing of the pellet. Furthermore, synaphin associated with the docking/fusion complex does not dissociate even in the presence of detergents and high salts as described above. Thus synaphin seems to exist both in the soluble and insoluble fractions in the brain. Since some components of the docking/fusion complex exist in synaptic vesicles, we examined the presence of synaphin in synaptic vesicles. Figure 2-7 shows the distribution of synaphin and synaptophysin (a synaptic vesicle-specific protein) in the fractions obtained by the procedure for the isolation of synaptic vesicles. In contrast to synaptophysin, synaphin was scarce in the LP2 fractions that is rich in synaptic vesicles, indicating that very little, if any, of the protein is associated with synaptic vesicles.

2.2.4 Presence of synaphin in the 7S complex

Söllner et al. (1993) have demonstrated that four proteins, synaptotagmin, syntaxin, SNAP-25, and VAMP/synaptobrevin, associate to form a 7S complex that may mediate the docking of vesicles with presynaptic plasma membranes. We investigated whether synaphin is also localized to this 7S complex, using a combination of glycerol gradient centrifugation and immunoprecipitation. Synaptosomal proteins were fractionated on glycerol gradients, and immunoprecipitated with anti-synaptotagmin, anti-syntaxin, anti-SNAP-25, anti-VAMP/synaptobrevin and anti-synaphin antibodies. The distribution of immunoprecipitated proteins was determined by Western blotting with these five antibodies (Fig.

2-8). Synaptotagmin, syntaxin, SNAP-25, VAMP/synaptobrevin, and synaphin all migrated to fraction 10-14 (7S position was at fraction 12). All antibodies immunoprecipitate synaptotagmin, syntaxin, SNAP-25, VAMP/synaptobrevin, and synaphin, demonstrating the presence of synaphin in the 7S complex.

```
                                         CGGGCCGCATCGCCCCCAAGCCAGGCCTG    29
CCTGGAGCGCCGCATGCAAATTCAGCTGCTGGCTAGGGCACACTAACGCACGTTGTAGGC    89
ATGGACTTCGTCATGAAGCAGGCCCTTGGAGGGGCCACCAAGGACATGGGGAAGATGCTG   149
 M  D  F  V  M  K  Q  A  L  G  G  A  T  K  D  M  G  K  M  L     20

GGCGGAGAGGAGGAGAAGGACCCTGACGCGCAGAAGAAGGAAGAGGAGCGGCAGGAGGCA   209
 G  G  E  E  E  K  D  P  D  A  Q  K  K  E  E  E  R  Q  E  A     40

CTTCGGCAGCAAGAGGAGGAACGCAAGGCCAAGCACGCCCGCATGGAGGCCGAGCGTGAG   269
 L  R  Q  Q  E  E  E  R  K  A  K  H  A  R  M  E  A  E  R  E     60

AAGGTCCGGCAGCAGATACGAGACAAGTATGGGCTGAAGAAAAAGGAGGAGAAGGAGGCC   329
 K  V  R  Q  Q  I  R  D  K  Y  G  L  K  K  K  E  E  K  E  A     80

GAGGAGAAGGCGGCCCTGGAGCAGCCCTGCGAGGGGAGCCTGACCCGGCCCAAGAAGGCC   389
 E  E  K  A  A  L  E  Q  P  C  E  G  S  L  T  R  P  K  K  A    100

ATCCCAGCAGGCTGCGGGGACGAGGAGGAGGAGGAAGAGGAGAGCATCCTGGACACGGTG   449
 I  P  A  G  C  G  D  E  E  E  E  E  E  E  S  I  L  D  T  V    120

CTCAAATACCTCCCCGGGCCGCTGCAGGACATGTTCAAGAAGTAACCAGCCCCCCCGCCC   509
 L  K  Y  L  P  G  P  L  Q  D  M  F  K  K  *                   134

CGACCCCCGGGCCATCCACTTGTTACTTTTCTTTTCTTTTTGGTTCTTTCTTTTCTTTTT   569
ATTCTGTTAAGTCTCAGTTCTGAAGGGGAAAACCTCAGTTGGCCTCTGCCCCACTTCCCC   629
GGCCAGGGGCTCCTCCCCCTCAGCACTCCTTCATGCCCTCCTTCATCCAGGGTATCCCGC   689
CTCCATCACCTCACTCCCAAGTTGCTTGAAAAGAAGAAGACAGCTTCTCTCCAACAGGAG   749
GTGTTAAGCCCAAGGCGGGAGGCACTGGAGGCCCCCTCCGGAAGCCTGAAGTCTGTGCTG   809
GTGTGGTGATGCCCATACATTCCTTCATGCGCCCCACTGCCAGGAGGACCACTGTCCCAG   869
CCAGCCAAAGTAATGACACATTCCAGCCCTGCCCAGCATGCTGACCTTTGGCCTCTAAAT   929
CCCCGTGGGCCTCCAGGTCAGGGCAGGGGTTGAGTGGCCTGGCTCTGAGAAAGGGAGTCA   989
GGGTAAGTCTGGCCTGTCAGGGCCTAGCCTGAGGCTGGCCTGCTCTGTTGTGGCCAGGCC  1049
TGGGGCTGGGCAGGAGGCTGGGGCTCTCCTTCCTGCCTCCCTCTAAAGACACCCGATCCA  1109
GGAGCACCCCTCTACCACCCG                                         1130
```

Figure 2-2. Nucleotide and deduced amino acid sequence of bovine synaphin (19a component).

Numbers of both nucleotide and amino acid residues are shown on the right. The asterisk indicates termination.

```
      ATTCCGGCTGTACGGCGTCAGGGCCTGGCTGAGGGGCGTGATTGGAGGGCGCCT    54
TCGGCATCCGCCCGCCCGCGGCAGCAGCCTCGGTCCCAGCTCGCCTGGCGGAGCCACACC   114
AGGCAGCGGCGCGAGCTGGCTGAGCACACCGCACTGCCTGCTGGAGTCAGCCGGAGCCCT   174
GCAGTGGCTTAGACGGTTGCTGGGACCACCAGGCGAGCTCATTCCCCGAAGGCAGACCAG   234
CCAGGAGTGCTGAATGCGAATTCAGTCACGGGCTAGGGAGCACTAACCAAAGCCTGTAGG   294
ATGGACTTCGTCATGAAGCAAGCCCTCGGAGGGGCCACCAAGGACATGGGGAAGATGCTG   354
 M  D  F  V  M  K  Q  A  L  G  G  A  T  K  D  M  G  K  M  L     20

GGGGGAGAGGAGGAGAAGGACCCAGATGCACAGAAGAAGGAGGAGGAGCGGCAGGAGGCC   414
 G  G  E  E  E  K  D  P  D  A  Q  K  K  E  E  E  R  Q  E  A     40

CTGAGGCAGCAGGAGGAAGAGCGCAAGGCCAAACATGCCCGCATGGAAGCGGAACGTGAG   474
 L  R  Q  Q  E  E  E  R  K  A  K  H  A  R  M  E  A  E  R  E     60

AAGGTCCGGCAGCAGATTCGAGATAAGTATGGGCTGAAGAAGAAAGAAGAGAAGGAGGCA   534
 K  V  R  Q  Q  I  R  D  K  Y  G  L  K  K  K  E  E  K  E  A     80

GAGGAGAAGGCAGCCCTGGAACAGCCCTGCGAGGGAAGCCTGACCAGACCCAAGAAGGCC   594
 E  E  K  A  A  L  E  Q  P  C  E  G  S  L  T  R  P  K  K  A    100

ATCCCTGCAGGCTGTGGGGACGAGGAGGAGGAAGAAGAGGAGAGCATCCTGGACACGGTG   654
 I  P  A  G  C  G  D  E  E  E  E  E  E  E  S  I  L  D  T  V    120

CTCAAATATCTGCCGGGGCCACTGCAGGACATGTTCAAGAAGTAACCTGTCCTCTTCCGG   714
 L  K  Y  L  P  G  P  L  Q  D  M  F  K  K  *                   134

CCCCGTCCACGTTATGACTTTTTTTTTTTTTTTTTTGGTGTTCCTAGTTTTTCTTTTCTT   774
TTTTATTCAGTTAAGTCTCAGTTCCAAAGGGGAAAACCTCAGTCGGCCTCTGCCCCCTTT   834
CCCTCAGCAACCCTTCACAAGCCCTTCATCCAGGGTTTCTAGTTCTGTTTCACTCCCAAG   894
TAGCTTGGAGCCGGAATT                                             912
```

Figure 2-3. Nucleotide and deduced amino acid sequence of rat synaphin 1.

Numbers of both nucleotide and amino acid residues are shown on the right. The asterisk indicates termination.

```
           AATTCCGGGCGCTGCTGTTGATCGTTCACCTTGGAAGGCAGAGGACTCC     49
AACCACACTTGAATGTGAAATCTGACCCCGGAGTGTCTCCTGAGGAACCAAGCCATCATC    109
ATGGAGTTCGTGATGAAACAAGCCCTGGGAGGGGCCACCAAGGATATGGGGAAGATGCTT    169
 M  E  F  V  M  K  Q  A  L  G  G  A  T  K  D  M  G  K  M  L      20

GGGGGTGATGAGGAGAAGGACCCCGATGCCGCCAAGAAGGAGGAGGAGCGGCAGGAGGCA    229
 G  G  D  E  E  K  D  P  D  A  A  K  K  E  E  E  R  Q  E  A      40

CTGCGGCAGGCAGAGGAGGAGCGCAAAGCAAAGTATGCCAAGATGGAGGCAGAACGTGAG    289
 L  R  Q  A  E  E  E  R  K  A  K  Y  A  K  M  E  A  E  R  E      60

GTCATGCGGCAGGGCATACGAGATAAGTATGGCATCAAGAAGAAGGAGGAGCGTGAGGCT    349
 V  M  R  Q  G  I  R  D  K  Y  G  I  K  K  K  E  E  R  E  A      80

GAGGCTCAGGCAGCCATGGAGGCCAACTCAGAAGGCAGCCTGACTCGACCCAAGAAGGCT    409
 E  A  Q  A  A  M  E  A  N  S  E  G  S  L  T  R  P  K  K  A     100

ATCCCACCAGGCTGTGGGGACGAGCCCGAGGAGGAAGATGAGAGCATCCTGGACACTGTC    469
 I  P  P  G  C  G  D  E  P  E  E  E  D  E  S  I  L  D  T  V     120

ATCAAGTACCTGCCTGGGCCACTGCAGGACATGTTCAAGAAGTAATGATACTGGGTCAGC    529
 I  K  Y  L  P  G  P  L  Q  D  M  F  K  K  *                    134

ACCAGGAGCCCTGCCCACTGTACAGACCCCCGCGGAGTCTCCCCCCAACAAGGGATGTTG    589
GGAGCAGATGCAGCCTCACCCCCACCCCGCAAAAATAAGCCATAGTCCTAGATCTATCCT    649
GCCCATGTCCCCCTCGCGCCTCGGGAGCCTCTGGCCACCCCTCCCCGTACTGCCATCACT    709
GACCCCACCAGGCAAGGGTGTGCCCCTCAGGCCCCCGTGCTGTGCATCCTTGCCTTTCGG    769
TGGTTGGACCCCCTCCCTACCAATCTTAAGTTCACCATTCCTACAGAGCCTGCCCAAGGT    829
CAGGCCCCGAGGGCCAGCCCTGACCACCCCTGGATGGGTGGCCACCAGTCAGGGCCTAAA    889
CTCAGGAGCACAGCCATAGTGGGGTGGGACTTGGGAAATGAGCAGTGGGTGCTTGGGGGC    949
CCCAGCCCTTCCCTGAGTCCATTGCCCCTGTTTCTGGACTTGTTGGACCTGCTGGACCAT   1009
TTGCTCTGTCTTCCTGTCTGTGAGCTACCACCCTAGTCTTCCCCAGCAGCCCTTGCATAC   1069
TTTGTCCAGCTGTCTCTTTCTCTGTTGCTCTGCAAGGTTTGGCTTAAGAATTCCACAGCC   1129
AGAGGAAACACAGCAATAGAGCTGGAGTGGGTGGGCTCGACACACTTGGAGTCCCTTGAC   1189
GTGGTCTGTAATGCACCCGGAAT                                        1212
```

Figure 2-4. Nucleotide and deduced amino acid sequence of rat synaphin 2.

Numbers of both nucleotide and amino acid residues are shown on the right. The asterisk indicates termination.

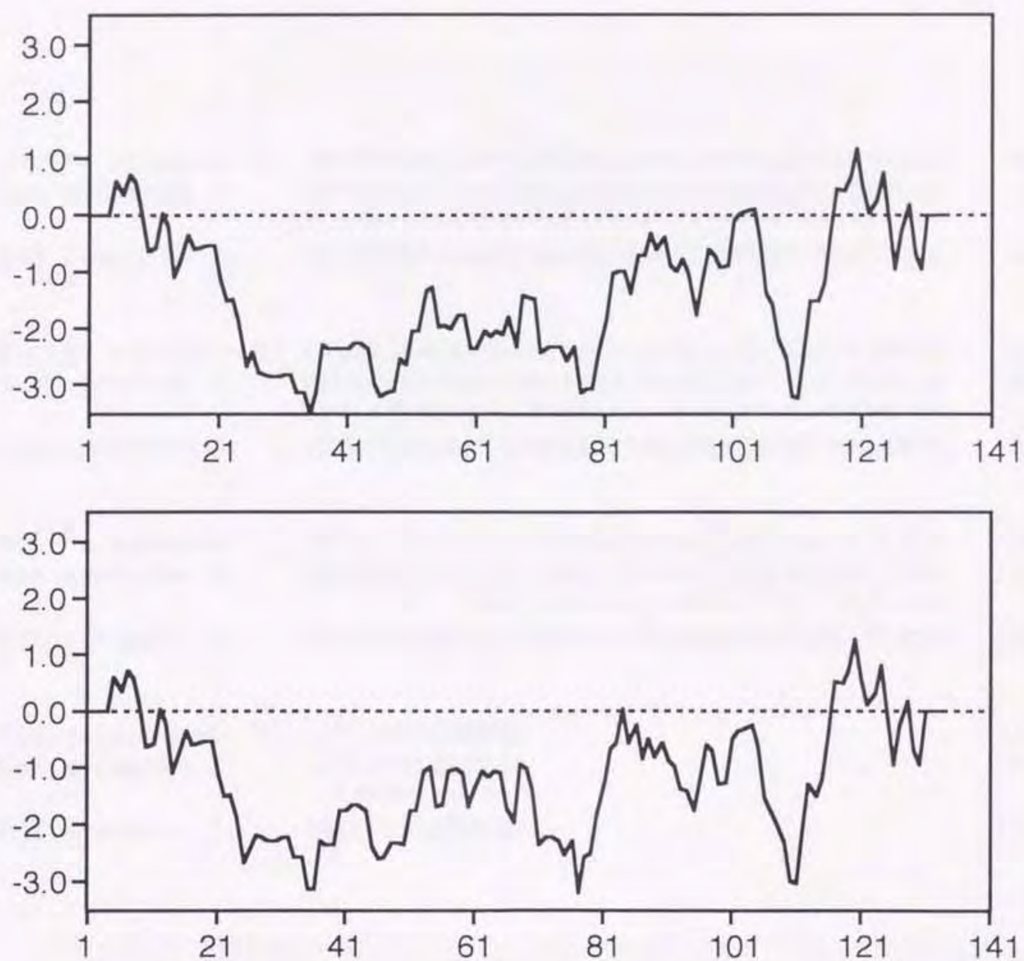


Figure 2-5. Hydropathy plots of synaphin 1 and synaphin 2.

Hydrophobicities were calculated for synaphin 1 (*upper panel*) and synaphin 2 (*lower panel*) using 9 amino acid windows along the different protein sequences (Kyte and Doolittle, 1982). Positive and negative hydropathy values indicate hydrophobic and hydrophilic regions, respectively.

```
Bovine synaphin 1: MDFVMKQALGGATKDMGKMLGGEEEKDPDAQKKEEERQEA    40
Rat synaphin 1:    MDFVMKQALGGATKDMGKMLGGEEEKDPDAQKKEEERQEA    40
                   * ******************** ******* *********
Rat synaphin 2:    MEFVMKQALGGATKDMGKMLGGDEEKDPDAAKKEEERQEA    40


Bovine synaphin 1: LRQQEEERKAKHARMEAEREKVRQQIRDKYGLKKKEEKEA    80
Rat synaphin 1:    LRQQEEERKAKHARMEAEREKVRQQIRDKYGLKKKEEKEA    80
                   *** ******* * ******  ** ****** ***** **
Rat synaphin 2:    LRQAEEERKAKYAKMEAEREVMRQGIRDKYGIKKKEEREA    80


Bovine synaphin 1: EEKAALEQPCEGSLTRPKKAIPAGCGDEEEEEEEESILDTV  120
Rat synaphin 1:    EEKAALEQPCEGSLTRPKKAIPAGCGDEEEEEEEESILDTV  120
                   *  ** *   ************ ***** *** *******
Rat synaphin 2:    EAQAAMEANSEGSLTRPKKAIPPGCGDEPEEEDESILDTV   120


Bovine synaphin 1: LKYLPGPLQDMFKK                             134
Rat synaphin 1:    LKYLPGPLQDMFKK                             134
                    *************
Rat synaphin 2:    IKYLPGPLQDMFKK                             134
```

Figure 2-6. Comparison of bovine synaphin 1 and rat synaphin 1 and 2 polypeptides.

The predicted amino acid sequences of both proteins were aligned. Amino acid sequences of bovine synaphin 1 and rat synaphin 1 are 100% identical. Identical amino acids among the bovine synaphin 1, rat synaphin 1 and 2 are shown in asterisks. Numbers of amino acid residues are shown on the right.

Figure 2-7. Distribution of synaphin in various subcellular fractions.

Each fraction (100 μg protein) was fractionated by SDS-PAGE and then analyzed by immunoblotting. Upper panel, synaptophysin (SP); lower panel, synaphin (19a). Lanes 1-5: S1, P2, LP1, LS1, LP2, respectively. The fractions were named exactly as in Huttner et al. (1983).

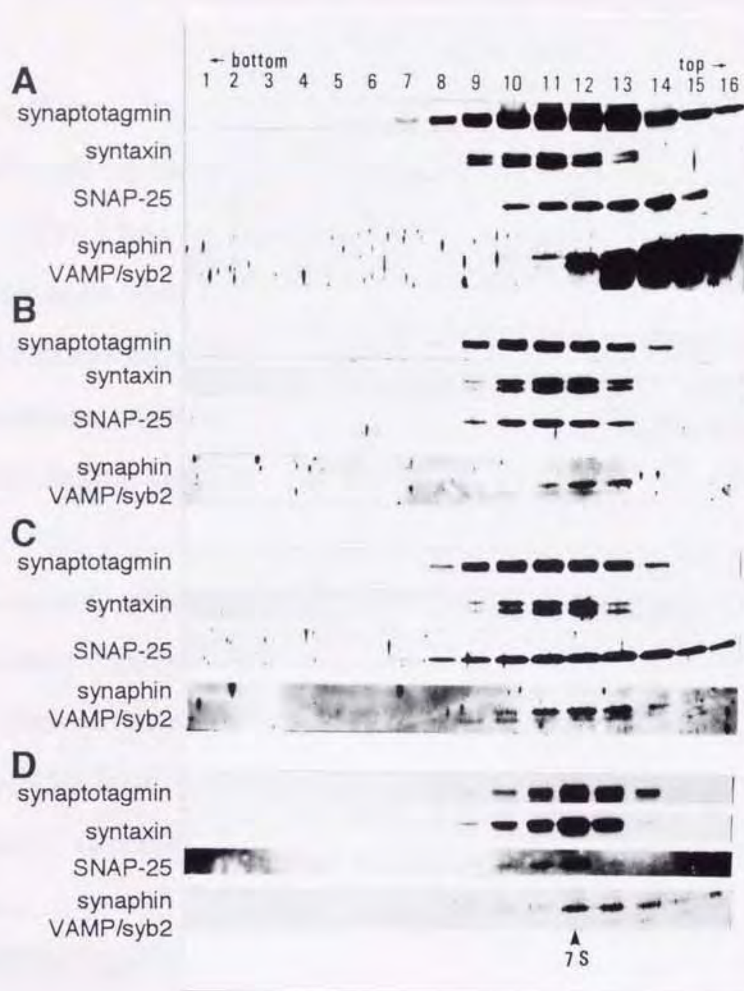


Figure 2-8. Association of synaphin with the 7S complex.

A Triton X-100 extract of rat brain LP2 was separated by glycerol gradient centrifugation (A) and the 7S complex was immunoprecipitated with anti-syntaxin (B), anti-SNAP-25 (C), or anti-synaphin (D) antibodies. Synaphin was immunoprecipitated with the 7S complex of synaptotagmin, syntaxin, SNAP-25, and VAMP/ synaptobrevin.

Chapter 3. Differential expression of synaphin isoform mRNAs

3.1 Experimental procedures

3.1.1 Materials

[^{35}S]dATP was obtained from DuPont NEN. [^{32}P]γ-ATP was from Amersham. RNA blots (Multiple Tissue Northern Blots) of rat tissues were purchased from Clontech. Terminal deoxynucleotidyl transferase and the kit for DNA labeling were obtained from TaKaRa Shuzo Ltd. Sephadex G-25 Spin Columns were from Boehringer Mannheim. All other reagents used were of the highest quality commercially available.

3.1.2 Preparation of oligonucleotide probes

Oligonucleotide probes (antisense, 50mer) of unique sequence were synthesized. The sequence of synaphin 1 probe (5'-CTACTTGGGAGTG AAACAGAACTAGAAACCCTGGATGAAGGGCTTGTGAA-3') corresponded to part of the 3' non-coding region of rat synaphin 1 mRNA, nucleotide positions (np) 848-897, while that of synaphin 2 probe (5'-CAC-CCACTCCAGCTCTATTGCTGTGTTTCCTCTGGCTGTGGAATTCTTAA-3') corresponded to part of the 3' non-coding region of rat synaphin 2 mRNA, np 1113-1162. For *in situ* hybridization, the oligonucleotide probes were 3'-end labeled with [^{35}S]dATP using a terminal deoxynucleotidyl transferase (TaKaRa), and purified by Sephadex G-25 Spin Columns. For northern blots, the oligonucleotide probes were ^{32}P-labeled at the 5'-end using [^{32}P]γ-ATP.

3.1.3 Northern blot analyses of various rat tissues

RNA blotting analysis was performed using commercially available

blots (Clontech) containing poly A^+ RNA from rat tissues. The membranes were prehybridized at 55 °C for 3 h in the hybridization solution containing 5x SSPE (1x SSPE = 0.15 M NaCl/10 mM sodium dihydrogenphosphate/1 mM EDTA), 2x Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrolidone/0.02% bovine serum albumin), 0.5% SDS and 100 μg/ml of heat-denatured salmon sperm DNA, then hybridized at 55 °C for 22 h in the hybridization solution containing 4 x 10^6 cpm/ml of ^{32}P-labeled oligonucleotide probe. The membranes were washed with 2x SSC/0.1% SDS at 55 °C for 20 min and autoradiographed.

3.1.4 Procedure for *in situ* hybridization

Brain tissues were removed from anesthetized rats and immediately embedded and frozen in O.C.T. compound (Miles, USA) .

Sagittal and coronal sections, 15 μm thick, were made on a cryostat, attached to glass slides and fixed in 4% paraformaldehyde/0.1% sodium phosphate-buffered saline (PBS) (pH 7.2) for 30 min. The sections were washed with PBS containing 2 mg/ml of glycine for 20 min and acetylated in 0.25% acetate/0.1 M Tris-HCl pH 8.0 for 10 min, followed by washing with 2x SSC (1x SSC = 0.15 M NaCl/0.015 M sodium citrate) three times (5 min each). The section were then prehybridized for 2 h at 42 °C in a prehybridization solution containing 50% formamide/5x SSC/50 mM sodium phosphate pH 6.5/2% Sarkosyl/1x Denhardt's solution/0.5 mg/ml heat-denatured herring sperm DNA/10 mM β-mercaptoethanol, and hybridized for 20 h at 42 °C in a hybridization solution containing 50% formamide/5x SSC/50 mM sodium phosphate pH 6.5/5x Denhardt's solution/1 mM EDTA/0.5 mg/ml heat-denatured herring sperm DNA/0.1 M dithiothreitol (DTT)/2% Sarkosyl/10% dextran sulfate and 100 pg/μl of radiolabeled oligonucleotide probe. The tissue sections were then washed 3 times in 0.1x

SSC containing 0.1% Sarkosyl/4 mM DTT at 42 °C for 40 min, dehydrated and exposed to Fuji X-ray film for 2 weeks at -80 °C, or autoradiographed using NTB2 nuclear track emulsion (Kodak, USA) for 1 month at 4 °C and counterstained with hematoxylin.

In the controls, sections were hybridized with a radiolabeled probe in the presence of a 20 molar excess of the unlabeled probe.

3.2 Results

3.2.1 Tissue distribution of mRNAs for synaphins 1 and 2

The tissue distribution of synaphin transcripts were investigated by Northern blotting (Fig. 3-1). To reduce the cross-hybridization between synaphin 1 and synaphin 2, oligonucleotide probes were generated to nonconserved 3' untranslated sequences. Each antisense nucleotide probe showed a single band. The synaphin 1 antisense probe revealed a single 4.8 kb band, while the synaphin 2 antisense probe showed a single 2.4 kb band. Both synaphins are highly enriched in brain. In addition, very low levels of synaphin 1 mRNA were observed in testis, lung and kidney, and a very small amount of synaphin 2 transcripts were found in testis.

3.2.2 Expression of synaphins 1 and 2 mRNAs in the adult rat brain

In order to localize synaphin 1 and synaphin 2 mRNAs more precisely, *in situ* hybridization of adult rat brain was performed.

Specificity of probes

The same antisense oligonucleotides as used for Northern blotting were employed for *in situ* hybridization. Probes for each of the two rat synaphins produced unique patterns of hybridization in the rat brain (Figs. 3-2~3-7). No labeling was observed on sections hybridized with a radiolabeled probe in the presence of 20-fold molar excess of its unlabeled probe (Fig. 3-2E, F).

In situ hybridization

Macroscopic distributions of synaphin mRNAs on sagittal and coronal sections of brain are shown in Fig. 3-2. Each probe showed regionally

distinct hybridization signals. Intense signals for synaphin 1 mRNA were detected in olfactory bulb, cerebral cortex, hippocampus and cerebellum. Synaphin 2 mRNAs were strongly expressed in olfactory bulb, cerebral cortex, thalamus, cerebellum and brain stem. Compared with synaphin 1 mRNA signals, synaphin 2 signals were more widely distributed throughout the brain.

Some of these structures were examined in more detail by studying the distribution of signals both oligonucleotide probes at the cellular level.

Olfactory bulb. Synaphin 1 and synaphin 2 mRNAs were detected in olfactory bulb tissue (Fig. 3-3). Intense signals for synaphin 2 were seen in the internal granule cells and mitral cells (Fig. 3-3B). Synaphin 1 signals also localized in the internal granule cells, but no significant signals were observed in mitral cells (Fig. 3-3A).

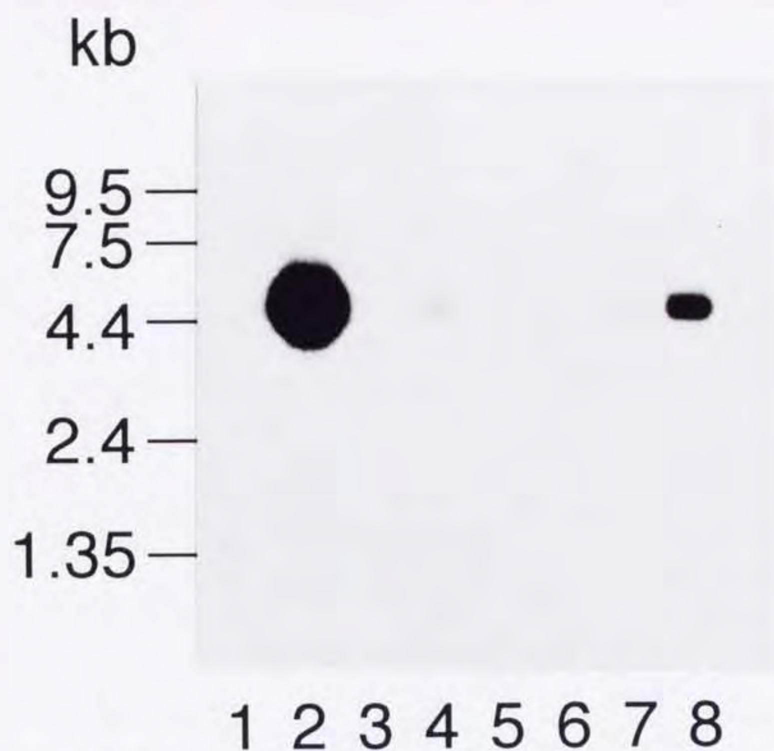
Cerebral cortex. Although both mRNAs were expressed in the cortex, their distributions were distinct. Intense signals of synaphin 1 mRNA were seen in superficial and deep cortex layers (Figs. 3-1A, C). On the contrary, intense signals of synaphin 2 mRNAs were observed in between these layers (Figs. 3-1B, D). Synaphin 1 transcript was predominantly present in cells of layer II/III and layer VI (Figs. 3-4A, C, E). Synaphin 2 mRNA was, however, abundantly expressed in cells of layer IV and pyramidal cells of layer V (Figs. 3-4B, D, F).

Hippocampus. In the hippocampus, distinct patterns of hybridization were observed for the different transcripts. Strong hybridization signals were observed by synaphin 1 probe in the CA2-CA3 areas and in the dentate gyrus (Fig. 3-2A, C). On the other hand, synaphin

2 transcript in these areas was much less (Figs. 3-2B, D). Localization of transcripts at the cellular level is shown in Figure 3-5. In the CA1 area, no significant signals for synaphin 1 mRNA were observed (Fig. 3-5A). However, weak but significant signals for synaphin 2 were detected in a number of the CA1 pyramidal cells (Figs. 3-5B and 3-2B, D).

Cerebellum. In cerebellum, the distributions of mRNAs for synaphin 1 and 2 were evidently different. Synaphin 1 transcript was abundant in granule cells, but not seen in Purkinje cells or in scattered cells in the molecular layer which might include stellate/basket cells (Fig. 3-6A). Synaphin 2 mRNA was widely expressed through all cell layers (Fig. 3-6B).

Facial nerve nucleus. Synaphin 2 mRNA was strongly expressed in the facial nerve nucleus (Fig. 3-7B), but synaphin 1 transcript was not observed (Fig. 3-7A).



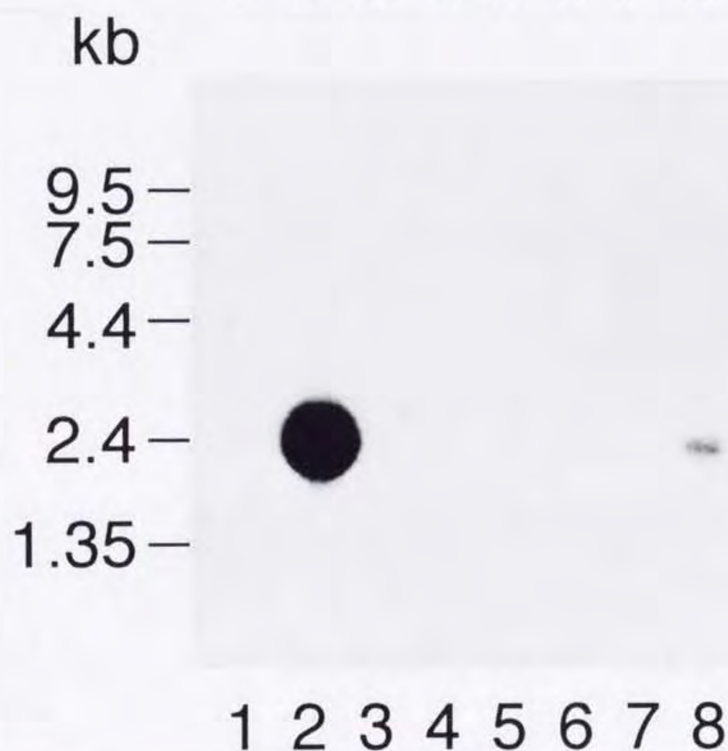


Figure 3-1. Northern blot analyses of two synaphin isoforms in various rat tissues.

The blot was hybridized with an antisense oligonucleotide probe specific for synaphin 1 (*upper panel*) or synaphin 2 (*lower panel*). Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis.

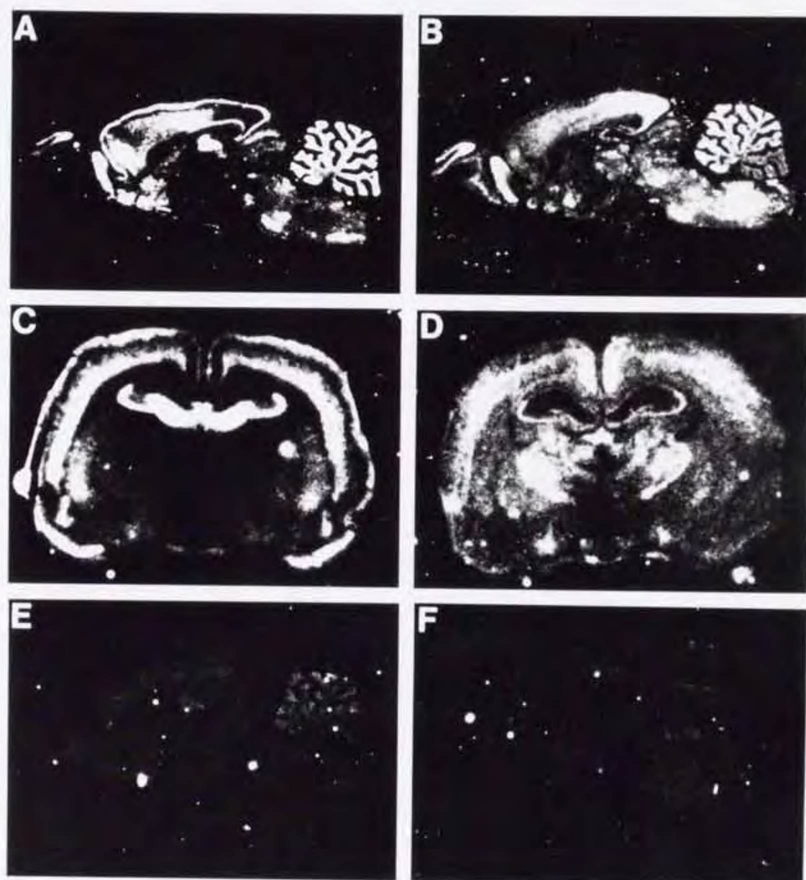


Figure 3-2. X-ray film images of sagittal (A, B) and coronal (C, D) sections of adult rat brain show distribution of mRNA as detected by *in situ* hybridization with ^{35}S-labeled antisense oligonucleotide probes specific for synaphin 1 (A, C) and synaphin 2 (B, D).

Control sections (E, F) hybridized with a mixture of labeled and a 20-fold excess of unlabeled oligonucleotide probe specific for synaphin 1 or synaphin 2, respectively. Exposure times were 2 weeks.

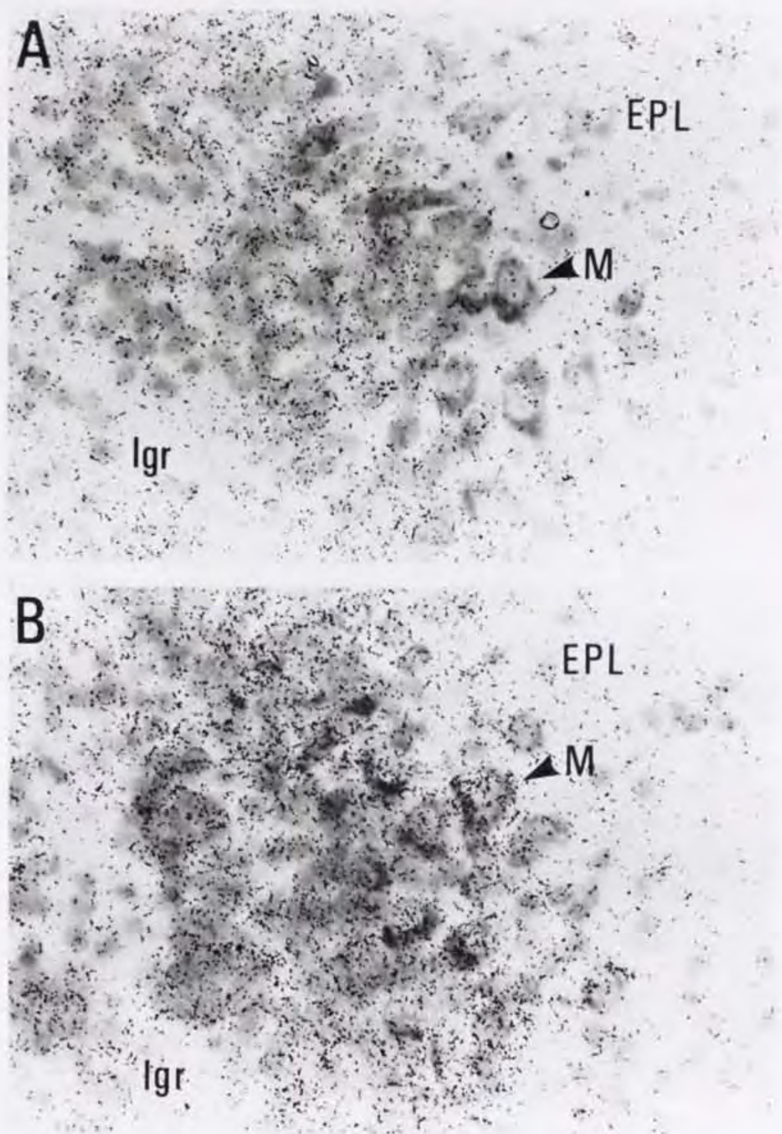


Figure 3-3. High-power bright-field photomicrographs of the olfactory bulb through the internal granular layer and mitral cell layers showing the distribution of synaphin 1 (A) and synaphin 2 (B) mRNAs.

Igr, internal granular layer; EPL, external plexiform layer; M, mitral cell layer. Exposure times of the emulsion-coated slides were 1 month.

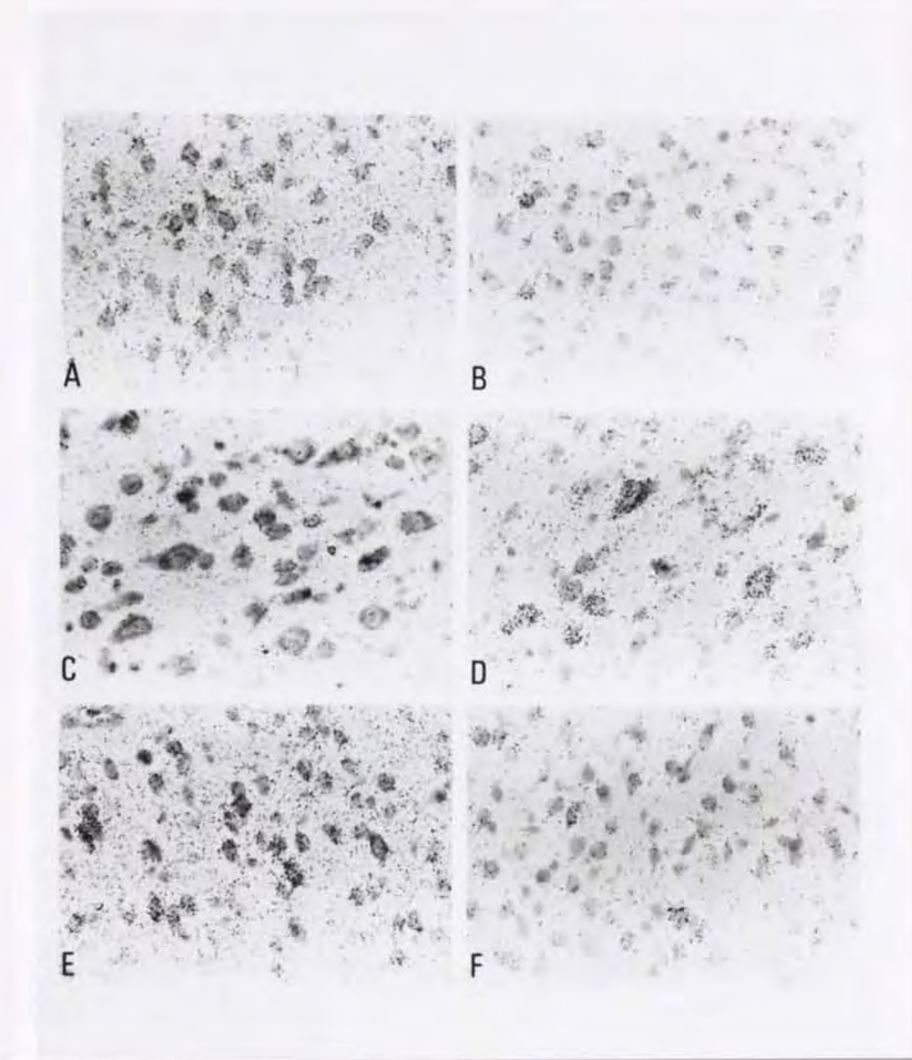


Figure 3-4. High-power bright-field photomicrographs of the cerebral cortex cell layer II (A, B), layer V (C, D) and layer VI (E, F) showing the distribution of synaphin 1 (A, C, E) and synaphin 2 (B, D, E) transcripts.

Exposure times were 1 month.

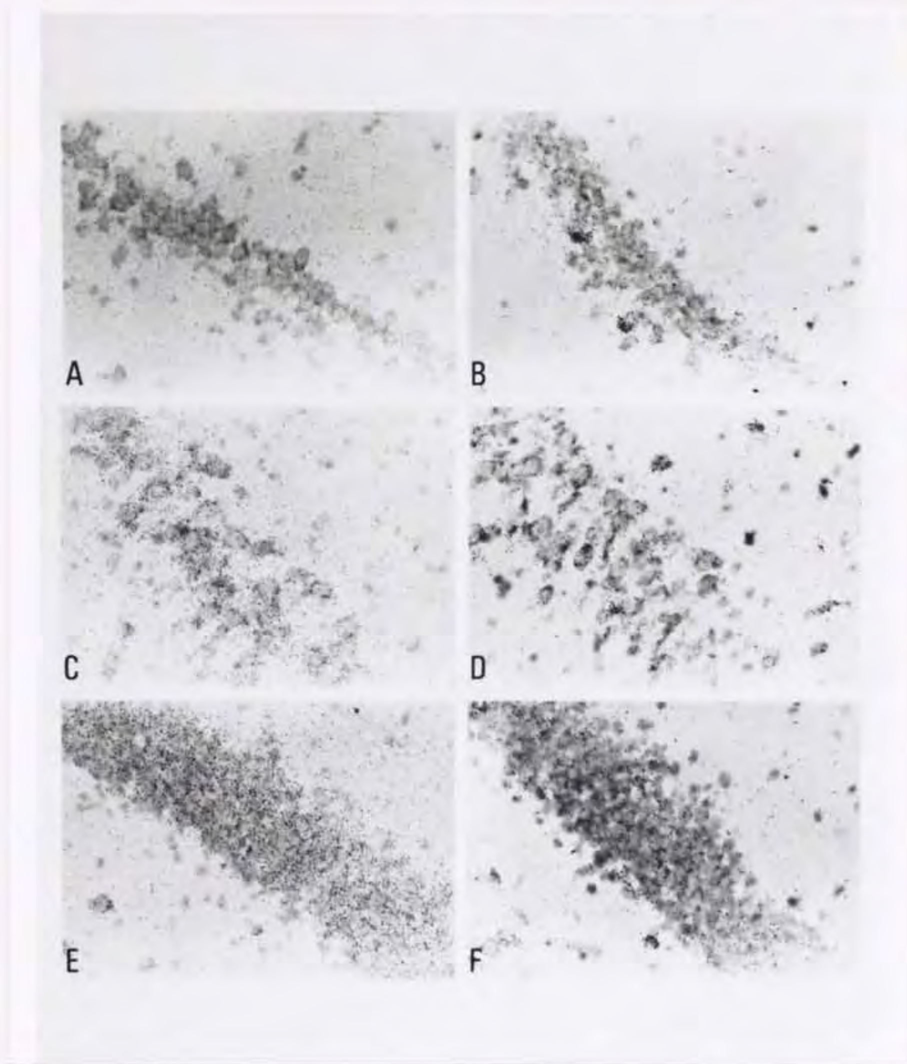


Figure 3-5. High-power bright-field photomicrographs of the hippocampus through the CA1 pyramidal cell layer (A, B), the CA3 pyramidal cell layer (C, D), and the dentate granule cells (E, F) illustrating the distribution of synaphin 1 (A, C, E) and synaphin 2 (B, D, F) transcripts.

Exposure times were 1 month.

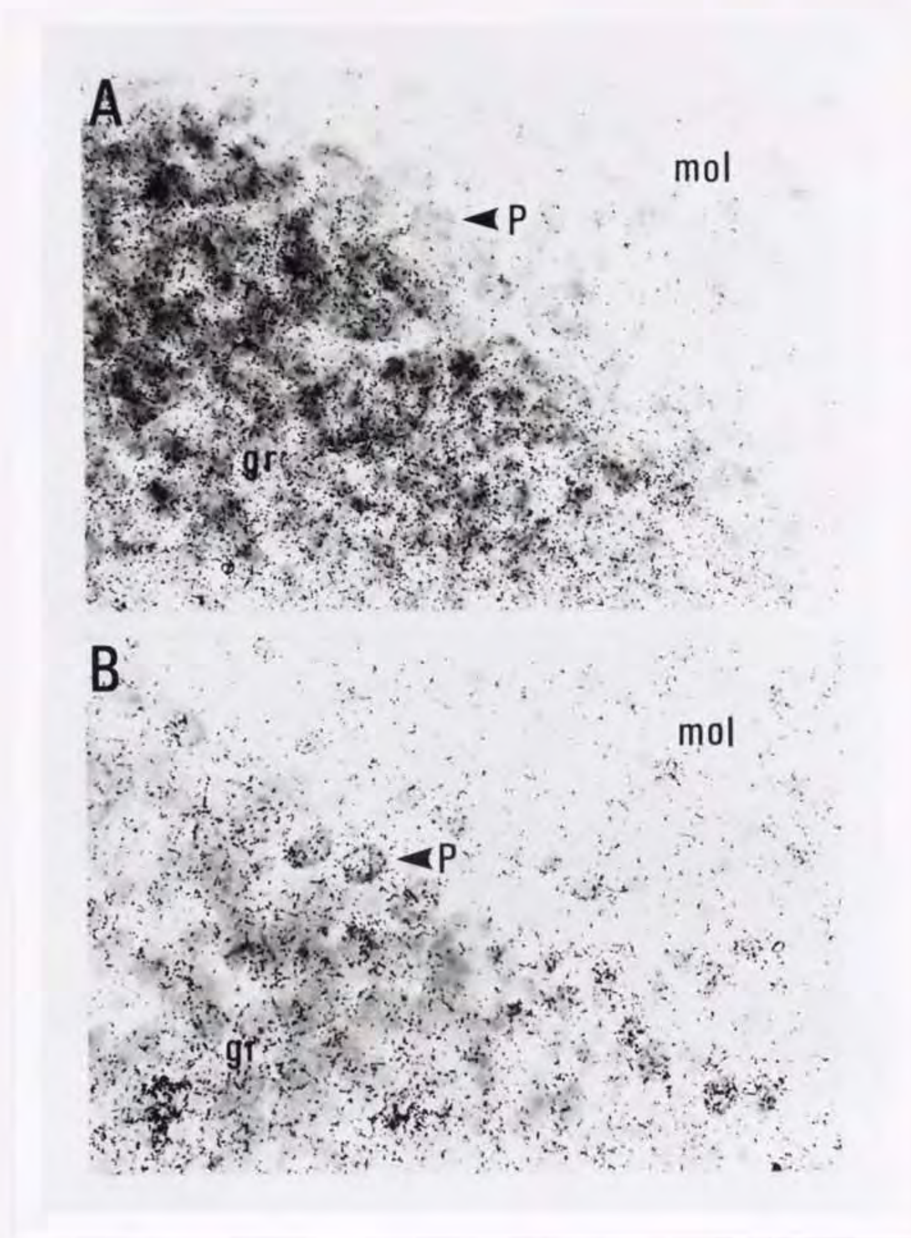


Figure 3-6. High-power bright-field photomicrographs of the cerebellum through the molecular and granular cell layer displaying the distribution of synaphin 1 (A) and synaphin 2 (B) transcripts.

gr, granular cell layer; mol, molecular layer; P, Purkinje cells (arrows). Exposure times were 1 month.

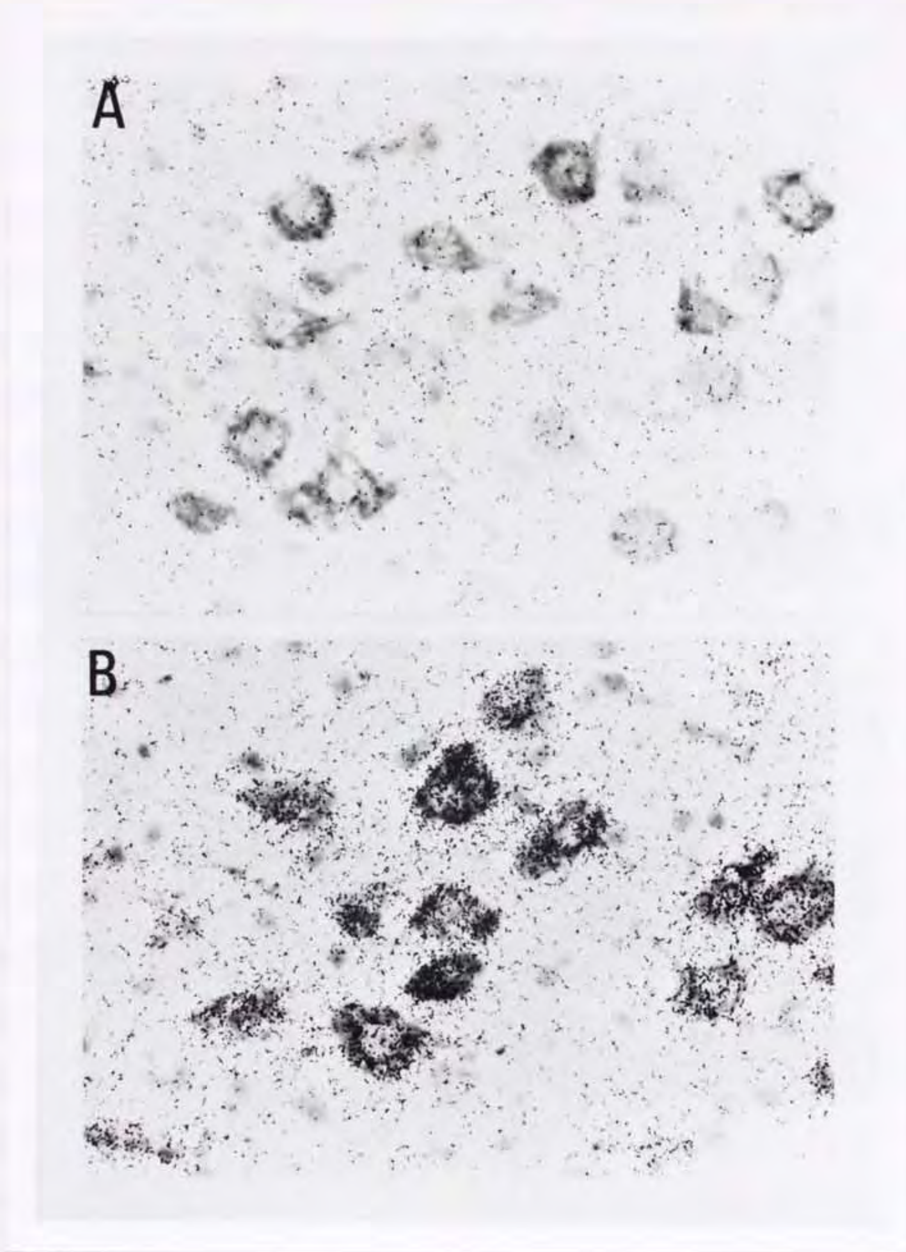


Figure 3-7. High-power bright-field photomicrographs of the facial nerve nucleus showing the distribution of synaphin 1 (A) and synaphin 2 (B) mRNAs.

Exposure times were 1 month.

Discussion

We have deduced the amino acid sequences of novel proteins (termed synaphins) that are associated with the docking/fusion complex critical for neurotransmitter release from presynaptic terminals. Synaphin is localized in the nervous system. Very little, if any, of the protein seems to be present in synaptic vesicles. Our preliminary investigations show that its density is higher in the synaptic plasma membrane fraction than in the lysed P2 fraction, suggesting its association with synaptic regions. It is an important possibility that synaphin regulates the efficiency of the docking/fusion complex and thereby contributes to the plasticity of synaptic transmission. Weak homologies of synaphin to myosin, troponin T and calreticulin may suggest its role related to cytoskeletons or a calcium-regulated process.

Synaphins are highly charged and seem to be mainly located in the cytoplasm. However, some of the protein definitely exists in the insoluble fraction. Since synaphins lack any membrane attachment domains, the proteins in the insoluble fraction may be bound to hydrophilic portions of a membrane protein forming a 7S complex. In this case, synaphin would resemble Munc-18/n-Sec1, a soluble protein that binds to syntaxins (Hata et al., 1993; Pevsner et al., 1994a). Alternatively, synaphin may carry linkers to the membrane such as fatty acids like SNAP-25 that is soluble by itself but tightly anchored to the presynaptic plasma membrane through palmytoyl moieties covalently attached to its cysteine residues (Hess et al., 1992). Concerning the function of synaphin, one interesting possibility is its translocation within presynaptic terminals. Synaphin may associate with or dissociate from the docking/fusion complex, depending on signals generated within cells by external and/or internal stimuli.

Northern blot analysis showed that the mRNAs for both synaphins are highly enriched in brain. Low levels of synaphin 1 mRNA were observed in testis, lung and kidney, and synaphin 2 mRNA was detected slightly in testis. Immunoblotting of various rat tissues with antibody against the partial sequence of the 19a component (synaphin) described in chapter 1 also indicates the presence of synaphins in cerebrum and cerebellum, but did not show the presence in kidney. Thus it was concluded that synaphin exists predominantly in brain.

The presence of two synaphin molecules in rat, and probably in other mammals as well, suggests that the different forms might serve discrete functions within the brain. As a step toward defining the function of synaphin in the brain, we have characterized the expression of mRNA of the two isoforms. *In situ* hybridization demonstrated that each of the two synaphin transcripts has a unique expression pattern. All brain structures expressed one or both forms of synaphin. I have examined the possibility that synaphin isoform expression correlates with the expression of other synaptic vesicle protein and presynaptic plasma membrane protein isoforms forming a docking/fusion complex. To date the distribution of three isoforms of synaptotagmin (Marquèze et al., 1995), two isoforms of VAMP/ synaptobrevin (Trimble et al., 1990) and two isoforms of SNAP-25 (Boschert et al., 1996) have been investigated in the rat brain. For example, the expression of synaptotagmin and VAMP/synaptobrevin isoforms appears to have a rostral-caudal pattern, with synaptotagmin I and VAMP/ synaptobrevin-2 expressed in more rostral brain structures, and synaptotagmin II and VAMP/synaptobrevin-1 are expressed in more caudal brain structures (Trimble et al., 1990; Marquèze et al., 1995). Differential expressions of two SNAP-25 isoforms in the rat brain was reported by Boschert et al. (1996). Expression patters of synaptic vesicle protein and

presynaptic plasma membrane protein mRNA previously reported were compared to those of synaphin 1 and 2 (Table 3-1). No striking correlations with the expression of the different isoforms were apparent. The expression of the synaphin isoforms appears to be independent of the expression of other synaptic vesicle protein and presynaptic plasma membrane protein isoforms forming the docking/fusion complex.

Synaphin appears to be part of a multicomponent complex involved in neurotransmitter release. Several synaptic vesicle and plasma membrane proteins are also present in multiple isoforms. The functional relevance of these variants is unknown, but it is likely that specific sets of protein variants localized in synaptic vesicle, cytosol, and plasma membrane will confer different properties on the docking/fusion machinery. Further studies on colocalization of these various isoforms will be necessary in order to sort out the potential combinations that may be used by individual neurons.

Table 3-1. Regional distributions in the brain of isoforms of proteins constituting the docking/fusion complex

	synaphin	synapto-tagmin	VAMP	SNAP-25
Olfactory bulb				
Mitral cell layer	2	I/III	ND	a/b
Internal granule cell layer	1/2	I/III	ND	a/b
Neocortex				
Layer II/III	1	I/II/III	2	a/b
Layer IV	2	I/II/III	2	b
Layer V	2	I/II/III	2	a/b
Layer VI	1	I/II/III	2	a/b
Hippocampus				
CA1 pyramidal cell layer	-	I/III	2	a/b
CA3 pyramidal cell layer	1/2	I/III	2	a/b
Dentate granule cells	2	I/III	2	a/b
Thalamus	2	I/II/III	2	a/b
Cerebellum				
Granule cell layer	1/2	I/II/III	1/2	a/b
Purkinje cell layer	2	II	-	b
Stellate/basket cells	2	I/II/III	-	-
Brain stem				
Pontine nuc.	1/2	I/II/III	1/2	b
Facial nuc.	2	ND	1	ND

The predominant isoforms of the indicated proteins constituting the docking/fusion complex are listed for selected brain regions. Synaptotagmin I, II, and III expression in rat brain was determined by *in situ* hybridization analysis (Marquèze et al., 1995). Expression of VAMP-1 and VAMP-2 in rat brain was surveyed by *in situ* hybridization (Trimble et al., 1990). Expression of SNAP-25a and SNAP-25b in rat brain was determined by *in situ* hybridization (Boschert et al., 1996). -, not detectable. ND, not determined.

General discussion

This study was carried out to elucidate the molecular mechanisms for neurotransmitter release.

Using two monoclonal antibodies (SPM-1 and SPM-2) that immunoprecipitate brain N-type Ca^{2+} channels, we have demonstrated that synaptic vesicle proteins, VAMP/synaptobrevin-2 and rab3A, synaptic plasma membrane proteins, syntaxins and SNAP-25, and cytosolic proteins, synaphins form a complex in the vicinity of N-type Ca^{2+} channels. This result suggests that this complex bridges the synaptic vesicles and plasma membranes, and may represent an essential machinery for rapid synaptic vesicle exocytosis.

Two independent approaches identified the three proteins, syntaxin, SNAP-25, and VAMP/synaptobrevin, as likely core components of the membrane fusion machinery (Südhof et al., 1993; Bennett and Scheller, 1994; Ferro-Novick and Jahn, 1994). First, studies on clostridial neurotoxins that block synaptic membrane fusion revealed that these constitute a family of metalloproteases which specifically cleave either VAMP/synaptobrevin, syntaxin, or SNAP-25 (Link et al., 1992; Schiavo et al., 1992; Blasi et al., 1993a, 1993b). Second, affinity chromatography on immobilized α-SNAP complexed to NSF results in the purification of the same three proteins. SNAPs (soluble NSF attachment proteins) are cytoplasmic proteins that bind to specific membrane receptors and mediate the membrane binding of NSF (N-ethylmaleimide-sensitive fusion protein), a protein required for membrane fusion reaction. Thus this result indicates syntaxin, SNAP-25, and VAMP/synaptobrevin-2 are SNAP receptors (Söllner et al., 1993). These findings strongly support that the complex we found functions as synaptic vesicle docking/fusion machinery.

We have isolated and identified a family of proteins named synaphins that associated with the synaptic vesicle docking/fusion complex for

neurotransmitter release. Synaphins are small, highly charged proteins of 134 amino acids and almost specifically expressed in brain. Subcellular fractionation studies demonstrated that synaphins are fractionated not only in the soluble fractions (S1 and LS1) but also in P2 (crude membrane fraction), LP1 (crude plasma membrane fraction) and LP2 (synaptic vesicle-containing fraction). Since synaphin lacks any membrane attachment domains, the protein in insoluble fractions may be bound to hydrophilic portions of a membrane protein(s) in the docking/fusion complex. In this case, synaphin would resemble Munc-18/n-sec1, a soluble proteins that binds to synaptic plasma membrane, syntaxins (Hata et al., 193; Pevsner et al., 1994a). Alternatively, like rab3A (Farnsworth et al., 1991) and SNAP-25 (Hess et al., 1992), the membrane attachment of synaphins may be due to acyl-modification at the cysteine residues. Concerning the function of synaphins, one interesting possibility is its translocation within presynaptic terminals. Synaphins may associate with or dissociate from the docking/fusion complex and regulates the efficiency of the docking/fusion complex .

The presence of two synaphin proteins in rat, and probably in other mammals as well, suggests that the different isoforms might serve discrete functions within the brain. *In situ* hybridizations demonstrate that each of two synaphin mRNAs has a unique expression pattern. All brain structures investigated expressed one or both forms of synaphin transcript. I have examined the possibility that synaphin isoform expression correlates with the expression of other synaptic vesicle protein and presynaptic plasma membrane protein isoforms forming the synaptic vesicle docking/fusion complex for neurotransmitter release. I have compared previously reported expression patters of synaptotagmin, SNAP-25, and VAMP/synaptobrevin transcript to that of synaphin 1 and 2, and found no striking correlation in

the expression of the different isoforms. The expression of synaphin isoforms appears to be independent of the expression of other protein isoforms forming the docking/fusion machinery in presynaptic terminals. The functional relevance of these isoforms is unknown, but it is likely that specific sets of protein variants localized in synaptic vesicle, cytosol, and plasma membrane will confer different properties on the docking/fusion machinery and thereby contribute the distinct functional properties onto synapse.

Summary

Two monoclonal antibodies (SPM-1 and SPM-2) immunoprecipitate brain N-type calcium channels. On immunoaffinity chromatography of digitonin or Triton X-100 extracts of bovine brain membranes on SPM-1- and SPM-2-Sepharose, proteins of 36 kDa (syntaxins), 28 kDa (SNAP-25 and rab3A), and 19 kDa are specifically retained by both columns. Here I show that the 19 kDa protein contains VAMP/synaptobrevin-2 and novel cytosolic proteins termed synaphins. We have isolated and cloned a bovine and two rat brain cDNAs for synaphins. Synaphins are very hydrophilic proteins consisting of 134 amino acid residues rich in glutamic acid and lysine residues. Bovine synaphin and rat synaphin 1 is 100% identical, and rat synaphin 2 exhibits 84% identity with bovine and rat synaphins 1. Immunoblot and Northern blot analyses in various rat tissues showed that synaphins predominantly exist in brain. *In situ* hybridization with synthetic 50 meric oligonucleotide probe specific to synaphins 1 or 2 demonstrated different localizations of the two mRNAs in the rat brain. Subcellular fractionation studies revealed that synaphins exist in both soluble and insoluble fractions. A Triton X-100 extract of the rat brain LP2 fraction was subjected to a glycerol density gradient, and the resultant fractions were immunoprecipitated with monoclonal antibodies that recognize syntaxin 1, SNAP-25 or synaphins. Synaphins fractionated in insoluble fraction were mostly present in the 7S complex together with synaptotagmin, syntaxin, SNAP-25, and VAMP/synaptobrevin. These results strongly suggest that the synaptic vesicle proteins, VAMP/synaptobrevin and synaptotagmin, the plasma membrane proteins, syntaxin and SNAP-25, and cytosolic protein, synaphin, form a tight complex probably essential to neurotransmitter release in brain presynaptic terminals. Functional relevance of two synaphin isoforms is unknown, but it is likely that specific sets of protein variants forming the complex will confer different properties on the docking/fusion complex and thereby contribute the distinct functions onto individual neurons.

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