

The Identification of a Novel Synaptosomal-associated Protein, SNAP-25, Differentially Expressed by Neuronal Subpopulations

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Abstract. cDNA clones of a neuronal-specific mRNA encoding a novel 25-kD synaptosomal protein, SNAP-25, that is widely, but differentially expressed by diverse neuronal subpopulations of the mammalian nervous system have been isolated and characterized. The sequence of the SNAP-25 cDNA revealed a single open reading frame that encodes a primary translation product of 206 amino acids. Antisera elicited against a 12-amino acid peptide, corresponding to the carboxy-terminal residues of the predicted polypeptide sequence, recognized a single 25-kD protein that is associated with synaptosomal fractions of hippocampal preparations. The SNAP-25 polypeptide remains associated with synaptosomal membrane components after hypoosmotic lysis and is released by nonionic detergent but not high salt extraction. Although the SNAP-25 polypeptide lacks a hydrophobic stretch of residues compatible with a transmembrane region, the amino terminus may form an amphiphilic helix that

may facilitate alignment with membranes. The predicted amino acid sequence also includes a cluster of four closely spaced cysteine residues, similar to the metal binding domains of some metalloproteins, suggesting that the SNAP-25 polypeptide may have the potential to coordinately bind metal ions. Consistent with the protein fractionation, light and electron microscopic immunocytochemistry indicated that SNAP-25 is located within the presynaptic terminals of hippocampal mossy fibers and the inner molecular layer of the dentate gyrus. The mRNA was found to be enriched within neurons of the neocortex, hippocampus, piriform cortex, anterior thalamic nuclei, pontine nuclei, and granule cells of the cerebellum. The distribution of the SNAP-25 mRNA and the association of the protein with presynaptic elements suggest that SNAP-25 may play an important role in the synaptic function of specific neuronal systems.

THE presynaptic terminal is a site of many specialized processes crucial to neuronal function. Presynaptic specializations include calcium channels that mediate the calcium signal for synaptic vesicle release, proteins involved in the storage and synthesis of neurotransmitters, and molecules that allow the movement and docking of synaptic vesicles with the presynaptic plasma membrane (see Reichardt and Kelly, 1983 for review). At the ultrastructural level, a variety of electronmicroscopic techniques have also revealed the specialized cytoarchitecture of the presynaptic terminal. The axoplasm in the region of the presynaptic terminal is differentiated from the other portions of the neuron by the presence of electron-dense thickenings on the inner surface of the presynaptic membrane (Gray, 1961), the concentration of synaptic vesicles (Palay, 1956), and the electronmicroscopic appearance of the cytoplasmic matrix (Landis et al., 1988). The region of the axonal terminal possessing these features, the active zone, is responsible for neurotransmitter release. The molecular composition that enables the active zone to fulfill these unique functions is only beginning to be

characterized. For example, the phosphoprotein synapsin I is believed to mediate the anchorage of synaptic vesicles to elements of the active zone matrix (DeCamilli et al., 1983; Huttner et al., 1983). Other proteins that are components of mammalian synaptic vesicles have been identified, including synaptophysin (Weidenmann and Franke, 1985; Jahn et al., 1985), a 65-kD calmodulin binding protein (Matthew et al., 1981; Fournier and Trifaro, 1988), the 95-kD transmembrane glycoprotein SV2 (Buckley and Kelley, 1985), integral membrane proteins p30 and p36 (Obata et al., 1987) and synaptobrevin/vesicle-associated membrane protein (VAMP)¹ (Sudhof et al., 1989; Elferink et al., 1989). With the exception of the differential expression of two species of VAMP proteins (Trimble, W. S., T. S. Gray, L. A. Elferink, M. C. Wilson and R. H. Scheller, manuscript in preparation), these

1. *Abbreviations used in this paper:* CRF, corticotropin-releasing factor; ORF, open reading frame; PB, phosphate buffer; SNAP-25, synaptosomal-associated protein; TE, 10 μ M Tris/1 mM EDTA, pH 8.0; VAMP, vesicle-associated membrane protein.

proteins are likely to be general to all neurons and related specifically to synaptic vesicle function.

The matrix of the active zone may contain a separate set of presynaptic proteins that mediate matrix functions such as synaptic plasticity, which involves the formation of new synapses and the removal of others. Additionally, to allow the rapid mobilization of synaptic vesicles, the matrix of the active zone must be able to assume a fluid state upon stimulus and yet maintain the localization of synaptic vesicles in quiescent periods. The identification of molecular components of the active zone, therefore, will be critical to understanding vesicle mobilization and synaptic plasticity. The diversity of neuronal cell subtypes, patterns of synaptic connectivity, and neurotransmitter utilization indicates that the components of the presynaptic terminal important to the function of specialized neuronal subgroups remain unidentified.

As an approach to understanding the molecular basis of neuronal diversity, we, as other groups, have applied recombinant DNA strategies to identify and characterize novel genes and their encoded proteins that are specifically expressed in the nervous system (Sutcliffe et al., 1983; Anderson and Axel, 1985; Branks and Wilson, 1986; Clayton et al., 1988; Travis and Sutcliffe, 1988; Oberdick et al., 1988). Our efforts have been directed towards identifying genes that are expressed in limited subsets of neuroanatomical structures. The analysis and characterization of genes differentially expressed among neuronal structures should identify proteins that contribute to the specialized function of neuronal subpopulations and provide new molecular probes for the investigation of the cell biology of nervous system function and differentiation. We have focused our investigation of expression and developmental regulation of differentially expressed neuronal specific genes on the hippocampal formation because of the structure's physiological properties, synaptic organization with laminar cellular distribution, and the sensitivity of the hippocampus to many disease processes, including Alzheimer's disease and temporal lobe epilepsy.

In a previous study designed to identify cDNA clones to genes operationally defined as "brain-specific" relative to other differentiated tissues, we observed that a cDNA probe designated pMuBr8 hybridized to a 2.2-kb mRNA present in brain and not in liver or kidney (Branks and Wilson, 1986). Initial *in situ* hybridization studies indicated that this mRNA is preferentially expressed in CA3 pyramidal neurons of the mouse hippocampus at a three- to fourfold higher level than in neurons of the CA1 field and the dentate gyrus, as well as within other specific structures of the brain. Because this cDNA appeared to discriminate between neurons of the hippocampal formation, and thus may represent a gene product that contributes to the specialization of hippocampal neuronal subtypes, we have isolated and sequenced cDNA clones representing virtually the entire length of the mRNA transcript and prepared antipeptide antisera to characterize the encoded polypeptide. We find that the sequence of the mRNA contains a single open reading frame encoding a 206-residue polypeptide. Antipeptide antisera generated against 12 amino acids at the carboxy terminus of the predicted polypeptide sequence reacts with a 25-kD protein in hippocampal protein preparations that is localized to the presynaptic mossy fiber boutons. Because of the apparent association of the protein with synaptic structures, we have designated the protein SNAP-25 (synaptosomal associated protein of 25 kD). The enrichment of the SNAP-25 mRNA in subsets of

neurons in the brain and the presynaptic localization of the protein indicate that SNAP-25 may have an important role in the synaptic function of circumscribed neuronal pathways of the nervous system.

Materials and Methods

Library Construction and Screening

Poly A⁺ RNA was selected by oligo(dT) cellulose chromatography of total RNA prepared from adult BALB/c mouse brain hippocampal tissue as previously described (Branks and Wilson, 1986). The gt10 recombinant phage library was constructed by oligo(dT)-primed synthesis of cDNA from the hippocampal poly A⁺ RNA template with avian myeloblastosis virus (AMV) reverse transcriptase and second strand synthesis with *Escherichia coli* Pol I Klenow fragment (Branks and Wilson, 1986). Internal Eco RI sites were protected by Eco RI methylase and the double-stranded cDNA was treated with S₁ nuclease to provide blunt ends before the addition of Eco RI linkers and ligation into the Eco RI sites of the vector lambda gt10 DNA. The complexity of the packaged library before amplification was 1 × 10⁶ independent clones with an average cDNA insert size of ~1,000 bp. Duplicate plaque lifts of ~20,000 plaques, plated at a density of 5,000 plaques per 150-mm petri dish were screened with the 800-nucleotide insert of pMuBr8 (Branks and Wilson, 1986) labeled by random oligonucleotide-primed synthesis (Feinberg and Vogelstein, 1983) with ³²P-α-dCTP (3,000 Ci/mmol; New England Nuclear, Boston, MA) to a specific activity of 5 × 10⁸ cpm/μg, essentially as described by Maniatis et al. (1982). The filters were washed in 2× SSC, 1× SSC, and 0.2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate) with 0.2% SDS at 67°C and autoradiographed. From 20 positives, one clone, lambda 8.1, was plaque purified and the 1.8-kb insert was subcloned into the Eco RI site of pBS KS(-) (Stratagene Corp., La Jolla, CA). To isolate cDNAs containing further 5' sequence, a 20-mer oligonucleotide probe A1 (5'-TCTGGCATCTCCTCCAGGTC-3') complementary to the sequence 14 bp from the 5' end of p8.1 was synthesized and labeled by T4 polynucleotide kinase reaction with ³²P-gamma ATP (3,000 Ci/mmol; New England Nuclear) (Maniatis et al., 1982). Nitrocellulose filter lifts of 50,000 plaques of the gt10 hippocampal cDNA library were prehybridized overnight with 6× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH₂PO₄, pH 7.4, 1 mM EDTA), 0.2% SDS, 100 μg/ml denatured salmon sperm DNA at 42°C and hybridized for 24 h with 5 × 10⁶ cpm/ml of labeled A1 oligonucleotide probe. Filters were washed in 2× SSC and 0.2% SDS at 37°C before autoradiography. Of the positives on this screen, four clones, lambda 8.23, 8.52, 8.53, and 8.71 were plaque purified and subcloned in pBS KS(-). To isolate clones containing the 3' end of the SNAP-25 mRNA, the cDNA insert of p8.1 was excised the Eco RI and labeled with ³²P-α-ATP (3,000 Ci/mmol; New England Nuclear) by filling in the recessed 3' termini of the Eco RI sites with DNA polymerase I Klenow fragment (Maniatis et al., 1982). The end-labeled p8.1 insert was digested with Hae III and the 3' end-labeled fragment was used to probe the filters. Of the positives obtained with this 3' probe, one clone lambda 8.51 was plaque purified and subcloned into pBS KS(-) to yield p8.51.

Sequencing

Double-stranded plasmid containing cDNA SNAP-25 inserts were used as template after alkali denaturation for dideoxy chain termination sequencing (Sanger et al., 1977). The clone p8.1 was completely sequenced from the 3' end with a series of progressive 3' deletion subclones prepared by exonuclease III and Mung bean nuclease digestion (Stratagene Corp.) with a modified T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH). Both the 3' and 5' ends of the clones p8.1, p8.23, p8.51, p8.52, p8.53, and p8.71 were sequenced using M13(+) and M13(-) primers (Stratagene Corp.).

Sequence Analysis

The amino acid sequence of the SNAP-25 ORF was compared to the NBRF protein sequence data base using the SEARCH (Dayhoff et al., 1983) and FASTP programs (Lipman and Pearson, 1985). The significance of similarities was assessed using the ALIGN program (Dayhoff et al., 1983). The hydrophobicity analysis of the SNAP-25 polypeptide was executed with the program of Rose and Roy (1980) using an averaging of five residues. The alpha helical potential, beta extend chain (averaging over five residues) and beta turn potential (averaging over four residues) were predicted with the programs of Chou and Fasman (1974) and Osguthorpe and Robson (1978).

Primer Extension

For primer extension, 20 pmol of a 20-mer oligonucleotide A52 (5'-GGTAGGGAGGGAAGGAAAGA-3') complementary to the sequence 19 bp from the 5' end of clone p8.52 was labeled by T4 polynucleotide kinase with 50 pmol of ^{32}P -gamma-ATP (3,000 Ci/mmol; New England Nuclear). The labeled probe was purified by G50 Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) chromatography, ethanol precipitation and resuspended in 10 μl of distilled H_2O . 2 pmol of the labeled A52 primer was added to 2 μg each of poly A⁺ RNA from murine BALB/c hippocampus, BALB/c hypothalamus, strain 129 whole mouse brain, strain 129 mouse liver, and 5 μg of yeast total RNA in 5 μl containing 200 mM KCl, 10 mM Tris, pH 7.5, 0.1 mM EDTA. The samples were heated to 90°C for 2 min and allowed to cool to room temperature for 15 min to allow annealing of the primer to RNA. 44 μl of reaction mix containing 50 mM Tris HCl pH 7.5, 75 mM KCl, 10 mM DTT, 3 mM MgCl_2 , 50 $\mu\text{g}/\text{ml}$ actinomycin D, 100 $\mu\text{g}/\text{ml}$ BSA, and 0.5 mM each of dATP, dCTP, dGTP, and dTTP were added with 1 μl AMV reverse transcriptase 20 U/ μl (Pharmacia Fine Chemicals) and the reaction mixture was incubated at 37°C for 1 h. The reactions were terminated by adding 50 μl 4 M ammonium acetate, ethanol precipitated, and the products were separated on a 6% polyacrylamide/7 M urea gel and visualized by autoradiography.

In Vitro Translation

Capped RNA transcripts were prepared from the plasmids p8.52, p8.71, p8.1 linearized either with Bam HI or Xba I within the polylinker sequence distal to the T7 or T3 RNA polymerase initiation site. Transcription reactions were performed with either T7 or T3 polymerase as described by Green et al. (1983) in the presence of 500 μM $^{7\text{m}}\text{GpppG}^{\text{m}}$ (Pharmacia Fine Chemicals), 100 mM GTP, CTP, ATP, UTP, and 5 μCi ^{32}P - α -GTP (3,000 Ci/mmol; New England Nuclear). The transcripts were extracted with phenol/chloroform, passed over G50 Sephadex, and ethanol precipitated. In vitro translation reactions were programmed with 200 ng of RNA transcript in an assay consisting of 18 μl of rabbit reticulocyte lysate (Amersham Corp., Arlington Heights, IL) and 20 μCi [^{35}S]methionine (1,127 Ci/mmol; New England Nuclear). The translation reactions were stopped by adding equal volumes of 0.1 M NaCl, 20 mM Tris pH 7.0 and 1% Triton X-100 and 2.5 mM PMSF and 4 μl of the reaction mixture containing stop solution was applied to 10–17.5% gradient SDS polyacrylamide gels. The translation products were visualized by impregnating the gel with EN³HANCE (New England Nuclear) and autoradiography.

Peptide-directed Antisera

A synthetic peptide corresponding to the carboxy terminal residues 195–206 of the SNAP-25 protein ($\text{NH}_3\text{-A-N-G-R-A-T-K-M-L-G-S-G-COOH}$) was generated on a protein synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) and purified by HPLC. 5 μg each of the synthetic peptide and the carrier protein edestrin (Sigma Chemical Co., St. Louis, MO) were conjugated with 0.1% glutaraldehyde and antisera was elicited in rabbits and assayed by ELISA as described by Green et al. (1982). To prepare affinity-purified antipeptide antisera, the immunoglobulin fraction of the serum was precipitated with 50% saturated ammonium sulfate, solubilized, and passed over an affinity column consisting of the immunizing peptide coupled to a mixture of Affigel-10 and Affigel-15 (Bio-Rad Laboratories, Richmond, CA). The bound immunoglobulin was eluted with 0.2 N glycine, dialyzed exhaustively against PBS, and concentrated to 1 mg/ml by filtration (Amicon Corp., Danvers, MA).

Protein Fractionation and Immunoblotting

A crude synaptosomal fraction of hippocampus and cerebral cortex dissected from adult BALB/c mouse brain was prepared as described by Krueger et al. (1977). The tissue was homogenized with a Dounce tight pestle in ice-cold 0.32 M sucrose, 5 mM Tris, pH 7.5, 2.5 mM PMSF and centrifuged at 1,000 g for 5 min at 4°C. The supernatant was removed and centrifuged at 12,500 g for 15 min at 4°C. The supernatant (S2) was collected as the cytosolic fraction. The pellet fraction was washed by resuspension in the same sucrose buffer, with centrifugation again at 12,500 g before finally resuspending in sucrose buffer as the crude synaptosomal pellet (P2).

Synaptic vesicles and membrane components were prepared from hypototically lysed synaptosomal preparations essentially as described by Huttner et al. (1983). Neocortex and hippocampus dissected from six adult BALB/c mice were homogenized in 10 ml of 0.32 M sucrose, 4 mM Hepes pH 7.4 with a motor driven glass-Teflon homogenizer at \sim 1,000 rpm. The

pellet was collected from the lysed synaptosome P2 fraction by centrifugation in the rotor (model Ti50; Beckman Instruments, Fullerton, CA) at 50,000 rpm for 2 h, resuspended in 0.6 ml in 40 mM sucrose, 4 mM Hepes pH 7.4, and applied to a 12.5-ml gradient of 50–800 mM sucrose. The gradients were centrifuged at 4°C for 3.5 h at 28,000 rpm in a rotor (model SW40; Beckman Instruments) and 0.5-ml fractions were collected from the top of the gradient.

A similar procedure was used to prepare the subcellular fractionation of whole rat brain except that all buffers were further supplemented with 1 mM EDTA, 1 mM EGTA, and 0.1 mM leupeptin. After hypotonic lysis, proteins from the synaptosomal membrane fraction (SM1) were extracted by sequentially resuspending pelleted fractions in buffers containing 0.5 M NaCl, 1 M NaCl, and finally 1 M NaCl and 1.25% Triton X-100. Soluble proteins were fractionated from pelleted membrane associated fractions by centrifugation at 39,000 g for 30 min at 4°C. All samples were dialyzed for 72 h against 10 mM Hepes, pH 7.4, 1 mM EDTA, and 1 mM EGTA. After dialysis, the protein concentration of each sample was determined using the Bio-Rad protein assay.

Electrophoresis was performed on SDS polyacrylamide gels (Laemmli, 1970) with 50 μg of protein from P2, S2, and subsequent salt and detergent extractions of hypototically lysed membrane fractions and 75 μl of each sucrose gradient fraction. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% [vol/vol] methanol and 0.01% SDS) overnight, blocked by a 30-min incubation in Blotto (5% nonfat dried milk in Tris buffer saline; Johnson et al., 1984) and probed with SNAP-25 synthetic peptide antisera at a 1/500 dilution in Blotto for 3 h at room temperature. The blots were washed three times in Blotto at room temperature and secondary reactions were carried out using either ^{125}I -labeled *Staphylococcus* protein A, a Vectastain avidin biotin complex kit (Vector Laboratories, Burlingame, CA), or alkaline phosphatase conjugates (Promega Biotec, Madison, WI).

Immunocytochemistry

For light microscopy immunohistochemistry, adult BALB/c mice were anesthetized with CO_2 and transcardially perfused with 4% paraformaldehyde in PBS. The brains were removed, postfixed for 4 h in 4% paraformaldehyde/PBS, and cryoprotected by immersion in a series of cold, graded PBS sucrose solutions to a final sucrose concentration of 25%. The brains were cut coronally as 40- μm sections on a cryostat, placed in ice-cold PBS, and transferred to a fresh solution of PBS containing 1% BSA, 0.25% Triton X-100, and 1 $\mu\text{g}/\text{ml}$ of affinity-purified synthetic peptide antisera with incubation overnight at 4°C. The sections were then washed three times for 15 min each in cold PBS, incubated with a biotinylated goat anti-rabbit secondary antibody for 6 hours and processed using a Vectastain avidin-biotin complex kit (Vector Laboratories). The sections were developed with 0.5 mg/ml diaminobenzidine and 0.003% H_2O_2 in 20% PBS for 4–15 min. The sections were then washed in PBS and mounted on chrome-alum-coated slides.

For electron microscopy-immunocytochemistry, anesthetized mice were perfused transcardially for 5 min with freshly depolymerized paraformaldehyde, 4% in 0.15 M sodium phosphate buffer, pH 7.4, containing either 0.5 or 1.0% glutaraldehyde. Brains were then removed, sliced at 5-mm intervals, postfixed for 6–8 h in 4% paraformaldehyde, and then transferred to PBS overnight. Tissue slabs containing the hippocampal formation and overlying cerebral cortex were sectioned on a vibratome at 50–70 μm thickness, collected in 50 mM NH_4Cl , and rinsed repeatedly in this solution over 2 h, then rinsed several more times in PBS, incubated for 60 min in 10% DMSO in PBS, and again rinsed in PBS. Free-floating sections were then incubated overnight with the rabbit polyclonal SNAP-25 antipeptide antiserum at 1 mg/ml, in PBS containing 1 mg/ml BSA, at room temperature, with constant mixing. Bound rabbit immunoglobulin was localized with avidin-biotin: (Vector Laboratories) tissue sections were first incubated with a biotinylated goat anti-rabbit Ig for 8–10 h, and then in horseradish peroxidase coupled avidin-biotin complex overnight at room temperature. After being rinsed with PBS, sections were postfixed 1 h in 1.5% glutaraldehyde in PBS, containing 5% sucrose, and again rinsed in PBS. Immunoreagents bound to the tissue sections were then localized by preincubation, 5 min in 0.05% diaminobenzidine hydrochloride, in 0.05 M phosphate buffer, pH 7.4, containing 7.5% sucrose. Sections were then transferred to a solution containing the same reagents freshly prepared with 0.003% hydrogen peroxide, and reacted for 10–20 min under observation and constant mixing, until judged to have reacted optimally. Sections were again rinsed in PBS 7% sucrose; CA3 and dentate gyrus regions of the hippocampal formation were excised and exposed on a slide to 1% osmium tetroxide in phosphate buffer for 1 h while being held flat beneath a coverslip. Lastly, sections were dehydrated and embedded in TAAB 812 resin (TAAB Lab Equipment,

Ltd., Reading, Berkshire, UK), sectioned at 800–1,000 Å, mounted on 200-mesh copper grids, and counterstained with uranyl acetate and lead citrate before viewing in an electron microscope (EM 10-A; Carl Zeiss, Inc., Thornwood, NY).

In Situ Hybridization

40- μ m cryostat cut sections of paraformaldehyde-fixed adult mouse brain, prepared as described for immunocytochemistry, were mounted on gelatin-coated slides and air dried. In situ hybridization was performed as essentially described previously (Higgins et al., 1988) with ³⁵S-labeled antisense RNA transcripts generated using the T7 promoter of the clone p8.52 inserted in the pBS vector (Stratagene Corp.) after linearizing the plasmid by Sal I digestion. Transcription was performed in 40 mM Tris-HCl pH 7.6, 6 mM MgCl₂, 2 mM DTT, five U of RNasin (Promega Biotec), 400 μ M each of ATP, CTP, and GTP, 25 μ M UTP 5'-[α -³⁵S]thiotriphosphate (800–1,000 Ci/mmol; New England Nuclear), 1–2 μ g of linearized template and 5–10 U T7 RNA polymerase (Stratagene Corp.). Probes were hydrolyzed to fragments ~100–200 nucleotides in length by treatment with 0.2 N NaOH at 0°C for 20 min, followed by neutralization with unbuffered 2 M Hepes and purification by G50 Sephadex chromatography. Before hybridization, slide-mounted sections were postfixed in 4% paraformaldehyde in phosphate buffer (PB) for 5 min at room temperature, rinsed twice in PB, and treated with proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 50 μ g/ml in 5 \times TE (1 \times TE = 10 mM Tris/1 mM EDTA, pH = 8.0) for 7.5 min. After further rinsing in PB and dehydration in graded alcohol rinses containing 0.33 mM ammonium acetate, the slides were air-dried at room temperature. The slides were then prehybridized for 2 h at 52°C in hybridization buffer containing 50% formamide, 0.75 M NaCl, 25 mM Pipes, pH 6.8 25 mM EDTA, 5 \times Denhardt's solution, 250 mM DTT, 0.2% SDS, 10% dextran sulfate, and denatured yeast RNA and salmon sperm DNA at 500 μ g/ml. For hybridization, 80 μ l of the same buffer containing 125 ng/ml ³⁵S-labeled probe was applied to each slide, sealed under a coverslip, and incubated at 52°C overnight. After hybridization, coverslips were removed in 4 \times SSC containing 300 mM 2-mercaptoethanol, and the slide-mounted sections were digested with pancreatic RNase (50 μ g/ml) in 0.5 M NaCl/1 \times TE for 30 min at 37°C and rinsed with 0.5 \times SSC at 42°C. The slides were air-dried and exposed to x-ray film (Cronex; Dupont, Wilmington, DE) for 3–6 h at room temperature. For emulsion autoradiography, the slides were coated with photographic emulsion (NTB-2; Eastman Kodak, Rochester, NY) (diluted 1:1 with H₂O), exposed for 1–3 d and developed.

Three-dimensional Reconstruction

In situ hybridization was performed on 76 serial coronal sections of adult mouse brain cut in 40- μ m thick sections, where every fourth section was collected. Image analysis and reconstruction was accomplished using programs developed by Hibbard et al. (1987). Autoradiographs of the hybridized sections were scanned and optical density values of the autoradiographs digitized at 50 μ m resolution with a computer-operated microdensitometer (P-1000; Optronics International, Elmsford, NY) set at 2 ODU maximum scale. The digitized images were displayed on an AED-1024 color terminal and scanning artifacts in the fields surrounding the image were removed. The images were aligned by the programs of Hibbard et al. (1987), run on a VAX 11/780 (Digital Equipment Corp., Marlboro, MA), whereby a center of mass is determined for each image and a set of principle axes established through the center of each section. Serial images were subsequently aligned by translation of the principle axes to a common origin of reference and rotated to place the longer principle axis horizontal. The aligned images were viewed either as a three-dimensional perspective or sections of the reconstructed brain were taken in the horizontal or sagittal planes. The optical density range corresponding to each color of the scale was established so that an equivalent number of pixels from an image would fall into each color bin. The effect of such a scaling technique is that the range of optical density values assigned to the colors of highest optical density are wider than in the middle range, reflecting the distribution of the optical density values.

Results

Nucleotide and Potential Amino Acid Sequence Encoded by SNAP-25 mRNA

To obtain a full-length sequence of the 2.2-kb SNAP-25

mRNA, a previously identified cDNA clone pMuBr8 (Branks and Wilson, 1986) was used to screen an adult murine hippocampal lambda gt10 cDNA library. Approximately 0.1% of the clones screened were positive, which corresponded well with the previous estimates of SNAP-25 mRNA abundance in total brain poly A⁺ RNA (Branks and Wilson, 1986). Of the isolated recombinant phage, one clone 8.1, contained a nearly full-length, 1.8-kb cDNA insert, which was subcloned and sequenced. To obtain further sequence of the 5' region of the mRNA, additional cDNA clones were identified by screening the same cDNA library with an oligonucleotide probe (A1) corresponding to the 5' end of the 8.1 cDNA. Although none of these cDNAs contained an insert spanning the entire length of the 2.2-kb mRNA, a composite sequence containing 2,052 continuous basepairs was obtained by assembling the sequence of p8.52 and p8.1 with the terminal sequence of other cDNA inserts (Fig. 1). The nucleotide sequence is terminated at the 3' end with a poly A⁺ tract of 13 A residues, which is preceded 13 nucleotides upstream by a potential polyadenylation signal, AUUAAA (Berget, 1984). This indicates that the sequence shown in Fig. 1 represents the 3' 2,039 encoded nucleotides contained within the 2.2-kb SNAP-25 mRNA. To evaluate the length of the 5' region not contained in the sequence presented in Fig. 1, an oligonucleotide probe (A52) was synthesized to the complement of the sequence from positions 18–38 and used for primer extension analysis of RNA isolated from several tissues. A primer-dependent extension product of 91 bp was generated with brain poly A⁺ mRNA but not with either liver poly A⁺ RNA or yeast total RNA as templates (data not shown). When the length of the oligonucleotide primer and its position are taken into account this result suggests that only 53 nucleotides at the 5' end are not represented in the composite sequence shown in Fig. 1.

Analysis of the SNAP-25 cDNA sequence for possible protein encoding regions revealed a single long open reading frame (ORF) extending from bp 125 to bp 781. The first AUG codon of this region (position 164–166, Fig. 1) has the surrounding sequence, CTACCATGG, which corresponds closely to the consensus translation initiation site (Kozak, 1984). The putative initiation site is preceded by two in-frame stop codons, suggesting that the 2,052 bp of cDNA contain the entire translated region. The potential initiation site is followed by two additional in-frame AUG codons (182–184 and 204–206 bp). These AUG codons are surrounded by sequences that do not correspond to the consensus initiation sequence, and are unlikely to be used for initiation in vivo (see below). The 618-nucleotide ORF is terminated by the translational stop codon, TAA, at position 781–783, and encodes a 206-residue polypeptide having a calculated molecular mass of 23,315 D and a theoretical pI of 4.38. The remaining cDNA sequence includes a 3' untranslated region of 1,258 nucleotides that contains two extensive stretches of repeated CA dinucleotides at positions 879–933 and 1,578–1,619.

The appropriate assignment of the predicted SNAP-25 polypeptide was evaluated by in vitro translation of capped SNAP-25 RNA transcripts. The transcripts were prepared from several cDNAs differing in the extent of their 5' and 3' sequence, described in the legend of Fig. 2. As shown in Fig. 2 a, transcripts from clones p8.52 and p8.71, which contain the entire predicted open reading frame but vary in their ex-

5' CCCGAGGTTTGGAGCTGTCTTTCCTCCCTCCCTACCCGGCGGCTCCTCCACTTGTACCTGCAGGGATC 72
 AGCGGACAGCATCCTCTGAAGAAGACAAGGTTCTTAACTAAGCACCCTGACTTGGTGGCCCCGGCCCA 144

GCAACCCCCACCACTACCATGGCCGAGGACGCAGACATGCGTAATGAACTGGAGGAGATGCAGAGGAGGGC 216
 MetAlaGluAspAlaAspMetArgAsnGluLeuGluGluMetGlnArgArgAla 18

TGACCAGCTGGCTGATGAGTCCCTGGAAAGCACCCGTCGCATGCTGCAGCTGGTGAAGAGAGTAAAGATGC 288
 AspGlnLeuAlaAspGluSerLeuGluSerThrArgArgMetLeuGlnLeuValGluGluSerLysAspAla 42

TGGCATCAGGACTTTGGTTATGTTGGATGAGCAAGGCGAACAACCTGGAACGCATTGAGGAAGGGATGGACCA 360
 GlyIleArgThrLeuValMetLeuAspGluGlnGlyGluGlnLeuGluArgIleGluGluGlyMetAspGln 66

AATCAATAAGGATATGAAAGAAGCAGAAAAGAATTTGACGGACCTAGGAAAATTCGCGGGCTTTGTGTGTG 432
 IleAsnLysAspMetLysGluAlaGluLysAsnLeuThrAspLeuGlyLysPheCysGlyLeuCysValCys 90

TCCCTGTAACAAGCTTAAATCCAGTGATGCTTACAAAAAGCCTGGGGCAATAATCAGGATGGAGTAGTGGC 504
 ProCysAsnLysLeuLysSerSerAspAlaTyrLysLysAlaTrpGlyAsnAsnGlnAspGlyValValAla 114

CAGCCAGCCTGCCGTGGTGGATGAACGGGAGCAGATGGCCATCAGTGGTGGCTTACCGCAGGGTAAC 576
 SerGlnProAlaArgValValAspGluArgGluGlnMetAlaIleSerGlyGlyPheIleArgArgValThr 138

AAACGATGCCCGGAAAATGAAATGGATGAAAACCTAGAGCAGGTGAGCGGCATCAGGAAACCTCCGTCA 648
 AsnAspAlaArgGluAsnGluMetAspGluAsnLeuGluGlnValSerGlyIleIleGlyAsnLeuArgHis 162

TATGGCCCTAGACATGGGCAATGAGATTGACACCCAGAATCGCCAGATTGACAGGATCATGGAGAAGGCTGA 720
 MetAlaLeuAspMetGlyAsnGluIleAspThrGlnAsnArgGlnIleAspArgIleMetGluLysAlaAsp 186

CTCCAACAAAACCAGAATTGATGAAGCCAACCAACGTGCAACAAAGATGCTGGGAAGTGGTTAAATCTGCCG 792
 SerAsnLysThrArgIleAspGluAlaAsnGlnArgAlaThrLysMetLeuGlySerGly*** 206

TTCTGCTGTGCTCTCCCAATGTTGTTGGACAAGAGAGAAGAGCTCCTTCATGCTTCTCTCATGGTATT 864
 ACCTAGTAAGACTTACAGAGTA 936
 GTCACCCCATGTAATGCTGTGTGGTTTGTGAGCTTCCCAATGATACCATGTGCTTTTGTCTTCCG 1008
 GCTCTCTTTTGGCCAAAGGTTGTACATAGTGGTCACTGTGTGACTTATTTCCGACTAAGAGTCTTG 1080
 GGCTCTCTCTTTCTTCTCAGTGGCGTTTGTGTAATGACAACAATTTAGGAATGCTCAATGTACTGTTGA 1152
 TTTTCTCAATACACAGTATTGTTCTTGTAAAACCTGTGACTTACCACAGAGCTACTACCACAGCTCTTCTT 1224
 AGGGTGTCAAGGCTCTGAATCTCTCCAAATGTGCTCTCTTGGTTCCTCAGTGTATTCTTTGTCTTTATGAT 1296
 TTCATAATTAGACAATGTGAATTACATAACAGGCATTGCACATAAAGTGATGTGATTATGCATTTATGCA 1368
 TGAGAATAAATAGACTTTTATAGTCTACTTAAACAAAACCTCCATGACAGTAGCATACTGACAAGAAAAC 1440
 ACACACAACAGCAACAATAACAAGCAACAACCTACGCATGCTCAGCATTGGGACACTGTCAAGATTAAGTCA 1512
 TACCAGCAAAACCTGCAGCTGTGTACCTTCTCTGTCAACATACAGACTGATCATAATGATCCCTCTTTA 1584
 CACACACACACACACACACACACACACACACACAATGGAATTTAACCAACTCCAGAAATTTATGAA 1656
 GCAATATATGTTTGGCTGAAACTATTGTAATGTTGTAATATAGGGTTTGTGCAATGCTTTTGAAGCTC 1728
 TGTTTTCCAGACAATACTCTGTGTGGAACCTGGAAGTCTTCTAAGTCTGGCTCTTGTGATCACCAAACC 1800
 CTGGTGCATCAGTACAACACTTTGGCTAATCTAGAGCTATGCACAACCAAAATGCTGAGATGTTTAGTAGC 1872
 TGATAAAGAAACCTTTAAAAAATTATATAAATGAATGAATATAGATAAATGTGAGATAAATATCATTACA 1944
 GCATGTATATTAATCCCTCTCTCTCTCTGTTGGTTGTGAAGTGATTGACATTTTGTAGCTAGTTTAA 2016
 AATTATTAATAAATATAGATGTTAAAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA 2052

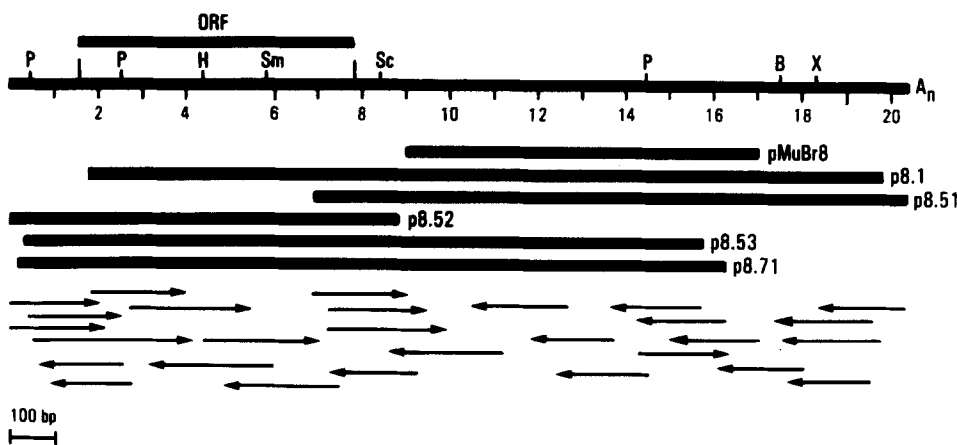


Figure 1. Nucleotide sequence of SNAP-25 cDNA and deduced amino acid sequence. The nucleotide sequence of cDNA representing 2,052 bp of the 2.2-kb SNAP-25 mRNA is shown. Primer extension experiments indicate that 53 nucleotides at the 5' end of the mRNA are not included in this sequence. The ORF predicted for the 206-amino acid SNAP-25 polypeptide is indicated and begins at position 164 and extends to position 781. The positions of hydrophobic residues within the amino-terminal domain are indicated with bars to demonstrate the three- to fourfold periodicity that may form an amphipathic alpha helix with a nonpolar face. The restriction map of the cDNA sequence is shown below the nucleotide sequence and the various cDNA clones isolated and used for sequence analysis are depicted. The arrows indicate the direction and extent of sequences determined by dideoxynucleotide sequencing either of cDNA progressively deleted by exonuclease III digestion or of subcloned fragments. P, Pst I; H, Hind III; Sm, Sma I; Sc, Sac I; B, Bam HI; X, Xba I.

tent of 3' untranslated sequence, both produced a major translation product migrating with an apparent molecular mass of 25.0 kD and a minor product of 24.3 kD. In contrast, transcripts of cDNA clone p8.1, which contain only the second AUG at position 182-184, produced only a single trans-

lation product, identical in mobility to the 24.3-kD secondary translation product of transcripts generated from clones p8.52 and p8.71. The difference in molecular mass between the primary and secondary products (0.7 kD) corresponds closely to the anticipated difference of six residues in length

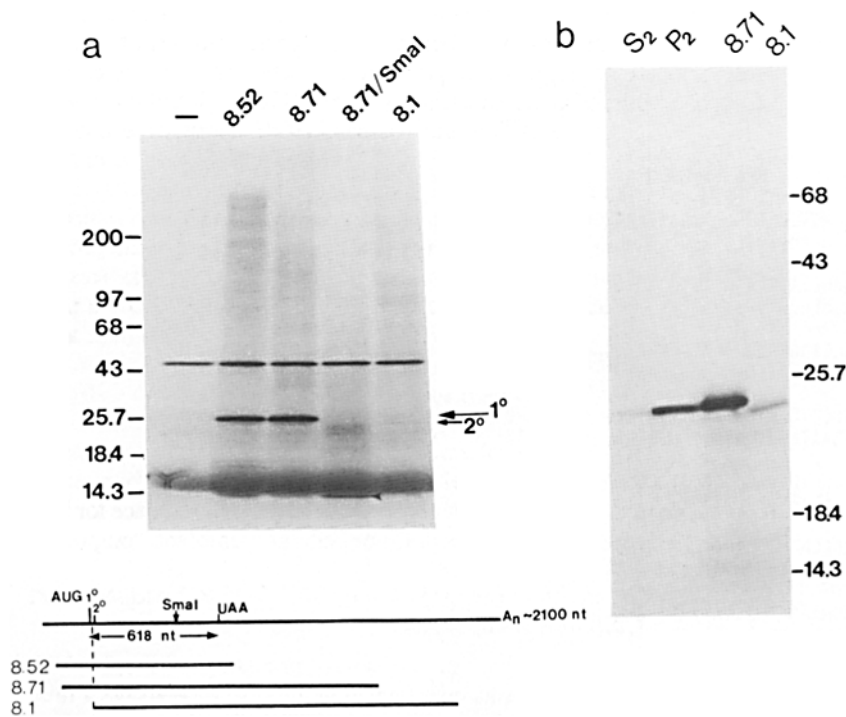


Figure 2. Autoradiographs of a polyacrylamide gel of *in vitro* translation products from SNAP-25 transcripts and immunoblots of mouse hippocampal synaptosomal and cytosolic protein fractions. RNA transcripts were generated from three independent SNAP-25 cDNA clones (8.52, 8.71, and 8.1). Clones 8.52 and 8.71 both contain the entire ORF of 618 nucleotides; clone 8.71 contains a more extensive portion of the 3' untranslated region as indicated by the schematic below. Clone 8.1, starting at bp 172, lacks the first AUG of the ORF (164–166 bp), but does contain the second in-frame AUG located at 182–184 bp along with most of the 3' untranslated region. An additional SNAP-25 transcript was generated from the 8.71 clone cut at a Sma I restriction site within the ORF, as shown in the diagram below. [³⁵S]Methionine-labeled translation products programmed by these transcripts in a rabbit reticulocyte lysate system were separated on a 15% polyacrylamide gel and autoradiographed (a). In the lanes containing the translation products from the 8.52 and 8.71 transcripts (labeled 8.52 and 8.71), a primary transcript dependent

translation product migrating at 25 kD and a secondary product migrating at 24.3 kD are seen. However, in the lane containing the translation product from the clone 8.1 transcript (which lacks the first AUG codon), only the 24.3-kD secondary translation product is seen. The translation product from the clone 8.71/Sma I clone migrated at 14.2 kD, reflecting the truncation of the transcript within the ORF (b). 75 μ g of total protein from the synaptosomal (P2) and the cytosolic (S2) fractions of adult mouse hippocampus were separated by SDS-PAGE along with the *in vitro* translation products from clone 8.71 and 8.1 transcripts. The gel was transferred to nitrocellulose and probed with SNAP-25 antipeptide antisera, which was detected on autoradiographs by ¹²⁵I labeled *Staphylococcus* protein A. A single immunoreactive protein was recognized by the antisera in the hippocampal fractions and was 5–10-fold enriched in the synaptosomal fraction. The immunoreactive protein from the hippocampal preparations comigrated with the transcript dependent *in vitro* translation product of clone 8.71 which contained the entire ORF (lane 8.71). The *in vitro* translation product of clone 8.1 transcripts (lane 8.1) migrated with slightly higher mobility than the hippocampal and clone 8.71 proteins reflecting the absence of the first AUG of the open reading frame in clone 8.1.

between the first and second AUG codons. The relatively greater intensity of the 25-kD primary translation product is consistent with more efficient initiation at the first AUG surrounded by a sequence corresponding to the consensus initiation sequence. Finally, translation of a transcript truncated within the ORF, synthesized from a cDNA template cleaved at a Sma I site at position 585–590 (Fig. 1) and 424 nucleotides downstream of the primary AUG initiation site, resulted in a 14-kD translation product, consistent with the anticipated reduction of the translated region.

Sequence Similarities and Structural Features of the SNAP-25 Polypeptide

The predicted amino acid sequence of SNAP-25 was compared with the National Biomedical Research Foundation protein sequence data base of April 1988 using the programs FASTP (Lipman and Pearson, 1985) and SEARCH (Dayhoff et al., 1983). Although sequences with identity to the SNAP-25 sequence were not found, amino acid residues 7–36 of the SNAP-25 polypeptide did show sequence similarity to the neuroactive 41-amino acid peptide corticotropin-releasing factor (CRF). With the introduction of three single-amino acid gaps in the CRF sequence, the amino-terminal

residues 7–36 of the SNAP-25 polypeptide exhibited a 40% identity and 76% similarity, considering conservative amino acid substitutions, to the carboxyl 27-amino acid sequence (residues 15–39) of the mature rat CRF (Jingami et al., 1985; data not shown). A functional relationship between SNAP-25 and CRF, however, is unlikely. Although the SNAP-25 polypeptide does not appear to be proteolytically cleaved and does not have the characteristics of a prepropeptide, the mature CRF peptide is cleaved from the carboxy terminus of a 186 residue prepropeptide (Rivier et al., 1983; Jingami et al., 1985). This region of sequence similarity between CRF and SNAP-25 resides in a domain of potential alpha-helical conformation for both peptides, discussed below, and may reflect similar sequence constraints of such secondary protein structure.

The SNAP-25 polypeptide sequence contains several consensus sites for phosphorylation by protein kinase C at serine or threonine residues: 28, 39, 138, 187, 190, and 200 (Kishimoto et al., 1985) and one candidate threonine for cAMP-dependent protein kinase phosphorylation at residue 138 (Edelman et al., 1987). The primary amino acid sequence also contains two potential sites for *N*-linked glycosylation, Asn-Leu-Thr and Asn-Lys-Thr at residues 77–79 and 188–190. Interestingly, all cysteine residues of the SNAP-25 peptide sequence were found clustered in the span of resi-

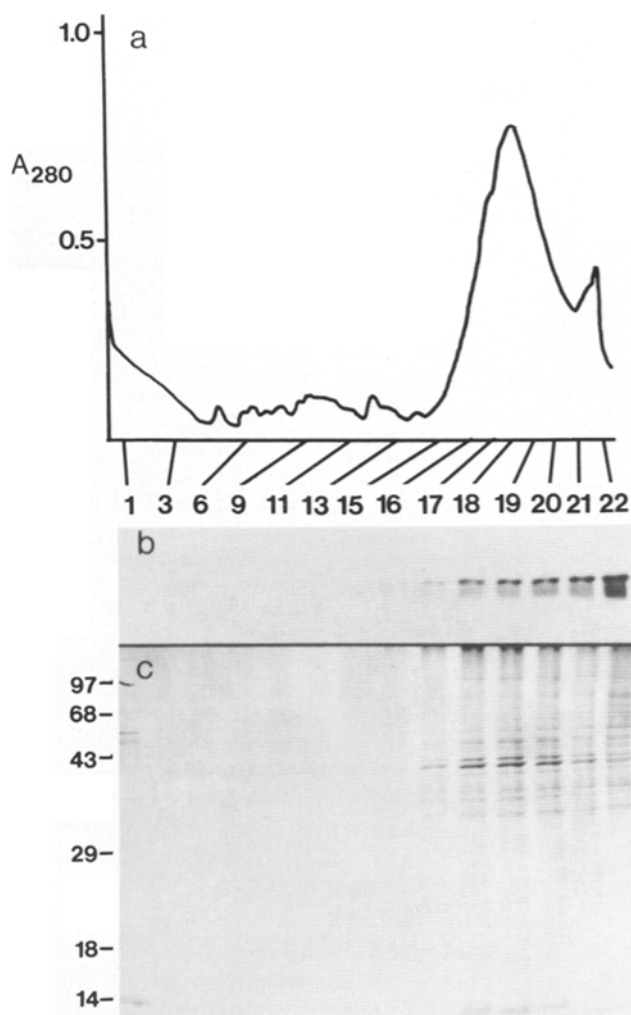


Figure 3. Sucrose gradient fractionation of SNAP-25 immunoreactive protein with synaptic vesicles and membrane components. Synaptic vesicles and membranes were released by hypoosmotic lysis of the P2 synaptosomal fraction of a hippocampal homogenate, collected by centrifugation and further fractionated by centrifugation through a 50–800-mM sucrose gradient as described in Materials and Methods. (a) Total optical density profile at 280 nm, representing total protein fractionated in the gradient. Sedimentation is from left to right. Aliquots of the indicated fractions were analyzed on 12.5% SDS-polyacrylamide gel followed by immunoblotting or silver staining of the fractionated proteins. (b) Immunoblot of SNAP-25 protein detected by antipeptide antisera and peroxidase reaction of the secondary antirabbit antisera. Only the region of the gel corresponding to 18–30 kD, where the immunoreactive SNAP-25 migrates is shown. (c) Silver-stained gel of the fractionated proteins to illustrate the peak of synaptosomal proteins in fractions 18–20.

dues 84–92: Cys-Gly-Leu-Cys-Val-Cys-Pro-Cys. This short Cys-containing region is similar to that found in a class of metal binding proteins including liver alcohol dehydrogenase, and metallothionein in which the Cys residues provide ligands for a metal ion (reviewed by Berg, 1986).

Analysis of the primary sequence of the SNAP-25 protein by hydrophobicity plots (Rose and Roy, 1980) indicated that the SNAP-25 peptide is a largely hydrophilic polypeptide, lacking any stretches of hydrophobic residues longer than four to five residues. Several polar amino acids are present

in the first 13 residues of the sequence, and consequently the amino terminus does not correspond well with the classic signal sequence directing translocation into the lumen of the endoplasmic reticulum (Blobel, 1980). Structural analysis programs (Chou and Fasman, 1974; Osguthorpe and Robson, 1978) were used to examine the SNAP-25 amino acid sequence for potential structural domains. The analysis suggested three domains of protein structure: an amino-terminal 80 residues consisting of six peaks of alpha-helical potential separated by five beta turns; an internal domain (residues 77–93 and 111–141) having high beta-extended chain potential; and a carboxy-terminal domain containing high alpha-helical potential. Within the amino terminal domain, residues 7–36, which showed sequence similarity to CRF, contain a series of hydrophobic residues separated by two to three polar or neutral amino acids (see Fig. 1). This suggests that the amino-terminal region has the potential to form an amphipathic helix that may provide a hydrophobic face for interaction with other proteins or cellular membrane components.

Subcellular Localization of SNAP-25 to Synaptosomal Components of Neurons

The expression and potential processing of the SNAP-25 polypeptide was investigated with polyclonal antisera raised against a synthetic peptide corresponding to the carboxy 12 residues (195–206) of the predicted amino acid sequence (see Material and Methods). As shown in Fig. 2 b, this antisera detected a single immunoreactive polypeptide of ~25 kD when used to probe protein transfer blots of cytosolic and synaptosomal fractions of mouse hippocampal homogenates. The immunoreactivity of the SNAP-25 synthetic peptide antisera was blocked by preincubation with the immunizing peptide and no immunoreactivity was detected on blots probed with preimmune sera (data not shown). The immunoreactive protein of hippocampal protein extracts, moreover, comigrated with the [³⁵S]methionine-labeled *in vitro* translation product of transcripts bearing the complete SNAP-25 ORF, suggesting that the SNAP-25 polypeptide does not undergo extensive posttranslational modification.

Immunoreactive SNAP-25 protein was enriched in the crude P2 synaptosomal fraction of hippocampal extracts prepared by differential centrifugation (Fig. 2 b). Similar enrichment of the 25-kD SNAP-25 protein was found in synaptosomal fractions of both mouse and rat neocortex preparations (data not shown). In order to evaluate whether the SNAP-25 polypeptide found in crude synaptosomal preparations is associated with structural components of the synaptosome, the P2 fraction of hippocampal and neocortex homogenates was subjected to hypotonic lysis and fractionation on continuous sucrose gradients as described by Huttner et al. (1983). As shown in Fig. 3, although a minor fraction of the immunoreactive SNAP-25 protein of hippocampal extracts was detected in fractions containing the major peak of synaptic vesicles, the SNAP-25 polypeptide was predominantly associated with faster sedimenting components, suggesting that it is not a free cytoplasmic protein but is complexed with macromolecular elements of the nerve terminal.

To evaluate the nature of SNAP-25 association with synaptosomal components, synaptosomal preparations of whole rat brain were treated with solutions of increasing ionic strength and detergent to dissociate membrane associated proteins. The majority of SNAP-25 protein was enriched in

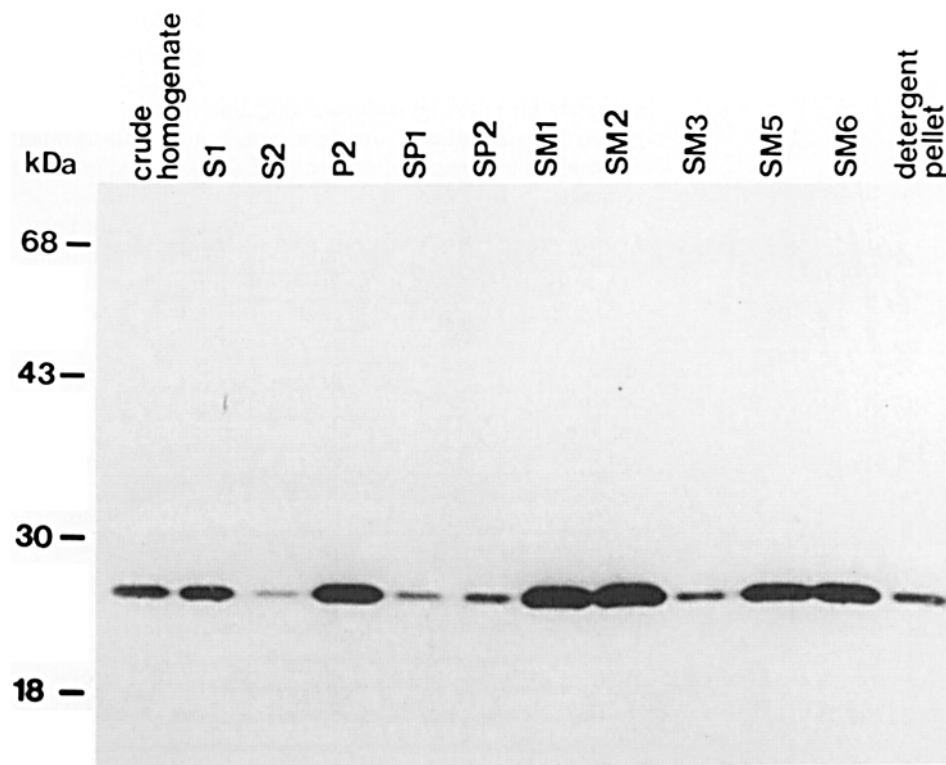


Figure 4. Subcellular fractionation of synaptosomal components prepared from whole rat brain. Brain homogenates were centrifuged at 1,000 g for 10 min and the cytosolic supernatant (S1) was further centrifuged at 39,000 g to provide S2 supernatant and P2 synaptosomal pellet fractions. After hypotonic lysis, the supernatants of the initial lysis (SP1) and wash in hypotonic buffer (SP2) and the final, synaptosomal membrane fraction pellet (SM1) were separated by centrifugation (39,000 g for 30 min). The following refer to fractions obtained by sequential salt and detergent extraction of SM1: SM2 and SM3, pellet and supernatant of the 0.5 M NaCl wash; SM5, pellet after 1 M NaCl wash; SM6, the supernatant fraction after treatment with 1.25% Triton X-100 in buffer containing 1 M NaCl. After dialysis, 50 μ g of each fraction were analyzed by SDS-PAGE, electrophoretically transferred to nitrocellulose, and incubated with SNAP-25 peptide antisera. The positions of molecular markers are indicated in kilodaltons on the left.

the P2 fraction of rat brain and remained associated with the synaptosomal membrane fraction (SM1) after hypotonic lysis (Fig. 4). The SNAP-25 protein in this membrane fraction, moreover, was not extracted by 0.5 and 1 M NaCl salt washes, remaining in the pelleted membrane fractions (SM2 and SM5). SNAP-25 protein was released into the supernatant (SM6) and depleted from the detergent pellet after treatment with 1.25% Triton X-100 in the presence of 1 M NaCl. These fractionation results suggest that a significant portion of SNAP-25 protein is tightly associated with detergent-soluble membrane components of the nervous system. The immunocytochemical analysis, described below, provides evidence that this subcellular association SNAP-25 is further localized in presynaptic terminals of certain neuronal subsets.

Cellular Localization of the SNAP-25 mRNA and Protein

SNAP-25 mRNA was previously observed to be differentially expressed in distinct groups of neurons of the hippocampal formation (Branks and Wilson, 1986). Consequently, we examined the hippocampus using *in situ* hybridization and immunocytochemistry with antipeptide antisera to compare the distribution of the protein and the mRNA on adjacent coronal sections. *In situ* hybridization with an antisense SNAP-25 RNA probe corresponding to the ORF showed a higher level of SNAP-25 in CA3 pyramidal neurons than in CA1 neurons or dentate granule cells (Fig. 5, *c* and *d*). This pattern of hybridization was consistent with our previous observation using a nick-translated DNA probe of the 3' untranslated region

of SNAP-25 (Branks and Wilson, 1986). The correspondence of the pattern of hybridization with probes for two different regions of the SNAP-25 mRNA indicates that this pattern is not due to nonspecific hybridization reflecting cell density. In contrast, the antipeptide antisera recognized immunoreactive protein located exclusively in neuronal processes of the hippocampal formation with intense immunoreactivity found at the CA3 mossy fiber terminal field (Fig. 5, *a* and *b*). No immunoreactivity was seen in the cell bodies of the CA3 pyramidal neurons at the level of light microscopy. The molecular layer of the dentate gyrus was immunoreactive, with the outer edge of the inner molecular layer staining most intensely. This pattern of immunoreactivity differed from the distribution seen with antibody to neurofilament proteins (data not shown), indicating that SNAP-25 is not uniformly expressed throughout neuronal processes. The immunoreactivity of the affinity-purified antipeptide antisera in these regions, moreover, was appropriately blocked by preincubation with the synthetic peptide used to elicit the antisera (data not shown).

The cellular localization of the SNAP-25 polypeptide was defined using immunoelectronmicroscopy with the same antipeptide antisera. When CA3 and adjacent regions of the hippocampal formation were sectioned at 0.5–1.0 μ m for orientation and selection, fine punctate deposits could be observed surrounding the perikarya and apical dendrites of pyramidal neurons, while the cytoplasm of these neurons appeared unreactive (Fig. 6). No clearcut immunoreactive structures were noted within the myelinated tracts. In dentate gyrus some neurons appeared to have modest immunoperoxidase deposits within their perikarya, and fiber like processes

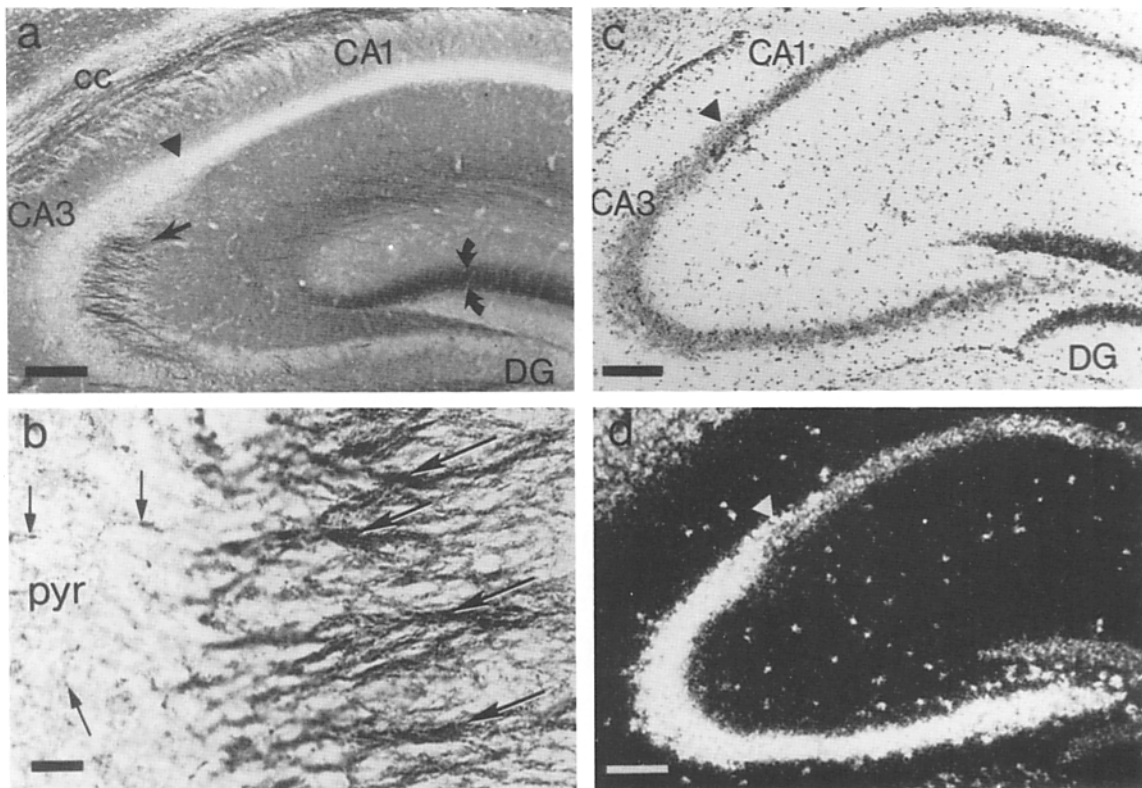


Figure 5. Immunohistochemistry and in situ hybridization for SNAP-25 in coronal sections of adult mouse hippocampus. Adjacent coronal sections through the hippocampal formation of an adult mouse brain were either immunostained with SNAP-25 antipeptide antisera (*a* and *b*) or hybridized with ^{35}S -labeled riboprobe for SNAP-25 mRNA (*c* and *d*). In *a*, intense staining is seen in the mossy fiber terminal fields as well as in the inner molecular layer of the dentate gyrus (DG). The apparent staining of corpus callosum fibers (cc) was not observed in other preparations or in other major white matter tracts. *b* represents an enlargement of the CA3 pyramidal cell layer (pyr) and the mossy fiber terminal fields; coarse immunoreactive fibers are indicated by arrows in the mossy fiber field. Immunoreactivity is largely absent from the pyramidal cell layer. In *c*, a bright-field photomicrograph of a cresyl violet-stained adjacent coronal section through the hippocampus is shown. A dark-field photomicrograph of an emulsion-coated adjacent section through the hippocampus hybridized with SNAP-25 ^{35}S riboprobe is shown in *d*. The signal level in the CA3 and CA4 fields of the pyramidal cell layer is greater than in CA1 pyramidal cells and the dentate gyrus. The autoradiographic grains are confined to the regions of neuronal cell bodies as indicated by the location of cresyl violet staining in *c*. Calibration bars in *a*, *c*, and *d*, 100 μm ; *b*, 5 μm .

were apparent deep to the granule cells as well. At the electron microscope level, sections taken close to the surface of the original vibratome section showed a very high density of immunoreactive nerve terminals and presynaptic axons grouped around unreactive dendritic shafts and medium-sized spines among a more sparse representation of unreactive boutons synapsing on the same dendrites. Most reactive terminals were relatively small, $\sim 1\text{--}2\ \mu\text{m}$ in diameter. Definite asymmetric postsynaptic specializations were observed opposite the immunoreactive terminals. Some reactive boutons were considerably larger, and also made asymmetric synaptic contacts on unreactive dendrites. Within the reactive terminals, immunoperoxidase deposits were generally confluent surrounding the synaptic vesicles, making more precise localization of the immune epitopes impossible. Very rarely, small deposits of immunoperoxidase reactivity were detected within the perikarya of pyramidal and granule neurons, associated with Golgi zones. This pattern of immunoreactivity indicates an association of the SNAP-25 peptide with the presynaptic terminals of mossy fiber axonal projections.

Distribution of SNAP-25 mRNA in the Mouse Forebrain

To provide a more comprehensive determination of the neuroanatomical distribution of SNAP-25 mRNA expression, an entire mouse forebrain was serially sectioned along the coronal axis at 40- μm intervals and every fourth section was selected for in situ hybridization with an ^{35}S -radiolabeled antisense RNA probe. To distinguish the relative levels of RNA, the extent of hybridization was determined from autoradiographic images of the sections by scanning optical density and establishing digital images. These images were assembled using a program devised by Hibbard et al. (1987). By representing the hybridization as a three-dimensional reconstruction and resectioning the generated image on a horizontal plane, hybridization to distinct neuronal nuclei could be traced through multiple sections and observed to have bilateral symmetry. Regions of brain containing high levels of SNAP-25 hybridization were the hippocampus and neocortex, particularly cortical layers III and V (Fig. 7). In

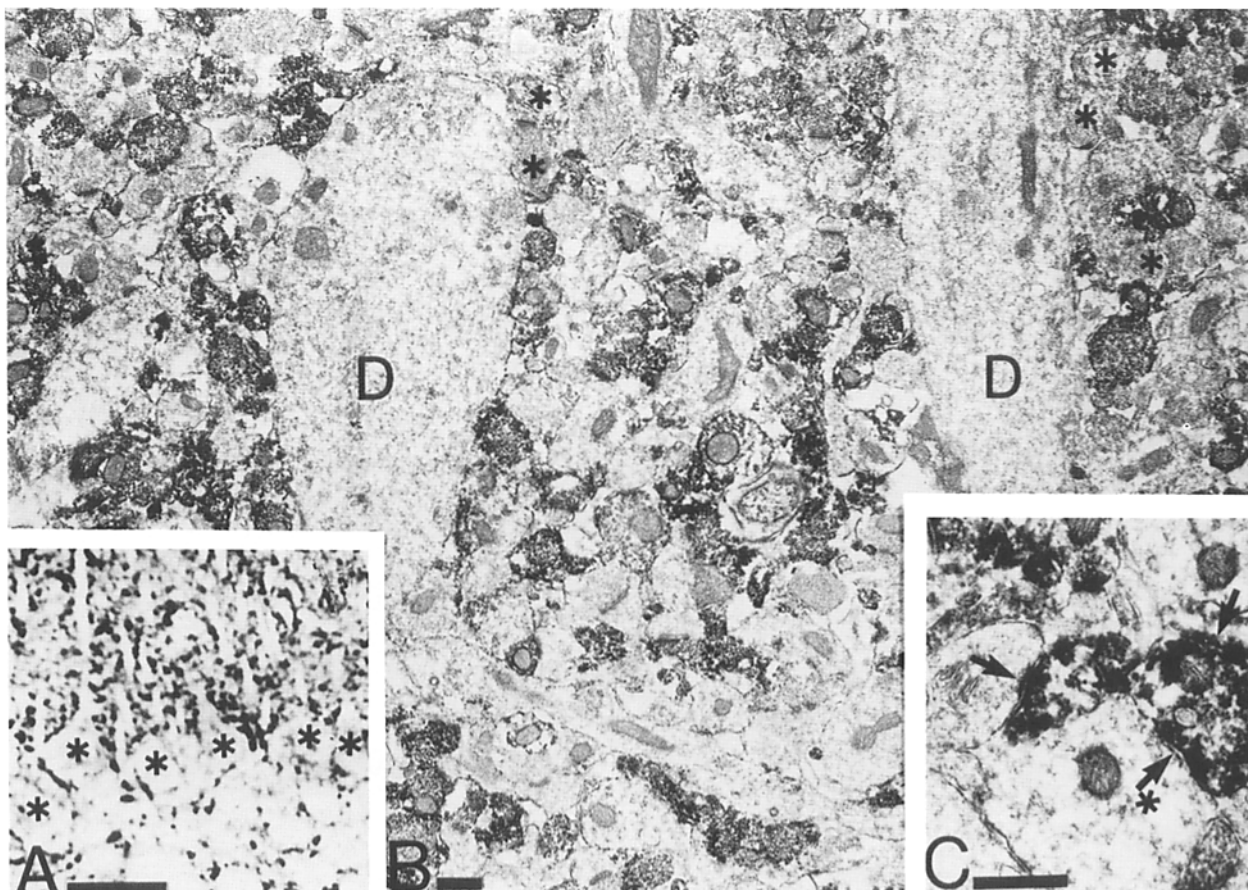


Figure 6. Immunocytochemical localization of SNAP-25 in mouse hippocampus. (A) Low-power, phase-contrast light micrograph of plastic-embedded specimen, illustrating the upper pyramidal cell layer of hippocampal field CA3, with nonimmunoreactive perikarya (*) and their apical dendrites extending upwards into the stratum radiatum where their outer surfaces are marked by multiple punctate immunoreactive elements, interpreted as nerve terminals. (B and C) Electronmicrographs of this region, in which two large, unreactive apical dendrites (D) can be seen to have multiple immunoreactive boutons in contact with their surfaces, although nonimmunoreactive boutons (*) can also be seen on these dendrites as well as on the axodendritic synapses in the intervening and surrounding neuropil. In C, two immunoreactive boutons are shown at higher magnification with immunoreactivity deposited on and around the synaptic vesicles; note that both asymmetric synaptic specializations (arrows) and symmetric specialization (arrow with *) are made by immunoreactive boutons. Calibration bars: A, 25 μm ; B and C, 0.5 μm .

the neocortex, both dorsal-to-ventral and rostral-to-caudal gradients of expression are evident, with lower levels of hybridization detected in the ventral and caudal regions. In other brain regions, a high level of hybridization was found in the piriform cortex, medial septal nuclei, pontine nuclei, the mammillary bodies, the granule cell layer of the olfactory bulbs, and regions of the thalamus. Within the thalamus, several nuclei exhibited either high or intermediate levels of the mRNA. The anterior thalamic nuclei showed high levels of hybridization, whereas the ventral posterior and ventral lateral thalamic nuclei contained intermediate mRNA levels. In contrast, low levels of the mRNA were present in the basal ganglia and in the hypothalamus, with the exclusion of the arcuate nucleus which appeared to have higher mRNA levels throughout several sections. Regions of white matter, such as the corpus callosum or anterior commissure, were devoid of the SNAP-25 mRNA, affirming the neuronal specificity of the gene. Although sections through the cerebellum were not included in this analysis, previous studies have demonstrated that SNAP-25 mRNA is expressed in granule cells

but not in Purkinje cells and is present in specific interneurons of the deep cerebellar nuclei (Branks and Wilson, 1986).

Discussion

The nucleic acid sequence of SNAP-25 cDNA revealed a single ORF encoding a 206-residue, 23.3-kD protein. Although three potential initiation sites exist within the ORF, *in vitro* experiments using rabbit reticulocyte lysate, indicated that the first in frame AUG was most efficiently used for translation. The *in vitro* translation product of synthesized SNAP-25 transcripts comigrated at 25 kD on SDS-PAGE with a protein from adult mouse brain recognized by antisera prepared to the predicted sequence of the SNAP-25 protein; this suggests that the polypeptide is not modified by proteolytic processing. Although two potential sites of *N*-linked glycosylation exist in SNAP-25, endoglycosidase F treatment of adult murine brain protein preparations did not alter the electrophoretic mobility of SNAP-25 immunoreactive protein,

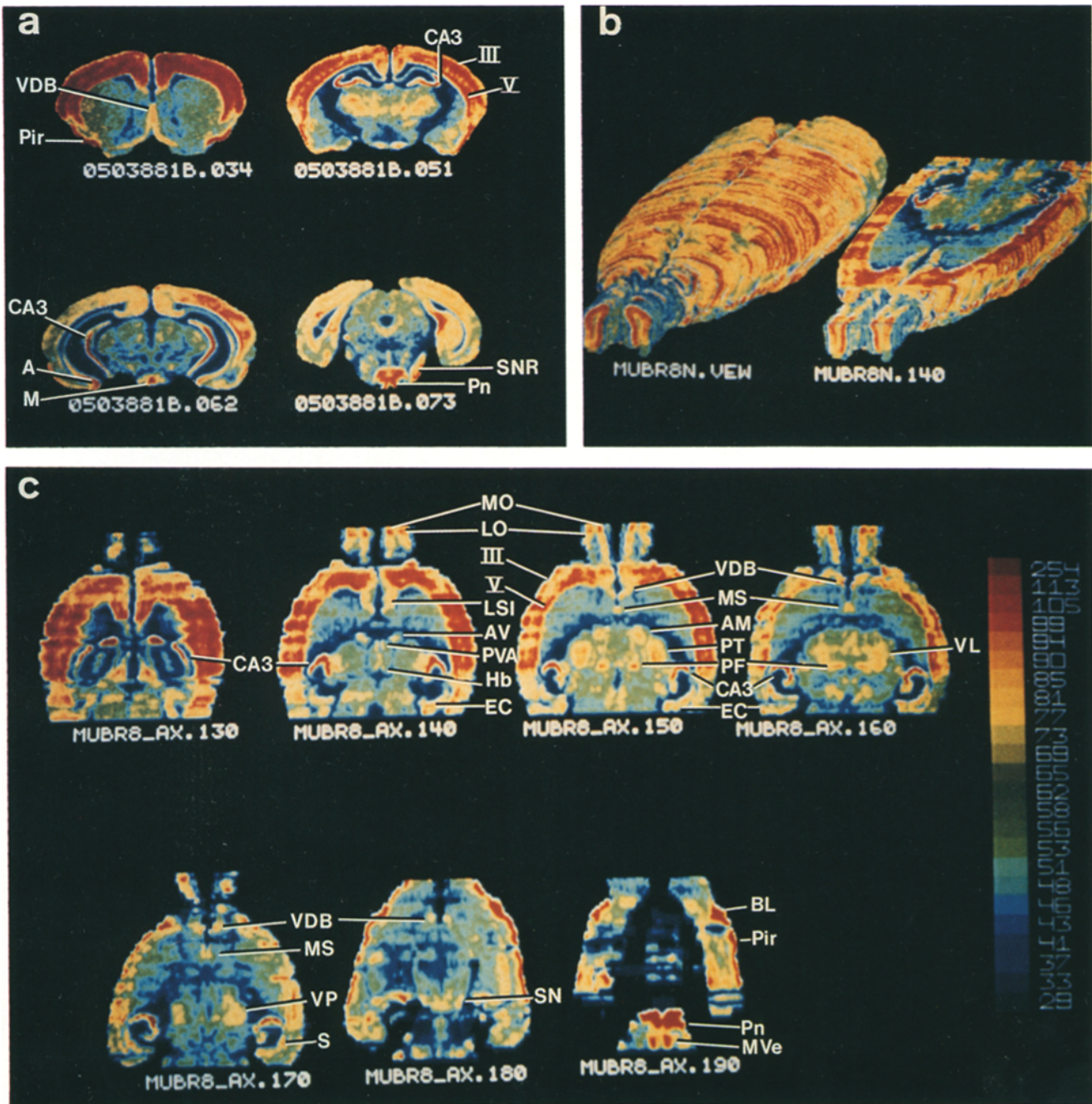


Figure 7. Color-enhanced three-dimensional reconstructions of SNAP-25 in situ hybridization. Autoradiographs of 76 serial coronal sections through the forebrain of an adult mouse were hybridized with ^{35}S labeled riboprobe for SNAP-25 mRNA, scanned with a computer operated optical densitometer, and depicted as color enhanced images. The colors of the images represent an optical density value of the autoradiograph; the optical density corresponding to each hue is shown in the scale in *c*. The numerical figures indicate the maximum optical density value assigned to a specific hue in millioptical density units. In *a*, four representative coronal sections are depicted. Starting at the upper left corner and proceeding clockwise, the sections were taken through the caudate, anterior thalamus, and hippocampus, the mid mesencephalon, the entorhinal cortex, and pons, respectively. Structures containing high to moderate levels of the SNAP-25 mRNA (reds and yellows) are labeled with arrows (VDB, nucleus of ventral limb diagonal band; Pir, piriform cortex; III and V, layers 3 and 5 of the neocortex; CA3, the CA3 field of Ammon's horn; A, amygdala; M, mammillary bodies; Pn, pontine nuclei; SNR, substantia nigra). In *b*, a three-dimensional view of the brain after aligning and reassembling the serial coronal sections is shown along with a reconstructed brain that has been sliced horizontally to reveal internal structures. To allow a more detailed analysis of the neuroanatomical distribution of the SNAP-25 mRNA, horizontal sections were taken through various levels of the three dimensional reconstruction and shown in *c*. Structures containing high to moderate levels of the SNAP-25 mRNA are labeled (LO/MO, medial and lateral granule cell layers of the olfactory bulb, CA3 region of the hippocampus; III and V, cortical layers 3 and 5; LSI, lateral septal nucleus, intermediate; AV, anterior ventral thalamic nucleus; ADT, anterior dorsal thalamic nucleus; PVA, paraventricular thalamic nucleus; Hb, habenular nucleus; EC, entorhinal cortex; VDB, ventral limb of the diagonal band; MS, medial septal nucleus; AM, anteromedial thalamic nucleus; PT, paratenial thalamic nucleus; PF, parafascicular thalamic nucleus; VL, ventral lateral thalamic; VDB, ventral limb diagonal band; MS, medial septal nucleus; VP, ventral posterior thalamic nucleus; S, subiculum; SN, substantia nigra; BL, basolateral amygdaloid nucleus; Pir, piriform cortex; Pn, pontine nucleus; MVe, medial vestibular nucleus).

indicating that glycosylation at these sites does not occur (Wilson, M. C., and J. H. Elder, unpublished results). Preliminary evidence (Oyler, G. A., and M. Billingsley, unpublished results) suggests that potential protein kinase C and cAMP-dependent protein kinase sites in SNAP-25 are not phosphorylated either *in vivo* or *in vitro*. The SNAP-25 protein appears highly conserved among diverse vertebrate species as sequence analysis of cDNA isolated from developing chick retina has shown that SNAP-25 of chick and mouse exhibit 100% amino acid identity (Catsicas, S., D. Larhammar, A. Blomqvist, P. P. Sanna, R. J. Milner, and M. C. Wilson, manuscript in preparation). This degree of conservation suggests that the SNAP-25 protein may play an important role in the function of the nervous system.

Distribution of the SNAP-25 Protein and mRNA

Within the hippocampus, the most intense immunoreactivity of the SNAP-25 antisera is found in the mossy fibers and the outer edge of the inner molecular layer of the dentate gyrus. This immunoreactivity was further localized to presynaptic terminals of the mossy fibers by immunoelectronmicroscopy. The SNAP-25 protein accumulating at the boutons of immunoreactive mossy fibers is likely to be expressed by dentate granule cells, which contain lower levels of the mRNA relative to the neurons of the CA3 field. The lack of significant staining of the mossy fiber axons transversing from dentate granule cells to the terminal fields is consistent with SNAP-25 being predominantly a component of presynaptic terminals rather than axons of these neurons. The axonal transport of SNAP-25 protein has been recently examined in rats lesioned in the dentate gyrus by localized colchicine injection, demonstrating a loss of immunoreactivity at mossy fiber terminals (Geddes, J. W., E. J. Hess, R. A. Hart, J. P. Kesslak, C. W. Cotman, and M. C. Wilson, in preparation). Within the hippocampus, the highest level of SNAP-25 mRNA appears to be expressed by CA3 pyramidal cells (see Figs. 6 and 7), where it is presumably transported to the presynaptic terminals of these neurons. In this immunocytochemical analysis, we observed only modest immunostaining in the terminal fields of Schaffer collateral fibers, which represent a major CA3 afferent projection to the CA1 pyramidal neurons (Swanson et al., 1978). We do, however, find significant SNAP-25 immunoreactivity within terminal zones of the inner molecular layer of the dentate gyrus, particularly at the outer edge, which is consistent with innervation by the commissural/associational pathway (Swanson et al., 1978). It will be of interest to determine whether the differential accumulation of SNAP-25 in different terminal fields is due to rapid turnover of the SNAP-25 polypeptide at specific nerve terminals, or reflects differential axonal processing or transport to extrahippocampal CA3 efferent projections. The localization of the SNAP-25 protein synthesized by the CA3 neurons is being investigated and initial results suggest a major site is the lateral septal nuclei, an important target of CA3 projection.

Neuronal cell groups expressing the SNAP-25 mRNA are distributed in a discrete pattern in several regions of the brain. To analyze the neuroanatomical complexities of the mRNA distribution, three-dimensional reconstructions of *in situ* hybridizations were used. The reconstruction and color enhancement of the SNAP-25 *in situ* hybridization allowed neuronal structures with abundant levels of SNAP-25 mRNA

to be viewed from perspectives different than the original sections. The neuroanatomical distribution of SNAP-25 gene expression was not characteristic of any known neurotransmitter system or neuronal class. Many elements of the limbic system, however, are enriched in the SNAP-25 mRNA including the hippocampus, anterior thalamic nuclei, and medial septal nuclei. The SNAP-25 gene product may define a new molecular relationship between previously unrelated neuronal groups such as neocortical layers III and V, limbic system structures, and pontine nuclei.

Potential Structures and Function of SNAP-25

The association of SNAP-25 with structural and potentially membrane bound complexes has been demonstrated by its sedimentation through sucrose density gradients, resistance of the complex to high (1 M) salt and solubilization after extraction with Triton X-100 detergent (Fig. 4). In other experiments, we have found that SNAP-25 partitions in Triton X-114 phase separations (Bordier, 1981) with integral membrane proteins (data not shown). Taken together with the immunocytochemical evidence indicating presynaptic localization of the SNAP-25 protein, this suggests possible roles for the protein in the structural specialization of subsets of presynaptic terminals. For example, SNAP-25 may contribute to the recognition at docking sites for vesicle fusion and neurotransmitter release or to membrane attachment sites for cytoskeletal elements within the presynaptic terminal. The transient localization of SNAP-25 in axons within a number of major white matter tracts in the developing brain (Oyler, G. A., J. W. Polli, M. C. Wilson, and M. L. Billingsley, manuscript in preparation), moreover indicates that SNAP-25 may be required for plasticity of developing axons and the development of specific presynaptic terminals in the adult brain.

Although the mechanism that promotes the strong association of SNAP-25 with synaptosomal membranes remains to be resolved, the primary amino acid sequence of SNAP-25 indicates regions of potential protein interactions. Hydrophobicity analysis indicated that the SNAP-25 protein lacks the stretch of ~20 hydrophobic residues characteristic of single transmembrane domains (Eisenberg, 1984). Several domains of the SNAP-25 protein, however, have high potential for forming amphiphilic alpha helices, particularly the amino-terminal residues 7-36. By presenting a hydrophobic face, these alpha-helical regions may permit hydrophobic association with membranes or integral proteins. Alternatively, the association of SNAP-25 in detergent solubilized complexes may result from posttranslational addition of fatty acid or phospholipid (reviewed by Sefton and Buss, 1987). The amino terminal glycine residue required for myristylation is not present in the SNAP-25 sequence, which precludes this mechanism for membrane binding. Initial experiments have shown further that SNAP-25 is not released by phospholipase C (Elder, J., and M. C. Wilson, unpublished observations) indicating that it is not attached by phospholipid. The internal cysteine residues at positions 84-92, however, are potential sites for linkage through thioester bonds of palmitic acid. Although palmitylation often occurs at the carboxy terminus of proteins attaching the protein to the cytoplasmic face of the membrane, the addition of palmitic acid can occur throughout the length of a polypeptide. For example, it has been demonstrated that palmitylation at adjacent cysteines

within the amino-terminal sequence of GAP-43, associated with axonal outgrowth and present in growth cones and synapses, contributes to its attachment to membranes (Skene and Virag, 1989).

The cysteine residues of the SNAP-25 polypeptide sequence could provide sulfhydryl groups for formation of disulfide bonds with other proteins. The clustering of cysteine residues, however, is similar to the arrangement of cysteine residues found in certain metalloproteins (reviewed by Berg, 1986). A metal binding capability for SNAP-25 would be important in light of the abundance of zinc within the mossy fibers of dentate granule cells indicated by Timm staining (Frederickson, 1983). Although the role of zinc in hippocampal function has not been clearly determined, zinc is released into the synapse by stimulation of the perforant pathway and may be involved in hippocampal damage during pathological states (Sloviter, 1985). The possibility that SNAP-25 may have a role in zinc mobilization in neurons of the hippocampus as well as in other brain regions serves as a model to further explore the function of this neuronal-specific protein. Biochemical and molecular genetic approaches, including the use of site-specific mutagenesis to alter specific residues of the SNAP-25 sequence, together with the evaluation of palmitoylation, metal binding, and membrane attachment should provide evidence of the nature of the association of SNAP-25 with synaptic membrane components and begin an evaluation of its function in neurotransmission.

By characterizing the novel neuronal mRNA SNAP-25, we have identified a new component of the presynaptic terminal that is localized to a subpopulation of synapses in the brain. Because the presynaptic terminal is a region of critical activity in the general function of the nervous system and has implications in the specialization of specific synaptic connections, the identification and characterization of such constituents of nerve terminals (see reviews by Kelly, 1988; and Trimble and Scheller, 1988) will be essential in understanding neuronal function at the molecular level.

We wish to thank Ellen Hess, Dan Larhammar, and Stefano Catsicas for critical comments; and Michelle Dietrich for expert assistance in the preparation of the manuscript. We wish to thank Jim Hogle and George Rose for discussions on the potential structural implications of protein sequence and Richard Hawkins, Lyndon Hibbard, and Gina DeJoseph for use of their computer facility at Pennsylvania State University and for their advice and guidance in generating the three-dimensional reconstruction of *in situ* hybridization images. We wish to thank Vito Quaranta and Anita Holstrom for advice and help in conducting the protein data base computer search and in preparing peptide affinity-purified antisera.

This work was supported in part by National Institutes of Health grants CA-33730 and NS-23028 (M. C. Wilson), NS-22347 (F. E. Bloom), AG-06337 (M. L. Billingsley); an American Heart Association training grant and March of Dimes predoctoral fellowship to G. A. Oyler; and a Hereditary Disease Foundation Fellowship to G. A. Higgins. This is publication 5369-MB from the Research Institute of Scripps Clinic.

Received for publication 1 May 1989 and in revised form 22 August 1989.

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