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The 25 kDa Synaptosomal-associated Protein SNAP-25 Is the Major Methionine-Rich Polypeptide in Rapid Axonal Transport and a Major Substrate for Palmitoylation in Adult CNS

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A conspicuous correlate of the developmental transformation of axonal growth cones to synaptic terminals is a marked increase in synthesis and axonal transport of a methionine-rich, acidic polypeptide of approximately 25 kDa. This polypeptide, designated "super protein" (SuP), is the most prominent species among methionine-labeled proteins conveyed by rapid axonal transport in mature CNS and PNS neurons of warm- and cold-blooded vertebrates. We show here that SuP is identical to SNAP-25, a highly conserved synaptic protein of known primary structure, by immunoprecipitation with anti-SNAP-25 antiserum of SuP labeled with ^{35}S-methionine and transported by retinal ganglion cells of rat and cat. In addition, we show that SNAP-25/SuP is the most prominent species among retinal polypeptides that incorporate ^{3}H-palmitate *in vivo,* that it is fatty acylated through a hydroxylamine-labile, thioester bond, and that palmitoylated SNAP-25/SuP is axonally transported. Thus, SNAP-25/SuP is a rapidly transported constituent of the presynaptic apparatus and a major neuronal substrate for long-chain fatty acylation.

Although mature synapses and axonal growth cones have some components in common (Bixby and Reichardt, 1985; Buckley and Kelly, 1985; Mason, 1986), the metamorphosis of axon terminals from growth cone to synapse involves discrete changes in the complement of proteins synthesized by developing neurons and committed to axonal transport. Expression of several growth cone components declines sharply, as additional synaptic proteins are induced (Simkowitz et al., 1989; Skene, 1989). Among proteins conveyed by rapid axonal transport, the most conspicuous positive correlate of this transition is a pronounced increase in expression of an acidic, membrane-bound polypeptide with an apparent molecular mass (M_r) of approximately 25 kDa (Skene and Willard, 1981b; Kalil and Skene, 1986; Moya et al., 1988; Simkowitz et al., 1989).

On the basis of its intense metabolic labeling with ^{35}S-methionine, markedly acidic isoelectric point (pI of approximately 4.4), and M_r on SDS-PAGE of 24–29 kDa (depending on the gel system employed), this protein may be identified as a prominent and characteristic component of rapid axonal transport in a wide range of vertebrate neurons (Willard et al., 1974; Estridge and Bunge, 1978; Stone et al., 1978; Padilla et al., 1979; Bisby, 1980; Levine and Willard, 1980; Padilla and Morell, 1980; Skene and Willard, 1981a,b; Benowitz et al., 1983; Redshaw and Bisby, 1984a,b; Kalil and Skene, 1986; Baitinger and Willard, 1987; Archer and McLean, 1988; Moya et al., 1988). Designated variously as polypeptide 20 (Willard et al., 1974; Lorenz and Willard, 1978; Levine and Willard, 1980) or band S1 (Bisby, 1980; Redshaw and Bisby, 1984a,b), it has come to be known as "super protein" (SuP) (Doster et al., 1991) due to its predominance among methionine-labeled group I proteins in adult animals. In many neurons, elevated expression of SuP is correlated with establishment and maintenance of differentiated synaptic terminals, during both development and axonal regeneration (Bisby, 1980; Redshaw and Bisby, 1984a,b; Kalil and Skene, 1986; Moya et al., 1988; Simkowitz et al., 1989).

In several of its distinguishing characteristics, SuP resembles a 25 kDa synaptosomal-associated protein (SNAP-25) identified recently with a cDNA clone corresponding to an abundant, neural-specific mRNA from rat brain and shown to be localized to synaptic terminals (Oyler et al., 1989). The primary structure of SNAP-25 is highly conserved across species, and indicates an acidic, methionine-rich protein (Oyler et al., 1989; Catsicas et al., 1991). In development, expression of SNAP-25 mRNA and protein corresponds with the onset of synaptogenesis (Oyler et al., 1989, 1991; Catsicas et al., 1991). These similarities suggest that SuP and SNAP-25 might be one and the same. In a recent study, Loewy et al. (1991) reported that the sequences of tryptic peptides derived from SuP were contained within the primary sequence of SNAP-25 described by Oyler et al. (1989). We demonstrate here that SuP, synthesized and transported by retinal ganglion cells (RGCs) of adult rat and cat, is recognized specifically by antibodies to SNAP-25.

The primary structure of SNAP-25 is predominantly hydrophilic and contains no apparent membrane binding domains (Oyler et al., 1989; Catsicas et al., 1991). SuP, however, is tightly associated with neuronal membranes during axonal transport and in synaptic terminals (Lorenz and Willard, 1978; Skene and Willard, 1981d; Oyler et al., 1989). This disparity suggests that SNAP-25/SuP may undergo posttranslational modification that subserves membrane anchoring. One candidate for such a modification is the addition of a hydrophobic lipid moiety (Sefton

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and Buss, 1987; James and Olson, 1990), and the deduced sequence of SNAP-25 contains cysteine residues that might be subject to covalent attachment of long-chain fatty acid (Oyler et al., 1989; Catsicas et al., 1991). We show here that SNAP-25/SuP is modified by fatty acylation, serving as the most prominent substrate in retina for incorporation of ^{3}H-palmitic acid *in vivo*.

Materials and Methods

Metabolic labeling with ^{35}S-methionine. ^{35}S-methionine (New England Nuclear; 1100 Ci/mmol) was administered intravitreally employing a microsyringe and 30 ga injection cannula, in adult Long–Evans hooded rats anesthetized with a combination of ketamine and chloral hydrate and in adult cats anesthetized with halothane. Each eye was injected with 500 μCi in 4 μl (rat) or 25 μl (cat) of physiological saline. Following appropriate survival intervals, rats were killed by exposure to concentrated carbon dioxide and cats were killed by barbiturate overdose. Tissue samples removed included the retinae (RET), optic nerves (ON), dorsal lateral geniculate nuclei (LGN), and superior colliculi (SC). Samples were frozen on dry ice immediately upon removal and stored at −70°C until processed.

Metabolic labeling with ^{3}H-palmitic acid. ^{3}H-palmitic acid (New England Nuclear; 60 Ci/mmol) was administered intravitreally in adult Long–Evans hooded rats. Each eye was injected with 1 mCi in 2–4 μl of dimethyl sulfoxide (DMSO; Aldrich, HPLC grade). Following survival intervals of 18–20 hr, samples were obtained as above and stored at −70°C until processed.

Preparation of membrane fraction. Samples were homogenized with a Potter–Elvejehm homogenizer in osmotic lysis buffer consisting of 10 mM Tris, pH 7.5, 5 mM EDTA, 5 mM dithiothreitol (DTT) and the following protease inhibitors: antipain (10^{-6} M), aprotinin (1.4×10^{-6} M), bacitracin (10^{-5} M), benzamidine (10^{-3} M), benzethonium chloride (10^{-4} M), leupeptin (10^{-5} M), 1,10-*O*-phenanthroline (1 mg/ml), pepstatin A (10 mg/ml), phenylmethylsulfonyl fluoride (10^{-3} M), and soybean trypsin inhibitor (5×10^{-6} M). Nuclei and cell debris were removed by centrifugation at 1000 × *g* for 5 min. The low-speed supernatant was subjected to centrifugation at 100,000 × *g* for 30 min. The resultant pellet, referred to as the "membrane fraction," was solubilized in 0.5% SDS, 5 mM DTT by heating in a boiling water bath for 1–3 min.

Chloroform:methanol extraction. SDS-solubilized membrane fractions of samples labeled with ^{3}H-palmitic acid were extracted overnight at −20°C with 50–100 vol of chloroform:methanol (2:1) to remove lipids not bound to protein. Precipitated material was collected by centrifugation at 12,000 × *g* for 30 min, and the resultant pellet was subjected to 20 min washes with chloroform:methanol (2:1) until liquid scintillation counts of wash aliquots were at background levels (generally after three washes). Extracted pellets were air dried and solubilized in 0.5% SDS, 5 mM DTT by heating in a boiling water bath for 1–3 min.

Immunoprecipitation. SDS-solubilized membrane fractions were subjected to immunoprecipitation with a previously characterized antiserum obtained from rabbit immunized with a synthetic peptide corresponding to carboxy terminal residues 195–206 of murine SNAP-25 (Oyler et al., 1989). Antiserum was preincubated at 100 μl/ml with a 10% (w/v) slurry of Protein A–coupled Sepharose beads (Pharmacia CL4B) in immunoprecipitation buffer (IP buffer: 150 mM NaCl, 10 mM Tris, pH 8.0, 0.5% Tween-20, 0.1% NP-40, 100 μg/ml ovalbumin) for 2 hr at 40°C with constant rotation. Beads were then washed three times in the original volume of IP buffer, recovered by centrifugation, and resuspended in IP buffer as a 10% (w/v) slurry. Protein samples in 0.5% SDS were added to a final concentration of <0.05% SDS, incubated overnight at 4°C, and washed as above. After a final wash (150 mM NaCl, 10 mM Tris, pH 8.0), bound protein was eluted in 0.5% or 1% SDS, 5 mM DTT at 100°C for 10 min. Samples prepared as above but with serum obtained prior to immunization served as controls for the specificity of immunoprecipitation.

Electrophoresis. Proteins were analyzed on one-dimensional (1-D) gels containing 12% or 12.5% acrylamide/0.36% or 0.33% bisacrylamide using the buffer system of Laemmli (1970) or on two-dimensional (2-D) gels essentially according to O'Farrell (1975) as described previously (Jacobson et al., 1986). Isoelectric focusing gels contained ampholines of pH 3.5–10 and of pH 4–6 in a 2:1 ratio. Gels were fixed and stained with Coomassie brilliant blue R (Fairbanks et al., 1971), then prepared for fluorography using the APEX method (Jen and Thach, 1982) and apposed to x-ray film preexposed to yield a background optical density of 0.1 (Laskey and Mills, 1975).

To examine the hydroxylamine lability of the SuP–palmityl bond, gels were fixed for 30 min in 25% isopropanol, rinsed for 30 min in water, and soaked for 4 hr at room temperature in at least 10 vol of 1 M hydroxylamine, pH 7.0, or 1 M Tris, pH 7.0. Gels were then rinsed for 1 hr in water, stained, and prepared for fluorography.

Identification of bound fatty acids. The membrane fraction derived from ^{3}H-palmitate–labeled adult rat retina was extracted with chloroform:methanol as described above to remove noncovalently bound fatty acids and other lipids. For analysis of total protein-bound fatty acids, the pellet derived from one retina was incubated for 4 hr at room temperature (21°C) in 1 ml of 0.1*N* KOH in 100% methanol, and the released fatty acids were processed for thin-layer chromatography as described below. For analysis of fatty acid bound specifically to SNAP-25/SuP, labeled membrane proteins derived from five retinae were separated on one-dimensional SDS-PAGE gels (10% acrylamide). Gels were fixed in three changes of 25 vol of 25% isopropanol for a total of 3 hr. The position of SNAP-25/SuP was estimated by reference to the position of prestained molecular weight standards (Bio-Rad, low range) separated in lanes at each end of each gel, and a gel band 5 mm in width, corresponding to the position of SNAP-25/SuP, was cut out of each gel. Samples were loaded in multiple gel lanes, and one lane was left intact to confirm the location of SNAP-25/SuP by fluorography after excision of SNAP-25/SuP from the other lanes. Excised gel bands were dehydrated in three changes of 10 ml of 100% methanol, for 1 hr, overnight, and 1 hr, respectively. Protein-bound fatty acid was released by incubating the gel pieces (approximately 1 cm^3) in 5 ml of 0.1*N* KOH in 100% methanol for 4 hr at room temperature (21°C).

Following alkaline methanolysis of unfractionated protein or of isolated SNAP-25/SuP, each supernatant was extracted with 2 vol of chloroform:water (1:1). The aqueous (upper) phase was removed and back-extracted with 1 vol of chloroform. The organic phases were combined and washed three times with an equal volume of chloroform:methanol:water (1:10:10). The final organic phase was dried and dissolved in methanol, warmed to 30°C to ensure solubility of fatty acid methyl esters. Samples were spotted onto reverse-phase thin-layer plates (Whatman KC-18) and developed essentially as described by Bizzozero and Lees (1986). Plates were developed in acetone:methanol:water (80:20:10) to a height of 10 cm, air dried, and developed a second time to the same height. Aliquots of each sample were analyzed alone, and after mixing with migration standards. Migration standards were visualized by spraying the plates with phosphomolybdic acid (10% w/v in ethanol) and heating at 150°C for 5–10 min (Heuser, 1968). Lanes used for fluorography were not sprayed with phosphomolybdic acid; duplicate lanes were used for staining and fluorography. For fluorography, plates were soaked with a solution of 10% diphenyloxazole in diethyl ether at 0°C, air dried, and exposed to preflashed x-ray film at −70°C.

Results

Identity between SuP and SNAP-25

In adult rats and cats, monocular injection of ^{35}S-methionine was employed to label proteins synthesized by RGCs and transported through their axons in the ON to synaptic terminals in primary optic targets. In membrane samples derived from ON, LGN, and SC of both rat (Fig. 1) and cat (Fig. 2), SDS-PAGE revealed a prominent labeled polypeptide with M_r of approximately 25 kDa. This polypeptide was precipitated by anti-SNAP-25 antiserum (Oyler et al., 1989) but not by preimmune serum (Figs. 1, 2). The absence of the polypeptide in samples derived from the ON contralateral to the injected eye indicates that it is present as a result of axonal transport without a contribution from local synthesis, and its arrival in the SC contralateral to the injected eye as early as 3.5 hr following intravitreal administration indicates that it is conveyed among group I proteins in the most rapid phase of transport (Fig. 1). The increase over time in the abundance of labeled polypeptide in the SC indicates that it accumulates in synapses formed by RGCs in primary optic targets (Fig. 1). The presence of substantial levels of labeled polypeptide in the ON as long as 33 hr following eye injection

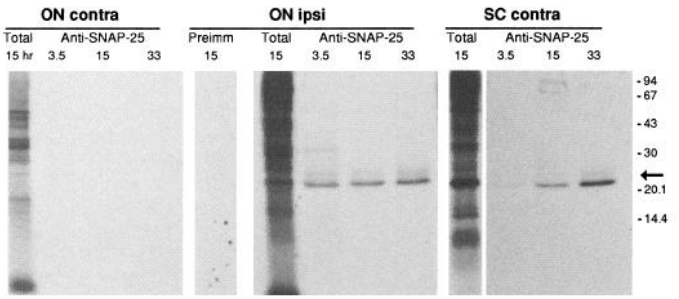

Figure 1. SuP is recognized by anti-SNAP-25 antiserum. ^{35}S-methionine was injected in one eye of adult rats followed by survival intervals of 3.5, 15, or 33 hr. Membrane fractions prepared from the ONs ipsilateral and contralateral and the SC contralateral to the injected eye were subjected to immunoprecipitation, SDS-PAGE, and fluorography. An *arrow* indicates the position of SuP (M_r = 25 kDa) among total membrane proteins derived from ipsilateral ON and contralateral SC, and immunoprecipitated by anti-SNAP-25 antiserum (but not by preimmune serum) from membrane proteins derived from SC contralateral and ON ipsilateral (but not contralateral) to the injected eye. In this and subsequent figures, the position and M_r (kDa) of molecular weight standards are indicated.

(Fig. 1) is consistent with entry of some fraction of transported protein into a relatively stationary phase within RGC axons, and/or gradual release of labeled protein from a relatively large pool within RGC somata. On the basis of its presence and abundance in rapid axonal transport, this polypeptide is SuP. Specific recognition of this polypeptide by antibodies to SNAP-25 indicates identity between SNAP-25 and SuP.

The identification of SNAP-25 as SuP was strengthened by high-resolution 2-D gel analysis. In the membrane fraction derived from ON or LGN of both cat (Fig. 2) and rat (Fig. 3), SuP was easily identified among transported species as a prominent labeled polypeptide with M_r of approximately 25 kDa and pI of approximately 4.4 (Figs. 2, 3, original). A polypeptide of similar electrophoretic mobility was recognized by SNAP-25–specific antibodies (Figs. 2, 3, anti-SNAP-25). Electrophoresis of a mixture of original membrane fraction and immunoprecipitate revealed that SuP and SNAP-25 comigrated precisely (Fig. 3, mixture).

Proteins rapidly transported by ganglion cells may constitute only a small fraction of all proteins synthesized by retinal neurons. Nonetheless, 2-D gel analysis of ^{35}S-methionine–labeled membrane proteins from whole RET revealed a prominent labeled species corresponding in position to axonally transported SuP in both cat (Fig. 2) and rat (Fig. 3), and this polypeptide was identified as SNAP-25 by immunoprecipitation (Figs. 2, 3). The prominence of SNAP-25/SuP among ^{35}S-methionine–labeled retinal proteins may reflect the fact that SNAP-25 found in RET is synthesized by retinal interneurons as well as by ganglion cells (Catsicas et al., 1991, 1992).

Fatty acylation of SNAP-25/SuP

Metabolic labeling with ^{3}H-palmitic acid was employed to examine fatty acylation of retinal proteins *in vivo*. In preliminary experiments, we observed that the complement of labeled polypeptides in the membrane fraction derived from adult rat RET was indistinguishable when ^{3}H-palmitic acid was injected intravitreally either with DMSO as vehicle or as an aqueous solution with BSA as carrier. However, incorporation of radioactivity into protein was greater with DMSO as vehicle. In addition, we examined the time course of incorporation of radioactivity and found a monotonic increase in the amount of label associated with protein over survival intervals of 4–18 hr without apparent change in either the complement of labeled polypeptides or the relative intensity of labeling of individual polypeptides, as revealed by fluorography of 1-D and 2-D gels. Accordingly, we routinely administered ^{3}H-palmitic acid with DMSO as vehicle and employed survival intervals of 18–20 hr. Monitoring of radioactivity extracted by chloroform:methanol

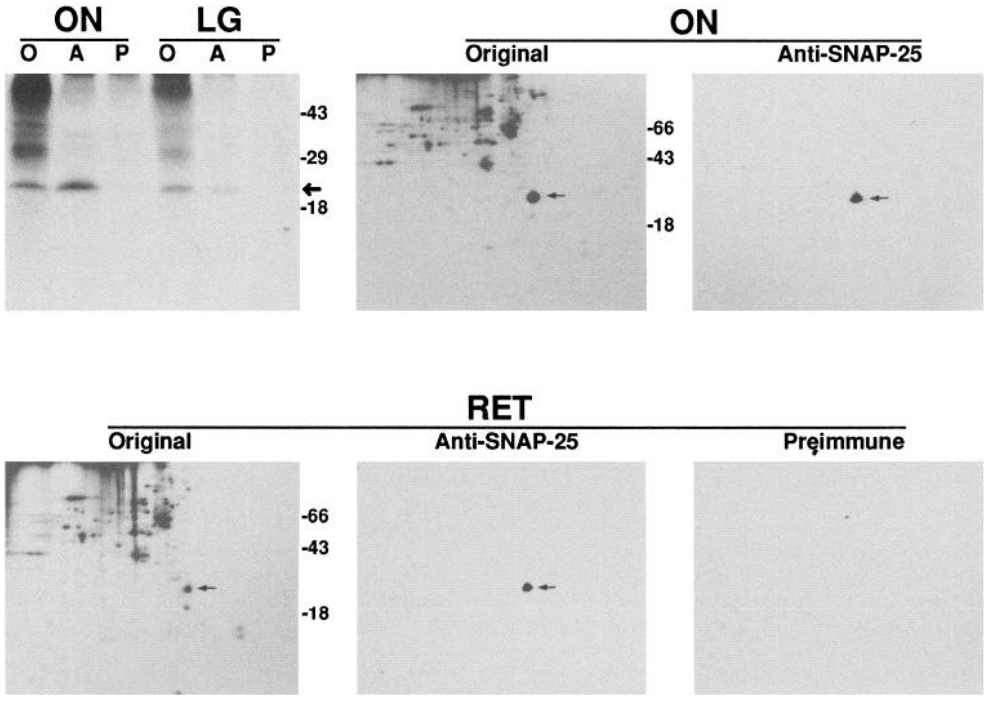

Figure 2. Identification of SuP as SNAP-25 in the cat. ^{35}S-methionine was administered intravitreally in an adult cat followed by a survival interval of 6.5 hr, and membrane fractions derived from RET, ON, and dorsal LGN (*LG*) were subjected to immunoprecipitation, 1-D or 2-D gel electrophoresis and fluorography. The *upper left panel* shows a fluorograph of a 1-D gel. An *arrow* indicates the position of SuP (M_r = 25 kDa) among the original complement of membrane proteins (*lanes O*), and immunoprecipitated by anti-SNAP-25 antiserum (*lanes A*) but not by preimmune serum (*lanes P*). In fluorographs of 2-D gels, *arrows* indicate the position of SuP (M_r = 25 kDa; pI = 4.4) among the original complement of membrane proteins, and immunoprecipitated by anti-SNAP-25 antiserum (but not by preimmune serum). In 2-D separations illustrated in this and subsequent figures, isoelectric focusing gels contained ampholines of pH 3.5–10 and of pH 4–6 in a 2:1 ratio, and the acidic end of the isoelectric focusing dimension is to the right.

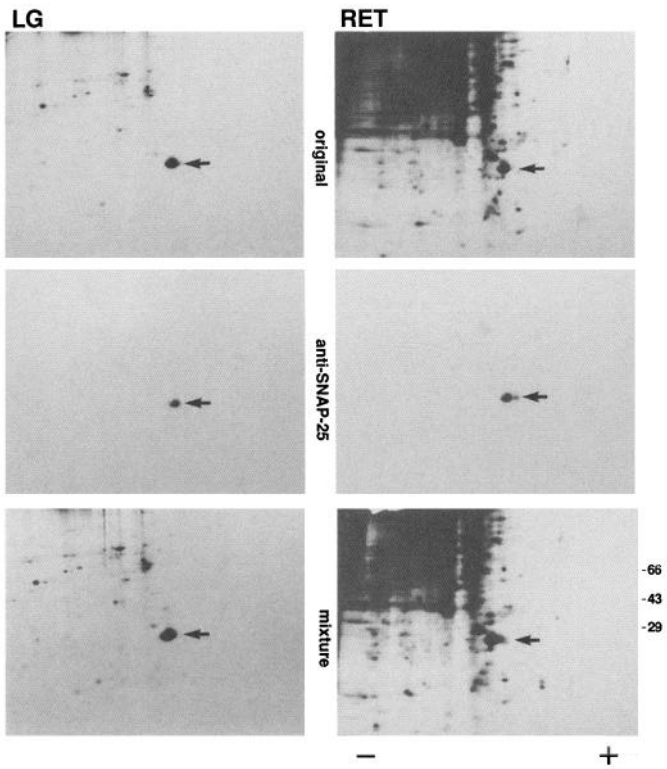

Figure 3. 2-D comigration of SuP and SNAP-25. ^{35}S-methionine was administered intravitreally in an adult rat followed by an 8 hr survival interval, and membrane fractions were derived from the LGN (*LG*) and RET. Eighty-two percent of each sample was subjected to immunoprecipitation with anti-SNAP-25 antiserum. For LG and RET, the remaining membrane sample and immunoprecipitate were analyzed as a triplet of 2-D gels, such that each gel contained either 12% of original sample, 50% of immunoprecipitate (derived from 41% of original sample), or a mixture of 6% of original sample and 50% of immunoprecipitate. Fluorographs of gels of immunoprecipitate and of the mixture of immunoprecipitate and total membrane protein were exposed to film for twice as long as fluorographs of gels of original membrane samples. Note that SuP and SNAP-25 (*arrows*) comigrate exactly (M_r = 25 kDa; pI = 4.4).

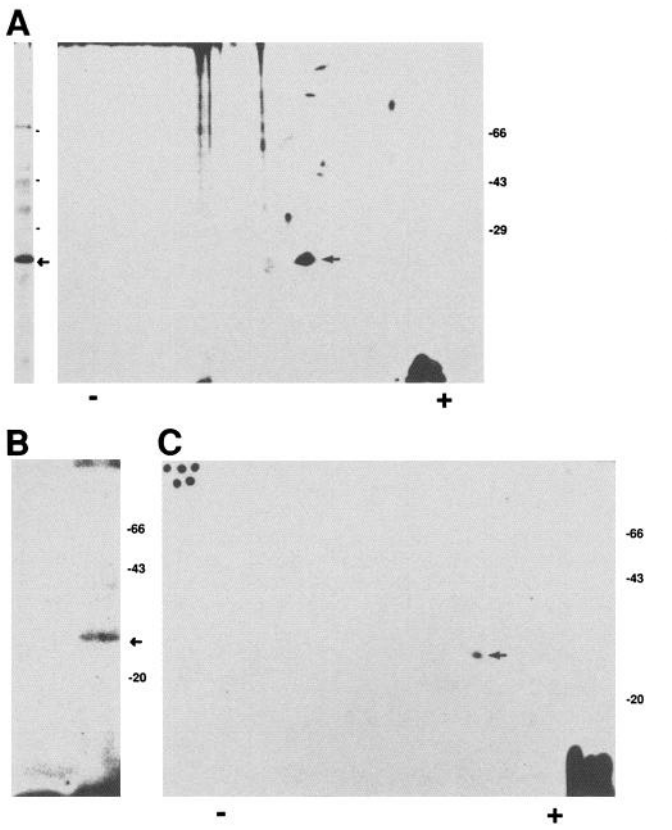

Figure 4. Labeling of SNAP-25/SuP by ^{3}H-palmitate. ^{3}H-palmitic acid was administered intravitreally in adult rats followed by 18–20 hr survival intervals. Fluorographs are shown in *A–C*. *A*, 1-D and 2-D separations of retinal membrane proteins. At *left*, the major labeled polypeptide following SDS-PAGE is indicated by an *arrow* (M_r = 25 kDa). At *right*, the position of SNAP-25/SuP following 2-D electrophoresis is indicated by an *arrow* (M_r = 25 kDa; pI = 4.4). *B*, Equal aliquots of retinal membrane protein were subjected to immunoprecipitation with anti-SNAP-25 antiserum or preimmune serum, followed by SDS-PAGE. The position of labeled SNAP-25 recognized by antiserum (*right lane*) but not by preimmune serum (*left lane*) is indicated by an *arrow*. *C*, 2-D separation of membrane proteins derived from the ON ipsilateral to intravitreal injection of ^{3}H-palmitic acid. The position of the major labeled polypeptide (M_r = 25 kDa; pI = 4.4) is indicated by an *arrow*.

and remaining in extracted samples indicated that approximately 0.1% of total radioactivity associated with the membrane fraction of RET was covalently linked to protein after survival intervals of 18–20 hr.

Following intravitreal administration of ^{3}H-palmitic acid, a single species with M_r of 25 kDa was found consistently to be most heavily labeled in fluorographs of 1-D gels of retinal membrane proteins (Fig. 4*A*). In 2-D electrophoresis, this 25 kDa species resolved predominantly as a single polypeptide of pI 4.4, with electrophoretic mobility indistinguishable from that of SNAP-25/SuP identified by labeling with ^{35}S-methionine (Fig. 4*A*). The ability of SNAP-25 to serve as a substrate for fatty acylation was confirmed by the finding that labeled SNAP-25 was precipitated from the membrane fraction of RET by anti-SNAP-25 antiserum (but not by preimmune serum) following intravitreal administration of ^{3}H-palmitic acid (Fig. 4*B*). In addition, following 2-D electrophoresis of membrane proteins derived from the ON ipsilateral to the eye injected with ^{3}H-palmitic acid (but not from the contralateral ON; data not shown), label was associated predominantly with a single polypeptide of electrophoretic mobility indistinguishable from that of the major labeled retinal polypeptide (Fig. 4*C*), demonstrating that at least some portion of SNAP-25/SuP fatty acylated within RGCs is committed to axonal transport.

Palmitic and other long-chain fatty acids are typically linked covalently to protein by thioester bonds with cysteine residues (Sefton and Buss, 1987; James and Olson, 1990), and the deduced primary structure of SNAP-25 contains a total of four, closely spaced cysteine residues (Oyler et al., 1989; Catsicas et al., 1991). To characterize the bond linking fatty acid to SNAP-25/SuP, we treated 2-D gels of ^{3}H-palmitic acid–labeled retinal membrane proteins with 1 M hydroxylamine at pH 7 to cleave labile ester bonds selectively (Kaufman et al., 1984). Label was removed almost quantitatively from SNAP-25/SuP by 1 M hydroxylamine at pH 7, but not by 1 M Tris at pH 7 (Fig. 5*A*). Hydroxylamine treatment also eliminated label from most other retinal polypeptides, including an acidic protein with M_r of approximately 43 kDa that was identified as GAP-43 (Fig. 5*A*). GAP-43 has been shown previously to incorporate palmitate through a hydroxylamine-sensitive thioester linkage (Skene and Virag, 1989).

Hydroxylamine treatment had little or no effect on label as-

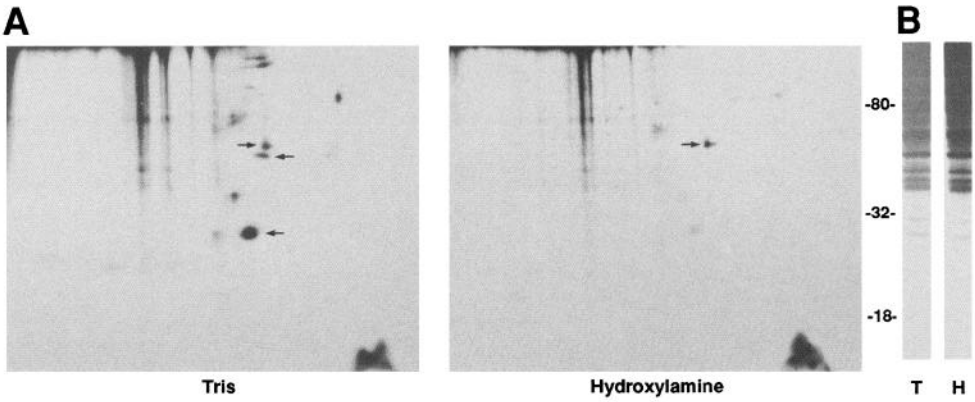

Figure 5. Hydroxylamine lability of the SNAP-25/SuP-palmityl bond. *A*, The membrane fraction derived from adult rat RET following *in vivo* labeling with ^{3}H-palmitic acid was subjected to 2-D electrophoresis, and identical gels were incubated for 4 hr in 1 M hydroxylamine, pH 7.0, or 1 M Tris, pH 7.0 prior to fluorography. Treatment with hydroxylamine (but not with Tris) removed label from SNAP-25/SuP (*lower arrow*) and from GAP-43 (M_r = 43 kDa; pI = 4.3; *middle arrow*). GAP-43 was identified by immunoprecipitation with monoclonal antibody 91E12 (Schreyer and Skene, 1991) followed by 2-D gel analysis (data not shown). Hydroxylamine treatment did not affect label associated with a polypeptide (*upper arrow*) shown previously to be myristoylated (see Results). *B*, An adult rat RET was labeled by intravitreal injection of 200 μCi of ^{35}S-methionine (18 hr survival). Membrane proteins were subjected to SDS-PAGE and individual lanes were treated for 4 hr with 1 M hydroxylamine (*H*), pH 7.0, or 1 M Tris (*T*), pH 7.0, prior to fluorography. Hydroxylamine had no effect on label associated with retinal polypeptides that had incorporated ^{35}S-methionine.

sociated with one retinal protein (indicated in Fig. 5*A*) that comigrates with a polypeptide found previously to be myristoylated (Skene and Virag, 1989; see their Fig. 7). Since myristoylation appears to occur exclusively through the formation of hydroxylamine-resistant amide bonds (Sefton and Buss; 1987; James and Olson, 1990), hydroxylamine-resistant labeling of this protein would be consistent with conversion of some ^{3}H-palmitic acid to shorter-chain fatty acid during *in vivo* labeling. However, the hydroxylamine lability of label associated with SNAP-25/SuP indicates that it is not myristoylated, as predicted by the absence of an NH_2-terminal glycine consensus for myristoylation in the deduced sequence of SNAP-25 (Oyler et al., 1989; Catsicas et al., 1991).

We also examined the possibility that label became associated with retinal polypeptides as a result of metabolic conversion of ^{3}H-palmitic acid to amino acids that were then incorporated into newly synthesized protein (Miller et al., 1987). Hydroxylamine treatment that removed ^{3}H label from acylated proteins had no effect on label associated with retinal polypeptides that had incorporated ^{35}S-methionine (Fig. 5*B*).

The identity of ^{3}H-labeled fatty acid linked to retinal polypeptides following *in vivo* administration of ^{3}H-palmitate was determined directly by reverse-phase thin-layer chromatography (Fig. 6). Organically extractable material liberated by alkaline methanolysis either from SNAP-25/SuP isolated by SDS-PAGE or from the entire complement of retinal membrane proteins was separated on KC-18 plates followed by fluorography. Label released either from isolated SNAP-25/SuP or from unfractionated retinal proteins comigrated predominantly with methyl palmitate (16:0). A small fraction of label comigrated with methyl stearate (18:0). Metabolic conversion of fatty acids by chain elongation is commonly observed in many cell types (Dhopheswarkar and Mead, 1973; Robert et al., 1983; Tabata et al., 1986), and it has been shown for other proteins that the enzymatic mechanisms subserving long-chain fatty acylation can employ fatty acids of chain lengths greater than 16 carbons (Berger and Schmidt, 1984; Bizzozero and Lees, 1986; James and Olson, 1990).

Discussion

We have shown that SuP, whose synthesis and transport is a conspicuous correlate of the transformation of axonal growth cones to synapses, is identical to SNAP-25, a highly conserved synaptic protein. In addition, we have demonstrated that SNAP-25/SuP is a major substrate for long-chain fatty acylation in the adult CNS.

Regulation of SNAP-25/SuP expression

The developmental time course of SNAP-25 expression has recently been described, and a number of studies have described changes in synthesis and transport of SuP during development and after axon injury. Establishment of identity between SNAP-25 and SuP makes it possible to concorporate these separate lines of evidence, to produce a clearer understanding of the regulation of expression of this protein.

In developing PNS and CNS, induction of SNAP-25 mRNA and protein corresponds with the onset of synaptogenesis (Oyler et al., 1989, 1991; Catsicas et al., 1991). This correspondence suggests a role for signals derived from axon–target interaction in regulating expression of SNAP-25/SuP. Consistent with this suggestion, the prominence of SuP in rapid axonal transport increases greatly in conjunction with target innervation and synapse elaboration by developing neurons (Skene and Willard, 1981b; Kalil and Skene, 1986; Moya et al., 1988; Simkowitz et al., 1989).

Experimental support for an influence of axon–target interaction on SNAP-25/SuP expression is provided by the finding that axotomy leads to a substantial reduction in transported SuP in the adult mammalian PNS (Bisby, 1980; Redshaw and Bisby, 1984a,b; Reh et al., 1987; Archer and McLean, 1988). Restoration of high levels of expression depends upon reinnervation by axons of their targets (Bisby, 1982; Redshaw and Bisby, 1985). However, even after axotomy, levels of SuP synthesis and transport remain substantially above those observed in developing neurons prior to synaptogenesis. Therefore, the importance of axon–target interaction may differ between initial

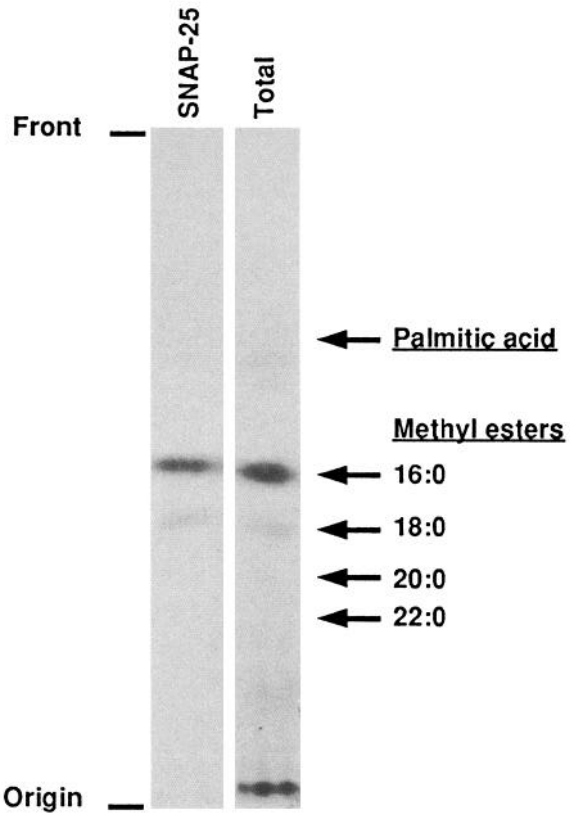

Figure 6. Identification of fatty acids bound to SNAP-25/SuP. The membrane fraction was prepared from adult rat RET following *in vivo* labeling with ^{3}H-palmitate and extracted with chloroform:methanol. Labeled fatty acids bound covalently to retinal proteins were released by alkaline methanolysis of unfractionated membrane proteins (*Total*) or of SNAP-25/SuP isolated by SDS-PAGE (*SNAP-25*). Released fatty acids were separated by reverse-phase thin-layer chromatography and visualized by fluorography. The origin of migration and the position of the solvent front are indicated at *left*. *Arrows at right* indicate the positions of palmitic acid, and of the methyl esters of palmitic (16:0), stearic (18:0), arachidic (20:0), and behenic (22:0) acids, separated in parallel thin-layer chromatography lanes. Fatty acids liberated from retinal proteins by alkaline methanolysis are expected to be converted to the corresponding methyl esters. Label released from SNAP-25/SuP comigrates predominantly with methyl palmitate (16:0), with a minor component corresponding to methyl stearate (18:0). An additional labeled component, released only from the unfractionated retinal pellet (*Total*), is visible as a prominent band near the origin of migration. This component may represent another lipid released from retinal proteins, or palmitate-containing lipid that was not removed fully in the original chloroform:methanol extraction.

induction and maintained expression of SNAP-25/SuP. Further, disconnection of adult mammalian CNS neurons from their targets does not result in downregulation of SuP, at least when axotomy occurs at a distance from the cell body (Skene and Willard, 1981b; Redshaw and Bisby, 1984b; Kalil and Skene, 1986; Reh et al., 1987). Thus, there may be significant differences between peripheral and central neurons in the signals that regulate expression of SNAP-25/SuP.

SNAP-25/SuP in mature neurons

The ubiquitous appearance of SNAP-25/SuP in rapid axonal transport in vertebrate neurons, and its highly conserved sequence among vertebrates and invertebrates (Oyler et al., 1989; Risinger et al., 1990; Catsicas et al., 1991) suggest that SNAP-25/SuP has an important functional role, exercised under strong structural constraints. The late appearance of SNAP-25/SuP in axonal development indicates that this role is carried out in axon terminals only after they have begun or completed the transition from growth cone to synapse. Because levels of expression differ significantly among neuronal populations in the adult CNS (Oyler et al., 1989), induction of SNAP-25/SuP appears to mark a transition that occurs in many, but not all, developing axon terminals.

In the adult CNS, SNAP-25/SuP is localized by immunohistochemistry largely to regions of neuropil (Oyler et al., 1989; Geddes et al., 1990), and metabolically labeled SuP accumulates in synaptic terminals (Kelly et al., 1980; Skene and Willard, 1981b; Simkowitz et al., 1989). However, labeled SuP persists at fairly constant levels in RGC axons for at least 33 hr following intraocular administration of ^{35}S-methionine (Fig. 1; Kelly et al., 1980; Levine and Willard, 1980; Skene and Willard, 1981c; Baitinger and Willard, 1987). One explanation for this persistence may be that some fraction of transported protein enters a relatively stationary axonal compartment, consistent with the immunohistochemical detection of SNAP-25 in some preterminal axons (Oyler et al., 1989; Geddes et al., 1990). Alternatively, newly synthesized SNAP-25/SuP may enter a pool within neuronal somata from which it is committed only gradually to axonal transport, as described for synapsin I (Baitinger and Willard, 1987). In either case, it is notable that the distribution of SNAP-25 within neurons can change with development (Oyler et al., 1991) and in response to activation of specific second messenger pathways (Sanna et al., 1991). Thus, the commitment of SNAP-25/SuP to axonal transport and/or its selective deposition may be subject to posttranslational regulation.

Long-chain fatty acylation of SNAP-25/SuP

Labeling with ^{3}H-palmitic acid reveals that SNAP-25/SuP is subject to modification by covalent addition of fatty acid. Indeed, in adult RET, SNAP-25/SuP is the most prominent substrate for palmitoylation *in vivo*. Our results also indicate that SNAP-25/SuP can incorporate ^{3}H-stearic acid (18:0) produced by metabolic conversion of labeled palmitate. Studies in other systems have shown that enzymes that palmitoylate proteins can also accept other long-chain fatty acids, but not myristate (14:0) or other shorter-chain fatty acids (Berger and Schmidt, 1984; Bizzozero and Lees, 1986; James and Olson, 1990). Thus, although palmitate—as the most abundant cellular fatty acid—is likely to be most frequently available as a substrate for protein acylation, the exact complement of fatty acids linked to SNAP-25/SuP *in situ* will depend on the local availability of individual long-chain fatty acids.

The bond linking long-chain fatty acid to SNAP-25/SuP is cleaved by hydroxylamine at neutral pH, which has proved to be diagnostic of a thioester linkage in other palmitoylated proteins (Kaufman et al., 1984; Magee et al., 1987; Skene and Virag, 1989; Pedraza et al., 1990). Therefore, our results indicate that palmitate is linked to SNAP-25/SuP by thioester bonds to some or all of its cysteine residues. SNAP-25 in mouse and chicken contains a total of four cysteines, organized as a cluster between positions 84 and 92 (Oyler et al., 1989; Catsicas et al., 1991). Clustered cysteines are conserved in similar positions within SNAP-25 in goldfish, *Torpedo*, and *Drosophila* (Risinger et al., 1990), indicating that these residues are important functionally. Recent analysis shows further that a 39 amino acid domain encompassing the clustered cysteine residues is the specific target of developmentally regulated alternative splicing in human

SNAP-25 (Bark and Wilson, 1991). Thus, the domain of SNAP-25/SuP that is the probable locus of posttranslational modification by long-chain fatty acid may have particular regulatory significance.

Functional implications of fatty acylation

The membrane affinity of a number of eukaryotic proteins is altered by posttranslational, covalent addition of one or more lipid moieties (Sefton and Buss, 1987; James and Olson, 1990). Long-chain fatty acylation in particular contributes to the membrane association of some members of the *ras* superfamily of small G-proteins (Willumsen et al., 1984; Magee et al., 1987; Hancock et al., 1989, 1990), and is required for membrane attachment by the neural-specific growth cone protein GAP-43 (Skene and Virag, 1989; Zuber et el., 1989; Liu et al., 1991).

The association of SNAP-25/SuP with the membrane compartment of axons and synapses is disrupted by detergents, but not by treatments known to release only peripheral proteins (Lorenz and Willard, 1978; Skene and Willard, 1981d; Oyler et al., 1989). The primary structure of SNAP-25/SuP, however, is unusually hydrophilic and lacks transmembrane or other strongly hydrophobic domains, although a moderately hydrophobic domain may emerge through formation of an amphiphilic helix at the amino terminus (Oyler et al., 1989). Covalent attachment of one or more long-chain fatty acid moieties to the clustered cysteine residues of SNAP-25/SuP would establish a strongly hydrophobic domain that could mediate or substantially enhance the interaction of this hydrophilic protein with neuronal membranes. In this respect, SNAP-25/SuP resembles GAP-43, which is predominantly hydrophilic but tightly membrane associated by virtue of palmitoylation (Skene and Virag, 1989; Zuber at al., 1989; Liu et al., 1991). If the hydrophobic domain created by fatty acylation anchors SNAP-25/SuP to the membrane, then more hydrophilic regions of the molecule would likely remain exposed to the cytoplasmic environment, facilitating interaction with cytoskeletal or other cytoplasmic elements.

SNAP-25/SuP en route to synaptic terminals, like other group I proteins, is conveyed in association with vesicular or other membranes (Lorenz and Willard, 1978; Grafstein and Forman, 1980). The appearance of labeled SNAP-25/SuP in the ON following intravitreal administration of ^{3}H-palmitic acid indicates that the protein is fatty acylated in ganglion cells prior to its commitment to axonal transport. Thus, fatty acylation might either directly mediate or enhance the association of SNAP-25/SuP with membranous structures targeted for rapid axonal transport. It has recently been shown that site-directed mutagenesis that eliminates palmitoylation of another group I protein, GAP-43, attenuates its delivery into growing neurites and prevents its localization to growth cones (Liu et al., 1991). Long-chain fatty acylation may play similar roles in modulating the commitment of SNAP-25/SuP to rapid axonal transport and its selective accumulation in synaptic terminals.

References

Archer DR, McLean GW (1988) Changes in fast axonally transported proteins in the regenerating rabbit vagus nerve. Neurosci Lett 87:151–156.

Baitinger C, Willard M (1987) Axonal transport of synapsin I-like proteins in rabbit retinal ganglion cells. J Neurosci 7:3732–3735.

Bark C, Wilson MC (1991) Alternative splicing generates a variant SNAP-25 protein during development. Soc Neurosci Abstr 17:531.

Benowitz LI, Lewis ER (1983) Increased transport of 44,000- to 49,000-dalton acidic proteins during regeneration of the goldfish optic nerve: a two-dimensional gel analysis. J Neurosci 3:2153–2163.

Berger M, Schmidt MFG (1984) Cell-free fatty acid acylation of Semliki Forest viral polypeptides with microsomal membranes from eukaryotic cells. J Biol Chem 259:7245–7252.

Bisby MA (1980) Changes in the composition of labelled protein transported in motor axons during their regeneration. J Neurobiol 11:345–360.

Bisby MA (1982) Prolonged alteration in composition of fast-transported proteins in axons prevented from regenerating after injury. J Neurobiol 13:377–381.

Bixby JL, Reichardt LF (1985) The expression and localization of synaptic vesicle antigens at neuromuscular junctions *in vitro*. J Neurosci 5:3070–3080.

Bizzozero OA, Lees MB (1986) Fatty acid acylation of brain myelin proteolipid protein *in vitro*: identification of the lipid donor. J Neurochem 46:630–636.

Buckley K, Kelly RB (1985) Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. J Cell Biol 100:1284–1294.

Catsicas S, Larhammar D, Blomqvist A, Sanna PP, Milne RJ, Wilson MC (1991) Expression of a conserved cell-type-specific protein in nerve terminals coincides with synaptogenesis. Proc Natl Acad Sci USA 88:785–789.

Catsicas S, Catsicas M, Keyser KT, Karten HJ, Wilson MC, Milner RJ (1992) Differential expression of the presynaptic protein SNAP-25 in mammalian retina. J Neurosci Res, in press.

Dhopheswarkar GA, Mead JF (1973) Uptake and transport of fatty acids into the brain and the role of the blood-brain barrier. Adv Lipid Res 11:109–142.

Doster SK, Lozano AM, Aguayo AJ, Willard MB (1991) Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. Neuron 6:635–647.

Estridge M, Bunge R (1978) Compositional analysis of growing axons from rat sympathetic neurons. J Cell Biol 79:138–155.

Fairbanks G, Steck TL, Wallach DFH (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606–2617.

Geddes JW, Hess EJ, Hart RA, Kesslak JP, Cotman CW, Wilson MC (1990) Lesions of hippocampal circuitry define synaptosomal-associated protein-25 as a novel presynaptic marker. Neuroscience 38: 515–525.

Grafstein B, Forman DS (1980) Intracellular transport in neurons. Physiol Rev 60:1167–1283.

Hancock JF, Magee AI, Childs JE, Marshall CJ (1989) All *ras* proteins are polyisoprenylated but only some are palmitoylated. Cell 57:1167–1177.

Hancock JF, Paterson H, Marshall CJ (1990) A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21*ras* to the plasma membrane. Cell 63:133–139.

Heuser D (1968) Thin layer chromatography of fatty acids on Silanised silica gel. J Chromatogr 33:62–69.

Jacobson RD, Virag I, Skene JHP (1986) A protein associated with axon growth, GAP–43, is widely distributed and developmentally regulated in rat CNS. J Neurosci 6:1843–1855.

James G, Olson EN (1990) Fatty acylated proteins as components of intracellular signaling pathways. Biochemistry 29:2623–2634.

Jen G, Thach RE (1982) Inhibition of host translation in encephalomyocarditis virus-infected L cells: a novel mechanism. J Virol 43: 250–261.

Kalil K, Skene JHP (1986) Elevated synthesis of an axonally transported protein correlates with axon outgrowth in normal and injured pyramidal tracts. J Neurosci 6:2563–2570.

Kaufman JF, Krangel MS, Strominger JL (1984) Cysteines in the transmembrane region of major histocompatibility complex antigens are fatty acylated via thioester bonds. J Biol Chem 259:7230–7238.

Kelly AM, Wagner JA, Kelly RB (1980) Properties of individual nerve terminal proteins identified by two-dimensional gel electrophoresis. Brain Res 185:192–197.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Laskey RA, Mills AD (1975) Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur J Biochem 56:335–341.

Levine J, Willard MB (1980) The composition and organization of

axonally transported proteins in the retinal ganglion cells of the guinea pig. Brain Res 194:137–154.

Liu Y, Chapman ER, Storm DR (1991) Targeting of neuromodulin (GAP-43) fusion proteins to growth cones in cultured rat embryonic neurons. Neuron 6:411–420.

Loewy A, Liu W-S, Baitinger C, Willard MB (1991) The major ^{35}S-methionine-labeled rapidly transported protein (superprotein) is identical to SNAP-25, a protein of synaptic terminals. J Neurosci 11: 3412–3421.

Lorenz T, Willard M (1978) Subcellular fractionation of intra-axonally transported polypeptides in the rabbit visual system. Proc Natl Acad Sci USA 75:505–509.

Magee AI, Gutierrez L, McKay IA, Marshall CJ, Hall A (1987) Dynamic fatty acylation of $p21^{N\text{-}ras}$. EMBO J 6:3353–3357.

Mason CA (1986) Axon development in mouse cerebellum: embryonic axon forms and expression of synapsin I. Neuroscience 19:1319–1333.

Miller JC, Gnaedinger JM, Rapoport SI (1987) Utilization of plasma fatty acid in rat brain: distribution of [^{14}C]palmitate between oxidative and synthetic pathways. J Neurochem 49:1501–1514.

Moya KL, Benowitz LI, Jhaveri S, Schneider GE (1988) Changes in rapidly transported proteins in developing hamster retinofugal axons. J Neurosci 8:4445–4454.

O'Farrell PH (1975) High-resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021.

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE, Wilson MC (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J Cell Biol 109:3039–3052.

Oyler GA, Polli JW, Wilson MC, Billingsley ML (1991) Developmental expression of the 25-kDa synaptosomal-associated protein (SNAP-25) in rat brain. Proc Natl Acad Sci USA 88:5247–5251.

Padilla SS, Morell P (1980) Axonal transport of ^{35}S-methionine-labeled proteins in two intra-brain tracts of the rat. J Neurochem 35: 436–443.

Padilla SS, Roger LJ, Toews AD, Goodrum JF, Morell P (1979) Comparison of proteins transported in different tracts of the central nervous system. Brain Res 176:407–411.

Pedraza L, Owens GC, Green LAD, Salzer JL (1990) The myelin-associated glycoproteins: membrane disposition, evidence of a novel disulfide linkage between immunoglobulin-like domains, and post-translational palmitylation. J Cell Biol 111:2651–2661.

Redshaw JD, Bisby MA (1984a) Proteins of fast axonal transport in the regenerating hypoglossal nerve of the rat. Can J Physiol Pharmacol 62:1387–1393.

Redshaw JD, Bisby MA (1984b) Fast axonal transport in central nervous system and peripheral nervous system axons following axotomy. J Neurobiol 15:109–118.

Redshaw JD, Bisby MA (1985) Comparison of the effects of sciatic nerve crush or resection on the proteins of fast axonal transport in rat dorsal root ganglion cell axons. Exp Neurol 88:437–446.

Reh TA, Redshaw JD, Bisby MA (1987) Axons of the pyramidal tract do not increase their transport of growth-associated proteins after axotomy. Mol Brain Res 2:1–6.

Risinger C, Blomqvist AG, Lundell I, Catsicas S, Wilson MC, Larhammar D (1990) Strong evolutionary conservation of SNAP-25 between *Drosophila*, goldfish, chicken and mouse. Soc Neurosci Abstr 16:355.

Robert J, Montaudon D, Hugues P (1983) Incorporation and metabolism of exogenous fatty acids by cultured normal and tumoral glial cells. Biochem Biophys Acta 752:383–395.

Sanna PP, Bloom FE, Wilson MC (1991) Dibutyryl-cAMP induces SNAP-25 translocation into the neurites of PC12 cells. Dev Brain Res 59:104–108.

Schreyer DJ, Skene JHP (1991) Fate of GAP-43 in ascending spinal axons of DRG neurons after peripheral nerve injury: delayed accumulation and correlation with regenerative potential. J Neurosci 11: 3738–3751.

Sefton BM, Buss JE (1987) The covalent modification of eukaryotic proteins with lipid. J Cell Biol 104:1449–1453.

Simkowitz P, Ellis L, Pfenninger KH (1989) Membrane proteins of the nerve growth cone and their developmental regulation. J Neurosci 9:1004–1017.

Skene JHP (1989) Axonal growth-associated proteins. Annu Rev Neurosci 12:127–156.

Skene JHP, Virag I (1989) Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. J Cell Biol 108:613–624.

Skene JHP, Willard M (1981a) Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. J Cell Biol 89:86–95.

Skene JHP, Willard M (1981b) Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. J Cell Biol 89:96–103.

Skene JHP, Willard M (1981c) Electrophoretic analysis of axonally transported proteins in toad retinal ganglion cells. J Neurochem 37: 79–87.

Skene JHP, Willard M (1981d) Characteristics of growth-associated polypeptides in regenerating toad retinal ganglion cell axons. J Neurosci 1:419–426.

Stone GC, Wilson PL, Hall ME (1978) Two-dimensional gel electrophoresis of proteins in rapid axoplasmic transport. Brain Res 144: 287–302.

Tabata H, Bell JM, Miller JC, Rapoport SI (1986) Incorporation of plasma palmitate into the brain of the rat during development. Dev Brain Res 29:1–8.

Willard M, Cowan WM, Vagelos PR (1974) The polypeptide composition of intra-axonally transported proteins: evidence for four transport velocities. Proc Natl Acad Sci USA 71:2183–2187.

Willumsen BM, Norris K, Papageorge AG, Hubbert NL, Lowy DR (1984) Harvey murine sarcoma virus p21 *ras* protein: biological and biochemical significance of the cysteine nearest the carboxy terminus. EMBO J 3:2581–2585.

Zuber MX, Strittmatter SM, Fishman MC (1989) A membrane-targeting signal in the amino terminus of the neuronal protein GAP-43. Nature 341:345–348.