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Organelles in Fast Axonal Transport

What Molecules Do They Carry in Anterograde vs Retrograde Directions, as Observed in Mammalian Systems?

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Abstract

The present minireview describes experiments carried out, in short-term crush-operated rat nerves, using immunofluorescence and cytofluorimetric scanning techniques to study endogenous substances in anterograde and retrograde fast axonal transport. Vesicle membrane components p38 (synaptophysin) and SV2 are accumulating on both sides of a crush, but a larger proportion of p38 (about 3/4) than of SV2 (about 1/2) is recycling toward the cell body, compared to the amount carried with anterograde transport. Matrix peptides, such as CGRP, ChrA, VIP, and DBH are recycling to a minor degree, although only 10–20% of surface-associated molecules, such as synapsins and kinesin, appear to recycle.

The described methodological approach to study the composition of organelles in fast axonal transport, anterograde as compared to retrograde, is shown to be useful for investigating neurobiological processes. We make use of the "in vivo chromatography" process that the fast axonal transport system constitutes. Only substances that are in some way either stored in, or associated with, transported organelles can be clearly observed to accumulate relative to the crush region.

Emphasis in this paper was given to the synapsins, because of diverging results published concerning the degree of affiliation with various neuronal organelles. Our previously published results have indicated that in the living axons the SYN I is affiliated with mainly anterogradely fast transported organelles. Therefore, some preliminary, previously unpublished results on the accumulations of the four different synapsins (SYN Ia, SYN Ib, SYN IIa, and SYN IIb), using antisera specific for each of the four members of the synapsin family, are described. It was found that SYN Ib clearly has a stronger affiliation to anterogradely transported organelles than SYN Ia, and that both SYN IIa and SYN IIb are bound to some degree to transported organelles.

Index Entries: Axonal transport; matrix peptides; CGRP; chromogranin A; VIP; synaptophysin; SV2; synapsin Ia and b; synapsin II a and b; kinesin; organelle association; immunofluorescence; cytofluorimetric scanning; confocal laser scanning.

Introduction

The occurrence of fast axonal transport in nerves was first suggested by Scott (1906), and based on circumstantial evidence, but was later demonstrated experimentally by Liliana Lubinska (1959). In her experiments the rapid accumulation of acetylcholine (ACh) esterase (AChE) proximal and distal to a sciatic nerve crush was visualized histochemically. Despite the prevailing attitude at that time, that the shift of material from the nerve cell body towards the periphery was a slow process, "a growth of the axoplasmic column," she emphasized a much more rapid process to explain her observations. Several observations in different types of mammalian nerves clearly showed the presence of a fast anterograde transport of axonal material (Dahlström and Fuxe, 1964; Lubinska, 1964; Dahlström and Häggendal, 1966; Ochs, 1966; Lasek, 1967,1968; Ochs et al., 1969). The retrograde transport was accepted some years later, when exogenous material, taken up at the nerve terminals, could be traced back to the cell body (Kristensson, 1970; Kristenson and Olsson, 1971; LaVail and LaVail, 1972). It is now generally assumed that there is a continous shuttle of membrane bounded organelles of various appearance from the cell body toward the terminals, and back to the soma (Tsukita and Ishikawa, 1980). This process is rapid in mammals, around several hundred mm/day, and is mechanistically different from the slow anterograde transport of formed cytoskeletal elements.

The general opinion of anterogradely transported material, initially synthesized in the gER, thereafter packed in the Golgi complex, loaded onto the transport track, and then transported distally in the axon, is an oversimplified view of the real events. The transported vesicles (1) are of many different species (small clear vesicles, large and small DCV, "sER tubules," mitochondria,

lysosomes, and so on); (2) have probably different tasks in the neuron; (3) are loaded with various substances; and (4) are destined for different, but probably specific, targets along the long neuronal processes. Organelles in retrograde transport are supposed to recycle worn material to the cell body, to carry molecules from the extracellular space around the nerve terminals (internalized via pinocytosis or receptor-mediated endocytosis) to the soma, and to mediate information from the distal parts of the neuron to the nucleus. The overall function of this traffic is to supply necessary material to the different processes of the neuron, and to carry trophic signals to and from the innervated cell (*see* Bisby, 1987).

This shuttle trafficking in the axon has recently been studied using video-enhanced interference contrast microscopy (developed and introduced by the late R.B. Allen at Woods Hole, cf. Allen et al., 1981). This technique enables the observation of even the small vesicles (40–50 nm) in the axon, and the number of individual particles passing a certain area in a particular axon can be recorded (*see* Snyder and Smith, 1992, this volume). A large number of investigations on protein transport, using labeled methionine or tritiated fucose (and other labeled precursors), administered to the cell body area, have been carried out. These investigations have shown that the organelle transport appears to continue for a comparatively long period of time, even if protein synthesis is inhibited and the anterograde transport of labeled macromolecules is severely depressed (Smith and Snyder, 1991). Therefore, there is no direct relationship between protein synthesis and transported organelles.

For a great number of years our group has been involved in the study of fast bidirectional axonal transport of endogenous material in the rat nervous system. The technique of "stop flow" or crushing, with the consequent piling up of material in transit proximal and distal to the stop, has been used. The crush interrupts the axon without interrupting the connective tissue sheaths. Material accumulates proximal or distal to the crush, and the rate of accumulation over time is a measure of the amounts of material in transit and the rate of transport. If clear accumulations of a particular substance can be observed during the first 3 h after crushing, and continues to accumulate during the following 6–8 h, this material must be in the fast moving phase in the living axon. The model can be looked upon as an "in vivo chromatography model," because only material rapidly transported in the living animal will show this pattern, whereas slowly moving components would not (cf. Dahlström and Bööj, 1988).

This paper will review previously obtained results concerning the difference in composition of anterogradely/retrogradely accumulated organelles, focusing on vesicle membrane components, matrix peptides, vesicle-associated synapsin I, and kinesin. In addition, some recent preliminary data on the accumulation pattern of the various synapsins (I a and b, II a and b) present in mammalian nerves will be described.

Material and Methods

Cytofluorimetric Scanning

The method for studying the accumulated material is immunofluorescence, which is potentially more sensitive than biochemistry and RIA. The reason for this claim is that presence of material in a tiny area of a large preparation can be observed as immunofluorescence in a microscope, but would be lost in the background noise in a homogenate. Amounts of immunoreactive material accumulated are recorded photographically and quantified using a fluorimetric method, applied on longitudinal sections of crush-operated nerves. This method, "cytofluorimetric scanning" (CFS), was developed some years ago (cf. Larsson et al., 1984,1986, Larsson, 1987, Dahlström et al., 1982,1987,1989). Using a motor-driven cross table, the longitudinally cut nerve sections, incubated with a variety of antibody preparations, are moved under the objective in an epifluorescence microscope (Fig. 1). The emitted fluorescence passes through a narrow insertion in the light

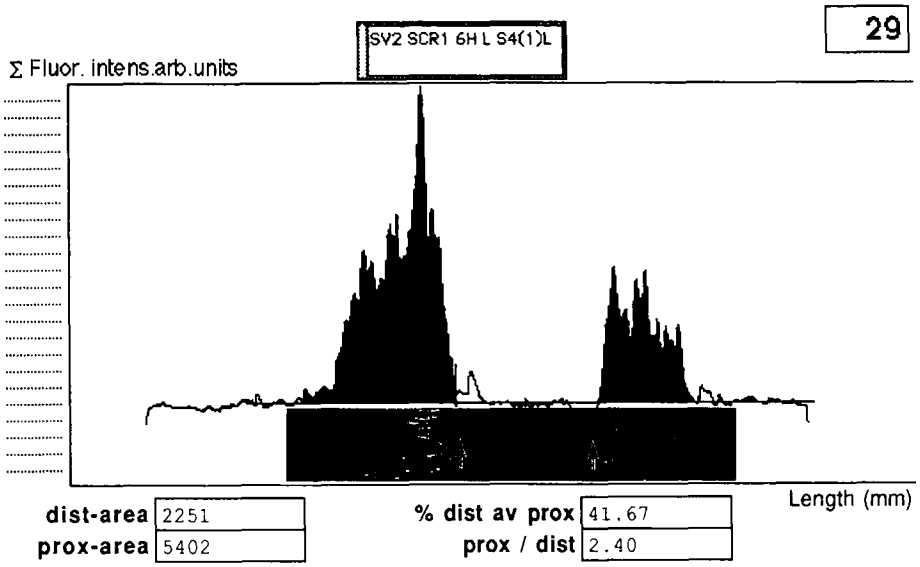


Fig. 1. Immunoincubated section of a rat sciatic nerve 6 h after double crushing (indicated by arrows). The section was incubated with antibody 10H recognizing the synaptic vesicle membrane marker SV2. The section was processed according to the cytofluorimetric scanning technique, where the section is passed under the fluorescence objective by a motor driven cross table in the microscope. The fluorescence escapes through a narrow slit inserted in the light path. The fluorescence is registered by a PM tube, and the signal fed into a MacIntosh II computer with the LabView program *(see* Dahlström et al., 1990). The black areas under the scanning curve are proportional to the immunofluorescence accumulated proximal and distal to the crush region, and are quantified by the software in pixel area and given in percent (%) of the proximal accumulation. The peaks of the upper scanning curve can be identified in the microscope photograph under the curve.

path, and is recorded with a PM tube. The signal is fed into a Macintosh II computer and translated into a curve, showing the intensity of emitted fluorescence along the length of the nerve section. The site of crush is obvious, and accumulations relative to the crush are indicated by the upward peaks above the basal line (background fluorescence). Using LabView software, an individually designed program digitally transforms the area under the peaks, which are relative to the fluorescence intensity, and to the relative amounts of accumulated fluorescent material (Dahlström et al., 1989). For this procedure a careful testing is carried out of appropriate concentrations of the various antisera to be used. Also, comparison between specimens must be done with care; consideration has to be given to section thickness, straightness of the section, experimental occasion, and so on *(see* discussions in Larsson, 1987; Dahlström et al., 1989). Only if the antigen can be obtained in pure form, and incorporated into gelatin models carrying different concentrations of antigen (standard curve), can accurate figures of antigen content be calculated (Larsson et al., 1986). In most cases a comparison against a 100% control specimen (Dahlström et al., 1989), or an evaluation of difference between anterogradely vs retrogradely accumulated material (Dahlström et al., 1991), is satisfactory.

The cytofluorimetric scanning technique has many advantages. Many different antisera can be tested on each nerve specimen, since 10–30 sections, 10 μm thick, can be collected from spinal roots or sciatic nerves of an adult rat. This means that the influence of biological variation can be minimized, and that the number of rats used in each experiment can be kept low. The average of 5–7 sections for each antibody is used as one observation, allowing 3–4 different antigens to be estimated per nerve specimen and for each rat.

The other advantage is that in addition to the scanning curve, the section is microphotographed, and, the quantitative information can be directly compared with the microscopic picture (Fig. 1).

Operative Procedures

Adult male Sprague-Dawley rats (170–200 g) were used. They were housed on a 12 h light/dark cycle with food and water *ad libitum*. Under deep ether anaesthesia the ventral roots or the sciatic nerves, bilaterally, were exposed. Spinal roots were crushed using a watchmaker's forceps (Bööj et al., 1989), while the sciatic nerves were double crushed (two crushes with 1–2 mm distance, *see* Dahlström et al., 1978) 1–12 h before sacrifice. The double crushes were performed in order to avoid "contamination" between anterogradely and retrogradely accumulated material. At sacrifice the rats were, under deep mebumal anaesthesia, exposed to transcardiac perfusion fixation with 4% paraformaldehyde (pH 7.4). The roots or sciatic nerves were dissected and postfixed for 4 h, before rinsing in PBS/10% sucrose. The nerve specimens were then frozen and sectioned longitudinally in a cryostat (10 µm sections). This procedure was carried out in accordance with the regulations of the Ethical Committee in Göteborg.

Immunofluorescence

The sections were placed on gelatin-coated glass slides, and incubated for indirect immunofluorescence using antisera to detect the following groups of antigens.

I: Vesicle Integral Membrane Antigens

(1) Rabbit-anti-p38, dil. 1:5,000, or mouse-anti-p38 dil. 1:100 (donated by P. Greengard, New York), recognizing p38, a 38 kD integral vesicle membrane protein (synaptophysin, Jahn et al., 1985; Navone et al., 1986), and (2) a mouse monoclonal antibody 10H, dil. 1:200, recognizing SV2, a 95 kD transmembrane glycoprotein (Buckley and Kelly, 1985; Pfeiffer and Kelly, 1985).

II: Matrix Peptides

(1) Rabbit anti-CGRP (AB08 or CA-08–220, Cambridge Res. Biochem, UK), and (2) rabbit–anti-chromogranin A (Chr-A), dil. 1:100, (donated by H. Winkler, Austria; Somogyi et al., 1984; Fischer-Colbrie et al., 1985; Lassman et al., 1986). These two peptides are present, in addition to other locations (e.g., Gibson et al., 1984; Ju et al., 1987), in mammalian motor axons (*see* e.g., Bööj, 1987; Bööj et al., 1989). For some colocalization experiments (3) anti-substance-P (SP) (donated by P. Keen, Bristol, UK; Harmar et al., 1981) was also used. In addition, (4) rabbit-anti-VIP (vasoactive intestinal peptide; donated by E. Theodorson, Stockholm, Sweden), dil. 1:100, and antisera against the two catecholamine synthesizing enzymes dopamine-β-hydroxylase (DBH) and tyrosine hydroxylase (TH), (5) goat-anti-DBH, dil. 1:200, and (6) rabbit-anti-TH (Goldstein, 1972; Goldstein et al., 1972), dil. 1:200, were used in some experiments to study sympathetic fibers in the sciatic nerve. (7) Sheep-anti-GAP-43 (donated by Larry Benowitz, Belmont, MA; USA), dil. 1:300, recognizing the growth-associated protein 43 (GAP-43; Skene et al., 1986), was also used, but it is not clear whether this protein is located in the matrix or adsorbed to the surface of transported organelles. However, the fact that this phosphoprotein is secreted to the surface of growth cones (Tetzlaff et al., 1989) may indicate that it is matrix-bound.

III: Surface Adsorbed Molecules

(1) Anti-SYN I, dil. 1:100, recognizing phospho- and dephospho-SYN I, the 85 kD endogenous substrate for Ca^{2+}/calmodulin-cAMP dependent protein kinases (Huttner et al., 1983; DeCamilli et al., 1983). SYN I is associated in nerve endings

with small synaptic vesicles (Navone et al., 1984; Nestler and Greengard, 1986). This antiserum, recognizing both Ia and Ib forms, was raised in rabbits (donated by P. Greengard, New York). In addition, (2) specific antisera recognizing SYN Ia (G306), dil. 1:200, (3) SYN Ib (G278), dil. 1:200, (4) SYN IIa (G281), dil. 1:800, and (5) SYN IIb (G211), dil. 1:200 (both phospho and dephospho forms; Südhof et al., 1989) were used in a set of preliminary studies to compare the pattern of each individual form of synapsin with the results obtained earlier, using the nondifferentiating anti-SYN I antiserum (*see* e.g., Dahlström and Bööj, 1989). (6) A mouse monoclonal anti-kinesin antibody, reacting with the heavy subunit of the ATPase, which is thought to be the anterograde transport motor, was donated by S. Brady and K. Pfister (Brady, 1990; Pfister et al., 1989). In immunoelectron microscopic studies (Hirokawa et al., 1989), kinesin has been demostrated to be located on the surface of vesicles as well as on microtubules.

After incubations with rabbit-polyclonal primary antisera, FITC-labeled goat–anti-rabbit IgG was used for the secondary incubation. In some experiments the secondary incubation was carried out with biotinylated secondary antisera (goat–anti-rabbit-IgG, horse–anti-mouse-IgG, or rabbit–anti-sheep-IgG), dil. 1:200 (Vector Lab. Burlingame, CA), followed by streptavidin labeled with FITC or Texas Red, dil. 1:200 (Amersham, Buckinghamshire, UK). Secondary incubations were carried out at room temperature for 1 h. All incubation solutions contained 0.1% Triton X-100. Double incubations were performed in some cases, but mainly consecutive sections, incubated with different antibodies, were studied. This was found to be of importance, since the emitted FITC fluorescence has a tail that may contaminate the Texas Red spectrum, and *vice versa*, and interfere with the cytofluorimetric registrations.

Approximately 20–30 sections were collected from the middle third of each sciatic nerve and placed in consecutive order on glass slides for incubation with the different antisera. One slide was always used for control incubation, omitting the primary antiserum. In these control sections, immunofluorescence was never observed. The antibody preparations used have been carefully characterized by the research groups that developed the antisera. Therefore, the antisera were considered to be specific for the peptides and vesicle markers studied. However, since it cannot be excluded that the antisera may recognize amino acid sequences present in unidentified peptides, the immunoreactivity observed is generally referred to as, e.g., CGRP-like immunoreactivity (LI).

The sections were first quickly examined in a fluorescence microscope with incident light (NIKON Microphot FXA), the basis of the scanning instrument, and scanned. After scanning, photography was performed using Kodak Tri-X Pan 400 ASA film. Specimens were then investigated and photographed in a confocal laser scanning instrument (BioRad, Lasersharp MRC 600). (For a technical description of this instrument, *see* e.g., Amos et al., 1987, and White et al., 1987.)

Results and Discussion

Vesicle Membrane Markers

The two antibodies recognizing integral membrane proteins, p38 and 10H (recognizing SV2, Buckley and Kelly, 1985), gave a similar picture in the fluorescence microscope. In sciatic nerves, as well as in ventral roots, immunoreactive material rapidly accumulated on both sides of the crush. Accumulations were already apparent 1 h after operation (Fig. 5G, Fig. 6E), and increased steadily for at least 12 h (Dahlström and Bööj, 1988; Bööj et al., 1989). This rapid appearance of retrogradely accumulating material is too quick to be explained by a crush-induced turnaround (Bisby and Bulger, 1977), but probably represents the arrest of material already in retrograde transport at the time of crushing. When cytofluorimetric scanning was applied, it was found that retrograde accumulations (recycling) of p38 amounted to approx 60–70% of the anterograde accumulations (*see* Fig. 2G). On the other hand, SV2 appeared to recycle to 40–60% (Dahlström

and Bööj, 1988; *see also* Fig. 2H). This is a constant finding, and although the results are based on the capacity of the antibody to recognize the antigen immunologically, and bearing in mind that recycling molecules may be modified as to be less immunoreactive, these results could indicate that there may be a somewhat different composition of the vesicle membranes in anterograde vs retrograde transport. Future experiments will include those where a turn-around is induced along the axon, and the ratio between SV2 and p38 will be measured. As indicated by the experiments of Smith and others (cf. Smith, 1987; Smith and Snyder, 1991,1992; this volume) turn-around and reversal of protein transport directions may be coupled to protease activites, and loss of proteins. It would therefore be of importance to study the effect of protease inhibition on integral membrane components in the recycling organelles.

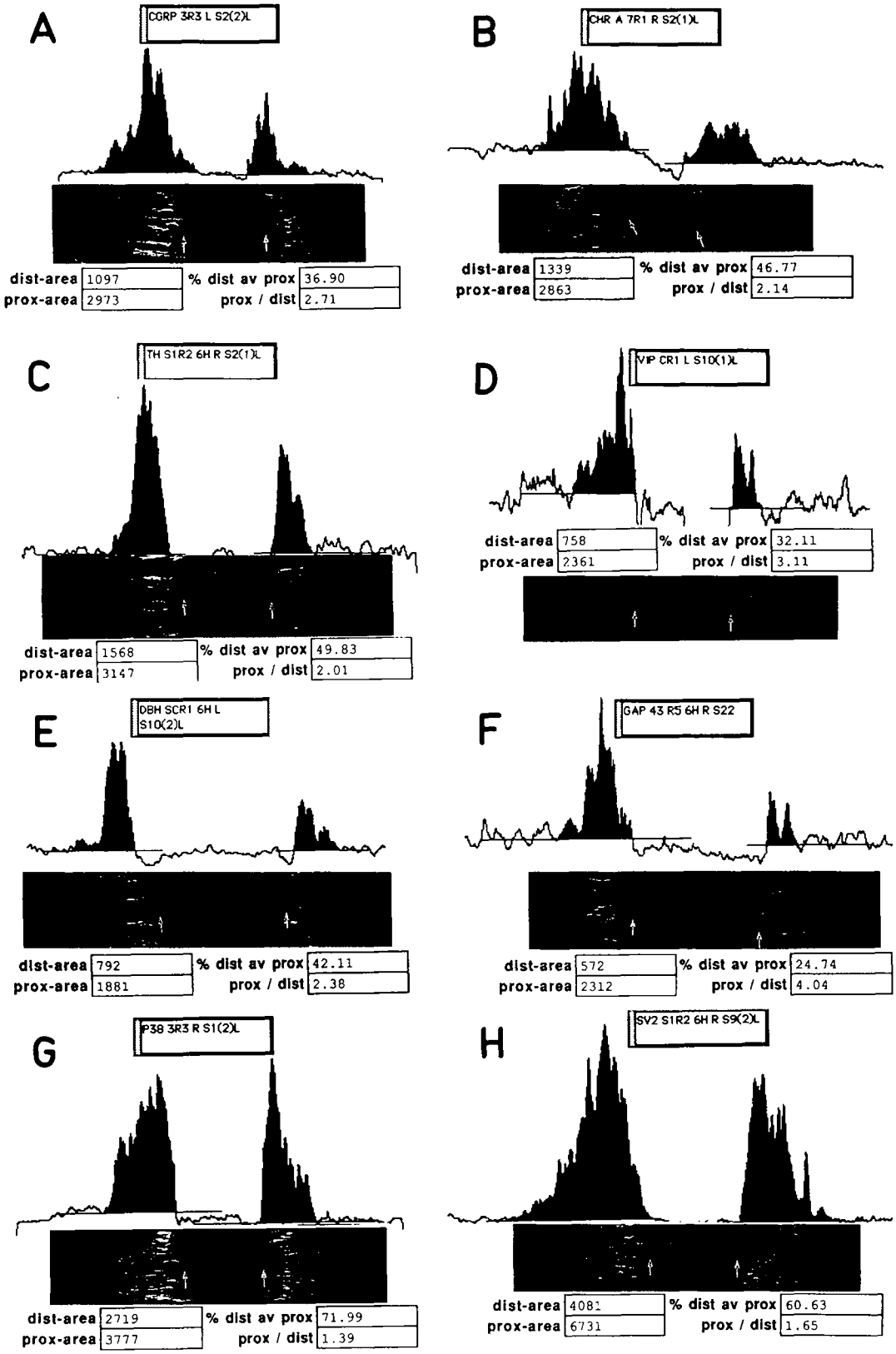
Matrix Peptides In Motor Axons

In the sciatic nerve the population of axons is mixed; motor, sympathetic, and sensory axons are bundled together. Sensory axons carry both substance P and CGRP (Lee at al., 1985; Ju et al., 1987), and these peptides in sensory axons clearly accumulate on both sides of a crush lesion (Bööj et al., 1989; *see* Fig. 2A). Sympathetic, unmyelinated axons contain Chr A (Neuman et al., 1984; Fischer-Colbrie et al., 1985), transported preferentially in a distal direction (Bööj et al., 1989; *see* Fig. 2B). Therefore, in order to study a comparatively pure population of axons, the axonal transport of CGRP and Chr A in ventral roots, which is composed of mainly motor axons, was also investigated. The results obtained indicate that there is a rapid anterograde transport of both CGRP and Chr A in motor axons, and that a very small fraction, around 5–8%, returns to the cell body, as judged from results obtained in ventral roots (Bööj et al., 1989). A larger proportion is observed in sciatic nerves, probably located, in this nerve, in sensory (CGRP) or sympathetic (Chr A) axons (Figs. 2A, 2B). These peptides can also be demonstrated in the motor endplates of the animals (for CGRP *see* e.g., Matteoli et al., 1990; for Chr A *see* Volknand et al., 1987) especially during postnatal development (Figs. 3A, 3B; *see also* Li et al., 1992). The peptides are probably of great importance in the proper development of endplate function. For example, CGRP has been demonstrated to influence cAMP levels in skeletal muscles (Takami et al., 1986), to stimulate adenylate cyclase (Kobayashi et al., 1987), and to regulate nicotinic ACh receptor clustering in the postsynaptic membrane (cf. New and Mudge, 1986; Fontaine et al., 1986; Laufer and Changeaux, 1987). Therefore, it is likely that most of the matrix peptides that have been shown to be present in the matrix of dense cored vesicles by immunoelectron microscopy, are secreted in the periphery, and therefore are not recycling.

In colocalization studies on accumulated axons, double labeling with one mouse monoclonal antibody and one rabbit-produced peptide antibody were performed, and the slides were studied using the confocal laser scanning instrument with dual channel registration. It has been observed that in the sciatic nerve some thin axons contained CGRP-LI in large granules, but no detectable SV2 (Fig. 4A). Other axons contained accumulations of both CGRP-LI and SV2, but in most cases the appearance of large CGRP-positive organelles was quite different from that of the very fine granular SV2-positive material (Fig. 4A). In some accumulated axons, the distribution of CGRP-LI and SV2 in some regions of the axon was very similar (Fig. 4B). The CGRP/SV2-positive axons may represent motor axons, since in the ventral root similar axon populations with both SV2 and CGRP were observed (eg. Bööj et al., 1989). Thin axons with large CGRP-positive granules but no detectable SV2, may be sensory, unmyelinated, axons.

Peptides and Enzymes in Sympathetic Axons and GAP-43

The results from a study on rat 6 h crushed sciatic nerves are shown in Fig. 2. Sections were incubated with antisera against CGRP, Chr A, TH and DBH, VIP, GAP 43 (the growth-associated


A
CGRP 3R3 L S2(2)L
dist-area 1097
% dist av prox 36.90
prox-area 2973
prox / dist 2.71
B
CHR A 7R1 R S2(1)L
dist-area 1339
% dist av prox 46.77
prox-area 2863
prox / dist 2.14
C
TH S1R2 6H R S2(1)L
dist-area 1568
% dist av prox 49.83
prox-area 3147
prox / dist 2.01
D
VIP CR1 L S10(1)L
dist-area 758
% dist av prox 32.11
prox-area 2361
prox / dist 3.11
E
DBH SCR1 6H L S10(2)L
dist-area 792
% dist av prox 42.11
prox-area 1881
prox / dist 2.38
F
GAP 43 R5 6H R S22
dist-area 572
% dist av prox 24.74
prox-area 2312
prox / dist 4.04
G
P38 3R3 R S1(2)L
dist-area 2719
% dist av prox 71.99
prox-area 3777
prox / dist 1.39
H
SV2 S1R2 6H R S9(2)L
dist-area 4081
% dist av prox 60.63
prox-area 6731
prox / dist 1.65

peptide), and for comparison with the above, antibodies directed against the vesicle membrane markers p38 and SV2 were used. All the antisera we used showed immunoreactivity toward the accumulated material on both sides of the crush. However, different fractions were observed in the retrograde accumulations. The most prominent "recycling" was demonstrated for p38 (72%), with SV2 as second highest (61%). TH-LI and ChrA-LI, both located in adrenergic axons, recycled to 50 and 47%, respectively. DBH showed a retrograde accumulation that was in this case 42% of proximal, while CGRP-LI, present in both motor and sensory axons, showed a "return" of 37%. The fraction found to accumulate the least distally was observed for GAP 43-LI, which probably is present in all types of axons in this nerve.

Surface Associated Antigens

I: The Synapsins

The first antiserum prepared against SYN I reacted with both forms a and b, and was demonstrated to be located on the surface of small synaptic vesides (DeCamilli et al., 1983; DeCamilli and Greengard, 1986). When used for axonal transport studies this antiserum recognized rapidly accumulating material proximal to the crush, but comparatively little was observed on the distal side (Bööj et al., 1986a,b). The contrast was striking when compared to the results after incubation with SV2 or p38; obviously the recycling organelles carried little SYN I-LI compared with organelles in anterograde transport (Dahlström et al., 1986,1987). Since a low background fluorescence was seen in the nerve sections, the results also suggested that the major fraction of SYN I in the axon was bound to organelles involved in fast axonal transport (Dahlström and Bööj, 1988). It was demonstrated that SYN I in the dephospho form had a higher affinity to vesicles than phosphorylated SYN I (cf. Schiebler et al., 1986). SYN I has serine residues in both the head and tail regions that are phosphorylated by cAMP and Ca^{2+}/-calmodulin dependent protein kinases (Nestler and Greengard, 1986). Such a phosphorylation reaction occurs in the synaptic region upon nerve activity. SYN I has been suggested to play a role in the movement of vesicles to the active sites in the presynaptic membrane, and thus play a regulatory role for transmitter release (DeCamilli et al., 1990). According to the results from Greengard's group, the phosphorylation process would imply that the association of SYN I to vesicles would be lost. It was previously suggested that this might explain the observation that recycling organelles would be comparatively poor in surface associated SYN I (Figs. 5E,5F).

However, later biochemical studies have indicated that in the mouse retinal ganglion, a major part of neuronal and axonal SYN I is axonally transported with the slow phase ScB (Batinger and Willard, 1987, Petrucci et al., 1991), and either soluble or associated with actin filaments and other constitutents of the cytoskeleton in the axon (cf. Petrucci and Morrow, 1987; Petrucci et al., 1991). This possible controversy to results in the sciatic nerve and motor axons, induced further studies concerning the axonal transport pattern of the synapsins, which is a family of four proteins, composed of SYN Ia, and Ib, and SYN IIa and IIb (Südhof et al., 1989; DeCamilli et al., 1990). Furthermore, SYN II, although lacking the phosphorylation site in the C-terminal tail region present in SYN I, has an N-terminal domain that binds to small synaptic vesicles (Thiel et al., 1990). Therefore, in a preliminary set of experiments, the patterns of anterograde and retrograde accumulations

Fig. 2. *(opposite page)* Montage of nerve sections and their respective scanning curves. The sections are from rat sciatic nerves that were double crushed 6 h before sacrifice. The sections were incubated with (A) anti-CGRP; (B) anti-Chr A; (C) anti-TH; (D) anti-VIP, (E) anti-DBH; (F) anti-GAP-43; (G) anti-p38; and (H) 10H recognizing SV2. The relative proportion of immunoreactive material in retrograde vs anterograde accumulations are shown in the upper right box of the scanning software print, shown below the microphotographs. The two crushes (double crushes to avoid contaminations between anterograde and retrograde accumulation measurements) are indicated by arrows.

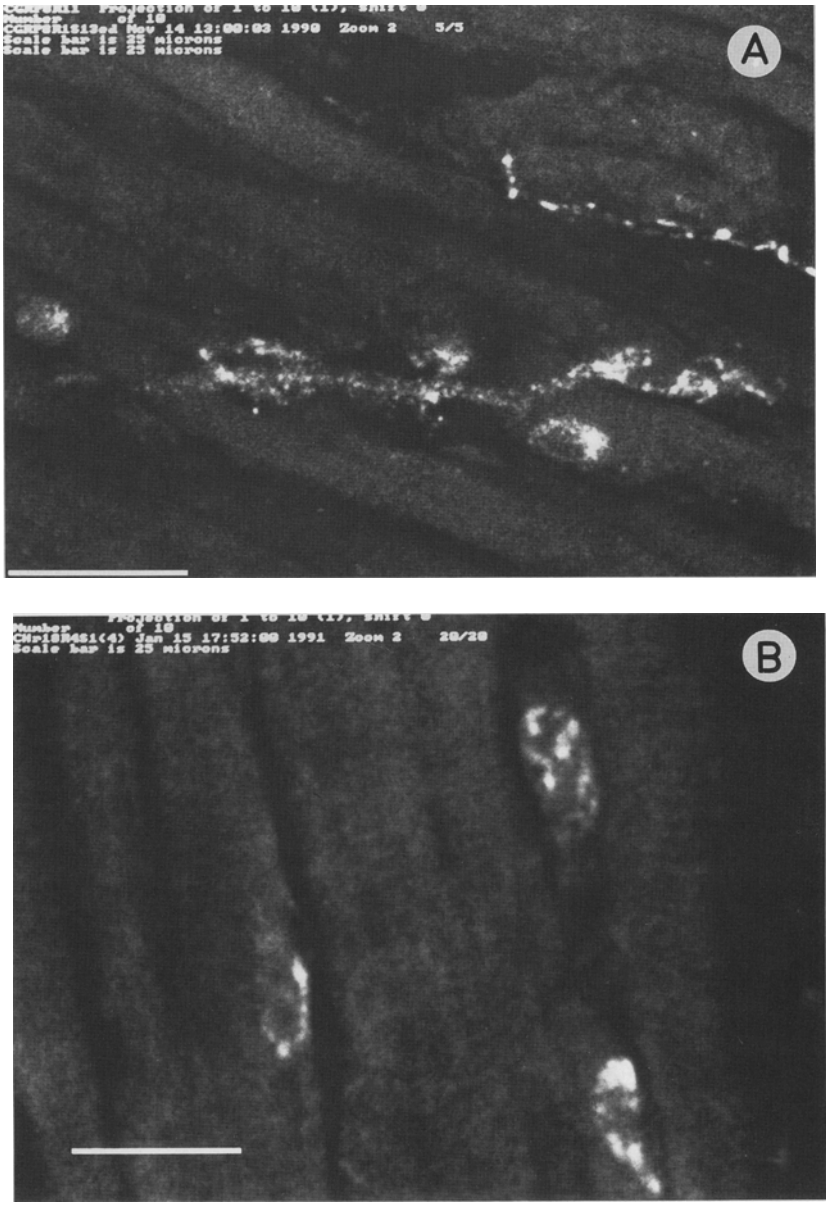


Fig. 3. Confocal laser scanning pictures of motor endplates in leg muscle of rat. (A) CGRP-LI in granular pattern in endplates and afferent motor axons of 8-d-old rat muscles. Scale bar is 25 μm. (B) Chr A-LI in motor endplates of 18-d-old rat muscles. Scale bar is 25 μm.

of the various SYN-family members in the rat sciatic nerve, using a number of different, isoform-specific anti-SYN antisera, were studied. Thus, anti-SYN Ia (G306), anti-SYN Ib (G278), anti-SYN IIa (G281), and anti-SYN IIb (G211) were tested in consecutive sections of crushed sciatic nerve, and compared with p38-antisera, to obtain information on the amounts of vesicle membrane-associated material accumulated in each individual nerve.

The results are shown in Figs. 5 and 6. SYN Ia-LI was very slow in accumulating; at 1 h after crushing no immunofluorescent accumulations

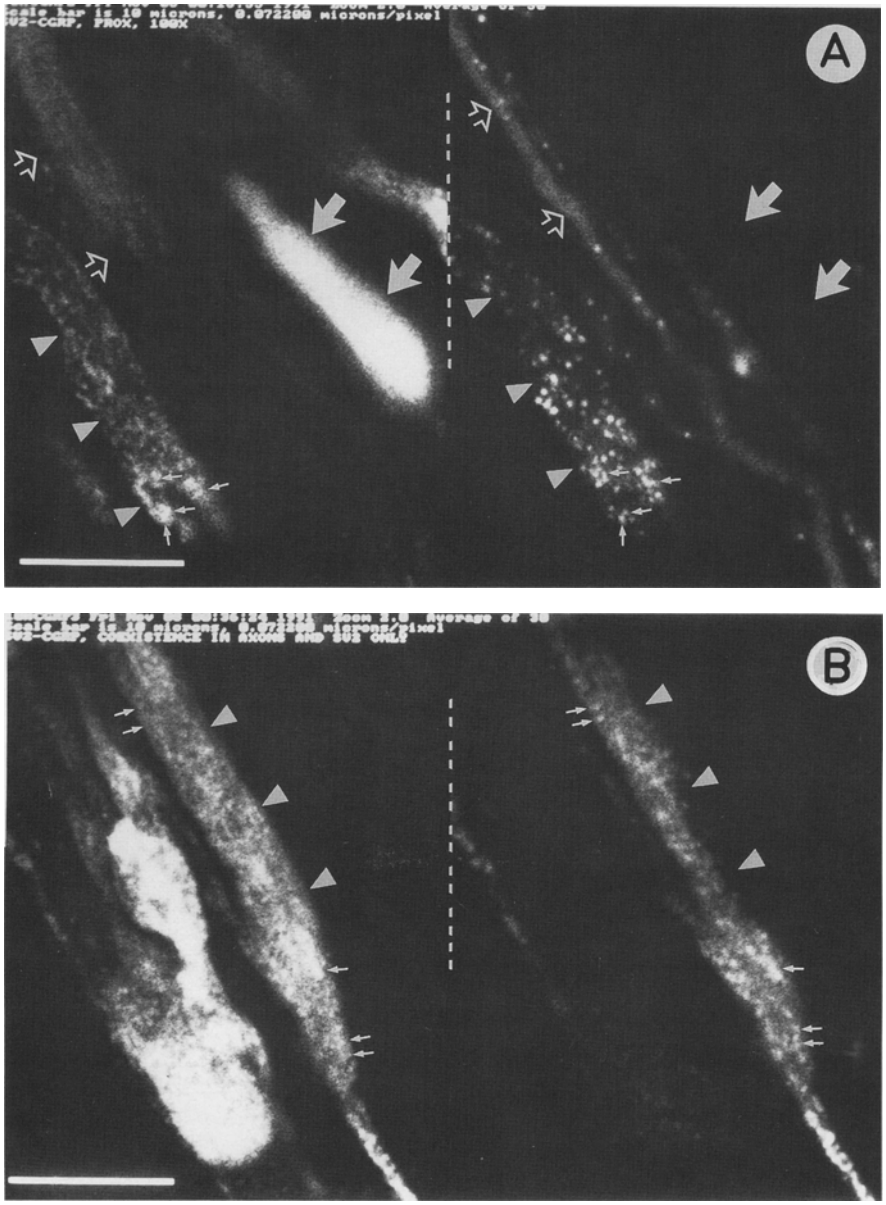
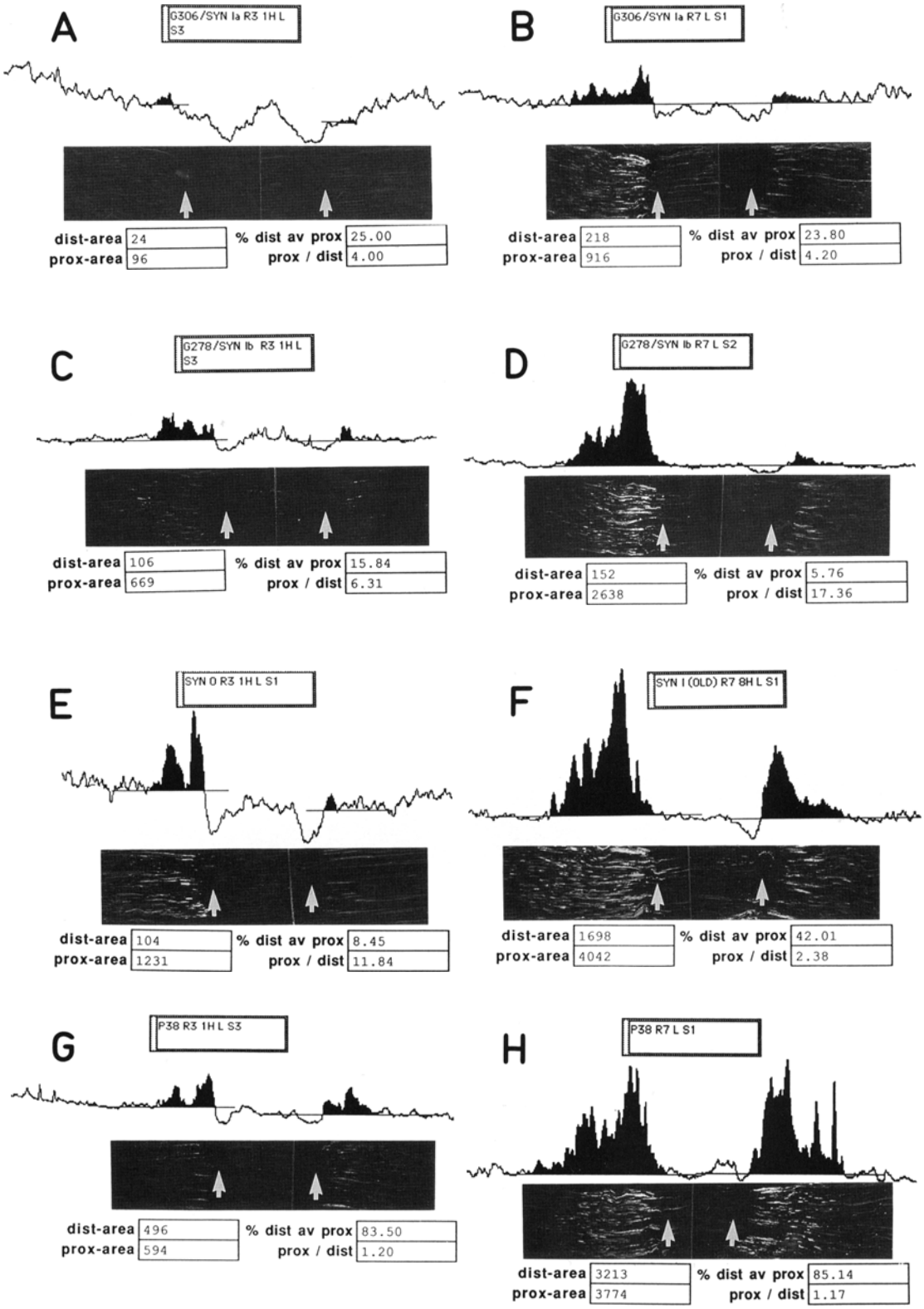


Fig. 4. Confocal laser scanning pictures of double incubated sciatic nerve sections. Dual channel scanning, with FITC-fluorescence representing SV2 in the left column, and Texas Red labeled CGRP-LI in the right lane. Dotted lines mark the borderline between the two channels. (A) A large myelinated axon with accumulated SV2 and CGRP-LI indicated by arrow head. In this axon the SV2 material is present in a finely granular pattern, while the CGRP-LI is localized in large flurescent granules (arrowheads). In most areas there appears to be no codistribution between the two antigens, but in some regions of this axon a codistribution is clearly indicated (small arrows). In diagonal from the upper left corner in the right lane is a thin unmyelinated axon with single CGRP-positive, large granules distributed along the axon. In the right lane no SV2-positive material can be observed (empty arrows). In the left lane a thick myelinated axon with accumulations of SV2, but without CGRP-LI (right lane) is indicated (large arrows). (B) In the left lane two axons with SV2 are seen to the low left; these axons have no detectable CGRP-LI. The indicated axon (arrow head) contains both SV2 and CGRP-LI (right lane). The CGRP-LI is located in large granules, and in some areas the codistribution with SV2 is apparent (arrows). Scale bars are 10 µm.


A
G306/SYN Ia R3 1H L S3
dist-area 24
prox-area 96
% dist av prox 25.00
prox / dist 4.00
B
G306/SYN Ia R7 L S1
dist-area 218
prox-area 916
% dist av prox 23.80
prox / dist 4.20
C
G278/SYN Ib R3 1H L S3
dist-area 106
prox-area 669
% dist av prox 15.84
prox / dist 6.31
D
G278/SYN Ib R7 L S2
dist-area 152
prox-area 2638
% dist av prox 5.76
prox / dist 17.36
E
SYN 0 R3 1H L S1
dist-area 104
prox-area 1231
% dist av prox 8.45
prox / dist 11.84
F
SYN I (OLD) R7 8H L S1
dist-area 1698
prox-area 4042
% dist av prox 42.01
prox / dist 2.38
G
P38 R3 1H L S3
dist-area 496
prox-area 594
% dist av prox 83.50
prox / dist 1.20
H
P38 R7 L S1
dist-area 3213
prox-area 3774
% dist av prox 85.14
prox / dist 1.17

were seen, neither proximal nor distal to the crush regions. On the other hand, SYN Ib-LI showed small but distinct accumulations, mainly on the proximal side. This was also the case for "total SYN I"-LI, using the "old" SYN-I(total) antibody. At 8 h, SYN Ib and "total SYN I" had accumulated markedly, giving the same picture proximally as p38, but distally p38 was more pronounced (85% of proximal., compared to 6 and 42% for SYN Ib and total SYN I, respectively). SYN Ia-LI showed rather weakly fluorescent proximal accumulations, with traces of immunofluorescence distally (Fig. 5B).

For SYN IIa-LI and SYN IIb-LI (Fig. 6) small but distinct accumulations were seen proximal to the crush at 1 h after crushing, and after 8 h the accumulations were evident also on the distal side (about 25% of proximal). There appeared to be more SYN IIa accumulating than SYN IIb. When compared with p38 the proportions of SYN IIa,b in the distal accumulations were much less for SYN II (25%) than for p38 (85%).

The accumulated amounts of immunoreactive SYN in rat sciatic nerve are compared in Fig. 7, where the relative increases with time after crushing are shown. Note that each antibody preparation can be related only to itself, since avidity and affinity may vary between the antisera. SYN Ib (Fig. 7A), SYN IIa and SYN IIb (Fig. 7B) showed a rapid accumulation proximal to the crush during the 8 h period studied. Distally, very little accumulated. The proximal accumulations were nearly parallel to the fast accumulations of p38, which were especially rapid during the first 3 h, and then appeared to level off. It is very clear that the distal accumulation of p38 is large, compared to the proximal amounts, and that the synapsins show comparatively little retrograde accumulation. The anterograde and retrograde accumulations after 8 h are indicated (Fig. 7C), in comparison with p38. SYN Ia, as judged from these results, appear to have a very poor affiliation with organelles in anterograde transport, whereas SYN Ib and SYN IIa seem to have a higher affiliation than SYN IIb. Also, it can be noted that SYN IIa may seem to have a somewhat higher affinity for retrogradely accumulating organelles than any of the other synapsin species.

The hypothesis presented earlier included the speculation that the degree of phosphorylation of the synapsins determined the degree of association with vesicle in transport. The present results offer no further insight with regard to this particular question. However, antisera that are differentiating between the different phosphorylation states of the synapsins have been developed, and it is hoped that these antisera can help to resolve this question.

II: Kinesin

The molecular motor for fast anterograde transport has been proposed to be the ATPase kinesin, which is a molecule built up from two heavy chains and two light subunits (e.g., Hirokawa et al., 1989). It was isolated from bovine brain (Brady, 1985; Vale et al., 1985; Kutznezov et al., 1988), and can support unilateral movement in an in vitro system of microtubules, vesicles (or latex beads), and ATP (Wagner et al., 1989). Movement starts at the moment the kinesin preparation is added. In freeze-etch EM studies, where antibodies to the heavy or light subunits were added, it was found that the antibodies decorated the sidearms that connected vesicles to tubules (Hirokawa et al., 1988). It was

Fig. 5. *(opposite page)* Montage of nerve sections and their respective scanning curves. Each column contains sections from one rat sciatic nerve, double crushed 1 h (left column) or 8 h (right column) before sacrifice. The sections were incubated with (A) and (B) antiserum G306 recognizing SYN Ia; (C) and (D) antiserum G278 recognizing SYN Ib; (E) and (F) antiserum reacting with "total SYN I" (the first anti-SYN I antiserum); and (G) and (H) anti-p38. The relative proportion of immunoreactive material in retrograde vs anterograde accumulations are shown in the upper right box of the scanning software print, shown below the microphotographs. The two crushes (double crushes to avoid contaminations between anterograde and retrograde accumulation measurements) are indicated by arrows.

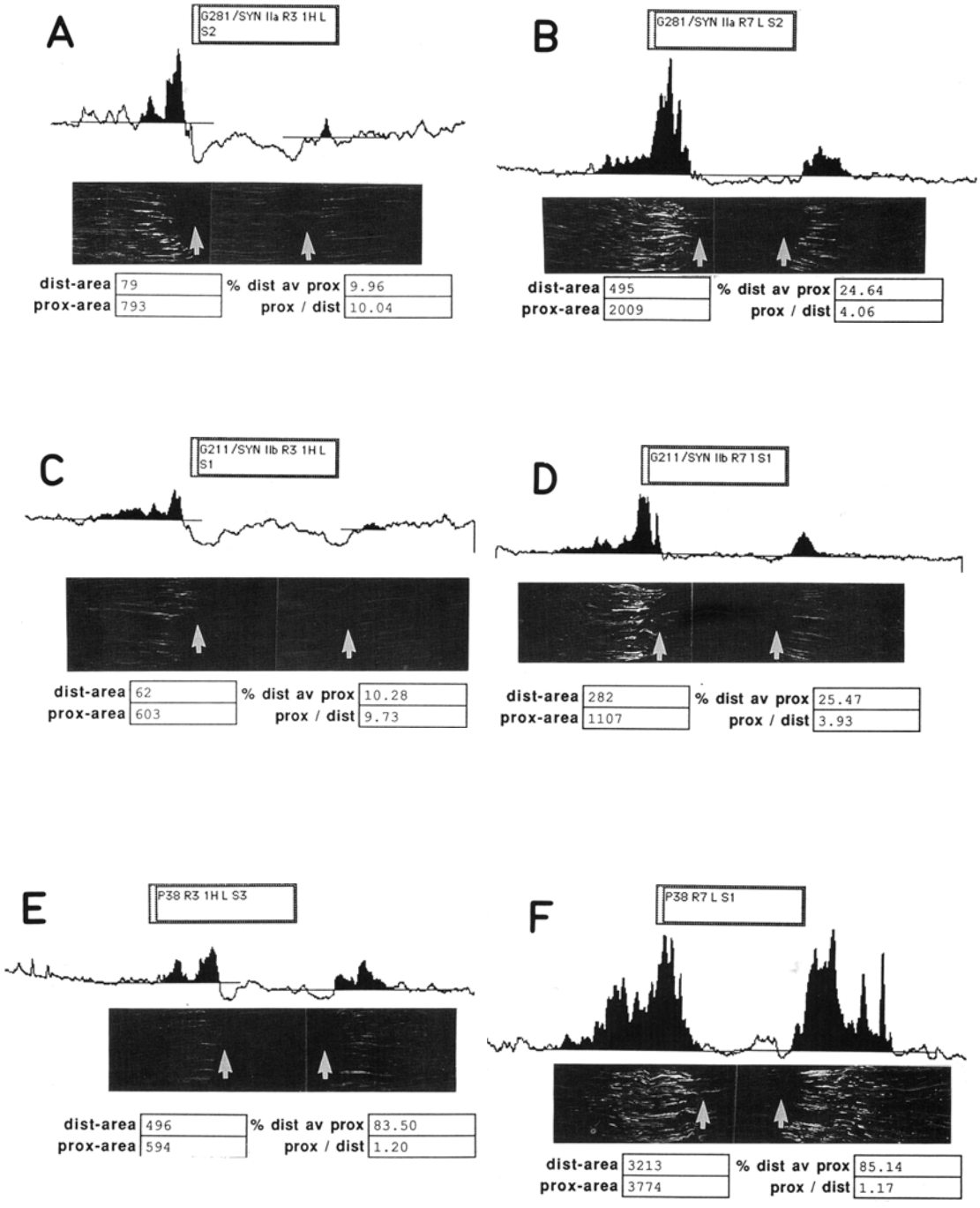


Fig. 6. Montage of nerve sections and their respective scanning curves. Each column contains sections from one rat sciatic nerve, double crushed 1 h (left column) or 8 h (right column) before sacrifice. The sections are from the same nerves as those shown in Fig. 5. The sections were incubated with (A) and (B) antiserum G281 recognizing SYN IIa; (C) and (D) antiserum G211 recognizing SYN IIb; and (E) and (F) anti-p38. The relative proportion of immunoreactive material in retrograde vs anterograde accumulations are shown in the upper right box of the scanning software print, shown below the microphotographs. The two crushes (double crushes to avoid contaminations between anterograde and retrograde accumulation measurements) are indicated by arrows.

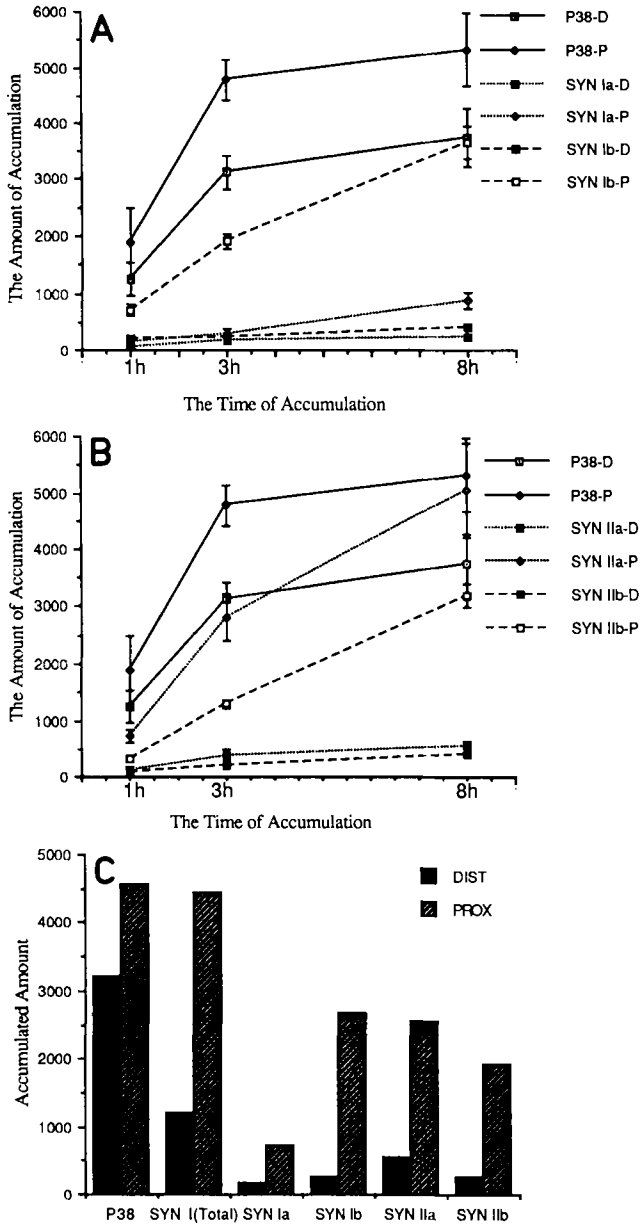


Fig. 7. (A) Time course of accumulations of SYN Ia-LI and SYN Ib-LI proximal and distal to a double crush performed 1–8 h before sacrifice. Also, the accumulation of p38 in consecutive sections is indicated. Key to the curves shown to the right, D = distal, and P = proximal accumulations. Means ± SEM of sections from four rats are shown. In each nerve four to five sections were incubated with each antibody, and the mean from these sections in each individual nerve was used to calculate the final mean. Antisera recognizing the SYN Ia and Ib, and p38 were used for incubating consecutive sections. It is clear that SYN Ib-LI is accumulating proximally more than SYN Ia-LI. Distally very little SYN Ia or SYN Ib has accumulated, in contrast to p38. (B) Time course of accumulations of SYN IIa-LI and SYN IIb-LI proximal and distal to a double crush performed 1–8 h before sacrifice. Also, the accumulation of p38 in consecutive sections is indicated. Key to the curves shown to the right, D = distal, and P = proximal accumulations. Means ± SEM of sections from four rats (four nerves) are shown. In each nerve four to five sections were incubated with each antibody, and the mean from these sections in each individual nerve was used to calculate the final mean. It is clear that SYN IIa-LI and SYN IIb-LI both have accumulated proximally, while distally very little SYN IIa or SYN IIb have accumulated, in contrast to p38. (C) The amounts os synapsin-LI material accumulated proximal (hatched bars) and distal (black bars) to the crush region, in comparison with p38 and "total SYN I." The relative proportions of immunoreactive material proximal and distal for each substance can be compared. The figure indicates that p38, total SYN I, SYN Ib-LI, and the SYN IIa and IIb are clearly associated with organelles in anterograde transport, while p38 has a marked association with both anterogradely and retrogradely transported organelles, in contrast to all the synapsins.

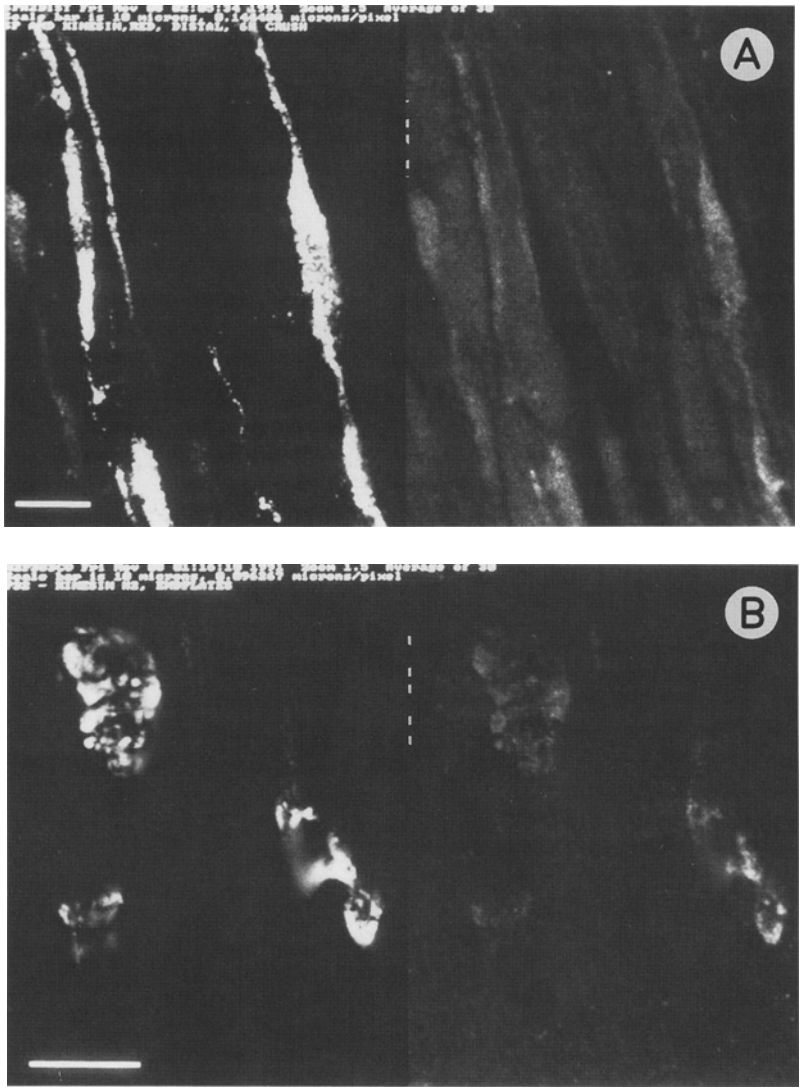


Fig. 8. (A) Confocal laser scanning picture (dual channel scanning) of accumulated sciatic nerve axons, after double incubations with antisubstance P (left lane) and antibody H2, recognizing the heavy subunit of kinesin. It is clear that the axons with SP-LI in large granules also contain obvious amounts of kinesin-LI. (B) Confocal laser scanning picture of hindlimb muscle from adult rat, double incubated with p38 (left lane) and anibody H2, recognizing the heavy subunit of kinesin. Although the kinesin-LI is weaker in fluorescence intensity, it is quite evident that kinesin-LI is present over groups of p38-containing organelles in the three motor endplates. Dotted white lines indicate the border between the two channels, registering FITC-fluorescence to the left and Texas Red fluorescence to the right. Scale bars are 10 μm.

also found that the light subunit anchored to the vesicle surface, while the heavy chain decorated the tubule surface. The first presented hypothesis for the interaction of kinesin with tubules and vesicles assumed that kinesin molecules were fixed to the tubules, and that vesicles interacted in a stepwise manner with the kinesin molecule. However, it was shown that in tissue cultured nonneuronal cells, kinesin-LI was clearly distributed in a punctate pattern, indicating association with rounded organelles, but no immunoreactivity was observed over tubular structures

(Pfister et al., 1989). A study on ligated sciatic nerve and crushed ventral root found that kinesin-LI accumulated rapidly proximal, but hardly at all distal to a crush (Dahlström et al., 1991). When compared with p38, the difference was dramatic; extremely little kinesin-LI accumulated distal to the crush (4–12%), whereas p38 accumulated to about 50–70% (*see* Dahlström et al., 1991). Kinesin-LI is also clearly colocalized both with p38-immunoreactive organelles (Dahlström et al., 1991) and with peptide-containing organelles in axonal transport, such as Substance P immunoreactive organelles (Fig. 8A).

There is also a clear association (colocalization) between kinesin-LI and p38 (synaptic vesicles) in the motor endplates (Fig. 8B). What specific events take place in the distal ends of neuronal processes, and which events detach, or degrade, kinesin before vesicles turn around for recycling are questions that arise. A number of suggestions should be tested, such as (1) local changes in pH or concentration of ions (Ca^{2+}), (2) digestion of "kinesin receptors," (3) conformational changes of existing "kinesin receptors" that result in lost affinity for kinesin, (4) protease digestion of kinesin outright, and (5) protease induced uncovering of preexisting, but hidden, binding sites for dynein, the proposed retrograde transport motor, (cf. Vale et al., 1985; Vallee and Shpetner, 1989).

Conclusion

The stop flow (crush) procedure, in combination with immunofluorescence and cytofluorimetric scanning, has been shown to be an important tool to investigate neurobiological events. As a consequence of the "in vivo chromatography" process, or the fast axonal transport machinery, many questions related to neuronal function in the living animal can now be suitably investigated. In this paper, differences in composition of anterogradely and retrogradely transported organelles, a matter of substantial importance for understanding the neurobiology of different neuronal populations, has been reviewed.

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