Molecular Neurobiology

ISSN0893-7648/92/6(2-3): 239-251/$2.60

Neuronal Compartments and Axonal Transport of Synapsin I

Paola Paggi [*,1] and Tamara C. Petrucci [2]

[1]Dipartimento di Biologia Cellulare e dello Sviluppo Università 'La Sapienza' P.le A. Moro 5, 00185 Rome, Italy; and [2]Laboratorio di Biologia Cellulare, Istituto Superiore di Sanità Viale Regina Elena 299, 00161 Rome, Italy

Contents

*Author to whom all correspondence and reprint requests should be addressed.

Abstract

Studies on the transport kinetics and the posttranslational modification of synapsin I in mouse retinal ganglion cells were performed to obtain an insight into the possible factors involved in forming the structural and functional differences between the axon and its terminals. Synapsin I, a neuronal phosphoprotein associated with small synaptic vesicles and cytoskeletal elements at the presynaptic terminals, is thought to be involved in modulating neurotransmitter release. The state of phosphorylation of synapsin I in vitro regulates its interaction with both synaptic vesicles and cytoskeletal components, including microtubules and microfilaments. Here we present the first evidence that in the mouse retinal ganglion cells most synapsin I is transported down the axon, together with the cytomatrix proteins, at the same rate as the slow component b of axonal transport, and is phosphorylated at both the head and tail regions. In addition, our data suggest that, after synapsin I has reached the nerve endings, the relative proportions of variously phosphorylated synapsin I molecules change, and that these changes lead to a decrease in the overall content of phosphorus. These results are consistent with the hypothesis that, in vivo, the phosphorylation of synapsin I along the axon prevents the formation of a dense network that could impair organelle movement. On the other hand, the dephosphorylation of synapsin I at the nerve endings may regulate the clustering of small synaptic vesicles and modulate neurotransmitter release by controlling the availability of small synaptic vesicles for exocytosis.

Index Entries: Neuronal compartments; posttranslational modification of synapsin I; slow axonal transport; synaptic vesicles; neuronal protein phosphorylation; mouse optic system; transport kinetics of synapsin I.

Introduction

Axons and their terminals are functionally and structurally very different, and the cytoskeleton provides the framework for their architecture. Pulse-labeling experiments indicate that cytoskeletal proteins synthesized in the neuronal cell body are metabolically stable during their passage through the axon, and essentially all of them reach the nerve endings (Hoffman and Lasek, 1975; Lasek and Black, 1977). It has been shown that most of the material anterogradely transported moves in three discrete groups that differ in both rate of transport and composition (Grafstein and Forman, 1980). The fast component (moving at 250–400 mm/d) primarily contains glycoproteins, membranes, vesicles, and other material related to the release of transmitters (Grafstein and Forman, 1980). The slow component comprises two major types: slow component a (SCa) and slow component b (SCb) (Lasek and Brady, 1982). SCa (moving at 0.2–1 mm/d) consists primarily of cytoskeletal proteins, including tubulin and the neurofilament proteins (Lasek et al., 1984). SCb (moving at 2–4 mm/d) is highly complex, being composed of microfilament-associated proteins, such as actin, the myosin-like M-2 protein, clathrin, and many of the metabolic enzymes, such as creatine phosphokinase, nerve-specific enolase, and calmodulin (Lasek et al., 1984; Paggi et al., 1990). Studies of the transport kinetics of pulse-labeled proteins have shown that the residence time for some cytomatrix proteins conveyed with SCb is much longer in the terminal region than in the axon shaft and that the residence times of proteins in the terminal regions also varies (Paggi and Lasek, 1987; Paggi et al., 1990). This suggests that, through their different effects on the residence time of proteins, the removal mechanisms operating at the nerve endings critically determine their structure and function, thus distinguishing them from the axon shaft. Moreover, studies on transport kinetics in axons and their terminals can be useful in determining not only the residence time of a protein, but also its structural and functional association with other proteins and, eventually, its posttranslational modifications after reaching the terminals.

Therefore, to obtain an insight into the factors that may control the association of proteins during their journey from the neuronal cell body, through the axon to the terminals with the various organelles present in the axon and its terminals, we decided to study the transport kinetics of synapsin I in mouse retinal ganglion cells. These studies call for an experimental model system that allows ready access to both axons and their terminals, such as the well-characterized mouse optic system. Synapsin I is particularly well suited to studies of this type: It appears to play a central role in the linkage of small synaptic vesicles to cytoskeletal elements at the nerve endings, where it contributes significantly to the mechanisms involved in modulating neurotransmitter release (De Camilli and Greengard, 1986). Synapsin I is also a major substrate for several protein kinases and undergoes multisite phosphorylation. One site in the head region, near the H_2-terminus, is phosphorylated by cAMP-dependent protein kinase and by calcium/calmodulin-dependent kinase I (CaM I), whereas two sites in the tail region are selectively phosphorylated by calcium/calmodulin-dependent kinase II (CaM II) (Huttner et al., 1981; Kennedy and Greengard, 1981).

In vitro studies have shown that the phosphorylation of synapsin I controls its interaction with several cytoskeletal proteins, including microtubules and microfilaments (Baines and Bennett, 1985; Bahler and Greengard, 1987; Petrucci and Morrow, 1987) and its association with small synaptic vesicles (Schiebler et al., 1986). Several indications suggest that the phosphorylation of synapsin I at the nerve terminals controls the binding of synaptic vesicles to actin filaments and the availability of small synaptic vesicles for exocytosis. Phosphorylation-associated translocation of synapsin I from synaptic vesicles/cytoskeleton to the cytosol compartment has been observed in response to synaptosomal depolarization (Sihra et al., 1989). The injection of dephosphorylated synapsin I has been shown to decrease neurotransmitter release at the squid giant synapse (Llinas et al., 1985) and to inhibit organelle movement along microtubules within the squid axoplasm (McGuinness et al., 1989). The present article reviews recent results obtained in our studies on the transport kinetics and the levels of phosphorylation of synapsin I during axonal transport and after reaching the nerve endings in the mouse optic system.

Materials and Methods

Radiolabeling of Retinal Ganglion Cell Proteins, and Quantitation of the Radioactivity of Transported Synapsin I

The detailed procedures have previously been fully described (Petrucci et al., 1991). Briefly, the retinal ganglion cells were labeled with either 0.19 mCi of ^{35}S-methionine or 100 µCi of ^{32}P-*ortho*-phosphate (SA > 800 mCi/m*M* and > 8500 Ci/mmol, respectively; New England Nuclear/Du Pont, de Hemann, Deutschland, GmbH) injected into the vitreous humor of the right eye of anesthetized male 6–8-wk-old C57Bl/6j mice. The mice were subsequently anesthetized and decapitated at intervals ranging from 1 h to 62 d after labeling. The following segments were collected from the dissected optic system (Fig. 1):

1. A 2-mm segment of the right optic nerve (N);
2. A 2-mm segment of the left optic tract (T); and
3. The left superior colliculus (SC).

The optic nerve (N) and tract (T) segments contain the axons of the retinal ganglion cells, and the superior colliculus segment (SC) contains these axons and their terminals.

Corresponding segments from 10 mice, sacrificed at each postinjection interval, were pooled, homogenized, and analyzed by two-dimensional (2-D) nonequilibrium pH/SDS-polyacrylamide gel electrophoresis (NEPHGE/SDS-PAGE) performed as described by O'Farrell (1975) using the standard range of ampholine (pH 3–7 and 5–7; LKB Instruments, Uppsala, Sweden) and 8%

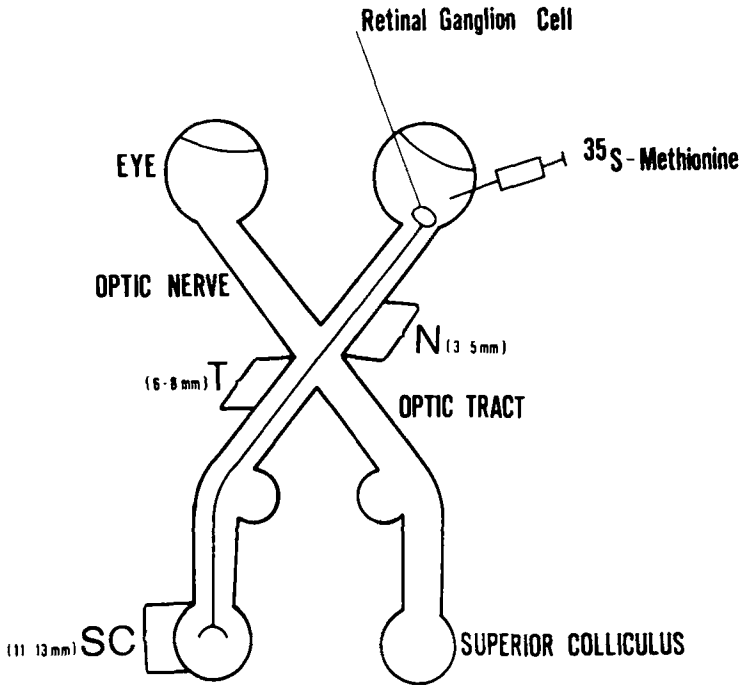

Fig 1. A schematic representation of the mouse optic system showing the retinal ganglion cell body axon and terminals. Segments N and T contain only axons, and extend 3–5 and 6–8 mm, respectively, from the posterior surface of the eyeball; the SC segment contains axons and their terminals, and is located 11–13 mm from the posterior surface of the eyeball.

acrylamide slab gels (Laemmli, 1970). The molecular-mass standards used were (expressed as kDa): β-galactosidase (116), phosphorylase b (97), bovine serum albumin (66), ovalbumin (45), and carbonic anhydrase (29). The radioactive polypeptides were visualized by fluorography according to the method of Bonner and Laskey (1974) and Laskey and Mills (1975) using XR-5 X-ray film (Kodak) stored at –70°C.

As previously described (Paggi et al., 1989), in order to deal with the unavoidable variability in the amount of labeled amino acid incorporated into the proteins of the retinal ganglion cells of different animals, we introduced an *a priori* correction. This *a priori* correction (Fisher, 1966) obviates any requirement for further normalization of the results and permits a direct comparison of data from 2-D NEPHGE/SDS-PAGE analysis. Regions of 2-D gel corresponding to synapsin Ia and Ib were excised using both the fluorograph to locate the position of the labeled polypeptides on gel and the Coomassie blue-stained spots of purified synapsin I. Gel slices were solubilized, and their radioactivity was counted (Paggi et al., 1989). Counts were corrected for quenching and were converted to disintegrations/min (DPM). Radioactivity values at each postinjection interval were corrected for ^{35}S-methionine decay. Data for individual segments (N, T, SC) were plotted as a function of the interval between labeling and collection of the segments for analysis. Synapsin I-associated radioactivity was also determined by densitometry of 2-D fluorographs (LKB, Ultroscan II, Uppsala, Sweden).

Determination of the Pulse Transient

The pulse transient (Paggi and Lasek, 1987; Paggi et al., 1989,1990) is a mathematical summary of the transport curve through an axonal

nerve segment: It is the area underneath the curve of radioactivity as a function of time. It measures all the radioactivity associated with a specific protein that has entered and cleared the axonal segment during the postlabeling period of observation. As previously reported (Paggi et al., 1989,1990), to compare the pulse transient of the optic nerve segment with the optic tract and superior colliculus segments, values for the optic tract and superior colliculus were corrected taking into account the percentages of retinal ganglion cell axons uncrossed at the optic chiasm and of axons projecting to the lateral geniculate nucleus rather than to the superior colliculus.

Cysteine-Specific Cleavage of Axonally Transported Proteins by S-Cyanylation

Segments from the left and right optic nerves and optic tracts of 15 mice, injected in both eyes with 100 μCi of ^{32}P-phosphate (SA > 8500 Ci/mmol; DuPont, de Hemann, Deutschland, GmbH) were dissected 2 d after the injection. Purified bovine synapsin I (1 mg) (Petrucci and Morrow, 1987) was added to 1 mL homogenate sample, dialyzed against 7.5*M* guanidine-HCl, 25 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.5 buffer, and cleaved with 2 m*M* 2-nitro-5-thiocyanobenzoic acid (NTCB) (Sigma, St. Louis, MO) at 37°C for 24 h (Petrucci and Morrow, 1987). The resulting peptide mixture was dialyzed in 10 m*M* Tris-HCl, 1 m*M* EDTA, 0.1 m*M* β-mercaptoethanol, pH 8.5 buffer, lyophilized, and analyzed by 2-D NEPHGE/SDS-PAGE. The labeled peptides were visualized as indicated above.

Results

Transport Kinetics of Synapsin I in Axons and Their Terminals

Figure 2 shows the transport kinetics curves of ^{35}S-methionine-labeled synapsin I in three different retinal ganglion cell axon segments: The optic nerve (N) and optic tract segments (T) contain axons; the superior colliculus segment (SC) contains axons and their terminals. The amounts of radioactivity present in the synapsin Ia and Ib spots were quantified by liquid scintillation spectroscopy and/or densitometry, and the results were plotted as a function of postlabeling time. A representative fluorograph of ^{35}S-methionine-labeled proteins separated by 2-D NEPHGE/SDS-PAGE from SC 14 d after labeling is shown in the insert (Fig. 2).

Synapsin I transport curves in N, T, and SC segments, like those of other SCb proteins (Paggi et al., 1989,1990), are asymmetrical and unimodal. Labeled synapsin I entered N, T, and SC segments between 3–6 h, 6–24 h, and 1–3 h, respectively (Fig. 2). The radioactivity reached a peak at 2 d in N and T, and at 7 d in SC. The peak was much broader in the optic tract than in the optic nerve. In fact, at 4 d, the radioactivity in N and T was about 10 and 68% of the respective maximum peaks. This suggests that the actual maximum should occur later in the tract (between 2 and 4 d) than in the nerve (about 2 d). Therefore, from the position of the observed peak in the three segments, we can estimate a peak rate of transport of 1.5–3 mm/d. This rate of transport indicates that synapsin I is associated with the SCb complex of proteins (Paggi et al., 1989,1990). The N and T curves reached background level at 7 d postinjection: This indicates that no radiolabeled synapsin I is permanently deposited within the axon segments. On the contrary, the SC curve, as already observed for other SCb proteins, had not yet reached the background level of radioactivity after 62 d. These observations indicate different residence times for synapsin I in N and in T, which contain only the retinal ganglion cell axons, and in SC, which contains axons and their terminals.

To quantify the differences within the amplitudes of the transport curves for N, T, and SC, we calculated the pulse transients. The pulse transient represents the area under the segment-analysis transport curve, and is a measure of the

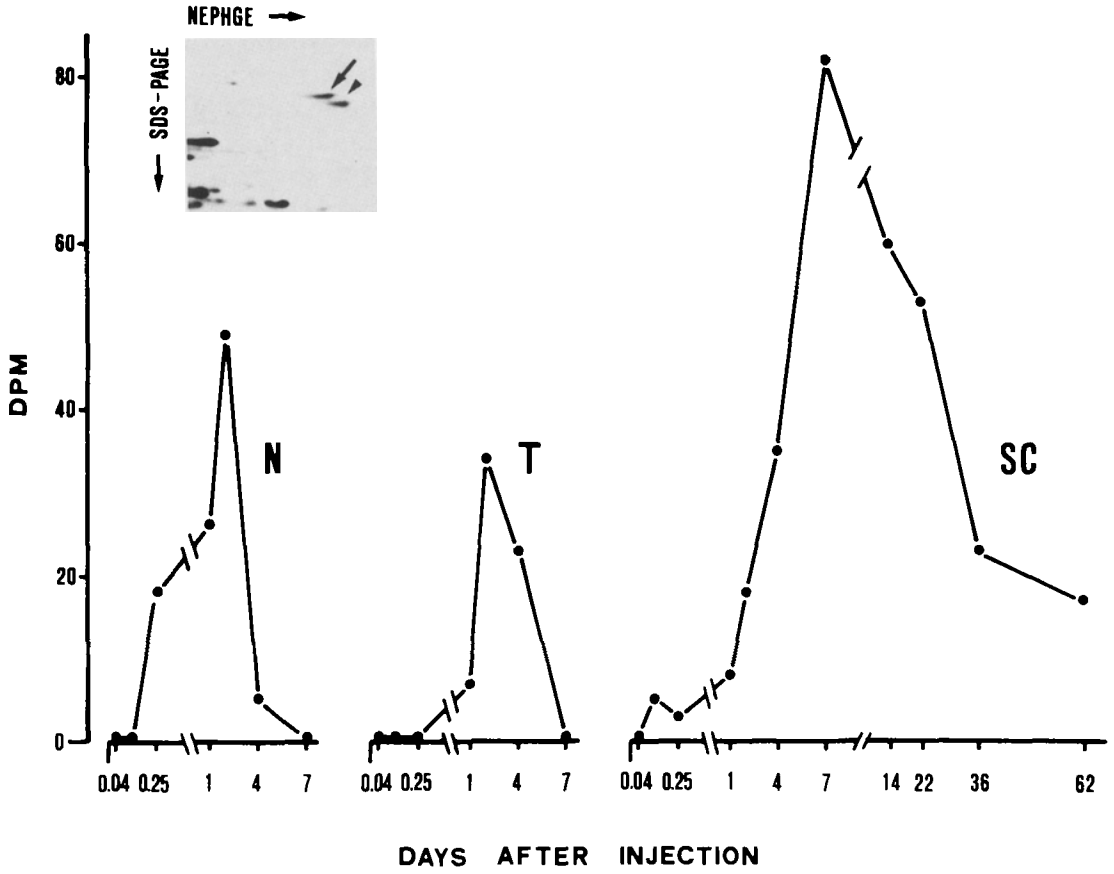

Fig. 2. Axonal transport kinetics of ^{35}S-methionine synapsin I after labeling of retinal ganglion cells. Fluorographs (*see* insert) were used to remove the gel regions containing synapsin I. Data from 2 mm of the optic nerve segment (N), the optic tract segment (T), and the superior colliculus (SC) are plotted as a function of the time interval between injection of the radioactive precursor and collection of the segments for analysis. Data points are the average of two or three observations, and each observation is a pool of 10 mice killed at 1, 3, and 6 h and 1, 2, 4, 7, 14, 22, 36, and 62 d for a total of more than 350 animals. The insert shows a section of a representative fluorograph showing labeled polypeptides from the SC separated by 2-D NEPHGE/SDS-PAGE 14 d after labeling the retinal ganglion cells. Spots corresponding to synapsin Ia (arrow) and Ib (arrowhead) are indicated. (Modified from Petrucci et al., 1991.)

radioactive synapsin I that entered and cleared the axonal segments N, T, and SC during the postlabeling period of observation. The pulse transient was 961 and 899 DPM/d for N and T, respectively, and 23,075 DPM/d for SC. Both the longer residence time and the higher pulse transient in SC agree with the detection of a higher amount of synapsin I in the terminal regions than in the axons by the immunogold labeling technique (De Camilli et al., 1983) and with the prevalently functional role suggested for synapsin I at nerve endings.

Phosphorylation of Axonally Transported Synapsin I and Identification of the Phosphorylated Sites

The phosphorylation of synapsin I has been shown to regulate the interaction of protein molecules with actin filaments (Bahler and Greengard, 1987; Petrucci and Morrow, 1987). Because synapsin I is transported with the slow component b, which comprises cytomatrix proteins, including actin, we investigated the phosphory-

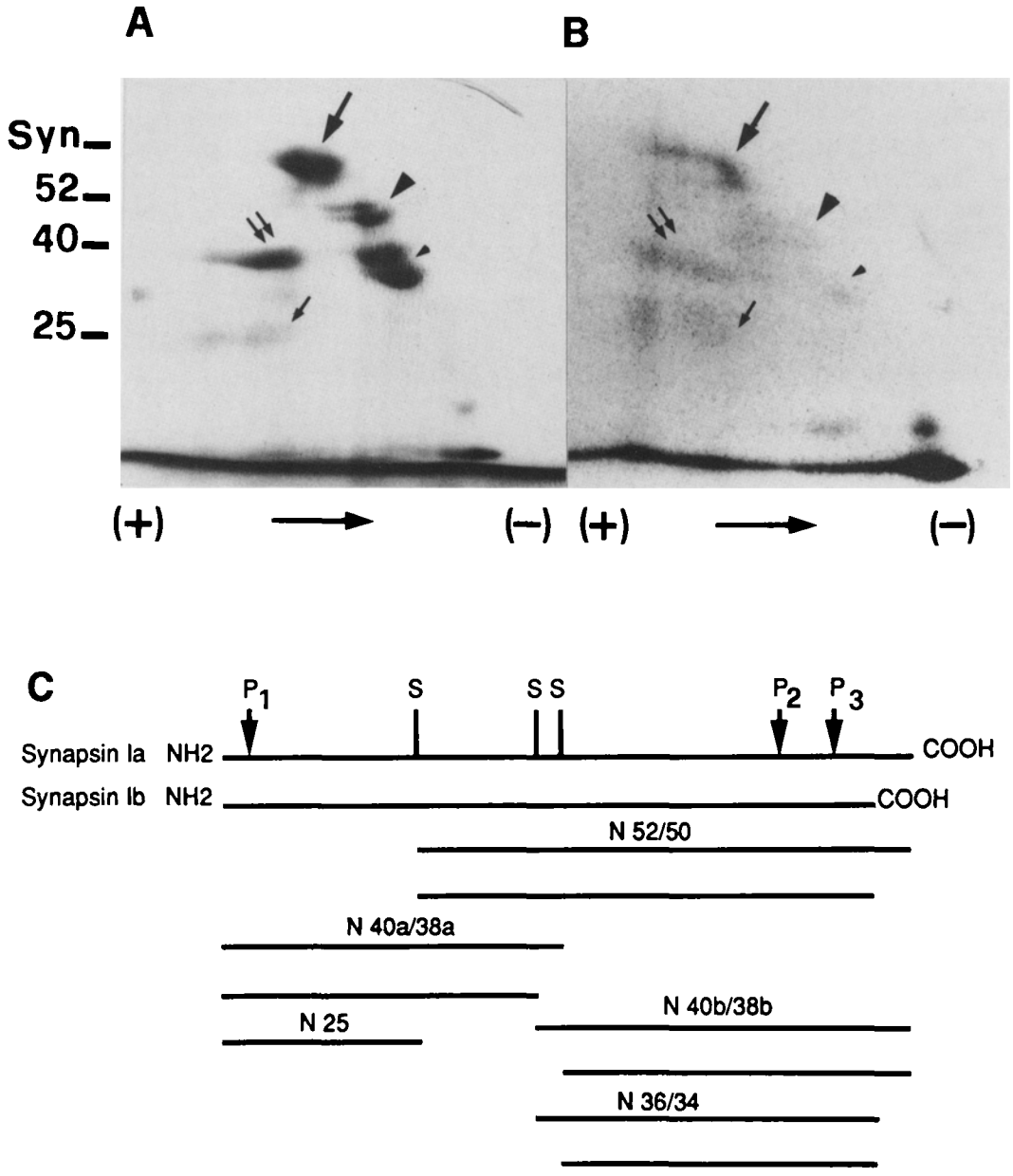

Fig. 3. Identification of the phosphorylated sites in axonally transported synapsin I. Purified synapsin I was added to ^{32}P-labeled axonally transported polypeptides and then subjected to NTCB cleavage. Analysis of the NTCB digestion mixture by 2-D NEPHGE/SDS-PAGE is indicated (A) by Coomassie-blue staining and (B) by autoradiography. The spots corresponding to intact synapsin I (large arrow), NTCB-derived peptides N52/50 (large arrowhead), N40a (double arrows), N40b, and N36/34 (small arrowhead), and N25 (small arrow) are indicated. All the NTCB-derived peptides that comprise the amino or carboxy terminal of synapsin I were radioactively labeled. The acidic (+) and basic (–) portions of the gel are indicated. (C) The relationship between NTCB-derived peptides and intact synapsin Ia and Ib is shown. The position of phosphorylated sites (P_{1-3}) and the cysteine residues (S) of the molecule are shown. The NH_2-terminal region of synapsin I comprises site P_1 phosphorylated by cAMP-dependent protein kinase and CaM I. Sites P_2 and P_3, near the COOH terminus of the molecule, are phosphorylated by CaM II (from Petrucci et al., 1991).

lation state of synapsin I during axonal transport by labeling mouse retinal ganglion cells with ^{32}P-*ortho*-phosphate in vivo. Two days after labeling, we found that synapsin I moving through N, T, and SC segments was labeled (Petrucci et al., 1991).

Synapsin I can be phosphorylated at different sites, in the "head" region by a cAMP-dependent protein kinase and CaM kinase I, and in the "tail" region by CaM kinase II (Huttner et al., 1981; Kennedy and Greengard, 1981). In order to determine which sites of axonally transported synapsin I were phosphorylated, labeled proteins from a pool of 30 optic nerves and tracts were subjected to chemical cleavage by a cysteine-specific reagent, NTCB (Petrucci and Morrow, 1987). NTCB-derived peptides of synapsin I have previously been characterized by 2-D NEPHGE and the relationship with the parent molecule established (Petrucci et al., 1988; Bahler et al., 1989; Petrucci and Morrow, 1991).

Synapsin I NTCB peptides were analyzed by 2-D NEPHGE (Fig. 3). Coomassie-blue staining and autoradiography (Fig. 3A,B) showed spots of NTCB-derived peptides whose relationship with the intact synapsin Ia and Ib is shown in Fig. 3C. The peptides comprising the amino terminal (N40a/38a and N25) or peptides comprising the carboxy terminal (N52/50, N40b/38b, and N36/34) were labeled as shown in Fig. 3B. This indicates that axonally transported synapsin I was phosphorylated in both the head and tail regions.

Two days after injection, when ^{32}P- or ^{35}S-labeled synapsin I was detectable in all segments (Petrucci et al.,1991), the relative amounts of phosphorylated synapsin I in N, T, and SC were comparable to the relative amounts of ^{35}S-labeled synapsin I in these segments. This indicates that synapsin I remains phosphorylated as it is transported along the axon and when it enters the nerve terminals. However, 14 d after injection, when a large amount of ^{35}S-labeled synapsin I (Petrucci et al., 1991; *see also* Fig. 2) was present in the superior colliculus, no ^{32}P-labeled synapsin I was detected in SC. This suggests that, after entering the nerve terminals in the superior colliculus, the initially incorporated phosphate groups are turned over, and the kinase and phosphatase systems operating at the nerve terminals change synapsin I axonal forms to forms that interact with small synaptic vesicles linking them to the cytoskeleton.

A decrease in the overall phosphate content of synapsin I in SC is demonstrated by the time-dependent changes in the electrophoretic resolution of transported ^{35}S-labeled synapsin I (Fig. 4) observed in SC, compared with N and T segments. Synapsin I spots on 2-D gels of N and T segments appeared as a streak of fairly uniform intensity and in which individual spots were indiscernible (Fig 4). This suggests the presence of several populations of molecules with different levels of phosphorylation, referred to here as α (the more acidic populations) and β forms (the more basic populations) (Fig. 4). In SC, the α forms of synapsin I were present until labeled synapsin I, moving along the axons, entered the nerve endings (Fig. 4). When all transported synapsin I had cleared the axon segments and entered the nerve terminals (Fig. 4, SC, 14 and 22d), the β forms were the only components present in SC. These region- and time-dependent changes in the electrophoretic resolution of synapsin I may be owing to the removal of phosphate from some or all sites. When 2-d post injection ^{35}S-labeled proteins prepared from the optic nerve, optic tract, and superior colliculus were incubated with alkaline phosphatase, the α form of synapsin I spots disappeared (Petrucci et al., 1991). By contrast, at 14 d postinjection, there were no evident differences in the electrophoretic resolution of ^{35}S-labeled synapsin I after alkaline phosphatase treatment of proteins from the superior colliculus. By postinjection day 7 ^{35}S-labeled synapsin I was no longer detected in the N and T segments (Fig. 2).

Discussion

Transport Kinetics of Synapsin I in Axons and Their Terminals

Our results show that, in the mouse optic system, synapsin I is transported principally with the slow component b (SCb) of axonal transport,

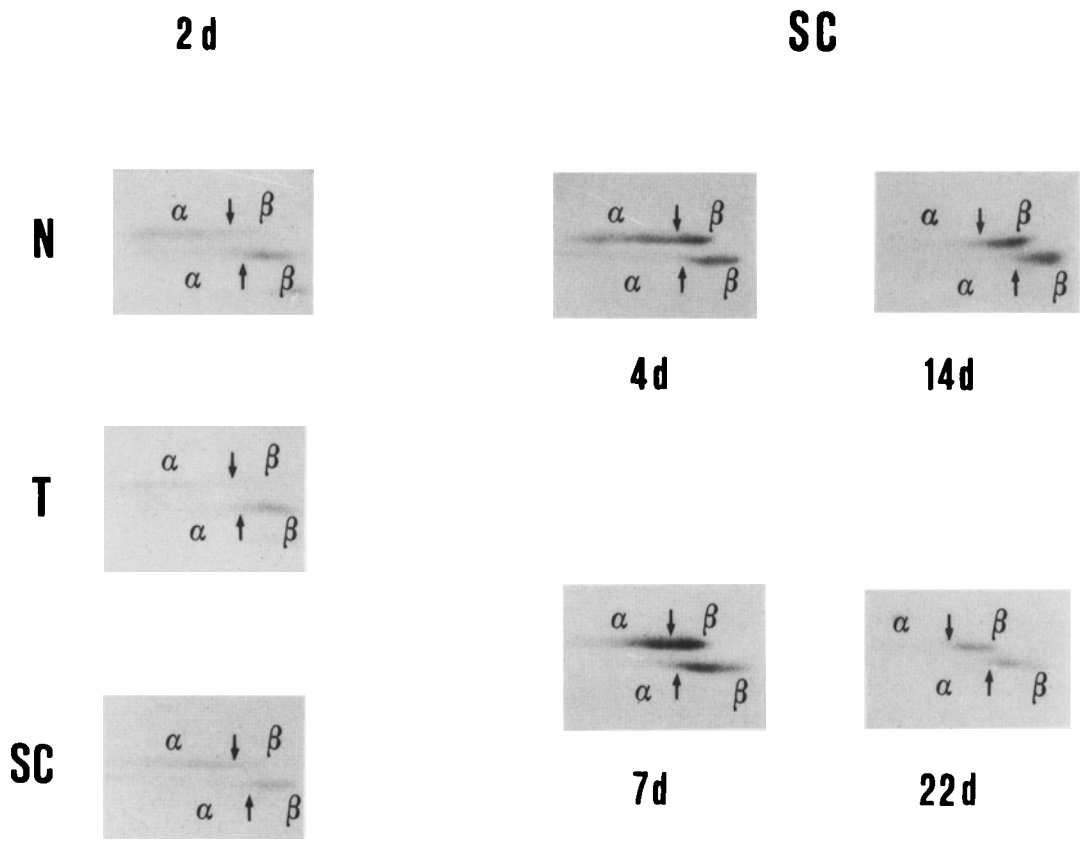

Fig. 4. Neuronal region-dependent and time-dependent changes in the spot morphology of axonally transported ^{35}S-methionine-labeled synapsin I. Fluorographs of 2-D NEPHGE/SDS-PAGE of the regions corresponding to ^{35}S-labeled synapsin Ia and Ib from samples obtained from the optic nerve (N) and tract (T) 2 d after injection, and from the superior colliculus (SC) 2, 4, 7, 14, and 22 d after injection, are shown. The arrows indicate the arbitrary boundary between the acidic and basic forms of synapsin I. The acidic forms of synapsin I, collectively labeled as α, are characteristic of the nerve fiber. The more basic forms, labeled as β, are predominant at the nerve endings after all the radioactive synapsin I has entered the terminals (from Petrucci et al., 1991).

as indicated by the peak rate of transport (1.5–3 mm/d) calculated on the basis of the observed peak in the optic nerve (N), optic tract (T), and superior colliculus (SC) segments. Therefore, the functional association of the bulk of synapsin I with the vesicles—transported with the fast component of axonal transport—must occur after the protein has entered the nerve endings. We could not detect any labeled synapsin I 1 h after labeling either in the retinal ganglion cell axon segments, N and T, or their terminals in SC. The presence of barely detectable labeled synapsin I in SC 3 h after labeling (Fig. 2), before its appearance in N and T, indicates that fast-transported synapsin I constitutes, at most, only a small pool. To demonstrate the presence of fast-transported synapsin I in N and T, observations at postlabeling intervals of <1 h would eventually be required. However, if the ratio of fast-transported to slow-transported synapsin I in mouse retinal ganglion cells is the same as that observed in rabbit retinal ganglion cells (Baitinger and Willard, 1987), the amount of fast-transported synapsin I in the axon would not be detectable by our methods of analysis.

As previously reported (Garner, 1979; Garner and Lasek, 1982; Paggi et al., 1989), differences have been found in the transport kinetics of SCb proteins. The present data show that synapsin I

is characterized by a faster overall transport rate than has previously been reported for the faster moving SCb proteins, such as clathrin (Paggi et al., 1989). In fact, synapsin I had already cleared the N and T windows between 4 and 7 d post injection, whereas clathrin was found moving through the same segments more than 21 d after labeling (Paggi et al., 1989).

In addition, it is important to point out that, as previously observed for clathrin (Paggi et al., 1989), the synapsin I pulse transients in the N and T segments remain essentially unchanged as the protein moves distally along the axon, i.e., from N to T. This indicates that synapsin I is not degraded or deposited along the axons. The constancy of the pulse transient in N and T may reflect the fact that both synapsin I and clathrin are proteins essentially devoted to nerve terminals, where they are associated with two different types of vesicles, the small synaptic vesicles (De Camilli et al., 1983; Hirokawa et al., 1989) and the coated vesicles (Pearse, 1976; Cheng et al., 1980; Puszkin et al., 1983), respectively.

In the present experiments, we found that the pulse transient, a measure of protein residence time (Paggi and Lasek, 1987; Paggi et al., 1989,1990), was about 20 times higher in the SC segment, which contains both the axons of retinal ganglion cells and their terminals, than in N and T segments. Different residence times have been found for proteins whose rates of transport along the axons are the same (Paggi et al., 1990). These differences among proteins cannot be fully accounted for by a uniform slowing of the rate of transport at the nerve endings. Therefore, the fact that synapsin I has a longer residence time at the nerve endings than other proteins, such as neurofilament proteins (Paggi and Lasek, 1987), suggests that the degrading mechanism operates more slowly on the former than on the latter.

The finding of a greater quantity of synapsin I in SC than in N and T segments agrees with the suggestion that the role of synapsin I is more specific to the axon terminals than to the axon proper (De Camilli and Greengard, 1986). It also agrees with the immunochemical finding of a greater proportion of synapsin I in the terminal region than in the axon (De Camilli et al., 1983; Goldenring et al., 1986; Hirokawa et al., 1989). A longer residence time in the nerve terminals than in the axons has also been reported for synapsin I in rabbit retinal ganglion cells (Baitinger and Willard, 1987).

Posttranslational Modification of Synapsin I

The phosphorylation of synapsin I regulates its interaction with cytoskeletal elements and small synaptic vesicles in vitro (Bahler and Greengard, 1987; Petrucci and Morrow, 1987; Schiebler et al., 1986). Our in vivo results show that synapsin I transported with the SCb remains phosphorylated as it moves along the axons. We speculate that the phosphorylation of synapsin I may prevent actin filaments from forming a dense meshwork along the axon, which could probably impair the whole process of axonal transport (McGuinness et al., 1989), and that it may facilitate the interaction of synapsin I with other cytomatrix components (Baines and Bennett, 1985,1986; Petrucci and Morrow, 1987; Hirokawa et al., 1989). Although phosphorylation inhibits the actin-bundling activity of synapsin I in vitro, it does not affect its ability to bind actin filaments and enhances its interaction with microtubules (Petrucci and Morrow, 1987).

It is relevant that phosphorylated synapsin I is modified at the nerve endings. A turnover of the initially incorporated radioactive phosphate groups after synapsin I has reached the nerve terminal compartment in the superior colliculus is suggested by the following evidence:

1. ^{32}P-labeled synapsin I was not detected 14 d after ^{32}P-*ortho*-phosphate injection, despite the fact that synapsin I accumulates at the terminals;
2. The more acidic α forms detected in the axons disappeared in the SC segment;
3. Only the α forms were affected by alkaline phosphatase treatment (Petrucci et al., 1991);
4. The portion of synapsin I dephosphorylated in the axon terminals is consistent with the fact

that the purified synapsin I, which is primarily dephosphorylated (Petrucci and Morrow, 1987), comprises only the β forms (Petrucci et al., 1991). The dephosphorylation of transported synapsin I may be functional in determining the cytoarchitecture of the presynaptic terminals by clustering small synaptic vesicles to microfilaments. Although neuronal region-dependent and time-dependent changes in the electrophoretic resolution of synapsin I spots have previously been reported in the rabbit optic system (Baitinger and Willard, 1987), our data indicate that the change in spot morphology could be owing to the disappearance of the more phosphorylated forms of synapsin I.

In conclusion, the data presented here suggest that, in vivo, the phosphorylation of axonally transported synapsin I may serve as a mechanism to regulate its interaction with cytomatrix components and synaptic vesicles. The bulk of synapsin I is axonally transported with the slow component b of axonal transport, and is phosphorylated at both the tail and head regions. Phosphorylation of synapsin I at the tail region by CaM II may be active in preventing the formation of a dense F-actin meshwork, which would restrict organelle movement in the axoplasm. After synapsin I has entered the nerve endings, a change occurs in its level of phosphorylation, and synapsin I axonal forms are converted into nerve terminal forms. The overall decrease in the phosphorus content of synapsin I at specific sites in the terminals would allow synapsin I to crosslink small synaptic vesicles to actin filaments, thus contributing to the formation of structural and functional features characteristic of the axon terminals. Depolarization of the terminals promotes translocation of synapsin I from the synaptic vesicles/cytoskeleton to cytosol (Sihra et al., 1989), thus modulating neurotransmitter release by controlling the availability of small synaptic vesicles. We do not yet know if, after synapsin I has reached the nerve endings, all sites are dephosphorylated. The role of the cAMP-dependent kinase phosphorylation of synapsin I is also unclear. The study of the phosphate turnover kinetics in the head and tail regions of the transported synapsin I after it has entered the nerve endings will possibly offer some insight into the mechanisms that cause synapsin I β forms to predominate at the synaptic terminals.

Acknowledgments

This work was partially supported by a NATO research grant 0195/90 (T.C.P.), and Ministero della Pubblica Istruzione—fondi40%—1989 and fondi 60%—1990 (P.P.), and CNR-progetto finalizzato invecchiamento to P. P. and T. C. P.

References

Bahler M. and Greengard P. (1987) Synapsin I bundles F-actin in a phosphorylation-dependent manner. *Nature (Lond.)* **326**, 704–770.

Bahler M., Benfenati F., Valtorta F., Czernik A. J., and Greengard P. (1989) Characterization of synapsin I fragments produced by cystein-specific cleavage: A study of their interactions with F-actin. *J. Cell Biol.* **108**, 1841–1849.

Baines A. J. and Bennett V. (1985) Synapsin I is a spectrin-binding protein immunologically related to erythrocyte protein 4.1. *Nature (Lond.)* **315**, 410–413.

Baines A. J. and Bennett V. (1986) Synapsin I is a microtubule-binding protein. *Nature (Lond.)* **319**, 145–147.

Baitinger C. and Willard M. (1987) Axonal transport of synapsin I-like proteins in rabbit retinal ganglion cells. *J. Neurosci.* **7**, 3723–3735.

Bonner W. M. and Laskey R. J. (1974) A film detection method for tritium labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83–88.

Cheng T. P. O., Byrd F. I., Whitaker J. N., and Wood J. G. (1980) Immunocytochemical localization of coated vesicle protein in rodent nervous system. *J. Cell Biol.* **86**, 624–633.

De Camilli P. and Greengard P. (1986) Synapsin I: a synaptic vesicle associated neuronal phosphoprotein. *Biochem. Pharmacol.* **35**, 4349–4357.

De Camilli P., Harris S. M., Jr., Huttern W. B., and Greengard P. (1983) Synapsin I (protein I), a nerve terminal-specific phosphoprotein. II. Its specific

association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.* **96**, 1337–1354.

Fisher R. A. (1966) *The Design of Experiments*, 8th ed., Ch. IX, Sect. 56, Haffener, New York, pp. 168–171.

Garner J. A. (1979) Cohesive movement of polypeptides characteristic of a distinct component of axonal transport. Ph.D. Thesis, Department of Anatomy, Case Western Reserve University, Cleveland, OH.

Garner J. A. and Lasek R. J. (1982) Cohesive axonal transport of the slow component b complex of polypeptides. *J. Neurosci.* **2**, 1824–1835.

Goldenring J. R., Lasher R. S., Vollano D. T.,Ueda T., Stenberg N. H., Stenberg L. A., and DeLorenzo R. J. (1986) Association of synapsin I with the neuronal cytoskeleton. *J. Biol. Chem.* **261**, 8495–8504.

Grafstein B. and Forman D. S. (1980) Intracellular transport in neurons. *Physiol. Rev.* **60**, 1167–1283.

Hirokawa N., Sobue K., Kanda K., Harada A., and Yorifuji H. (1989) The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin I. *J. Cell Biol.* **108**, 111–126.

Hoffman P. N. and Lasek R. J. (1975) The slow component of axonal transport: identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* **66**, 351–366.

Huttner W. B., DeGennaro L. J., and Greengard P. (1981) Differential phosphorylation of multiple sites in purified protein I by cyclic AMP-dependent and calcium-dependent protein kinases. *J. Biol. Chem.* **256**, 1482–1488.

Kennedy M. B. and Greengard P. (1981) Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylated protein I at distinct sites. *Proc. Natl. Acad. Sci. USA* **78**, 1293–1297.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lasek R. J. and Black M. M. (1977) How do axons stop growing? Some clues from the metabolism of the proteins in the slow component of the axonal transport, in *Mechanisms, Regulation and Special Functions of Protein Synthesis in Brain*, Roberts S., Rajtha A., and Gispen K. H., eds., Elsevier, North-Holland, New York, pp. 161–169.

Lasek R. J. and Brady S. T. (1982) The structural hypothesis of axonal transport: Two classes of moving elements, in *Axoplasmic Transport*, Weiss D. G., ed., Springer-Verlag, Berlin, pp. 397–405.

Lasek R. J., Garner J., and Brady S. T. (1984) Axonal transport of the cytoplasmic matrix. *J. Cell Biol.* **99**, 212s–221s.

Laskey R. A. and Mills A. D. (1975) Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gel fluorography. *Eur. J. Biochem.* **57**, 335–341.

Llinas R., McGuinnes T., Leonard C. S., Sugimori M., and Greengard P. (1985) Intraterminal injection of synapsin I or calcium/calmodulin dependent kinase II alters neurotransmitter release at the squid giant synapse. *Proc. Natl. Acad. Sci. USA* **82**, 3035–3039.

McGuinness T. L., Brady S. T., Gruner J. A., Sugimori M., Llnas R., and Greengard P. (1989) Phosphorylation-dependent inhibition by synapsin I of organelle movement in squid axoplasm. *J. Neurosci.* **9**, 4138–4149.

O'Farrell P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4004–4021.

Paggi P. and Lasek R. J. (1987) Axonal transport of cytoskeletal proteins in oculomotor axons and their residence times in the axon terminals. *J. Neurosci.* **7**, 2397–2411.

Paggi P., Lasek R. J., and Katz M. J. (1989) Slow component B protein kinetics in optic nerve and tract windows. *Brain Res.* **504**, 223–230.

Paggi P., Lasek R. J., and Katz M. J. (1990) Cytomatrix protein residence times differ significantly between the tract and the terminal segments of optic axons. *Brain Res.* **517**, 143–150.

Pearse B. M. F. (1976) Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc. Natl. Acad. Sci. USA* **73**, 1255–1259.

Petrucci T. C., Morrow J. S. (1987) Synapsin I: an actin-bundling protein under phosphorylation control. *J. Cell Biol.* **105**, 1355–1363.

Petrucci T. C. and Morrow J. S. (1991) The actin and tubulin binding domains of synapsin Ia and Ib. *Biochemistry* **30**, 413–422.

Petrucci T. C., Macioce P., and Paggi P. (1991) Axonal transport kinetics and posttranslational modification of synapsin I in mouse retinal ganglion cells. *J. Neurosci.* **11**, 2938–2946.

Petrucci T. C., Mooseker M.S., and Morrow J. S. (1988) A domain of synapsin I involved with actin bundling shares immunologic crossreactivity with villin. *J. Cell. Biochem.* **36**, 25–35.

Puszkin S., Andrés A., Ores C., Lisanti M. P., and Schook W. J. (1983) Immunochemical characterization of clathrin-associated proteins (CAPs). *Cell Tissue Res.* **231,** 495–505.

Schiebler W., Jahn R., Doucet J. P., Rothlein J., and Greengard P. (1986) Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.* **261,** 8383–8390.

Sihra T. S., Wang J. K. T., Gorelik F. S., and Greengard P. (1989) Translocation of synapsin I in response to depolarization of isolated nerve terminals. *Proc. Natl. Acad. Sci. USA* **86,** 8108–8112.