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**CYTOSKELETAL INTERACTIONS OF SYNAPSIN I IN NON-NEURONAL
CELLS**

by

Sandra L. Hurley

**A thesis submitted to the
Faculty of Graduate Studies and Research
Carleton University
in partial fulfillment of the requirements
for the degree of
Master of Science
Ottawa-Carleton Institute of Biology**

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**CYTOSKELETAL INTERACTIONS OF SYNAPSIN I
IN NON-NEURONAL CELLS**

Submitted by

Sandra L. Hurley, B.Sc. (Hons.)

in partial fulfilment of the requirements
for the degree of Masters of Science

Chair, Department of Biology
Thesis Supervisor

Carleton University

ABSTRACT

To test the hypothesis that synapsin I binds to the cytoskeleton *in vivo*, it was transiently expressed in HeLa and NIH/3T3 cells. Using fluorescence microscopy and immunofluorescent and fluorescent labeling of simultaneously fixed and extracted cells, synapsin Ia tagged with enhanced green fluorescent protein (EGFP) was found colocalized with F-actin in both cell lines. It did not colocalize with microtubules nor with vimentin and it did not alter cytoskeletal organization. Synapsin Ia-EGFP colocalized with microtubule bundles in taxol-treated HeLa cells and with F-actin spots in cells treated with cytochalasin B. It did not overtly affect F-actin reassembly following drug removal. Synapsin Ia-EGFP remained colocalized with F-actin in cells treated with nocodazole, and it did not affect reassembly of microtubules following drug removal. These *in vivo* results provide evidence that synapsin I binds to F-actin and suggest that synapsin I may bind to microtubules with low affinity.

To Emily.

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TABLE OF CONTENTS

LIST OF FIGURES.....	xiii
-----------------------------	-------------

ABBREVIATIONS.....	xv
---------------------------	-----------

1. INTRODUCTION

1.1	Synaptic transmission.....	1
1.2	Actin and microtubule organization in neurons.....	4
1.3	The Synapsins	
	1.3.1. Synapsin genes and isoforms.....	5
	1.3.2. Domain model for the synapsins.....	7
	1.3.3. Synapsin interactions with synaptic vesicles.....	8
	1.3.4. Synapsin interactions with cytoskeletal proteins.....	8
	1.3.5. A model for synapsin I regulation of the reserve pool of SVs.....	12
	1.3.6. Synapsin involvement in neurotransmission.....	15
	1.3.7. Synapsin involvement in neuronal formation and maintenance.....	18
1.4	Rationale for experiments.....	19

1.5	Hypothesis.....	20
1.6	Test of hypothesis	
	1.6.1. Localization of synapsin Ia expressed transiently in HeLa and NIH/3T3 cells.....	20
	1.6.2. Localization of synapsin Ia-EGFP in cells treated with taxol.....	20
	1.6.3. Localization of synapsin Ia-EGFP in cells treated with microtubule or F-actin depolymerizing drugs.....	21
	1.6.4. Effects of synapsin Ia-EGFP overexpression on reassembly of depolymerized F-actin and microtubules.....	21
2.	MATERIALS AND METHODS	
	2.1. General molecular cloning techniques	
	2.1.1. Agarose gel electrophoresis.....	23
	2.1.2. Small-scale plasmid DNA purification	23
	2.1.3. Restriction digestions.....	24
	2.1.4. Agarose gel purification of DNA.....	24
	2.2. Generation of plasmid constructs	
	2.2.1. PCR amplification of synapsin Ia cDNA.....	27

2.2.2. Cloning of synapsin Ia cDNA into pBAD-TOPO®.....	27
2.2.3. Cloning of synapsin Ia cDNA into pEGFP-N1.....	29
2.3. Large-scale purification of plasmid DNA for transfection	31
2.4. Expression of pSynIa-EGFP in HeLa and NIH/3T3 cells	
2.4.1. Cell culture.....	31
2.4.2. DNA transfections.....	33
2.5. Drug treatments	
2.5.1. Nocodazole.....	33
2.5.2. Cytochalasin B.....	34
2.5.3. Taxol.....	34
2.6. Western blotting of whole cell extracts	
2.6.1. Total protein extractions from transfected cells.....	35
2.6.2. Protein quantitation.....	35
2.6.3. SDS-PAGE of total protein from transfected cells.....	36
2.6.4. Electrophoretic transfer and probing of total protein with anti-EGFP-HRP	37
2.7. Microscopy	
2.7.1. Fixation.....	38

2.7.2. Fluorescent and immunofluorescent	
labeling.....	38
2.7.3. Fluorescence microscopy.....	39
2.7.4. Confocal laser scanning microscopy	39

3. RESULTS

3.1. Generation and expression of synapsin Ia-EGFP	
expression construct pSynIa-EGFP	
3.1.1. PCR amplification of synapsin Ia rat cDNA	
from pET15B-SynIa AND pBAD-TOPO®	
cloning of PCR product	40
3.1.2. Cloning of synapsin Ia cDNA from pBAD-	
SynIa into pEGFP-N1.....	40
3.1.3. Fidelity of expression of synapsin Ia-EGFP	
(SynIa-EGFP) in HeLa and NIH/3T3	
cells.....	43
3.2. Comparison of SynIa-EGFP and F-actin localizations in	
HeLa and NIH/3T3 cells	
3.2.1. SynIa-EGFP colocalizes with F-actin in HeLa	
cells but does not alter F-actin	
organization	44

3.2.2.	Synla-EGFP colocalizes with F-actin in stress fibers in NIH/3T3 cells but does not alter F-actin organization.....	47
3.3.	Comparison of Synla-EGFP and microtubule localizations in HeLa and NIH/3T3 cells	
3.3.1.	Synla-EGFP does not colocalize with microtubules in either HeLa or NIH/3T3 cells nor alter their organization	54
3.4.	Comparison of Synla-EGFP and vimentin (intermediate filament) localizations in HeLa cells	
3.4.1.	Synla-EGFP does not colocalize with vimentin in HeLa cells nor alter its organization.....	61
3.5.	Cytochalasin B treatment of HeLa cells transfected with pSynla-EGFP	
3.5.1.	Synla-EGFP colocalizes with cytochalasin B-treated F-actin.....	61
3.5.2.	Synla-EGFP overexpression in HeLa cells does not affect reassembly of F-actin following cytochalasin B treatment but colocalizes with F-actin throughout reassembly.....	66

3.6.	Nocodazole treatment of HeLa cells transfected with pSynla-EGFP	
3.6.1.	Synla-EGFP does not colocalize with microtubules nor vimentin in nocodazole-treated HeLa cells, but does colocalize with F-actin.....	69
3.6.2.	Synla-EGFP overexpression does not affect reassembly of microtubules following nocodazole treatment.....	69
3.7.	Taxol treatment of HeLa cells transfected with pSynla-EGFP	
3.7.1.	Synla-EGFP colocalizes with microtubule bundles in taxol-treated HeLa cells	76

4. DISCUSSION

4.1.	Interaction of synapsin Ia-EGFP with F-actin in HeLa and NIH/3T3 cells.....	79
4.2.	Interaction of synapsin Ia-EGFP with microtubules in HeLa and NIH/3T3 cells.....	83
4.3.	Interaction of synapsin Ia-EGFP with vimentin intermediate filaments in HeLa cells.....	85
4.4.	Implications for involvement of synapsin I in neurotransmission.....	86

4.5.	Implications for synapsin I involvement in neuronal formation and maintenance.....	87
4.6.	Future prospects.....	88
5.	REFERENCES.....	91

LIST OF FIGURES

- Figure 1.** The synaptic vesicle cycle.
- Figure 2.** A domain model for the synapsins.
- Figure 3.** Model for synapsin I involvement in neurotransmission.
- Figure 4.** Representation of the pSynla-EGFP construct.
- Figure 5.** Cloning of rat synapsin Ia cDNA into pEGFP-N1.
- Figure 6.** Fidelity of expression of synla-EGFP in HeLa cells and NIH/3T3 cells as demonstrated by Western blotting.
- Figure 7.** Colocalization of synla-EGFP with F-actin in HeLa cells as demonstrated by conventional fluorescence microscopy.
- Figure 8.** Colocalization of synapsin Ia-EGFP with F-actin in HeLa cells as demonstrated by confocal laser scanning microscopy.
- Figure 9.** Colocalization of synapsin Ia-EGFP with F-actin in NIH/3T3 cells as demonstrated by conventional fluorescence microscopy.
- Figure 10.** Lack of colocalization of synapsin Ia-EGFP with microtubules in HeLa cells as demonstrated by conventional fluorescence microscopy.
- Figure 11.** Lack of colocalization of synapsin Ia-EGFP with microtubules in NIH/3T3 cells as demonstrated by conventional fluorescence microscopy.
- Figure 12.** Control for non-specific binding by donkey anti-mouse-cy3 secondary antibody.

- Figure 13.** Lack of colocalization of synapsin Ia-EGFP with vimentin in HeLa cells as demonstrated by conventional fluorescence microscopy.
- Figure 14.** Colocalization of synapsin Ia-EGFP with F-actin spots in cytochalasin B-treated HeLa cells as demonstrated by conventional fluorescence microscopy.
- Figure 15.** Colocalization of synapsin Ia-EGFP with F-actin and lack of effect of synapsin Ia-EGFP overexpression on F-actin reassembly over a time-course of cytochalasin B wash-off in HeLa cells as demonstrated by conventional fluorescence microscopy.
- Figure 16.** Localization of synapsin Ia-EGFP compared to microtubules in HeLa cells treated with nocodazole as demonstrated by conventional fluorescence microscopy.
- Figure 17.** Colocalization of synapsin Ia-EGFP with F-actin and lack of colocalization of synapsin Ia-EGFP with vimentin in nocodazole-treated HeLa cells as demonstrated by conventional fluorescence microscopy.
- Figure 18.** Lack of effect of synapsin Ia-EGFP overexpression on microtubule reassembly over a time-course of nocodazole wash-off in HeLa cells as demonstrated by conventional fluorescence microscopy.
- Figure 19.** Colocalization of synapsin Ia-EGFP with microtubule bundles in taxol-treated HeLa cells as demonstrated by conventional fluorescence microscopy.

ABBREVIATIONS

α-MEM	alpha-modified Eagle's minimum essential medium
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BES	N,N-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid
bp	base pairs
BSA	bovine serum albumin
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CCD	charged couple display
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
cy3	indocarbocyanine
ddH₂O	double distilled and deionized water
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EEO	electroendosmosis

EGTA	ethylene glycol bis (β-aminoethylether) N,N,N',N' tetraacetic acid
EGFP	enhanced green fluorescent protein
F-actin	filamentous actin
FBS	fetal bovine serum
G-actin	globular actin
HRP	horseradish peroxidase
Hz	Hertz
kb	kilobase
K_d	equilibrium dissociation constant
KDa	kilodaltons
LB	Luria-Bertani
LMP	low melting point
MAP	microtubule-associated protein
MCS	multiple cloning site
mRNA	messenger ribonucleic acid
NF-L	light neurofilament subunit
P	phosphate group
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEM	PIPES/EGTA/MgCl₂ buffer
PIPES	piperazine-N,N,-bis(2-ethanesulfonic acid)

PPF	paired-pulse facilitation
PPtase	phosphatase
PKA	protein kinase A (cAMP-dependent protein kinase)
PTP	post-tetanic potentiation
SDS	sodium dodecyl sulfate
SV	synaptic vesicle
syn Ia	synapsin Ia
TAE	Tris acetate/EDTA buffer
<i>Taq</i>	<i>Thermus aquaticus</i>
TC-PBS	tissue culture phosphate-buffered saline
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet

1. INTRODUCTION

1.1. SYNAPTIC TRANSMISSION

Neuronal communication is mediated by synaptic transmission, which involves the quantal release of neurotransmitters into the synaptic cleft (Greengard *et al.*, 1993 and Sudhof, 1995 for review). When an action potential arrives at the nerve terminal, an influx of Ca^{2+} through voltage-gated Ca^{2+} channels occurs. This signals the fusion of synaptic vesicles (SVs) with the presynaptic plasma membrane to which they are predocked. Only a subset of SVs that have been primed through an ATP-dependent and rate-limiting maturation process are competent for fusion. When fusion occurs, the contents of these SVs, neurotransmitters, are released into the synaptic cleft. Fused SVs are then endocytosed and recycled, so that they may once again accumulate neurotransmitter and be made available for exocytosis.

Synaptic transmission is necessarily tightly regulated and this regulation contributes to synaptic plasticity (Greengard *et al.*, 1993; Sudhof, 1995 and Hilfiker *et al.*, 1999 for review). One important means by which neurons control neurotransmitter release is the regulation of the relative number of SVs that are kept in two distinct, functional pools (Figure1; Greengard *et al.*, 1993). These pools are: i) the releasable pool of SVs, some of which will fuse with the plasma membrane when there is an action potential and ii) the reserve pool of SVs, which are tethered to the cytoskeleton and may be enlisted to the releasable pool as needed. The synapsins are candidate mediators of the regulation of SV transition from the reserve pool to the releasable pool (Hilfiker *et al.*, 1999).

Figure 1. The synaptic vesicle cycle. When an action potential arrives at the nerve terminus, primed SVs from the releasable pool fuse with the presynaptic plasma membrane and release neurotransmitters into the synapse. These vesicles are endocytosed and recycled. There is also a reserve pool of SVs distal to the presynaptic membrane and these SVs are tethered to the cytoskeleton, putatively by synapsins. An influx of Ca^{2+} signals the release of reserve pool SVs from the cytoskeleton, and they in turn enter the releasable pool. (Source of figure:

<http://www.carleton.ca/~jcheetha/labpage/synapsins.html>).

1.2. ACTIN AND MICROTUBULE ORGANIZATION IN NEURONS

Neurons are highly polarized in morphology, typically with a single long axon and many smaller dendrites (Mattson, 1999 for review). This polarity enables them to form contacts with other neurons and to mediate rapid signalling. The neuronal cytoskeleton, which consists of intermediate filaments (neurofilaments), microfilaments and microtubules, is involved in the formation of specific neuronal connections, in the maintenance of neuronal structure, in the transport of cellular components to the axonal terminal and in the regulation of neurotransmitter release (Hirokawa, 1994; Brandt, 1998 and Mattson, 1999 for review).

During neurite growth, actin filaments are undergoing constant rearrangements in the filopodia and lamellipodia of growth cones, while there is a net microtubule-associated protein (MAP)-dependent promotion of microtubule polymerization in the shaft of the neurite (Brandt, 1998 and Mattson, 1999 for review). During axonal outgrowth, the former process is key in determining the orientation of the growth cone, while the latter process is the basis for the actual outgrowth of the axonal shaft. However, some microtubules do splay into the growth cone and appear to be necessary for determining final growth cone orientation (Tanaka and Kirschner, 1995). It has been suggested that this is mediated by the selective stabilization of some of these growth cone microtubules (Brandt, 1998).

As indicated, microtubules are highly concentrated in axons, where their fast-growing (plus) ends are all oriented distal to the cell body (Heidemann et

al., 1981). They exhibit a mixed orientation in dendrites (Baas *et al.*, 1988). In the axon of a mature neuron, microtubules are relatively resistant to depolymerization (Lim *et al.*, 1989). However, this stability decreases with increasing distance from the cell body (Donoso, 1986).

Actin filaments form a network in the presynaptic terminal, and SVs are captured in this network (Hirokawa *et al.*, 1989). Some of these filaments associate with the presynaptic plasma membrane at active zones (Hirokawa *et al.*, 1989).

1.3. THE SYNAPSINS

Synapsins are neuron-specific phosphoproteins that associate with the cytoplasmic side of synaptic vesicles (De Camilli *et al.*, 1983a,b; Thiel *et al.*, 1990; Kao *et al.*, 1998). Accounting for 9% of total vesicle protein, they are essential in the regulation of neurotransmitter release (Rosahl *et al.*, 1995) and have also been implicated in synaptogenesis, axonogenesis and neuronal formation and maintenance (Ferreira *et al.*, 1995; Takei *et al.*, 1995; Chin *et al.*, 1995; Lu *et al.*, 1996; Ferreira *et al.*, 1998; Zhong *et al.*, 1999).

1.3.1. SYNAPSIN GENES AND ISOFORMS

Invertebrates possess a single synapsin gene, while vertebrates have at least three functional synapsin genes (I, II and III) that likely arose from gene duplication events (Kao *et al.*, 1999). Synapsin I is composed of two isoforms, Ia and Ib, which are generated by alternative mRNA splicing (Sudhof *et al.*, 1989). It has an amino-terminal globular head region and an elongated, basic,

glycine and proline-rich carboxy-terminal tail (Sudhof *et al.*, 1989). Synapsin II, also composed of isoforms a and b, is similar to synapsin I in the head region but lacks the tail region (Sudhof *et al.*, 1989). Both synapsins I and II are present early in neuronal development but levels peak with synapse formation and remain high thereafter (Ferreira *et al.*, 1998). Prior to synaptogenesis, synapsin I is localized in axons, particularly in the distal axon and growth cone (Fletcher *et al.*, 1991). After synaptogenesis, it is specifically localized to the nerve terminal on SVs, mostly those of the reserve pool (Pieribone *et al.*, 1995).

Synapsin III was only recently discovered (Kao *et al.*, 1998). It is known that its gene gives rise to at least six transcripts: synapsins IIIa-III f (Porton *et al.*, 1999). Synapsins IIIa, IIIb and IIIc are expressed at low levels in fetal brain and at even lower levels in adult brain, while synapsin IIId is expressed only in fetal brain (Porton *et al.*, 1999). All synapsin III isoforms are also expressed in non-neuronal tissue, with synapsins IIIe and III f not being expressed at all in neuronal tissue (Porton *et al.*, 1999). Synapsins IIIa-IIIc share a similar head region to synapsins I and II, and synapsin IIIa, the best characterized synapsin III, is highly concentrated in growth cones (Kao *et al.*, 1998; Hosaka and Sudhof, 1999; Porton *et al.*, 1999; Ferreira *et al.*, 2000).

There is evidence for differential expression of synapsins in subsets of neurons (Sudhof *et al.*, 1989; Mandell *et al.*, 1990; Mandell *et al.*, 1992). In particular, a high level of expression of synapsin I relative to synapsin II may be

associated with inhibitory synapses, while the reverse case may be associated with excitatory synapses (Mandell *et al.*, 1992; Hilfiker *et al.*, 1999).

1.3.2. DOMAIN MODEL FOR THE SYNAPSINS

Synapsins I, II and III (a-c) are mosaics of shared and individual domains (Figure 2; Sudhof *et al.*, 1989; Kao *et al.*, 1998; Porter *et al.*, 1999). They have highly similar A and C domains (part of the head region) and somewhat conserved B domains (also part of the head region) (Sudhof *et al.*, 1989; Kao *et al.*, 1998). Domains A, C and E are the most evolutionarily conserved among vertebrate synapsins (Kao *et al.*, 1999). Among invertebrate synapsins, domains C and E are also highly conserved, but domain A is not (Kao *et al.*, 1999). Domain A contains a conserved phosphorylation site for Ca^{2+} /calmodulin-dependent protein kinase I and protein kinase A (Schiebler *et al.*, 1986; Hosaka *et al.*, 1999). Domain C binds to ATP in a Ca^{2+} -dependent manner and is structurally similar to ATP-utilizing enzymes (Esser *et al.*, 1998). It enables synapsin self-association and homo- and heterodimerization of the various synapsin isotypes (Hosaka and Sudhof, 1999). All "a" isoforms (i.e. Ia, IIa and IIIa) share the carboxyl terminal E domain (Sudhof *et al.*, 1989; Kao *et al.*, 1998). Domain E appears to be involved in the regulation of neurotransmitter release in the case of these "a" isoforms (see below; Hilfiker *et al.*, 1998). Synapsins Ia and Ib differ only at this carboxyl terminal domain, which is designated F in 1b (Sudhof *et al.*, 1989).

1.3.3. SYNAPSIN INTERACTIONS WITH SYNAPTIC VESICLES

Synapsins interact with high affinity ($K_d = 10.7$ nM) and high saturability *in vitro* with phospholipid vesicles that approximate the composition of synaptic vesicles (Benfenati *et al.*, 1989a). Domains A and C bind on their own to the vesicles (Benfenati *et al.*, 1989b; Hosaka *et al.*, 1999), with certain peptide sequences of the hydrophobic C domain able to insert into lipid membranes (Cheetham *et al.*, 2001). The interaction of synapsin I with synaptic vesicles is generally thought to be mediated by binding of the head region to lipid components and of the tail region to protein vesicular components including CaMKII (Benfenati *et al.*, 1989b; Valtorta *et al.*, 1992a). Domain B in synapsin II may also bind to protein vesicular components (Thiel *et al.*, 1990). Phosphorylations in the A domain of all synapsins by cAMP-dependent protein kinase or CaM kinase I (CaMKI) and in the D domain of synapsin I by CaM kinase II (CaMKII) reduce the affinity of synapsins for synaptic vesicles (Schiebler *et al.*, 1986; Hosaka *et al.*, 1999).

1.3.4. SYNAPSIN INTERACTIONS WITH CYTOSKELETAL PROTEINS

Interactions of synapsin I with the cytoskeleton are well-characterized *in vitro* and, like interactions with synaptic vesicles, are regulated by phosphorylation. Synapsin I binds *in vitro* to actin (Petrucci and Morrow, 1987; Bahler and Greengard, 1987) microtubules (Baines and Bennett, 1986; Bennett and Baines, 1992), brain spectrin (Sikorski *et al.*, 1991; Iga *et al.*, 1997) and neurofilaments (Goldenring *et al.*, 1986; Steiner *et al.*, 1987). Synapsin II has also been shown to bind *in vitro* to actin filaments (Chilcote *et al.*, 1994;

Figure 2. A domain model for the synapsins. All synapsins share similar A and C domains, and the “a” isoforms share similar E domains. Synapsins Ia and Ib are coded for by the same open reading frame and are generated by alternative mRNA splicing. The same is true for synapsins IIa and IIb. (Source of figure: <http://www.carleton.ca/~jcheetha/labpage/synapsins.html>).

Nieler *et al.*, 1997). It is not yet known whether or not synapsin III interacts with proteins of the cytoskeleton.

Synapsin I not only binds to actin filaments ($K_d = 1-2 \mu\text{M}$) but also bundles them; binding and bundling are reduced upon phosphorylation by cAMP-dependent protein kinase (in domain A) and mitogen-activated protein kinase (at 3 sites: two in domain B and one in domain D distinct from CaM kinase II sites), and are virtually abolished upon phosphorylation by CaM kinase II (two sites in domain D) (Petrucci and Morrow, 1987; Bahler and Greengard, 1987; Ceccaldi *et al.*, 1995; Matsubara *et al.*, 1996; Jovanovic *et al.*, 1996; Hilfiker *et al.*, 1999). Synapsin II also binds and bundles F-actin and these effects are markedly inhibited by phosphorylation by cAMP-dependent protein kinase (Chilcote *et al.*, 1994; Nieler *et al.*, 1997).

Dephosphorylated synapsins I and II also bind to G-actin (Ceccaldi *et al.*, 1993; Nieler *et al.*, 1997) and these complexes behave as F-actin nuclei, accelerating the initial rate of actin polymerization (Valtorta *et al.*, 1992a; Fesce *et al.*, 1992; Valtorta *et al.*, 1992b). These effects are also strongly inhibited by phosphorylation by CamKII or mitogen-activated protein kinase in synapsin I (Valtorta *et al.*, 1992b; Jovanovic *et al.*, 1996) and by cAMP-dependent protein kinase in synapsin II. Calmodulin suppresses binding, bundling and nucleating of actin by synapsin I in any of its phosphorylation states (Baines *et al.*, 1994; Nicol *et al.*, 1998).

Binding of synapsin I to F-actin *in vitro* is mediated by an actin-binding site in domain C (Bahler *et al.*, 1989; Valtorta *et al.*, 1992a). This site is distinct

from the putative vesicle-binding site in the same domain (Bahler *et al.*, 1989; Valtorta *et al.*, 1992a). There is also evidence for an additional actin-binding site in domain D, which might explain the bundling effect (Bahler *et al.*, 1989; Valtorta *et al.*, 1992a) although synapsin II does not contain this domain.

Synapsin I also binds to microtubules *in vitro*, albeit with less affinity ($K_d = 5 \mu\text{M}$) (Baines and Bennett, 1986; Hilfiker *et al.*, 1999). It can be isolated from brain as part of a microtubule-associated complex of proteins (Farrell and Keates, 1990). Synapsin I does not reduce the critical concentration for microtubule assembly nor bind to unpolymerized tubulin dimer; however at high concentrations it will induce the formation of microtubule bundles (Baines and Bennett, 1986). This may be due to the presence of microtubule binding regions in both the head and tail regions of synapsin I (Hirokawa *et al.*, 1989; Bennett *et al.*, 1991; Bennett and Baines, 1992). Alternatively, both microtubule and actin bundling by synapsin I may be due to its ability to self-associate (Hosaka and Sudhof, 1999).

1.3.5. A MODEL FOR SYNAPSIN I REGULATION OF THE RESERVE POOL OF SVs

Synapsin I is the best characterized of the synapsins and therefore a model for its involvement in neurotransmission has evolved (Figure 3). As mentioned, a network of actin filaments exists in the presynaptic terminal, and SVs are captured in this network (Hirokawa *et al.*, 1989). The interaction of synapsin I with actin in particular suggests that it forms cross-bridges among SVs and the actin-based cytoskeleton, regulating the transition of the vesicles

Figure 3. Model for synapsin I involvement in neurotransmission. Synapsin I tethers SVs to the actin-based cytoskeleton, forming a reserve pool. When nerve endings are depolarized, cAMP-dependent and CaM kinases phosphorylate synapsin I, thereby substantially lowering its affinity for both SVs and F-actin. The SVs are released from the cytoskeleton and made available to enter the releasable pool. Primed SVs in the releasable pool may undergo exocytosis when an action potential arrives at the neuronal terminus (Source of figure: <http://www.carleton.ca/~jcheetha/labpage/synapsins.html>).

from the reserve pool to the releasable pool (Greengard *et al.*, 1993; Takei *et al.*, 1995). Once phosphorylated by cAMP-dependent and CaM kinases, an event signalled by depolarization of nerve endings, synapsin I releases the vesicles from the cytoskeleton so that they are available for exocytosis and release of neurotransmitters into synapses (Baines and Bennett, 1986; Petrucci and Morrow, 1987; Bahler and Greengard, 1987; Greengard *et al.*, 1993). Because phosphorylation of synapsin also inhibits its actin-nucleating activity, recapture of synaptic vesicles in the actin meshwork is prevented (Greengard *et al.*, 1993). Videomicroscopic studies support this model, showing that the presence of dephosphorylated synapsin I is necessary for SVs to bind *in vitro* to actin filaments (Ceccaldi *et al.*, 1995).

There is some evidence for the presence of microtubules in the presynaptic terminus (Hirokawa *et al.*, 1989) and so microtubules may also be involved in regulating the reserve pool of SVs. It is also possible that synapsin I-microtubule interactions are important in neuronal development. Further investigation is needed to clarify the functional significance of these interactions if they do indeed exist *in vivo*.

1.3.6. SYNAPSIN INVOLVEMENT IN NEUROTRANSMISSION

Physiological studies support the involvement of synapsins in synaptic transmission. Injection of dephosphorylated synapsin I into the squid giant synapse reduces the amplitude and rate of rise of the postsynaptic potential and the rate of spontaneous and enhanced quantal transmitter release (Llinas

et al., 1985; Lin *et al.*, 1990). It now appears that regulation of the reserve pool of SVs by synapsins prevents synaptic depression under conditions of high frequency stimulation. When synapsin antibodies are injected into living lamprey reticulospinal axons, the reserve pool of SVs is depleted, and there is a concomitant depression in neurotransmitter release evoked by high frequency (18-20 Hz) but not low frequency (0.2 Hz) stimulation (Pieribone *et al.*, 1995). These results suggest that synapsins and the reserve pool of SVs with which they are associated enable neurons to respond in a highly reproducible manner during bursts of neuronal stimulation (Pieribone *et al.*, 1995; Hilfiker *et al.*, 1999).

Domain E appears to be involved in the regulation of neurotransmitter release by the “a” synapsin isoforms (Hilfiker *et al.*, 1998). Presynaptic injection of a peptide fragment of domain E at the squid giant synapse leads to a reduction in the number of SVs distal to the presynaptic membrane and to synaptic depression (Hilfiker *et al.*, 1998). This domain may also be involved in a direct regulation of neurotransmitter release. Presynaptic injection of domain E peptide has no effect on the number of docked vesicles, but it does reversibly inhibit and slow the kinetics of neurotransmitter release, indicating that synapsins may also be involved in the actual SV release and fusion processes (Hilfiker *et al.*, 1998).

The involvement of synapsins in synaptic transmission is also supported by knock-out mice studies. Surprisingly, mice lacking both synapsins I and II are viable and fertile and have no gross anatomical abnormality of the nervous

system (Rosahl *et al.*, 1995). They do however exhibit increased late-onset seizure propensity (Rosahl *et al.*, 1995). As expected, synaptic vesicle clustering is impaired in synapsin I-deficient mice, and in addition, the induced release of glutamate from their nerve endings is significantly decreased (Li *et al.*, 1995). The number of synaptic vesicles distal from the presynaptic plasma membrane is significantly reduced in their mossy fiber giant terminals (Takei *et al.*, 1995). During brief action potential trains (e.g. 90 seconds at 10 Hz) at synaptic boutons of hippocampal cell cultures from these mice, the number of exocytosed vesicles and the total recycling vesicle pool become reduced (Ryan *et al.*, 1996).

Deficiencies in synapsins also appear to affect short-term synaptic plasticity, although the significance of these observations is unclear. Synapsin I-deficient mice show an increase in paired-pulse facilitation (PPF) (Rosahl *et al.*, 1995; Terada *et al.*, 1999). PPF, which is very short-lived, occurs when two stimuli are applied within an interval in the millisecond range. The second response is larger than the first because of increased neurotransmitter release (Hilfiker *et al.*, 1999). On the other hand, synapsin II-deficient mice and mice lacking both synapsins I and II have decreased post-tetanic potentiation (PTP) but normal PPF (Rosahl *et al.*, 1995; Silva *et al.*, 1996). Like PPF, PTP results in an increased postsynaptic response but this response can last for several seconds and may be elicited by trains of presynaptic stimuli (Hilfiker *et al.*, 1999). Long-term potentiation, which is involved in learning and memory, is unaffected by any of the above knock-outs (Li *et al.*, 1995; Rosahl *et al.*, 1995).

Synapsin III is not upregulated in synapsin I and II double-knockout mice and therefore does not seem to compensate for the loss of synapsins I and II (Hosaka and Sudhof, 1999).

While excitatory synaptic transmission is not significantly affected by synapsin I depletion, there is a significant impairment in inhibitory synaptic transmission in synapsin I knock-out mice and this may contribute to their increased seizure propensity (Terada *et al.*, 1999). Following repeated application of a hypertonic solution, synaptic depression and depletion of reserve pool vesicles are observed in cultured inhibitory neurons from these mutants, while the same is not seen in cultured excitatory neurons from the same animals (Terada *et al.*, 1999). This correlates with high levels of expression of synapsin I relative to synapsin II at inhibitory synapses (Mandell *et al.*, 1992) and it is possible that synapsin II is able to compensate for the loss of synapsin I at excitatory synapses but not inhibitory ones (Terada *et al.*, 1999).

1.3.7. SYNAPSIN INVOLVEMENT IN NEURONAL FORMATION AND MAINTENANCE

Studies where synapsins are deficient also implicate them in the development and maintenance of synapses and axons. These functions may be related to their interactions with cytoskeletal components. Neuronal cultures from mice lacking synapsin I show greatly retarded synapse formation (Ferreira *et al.*, 1998). Hippocampal neurons of embryonic synapsin I-knock-out mice show delayed synapse formation and severely retarded outgrowth of

predendritic neurites and axons (Chin *et al.*, 1995). Mossy fiber giant terminals from the hippocampus of synapsin I knock-out mice are significantly smaller (Takei *et al.*, 1995). Depletion of synapsin II in rat hippocampal cultures using antisense oligonucleotides leads to aberrant neurite growth, failure to elongate axons, an abnormal distribution of F-actin and an inhibition of synapse formation and maintenance (Ferreira *et al.*, 1994; Ferreira *et al.*, 1995).

Overexpression studies also suggest that the synapsins are involved in neuronal formation and maintenance. Overexpression of synapsin IIb in NG108-15 neuroblastoma cells leads to the appearance of synapse-like cell-cell contacts and to marked increases in the number of neuritic varicosities and in the numbers of vesicles in those varicosities (Han *et al.*, 1991).

Overexpression of either synapsin I or II in these same cells enhances functional synapse formation with myotubes (Zhong *et al.*, 1999).

1.4. RATIONALE FOR EXPERIMENTS

Interactions of synapsins with the cytoskeleton are not characterized *in vivo*. *In vivo* studies of these interactions would help in ascertaining synapsin function, either by confirming or contradicting *in vitro* findings. I am interested in synapsin I, which has already been shown to bind *in vitro* to both actin and microtubules. If synapsin I is indeed shown to interact with these cytoskeletal components *in vivo*, its functional implication in both synaptic vesicle tethering and in neuronal development will have more credibility.

1.5. HYPOTHESIS

Synapsin I binds to both F-actin and microtubules *in vivo* and also induces them to bundle, causing changes in cell morphology.

1.6. TEST OF HYPOTHESIS

1.6.1. LOCALIZATION OF SYNAPSIN IA EXPRESSED TRANSIENTLY IN HELA AND NIH/3T3 CELLS

To determine whether or not synapsin I binds to F-actin and microtubules *in vivo*, the rat cDNA coding for full-length synapsin Ia was cloned into pEGFP-N1 so that expression of the resulting construct (pSynIa-EGFP) produced synapsin Ia as a fusion protein with EGFP at its carboxyl terminus. The construct was transiently transfected into both HeLa cervical carcinoma cells and NIH/3T3 mouse fibroblast cells which should express no or low levels of synapsins. Cells were fixed to their coverslips by a simultaneous fixation/extraction method (Falconer *et al.*, 1992) to determine whether or not colocalization of synapsin I with F-actin or microtubules and changes in cell morphology were occurring. Using fluorescence and immunofluorescence staining along with fluorescence microscopy, the colocalization (or lack thereof) of synapsin Ia-EGFP with cytoskeletal components was examined.

1.6.2. LOCALIZATION OF SYNAPSIN IA-EGFP IN CELLS TREATED WITH TAXOL

HeLa cells transfected with pSynIa-EGFP were treated with taxol, which

enhances polymerization of tubulin and inhibits depolymerization of microtubules (Marty *et al.*, 1994). Microtubule bundles form and this causes microtubule-bound proteins to also come closer together so that they are more easily detected (Vaillant *et al.*, 1998). Immunofluorescence and fluorescence staining and fluorescence microscopy were used to determine whether or not colocalization of synapsin Ia-EGFP with taxol-stabilized microtubules was occurring.

1.6.3. LOCALIZATION OF SYNAPSIN IA-EGFP IN CELLS TREATED WITH MICROTUBULE OR F-ACTIN DEPOLYMERIZING DRUGS

To determine whether or not the depolymerization of F-actin or microtubules led to a redistribution of synapsin Ia-EGFP, transfected HeLa cells were treated with cytochalasin B to depolymerize F-actin (Maruyama *et al.*, 1980) and nocodazole to depolymerize microtubules (Lee *et al.*, 1980). Fluorescence and immunofluorescence labeling and fluorescence microscopy were used to this end.

1.6.4. EFFECTS OF SYNAPSIN IA-EGFP OVEREXPRESSION ON REASSEMBLY OF DEPOLYMERIZED F-ACTIN AND MICROTUBULES

To test the effects of synapsin Ia-EGFP overexpression on the reassembly of depolymerized F-actin and microtubules, transfected HeLa cells were treated with cytochalasin B or nocodazole respectively. The drugs were washed off and reassembly of the cytoskeletal components was followed by fixation at various intervals after drug removal. Fluorescence and

immunofluorescence labeling and fluorescence microscopy were used to follow the reassemblies.

2. MATERIALS AND METHODS

2.1. GENERAL MOLECULAR CLONING TECHNIQUES

All molecular cloning and electrophoretic techniques were adapted from Sambrook *et al.* (1989).

2.1.1. AGAROSE GEL ELECTROPHORESIS

Agarose was supplied by either Gibco BRL (Burlington, ON) (Low Melting Point, LMP) or Sigma (Oakville, ON) (Low Electroendosmosis, Low EEO). Agarose gels were run for 1.5 hours at 60 volts in TAE buffer (0.04 M Tris-Acetate (pH 8.0) and 0.001 M EDTA in ddH₂O) in the 8.5 x 8 cm Owl Scientific (Portsmouth, NH) minigel Easy-Cast system. DNA samples for agarose gel electrophoresis were diluted 1:6 in DNA sample buffer IV (0.25 % (w/v) bromophenol blue (Sigma) and 40% (w/v) sucrose in ddH₂O). Gels were stained with 0.5 µg/ml ethidium bromide (in TAE running buffer) and viewed with UV light (312 nm). They were photographed using the Polaroid MP4+ instant camera and Polaroid Polapan 667 ISO 3000 film. Sizes of DNA were determined by simultaneously running Supercoiled DNA ladder (Gibco) for plasmids or 1-Kb DNA ladder (Gibco) for linear DNA.

2.1.2. SMALL-SCALE PLASMID DNA PURIFICATION

For small-scale purification of plasmid DNA (pET15b-synla, pBAD-synla and pSynla-EGFP), the Wizard® *Plus* miniprep DNA purification system (Promega Corporation, Madison, WI) was used and the protocol supplied by Promega (<http://www.promega.com/tbs/tb117/tb117.html>) was followed.

2.1.3. RESTRICTION DIGESTIONS

Simultaneous restriction digestion with *Xho*I (Gibco) and *Acc*65I (MBI Fermentas, Burlington, ON) was carried out for 5 hours at 37°C in a 20 µl reaction volume consisting of 0.2 to 1 µg of DNA, 4 µl Y⁺/TANGO buffer (MBI Fermentas; 66 mM Tris Acetate, 20 mM Magnesium nitrate, 132 mM Potassium acetate and 0.2 mg/ml BSA in ddH₂O (pH 7.9)), 5 units of *Xho*I and 5 units of *Acc*65I in ddH₂O. The digestions were stopped by adding 70 µl ddH₂O and 210 µl TE (10 mM Tris-Cl (pH 8.0) and 1 mM EDTA in ddH₂O). The DNA was then ethanol-precipitated by adding 1 ml of ice-cold 95% (v/v) ethanol/0.16 M sodium acetate solution and 10 µl of 2 µg/ml glycogen (in ddH₂O). After storing the mix at -20°C overnight, the DNA was pelleted at 10 000 x g at 4°C for 15 minutes. The pellets were washed with 70% ethanol and then dissolved in 25 µl TE buffer. All DNA was stored at -20°C.

2.1.4. AGAROSE GEL PURIFICATION OF DNA

Agarose gel purification of desired DNA was carried out as follows: DNA was run on a LMP agarose gel and the desired band was excised under 312 nm UV light. After melting the agarose at 70°C, the DNA was purified using the Wizard® PCR Preps DNA purification system (Promega) and following the protocol supplied by Promega (<http://www.promega.com/tbs/tb118/tb118.html>)

Figure 4. Representation of the pSynla-EGFP construct. The cDNA for rat synapsin Ia was cloned directly into pEGFP-N1 using *Xho*I and *Acc*651 restriction sites in the multiple cloning site (MCS). (Vector image obtained from:

<http://www.clontech.com/products/catalog01/Sec5/p178nterminalefpv.html>).

2.2. GENERATION OF PLASMID CONSTRUCTS

2.2.1. PCR AMPLIFICATION OF SYNAPSIN IA cDNA

The 2100-bp cDNA encoding full-length rat synapsin Ia was PCR-amplified from pET15b-synIa (a gift from Dr. Paul Greengard, Rockefeller University, NY). Upper and lower oligonucleotide PCR primers (5'-CCGCC-TCGAGGCCATATGAACTA-3' and 5'-CTAGGGGTACCTCGGAGAAGAGG-3' respectively) were synthesized by Gibco. These were engineered with *Xho*I (upper) and *Acc*65I (lower) restriction sites for direct cloning into the pEGFP-N1 vector (Figure 4; Clontech Laboratories Inc., Palo Alto, CA). This vector contains the constitutively active immediate early pCMV promoter. The PCR reaction was carried out in an AmpliTron II thermocycler from Barnstead/Thermolyne (Dubuque, IO) with a pre-dwell step (94°C for 4 minutes), 30 cycles of denaturation (94°C for 1 minute), annealing (50°C for 1 minute) and synthesis (72°C for 2.5 minutes) and a final post-dwell step (72°C for 10 minutes). The PCR reaction consisted of 100 pmol of each of the upper and lower primers, 3.0 µl of 10 mM dNTP mix, 3.0 µl of 50 mM MgCl₂, 20 ng of pET15b-synIa template and 2.5 units of *Taq* DNA polymerase in a PCR reaction buffer (50 mM KCl, 10 mM Tris-Cl (pH 8.3) and 0.01% (w/v) gelatin in ddH₂O). All reagents for PCR were purchased from Gibco.

2.2.2. CLONING OF SYNAPSIN IA cDNA INTO pBAD-TOPO®

The 2100 bp PCR product (with single 3' adenosine overhangs generated by *Taq* DNA polymerase terminal transferase activity) was gel-purified from a 0.7% (w/v) LMP agarose gel and then cloned directly into the

pBAD-TOPO® vector (Invitrogen Corporation, Carlsbad, CA). This vector is supplied linearized with 3' single thymidine overhangs and uses DNA Topoisomerase I for ligation. The resulting pBAD-synla plasmid was constructed to facilitate restriction digestion of the PCR product. The cloning reaction consisted of a mixture 4 µl of PCR product and 1 µl pBAD-TOPO® vector. After a 5-minute incubation at room temperature, the reaction was stopped by adding 1 µl of 6x TOPO® cloning stop solution (0.3 M NaCl and 0.06 M MgCl₂ in ddH₂O) and placement on ice. A sample of 2 µl of the reaction was added to a vial of One Shot™ *Escherichia coli* cells (Invitrogen), which was then incubated on ice for a further 25 minutes. The cells were heat shocked at 42°C for 30 seconds and then immediately transferred to ice and resuspended in 250 µl of SOC medium (Invitrogen; 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose in ddH₂O). After incubating the vial for 30 minutes at 37°C and 150 rpm, it was transferred to ice for 30 minutes and then a 50-µl sample was spread onto LB plates (1% (w/v) tryptone (Oxoid, Ottawa, ON), 0.5% (w/v) yeast extract (Oxoid), 1 % (w/v) NaCl and 1.5% (w/v) agar (BDH, Toronto, ON) in ddH₂O (pH 7.5)) supplemented with 50 µg/ml ampicillin (Sigma). Plasmid DNA was isolated from colonies by miniprep purification and its desired size (6200 bp) was confirmed on a 0.7% (w/v) Low EEO agarose gel. It was also simultaneously digested with *Xho*I and *Acc*65I to confirm the presence of two fragments with sizes of 4100 bp (vector) and 2100 bp (PCR product insert).

2.2.3. CLONING OF SYNAPSIN IA cDNA INTO PEGFP-N1

The 2100 bp *Xho*I-*Acc*65I synapsin Ia cDNA fragment from pBAD-synIa as well as pEGFP-N1 (4700 bp), also digested with *Xho*I and *Acc*65I, were each gel-purified from a 0.7% (w/v) LMP agarose gel. The gel-pure products were then ligated. The 10- μ l ligation reaction consisted of an excess of insert to vector DNA, 2 μ l of T4 DNA Ligase Buffer (Gibco; 100 mM Tris-HCl, 25 mM MgCl₂, 25 mM dithiothreitol, 250 μ g/ml BSA and 0.25 mM ATP in ddH₂O, pH 7.6) and 0.5 unit of T4 DNA ligase (Gibco) in ddH₂O. The reaction took place overnight at 16°C. Electrocompetent Top10F' *E. coli* (Invitrogen; 40 μ l sample) were transformed by electroporation with 2 μ l of the ligation reaction product (or no DNA as a negative control for transformation). The DNA/cells mixture was incubated on ice for 15 minutes and then transferred to 0.2-cm electroporation cuvettes (Invitrogen). Electroporation took place in a Bio-Rad (Mississauga, ON) *E. coli* Gene Pulser apparatus set at 1.8 volts. Immediately after electroporation, 950 μ l of SOC medium was added to the cells, and they were then incubated at 37°C and 250 rpm for 30 minutes. Samples (50 μ l) were plated on LB plates supplemented with 25 μ g/ml kanamycin (Sigma), and plates were grown overnight at 37°C.

Transformants were screened for the presence of the desired ligation product. To this end, colonies were used to inoculate 10 ml of LB with 25 μ g/ml kanamycin, and the cultures were grown overnight at 37°C and 250 rpm. Plasmid sizes were analysed on a 0.7% (w/v) Low EEO agarose gel (in TAE).

To restriction analyze putative pEGFP-synla plasmid DNA, DNA was digested with either *Acc65I* or *HindIII* (Gibco) or both simultaneously in a 8- μ l reaction volume. *Acc65I* would digest pEGFP-synla once at the 3' ligation site, and *HindIII* would cut the vector once within the insert. For digestion with *Acc65I*, the reaction consisted of 5 units of *Acc65I* and ~0.3 μ g DNA in Y+/TANGO buffer. For digestion with *HindIII*, the reaction consisted of 5 units of *HindIII* and 0.3 μ g DNA in REact® 2 buffer (50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 50 mM NaCl in ddH₂O; Gibco). For simultaneous digestion with *Acc65I* and *HindIII*, the reaction consisted of 5 units of each restriction enzyme and 0.3 μ g of DNA in Y+/TANGO buffer. All of these reactions took place for 5 hours at 37°C. Digestion products were analyzed on a 0.7% (w/v) Low EEO agarose gel (in TAE).

The vector pSynla-EGFP was obtained. This expresses rat synapsin Ia with the terminal aspartic acid converted to a glutamic acid and it is followed at its carboxy terminus by an 11-amino acid linker sequence (VPRARDPPVAT), and the EGFP tag. The sequence and frame of pSynla-EGFP were verified by automated DNA sequencing (carried out by Canadian Molecular Research Services, Ottawa, ON). The desired clones were restreaked on LB plates with 25 μ g/ml kanamycin to ensure clone purity. Glycerol stocks (15% (v/v) glycerol and 85% (v/v) overnight culture in LB with 25 μ g/ml kanamycin) were made of these clones by snap-freezing in liquid nitrogen and storage at -80°C.

2.3. LARGE-SCALE PURIFICATION OF PLASMID DNA FOR TRANSFECTION

For large-scale purification of pSynla-EGFP for transfection, samples from Top10F' *E. coli* glycerol stocks harbouring the pSynla-EGFP construct were used to inoculate 10 ml of LB supplemented with 25 µg/ml kanamycin. The starter cultures were grown for 8 hours at 37°C and 250 rpm. These were diluted 1:1000 in 500 ml of LB with 25 µg/ml kanamycin and grown overnight at 37°C and 250 rpm. Plasmid DNA was purified using the Wizard® megaprep DNA purification kit (Promega) following the protocol supplied by Promega (<http://www.promega.com/tbs/tb140/tb140.html>). The final product was dissolved in TE buffer that had been diluted 1:10 in ddH₂O. DNA concentration and purity were determined from absorbances at 260 nm and 280 nm using a Genequant® spectrophotometer (Amersham-Pharmacia Biotech, Baie d'Urfe, PQ).

2.4. EXPRESSION OF pSYNIA-EGFP IN HELA AND NIH/3T3 CELLS

2.4.1. CELL CULTURE

HeLa CCL-2 cells (American *Type Culture* Collection (ATCC), Rockville, MD) and NIH/3T3 cells (ATCC) were passaged every two days (when they had almost reached confluency). HeLa cells were seeded at 1×10^6 cells and NIH/3T3 cells at 5×10^5 cells per 100-mm tissue-culture grade dish (Corning, Corning, NY) in 10 ml of growth medium. HeLa cells were grown in α -modified

Eagle's medium (α -MEM; Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Cansera International Inc., Rexdale, ON) and 1% (v/v) of a 100x penicillin-streptomycin-fungizone antibiotic stock (Gibco). NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic stock.

When passing cells, the growth medium was removed, and after a brief rinse with TC-PBS (0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.02% (w/v) KH_2PO_4 and 0.115% (w/v) Na_2PO_4 in ddH₂O, pH 7.4), cells were lifted from the plates by 5- minute incubation with Trypsin-EDTA (1 mM EDTA (Sigma) and 0.025% (w/v) trypsin (Sigma) in TC-PBS) at 37°C. The action of trypsin was stopped by addition of growth medium (4 ml) and the cells were resuspended by vigorous pipetting to generate a single-cell suspension. Cells were counted using a BrightLine haemocytometer and centrifuged for 5 minutes at 1500 rpm. Cells were then resuspended in growth medium and plated.

For fluorescence microscopy, cells were seeded onto 22 x 22 mm glass coverslips (VWR Canlab, Mississauga, ON) with 4×10^4 cells per coverslip in 6-well Costar® tissue culture plates (Corning). For extractions, cells were seeded onto 60-mm tissue culture-grade dishes (Corning) at 2×10^5 cells per dish.

2.4.2. DNA TRANSFECTIONS

Expression vectors (either pSynla-EGFP or pEGFP-N1 (vector without insert)) were introduced into HeLa and NIH/3T3 cells by a calcium phosphate-mediated transient transfection method (Chen and Okayama, 1987) 24 hours after seeding onto coverslips or 60-mm dishes. The transfection cocktail was made as follows: after gentle mixing of 40 μ g of plasmid DNA with 50 μ l 2.5 M CaCl_2 (in ddH₂O) and ddH₂O to a 500- μ l volume, 500 μ l of BES buffer (50 mM N,N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (Sigma), 280 mM NaCl and 1.5 mM Na_2HPO_4 in ddH₂O, pH 6.86) was added dropwise and slowly to form a calcium phosphate-DNA precipitate. The mixture was incubated at room temperature for 20 minutes. After gentle mixing, 500 μ l of the mixture (20 μ g of DNA) was added dropwise to the cells on a single coverslip without removing the growth medium. The cells were incubated with the transfection mix for 7 hours at 37°C and 5% CO₂ in a humidified incubator. The precipitate was then removed by rinsing three times with TC-PBS and addition of fresh growth medium, and the cells were finally replaced in the incubator. After 40 hours, cells were fixed, treated with drugs and then fixed or extracted for total protein.

2.5. DRUG TREATMENTS

2.5.1. NOCODAZOLE

A stock solution of 5 mg/ml nocodazole in DMSO (both from Sigma) was kept at -20°C. For treatment of cells, growth medium was removed and replaced with growth medium supplemented with 10 μ g/ml nocodazole or with

0.2% (v/v) DMSO (as a control). After 1-hour incubation at 37°C and 5% CO₂ in a humidified incubator and in the presence of the drug, the cells were either fixed or the drug was washed off to follow the reassembly of depolymerized microtubules. The drug was washed off by rinsing cells three times with TC-PBS and adding fresh medium without drug. The cells were then replaced in the incubator and fixed at the desired intervals following the wash-off.

2.5.2. CYTOCHALASIN B

A stock solution of 10 mg/ml cytochalasin B (Sigma) in DMSO was kept at -20°C. For treatment of cells, growth medium was removed and replaced with growth medium supplemented with 10 µg/ml cytochalasin B or with 0.1% (v/v) DMSO (as a control). After 45-minute incubation at 37°C and 5% CO₂ in a humidified incubator and in the presence of the drug, the cells were either fixed or the drug was washed off to follow the reassembly of depolymerized F-actin. The drug was washed off by rinsing cells three times with TC-PBS and adding fresh medium without drug. The cells were then replaced in the incubator and fixed at the desired intervals following the wash-off.

2.5.3. TAXOL

A stock solution of 10 mM Paclitaxel (taxol; Sigma) in DMSO was kept at -20°C. For treatment of cells, growth medium was removed and replaced with growth medium supplemented with 5 µM taxol or with 0.05% (v/v) DMSO (as a control). The cells were fixed after 7-hour incubation at 37°C and 5% CO₂ in a humidified incubator and in the presence of the drug.

2.6. WESTERN BLOTTING OF WHOLE CELL EXTRACTS

2.6.1 TOTAL PROTEIN EXTRACTIONS FROM TRANSFECTED CELLS

To isolate total protein from transfected cells on 60-mm dishes, the cells were rinsed with PBS and then lysed on ice using 250 μ l of whole cell extraction buffer (25 mM Na_2PO_4 , 400 mM NaCl, 0.5% (w/v) SDS, 4 μ M phenylmethylsulfonyl fluoride, 40 μ g/ml aprotinin, 40 μ g/ml leupeptin and 40 μ g/ml pepstatin A (all from Sigma) in ddH₂O, pH 7.2). After scraping the viscous lysates into microfuge tubes, the samples were boiled for 10 minutes and then spun for another 10 minutes at 10 000 rpm and 4°C. The protein-containing supernatants were stored at -80°C.

2.6.2. PROTEIN QUANTITATION

Concentrations of protein samples were determined using the Bicinchoninic Acid (BCA) assay (Pierce). Briefly, a standard curve for absorbance at 562 nm was constructed using known quantities of BSA from a 2 mg/ml stock (Pierce). Samples were diluted 1:5 in ddH₂O. Standards and samples (20- μ l aliquots in triplicate) were loaded into a 96-well plate (Nalge Nunc International, Denmark) and then 200 μ l of Reagent A/B mix (Pierce) was added to each well for reaction. This mix had been made fresh from 49 parts reagent A (proprietary mix of sodium carbonate, sodium bicarbonate, sodium tartrate and 0.2 N sodium hydroxide) and 1 part reagent B (4% w/v copper sulfate in ddH₂O). The plate was incubated at 60°C for 30 minutes, and cooled to room temperature by brief incubation at -20°C. Absorbances at 562 nm

were measured using a Ceres 900 plate reader (Mandel Scientific Company, Guelph, ON).

2.6.3. SDS-PAGE OF TOTAL PROTEIN FROM TRANSFECTED CELLS

For separation of proteins, a discontinuous SDS-PAGE gel (Laemmli, 1970) was constructed in a Bio-Rad Mini-Protean® II apparatus equipped with 1.5-cm comb and spacers. Gels were composed of a 7.5 % separation gel (0.375 M Tris-Cl (pH 8.6), 0.1% (w/v) SDS, 7.5% (v/v) acrylamide(37.5)/bis-acrylamide(1), 0.1% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED, Boehringer-Mannheim, Laval, PQ) and 0.025% (w/v) ammonium persulfate (Sigma) in ddH₂O) and a 4% stacking gel (0.125 M Tris-Cl (pH 6.8), 0.1% (w/v) SDS, 4% (v/v) acrylamide(37.5)/bis-acrylamide(1), 0.1% (v/v) TEMED and 0.025% (w/v) ammonium persulfate). Total protein (30 µg) from transfected cells was diluted 1:2 in SDS gel-loading buffer (100 mM Tris-Cl (pH6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol (Sigma) in ddH₂O), and these mixtures were boiled for 5 minutes. These samples were loaded onto the gel alongside 7 µl of Biotinylated Broad Range Protein Ladder (Bio-Rad), also diluted 1:2 in loading buffer. Samples were run at 90 volts through the stacking gels and at 200 volts through the separation gels in Tris-glycine electrophoresis buffer (25 mM Tris-Cl, 250 mM glycine (Sigma) (pH 8.3) and 0.1% (w/v) SDS in ddH₂O).

2.6.4. ELECTROPHORETIC TRANSFER AND PROBING OF TOTAL PROTEIN WITH ANTI-EGFP-HRP

The gels were transferred overnight in a Bio-Rad mini-blotting apparatus to Biotran nitrocellulose (Scheichler and Schuell, Keene, NH) at 30 volts and 4°C in Transfer buffer (39 mM glycine, 48 mM Tris-Cl, 0.037% (w/v) SDS and 20% (v/v) methanol in ddH₂O). After briefly rinsing the blot with ddH₂O, the success of the transfer was assessed by staining for 2 minutes with 0.5 % (w/v) Ponceau S Red in 3% (v/v) trichloroacetic acid and ddH₂O. The blot was then destained for 10 minutes in PBS (with gentle agitation) and blocked for 1 hour in 5% (w/v) Carnation skim milk powder in PBS (with gentle agitation). Next, the blot was incubated for 1 hour on a nutator with the antibody-enzyme complex anti-EGFP-HRP (Clontech) diluted 1:300 in 2% (w/v) skim milk (in PBS). Unbound antibody-enzyme was washed away with three 5-minute washes in PBS. The biotinylated ladder was detected by incubating the blot for 1 hour in streptavidin-biotin-HRP (Amersham; diluted 1:5000 in PBS). After three final 5-minute washes in PBS, antibody binding was detected by enhanced chemiluminescence (ECL plus; Amersham) using the protocol supplied by the manufacturer and Hyperfilm™ ECL film (Amersham). Exposed films were digitized using a Plustek flatbed scanner. Digitized JPEG images were processed for optimal brightness and contrast using Adobe PhotoDeluxe v2.0.

2.7. MICROSCOPY

2.7.1. FIXATION

Cells were simultaneously fixed and extracted using a protocol described by Falconer *et al.* (1992) with slight modifications. Coverslips were rinsed briefly with PEM buffer (80 mM PIPES (pH 6.8), 10 mM EGTA and 2 mM MgCl₂ (all from Sigma) in ddH₂O) followed by a 10-minute fixation/extraction step in 4% (v/v) paraformaldehyde (BDH), 0.25% (v/v) glutaraldehyde (Pierce, Rockford, IL) and 0.5% (v/v) Triton X-100 (Pierce) in PEM buffer and finally three five-minute washes in PBS (13 mM NaCl, 0.5 mM Na₂HPO₄ and 0.15 mM KH₂PO₄ in ddH₂O, pH 7.0).

2.7.2. FLUORESCENT AND IMMUNOFLUORESCENT LABELING

After fixation of the samples, free aldehyde groups were reduced by washing with 0.1% (w/v) NaBH₄ (three times, 4 minutes per wash) in PBS. This was followed by three 5-minute washes in PBS. Antibody, rhodamine-phalloidin (Molecular Probes Inc., Eugene, OR), and Alexa™ (350)-phalloidin (Molecular Probes Inc.) incubations were for 45 minutes in a humidified chamber. Three 5-minute PBS washes followed every antibody or phalloidin incubation. Monoclonal mouse anti- α -tubulin 5A6 IgG (Aitchison and Brown, 1986) was diluted 1:10000 in PBS, both rhodamine-phalloidin and Alexa™ (350)-phalloidin were diluted 1:25 in PBS and monoclonal mouse anti-vimentin IgG₁ clone V9 (Sigma) was diluted 1:4000 in PBS. All coverslips treated with mouse monoclonal antibodies were next treated with the secondary antibody

polyclonal donkey anti-mouse conjugated to cy3 (diluted 1:400 in PBS) (Jackson ImmunoResearch Inc., West Grove, PA). To visualize DNA, some samples were treated with 1 mg/ml Hoechst 33258 (diluted 1:3000 in PBS) for 1 minute, followed by 3 five-minute washes in PBS. All coverslips were mounted in mounting medium (0.1% (w/v) p-phenylenediamine, 79.2% (v/v) glycerol (pH 7.0) in PBS).

2.7.3. FLUORESCENCE MICROSCOPY

Samples were visualized using the Zeiss Universal epifluorescence microscope which was equipped with a 50 watt mercury burner. Images were digitally captured using the Hamamatsu integrating chilled CCD camera and MetaMorph® v4.0 software (Universal Imaging Corporation, Downingtown, PA). Tagged image file format (TIFF) images were processed for optimal brightness and contrast in MetaMorph® v4.0 and Adobe Photoshop v4.0.1.

2.7.4. CONFOCAL LASER SCANNING MICROSCOPY

For confocal microscopy, specimens were observed using a 63x-oil objective lens on a Leitz Confocal Laser Scanning Microscope equipped with a 50 milliWatt Argon/Krypton mixed gas laser. Optical sections through the z-axis of cells were taken at 0.5- μ m intervals. Images were recorded as 8-bit bitmap images and then converted to 8-bit tagged image file format images using MetaMorph v4.0 software. Image processing, which included superimposition of optical sections and optimization of brightness and contrast, was carried out using MetaMorph v4.0 and Adobe Photoshop v4.0.1.

3. RESULTS

3.1. GENERATION AND EXPRESSION OF SYNAPSIN IA-EGFP

EXPRESSION CONSTRUCT pSYNIA-EGFP

3.1.1. PCR AMPLIFICATION OF SYNAPSIN IA RAT cDNA FROM pET15B-SYNIA AND pBAD-TOPO CLONING OF PCR PRODUCT.

In order to obtain synapsin Ia rat cDNA for cloning into pEGFP-N1, it was PCR amplified from pET15b-synIa. Upper and lower PCR primers were engineered with *Xho*I and *Acc*65I restriction sites respectively so that the product could be cloned directly into the multiple cloning site of pEGFP-N1. A 2.1-kb PCR product (the size of rat synapsin Ia cDNA) was obtained (data not shown). The PCR product was gel-purified and cloned directly into the pBAD-TOPO vector to yield pBAD-synIa (figure 5A). This step facilitated PCR product restriction digestion with *Xho*I and *Acc*65I.

3.1.2. CLONING OF SYNAPSIN IA cDNA FROM pBAD-SYNIA INTO pEGFP-N1.

Both plasmids pBAD-synIa and pEGFP-N1 were simultaneously digested with *Xho*I and *Acc*65I. Digestion of pBAD-synIa yielded the two expected fragments of 4.1-kb (linearized vector) and 2.1-kb (insert) (data not shown). Digestion of pEGFP-N1 produced a single 4.7-kb linear fragment (data not shown). The 2.1-kb synapsin Ia fragment and the 4.7-kb pEGFP-N1 fragment were gel-purified (figure 5B) and the gel-pure products were ligated.

After transforming Top10F' *E. coli* with the ligation mix, plasmid DNA was isolated from eight positive clones for size analysis. All eight clones

Figure 5. Cloning of rat synapsin Ia cDNA into pEGFP-N1 (Clontech).

Agarose (0.7%) gels were stained with 0.5 µg/ml ethidium and photographed under 312 nm UV light. (A) ~1 µg of pBAD-synIa (6.2 kb). This plasmid was constructed by direct cloning of rat synapsin Ia cDNA (2.1 kb) into pBAD-TOPO (4.1 kb) (Invitrogen). Synapsin Ia cDNA had been PCR amplified from pET15b-synIa using upper and lower primers engineered with *Xho*I and *Acc*65I restriction sites respectively. (B) *Xho*I and *Acc*65I digests of synapsin Ia cDNA (2.1 kb; from pBAD-synIa) and pEGFP-N1 (4.7 kb). ~0.1 µg of gel-purified samples of each were loaded. These digestions enabled direct cloning of synapsin Ia cDNA into the MCS of pEGFP-N1. (C) ~1 µg of putative pSynIa-EGFP construct (~6.8 kb). The two samples shown in B were ligated using T4 DNA ligase (Gibco) and the ligation mix was transformed into competent Top10F' *E. coli*. This miniprep-purified plasmid DNA from a kanamycin-resistant clone was the expected size. (D) Restriction analysis of putative pSynIa-EGFP from C. The plasmid was digested with *Acc*65I (which should cut at the 3' ligation site yielding one 6.8-kb fragment), *Hind*III (which should cut once within the insert yielding one 6.8-kb fragment), or both (which should yield one 1.4-kb fragment and one 5.4-kb fragment). ~1 µg samples of the digests were loaded.

harboured a plasmid of the desired size (6.8-kb; see for example one in figure 5C). One of these plasmids was restriction analyzed for the presence of the insert by digestion with *Acc65I* or *HindIII* or both. *Acc65I* would digest pSynla-EGFP (the desired construct) at the 3' site of ligation, while *HindIII* would cut once within the synapsin Ia cDNA insert. If pSynla-EGFP was cut with both enzymes, one 1.4-kb fragment and one 5.4-kb fragment would be produced. After confirming that the expected restriction patterns had been obtained (figure 5D), the sequence and frame of the construct were confirmed by automated DNA sequencing (data not shown).

3.1.3. FIDELITY OF EXPRESSION OF SYNAPSIN IA-EGFP (SYNIA-EGFP) IN HeLa AND NIH/3T3 CELLS.

HeLa human cervical carcinoma and NIH/3T3 mouse fibroblast cells were transfected with the pSynla-EGFP construct. Transfected cells were extracted for total protein to enable verification of both the molecular weight and integrity of the expressed syn Ia-EGFP fusion protein. As positive and negative controls, total protein was extracted from cells transfected with pEGFP-N1 (vector without insert) and from untransfected cells respectively. Total cell protein in all cases was separated by SDS-PAGE and then transferred electrophoretically to nitrocellulose. These blots were probed with an anti-EGFP-horseradish peroxidase antibody-enzyme complex, and signals were detected by enhanced chemiluminescence (ECL). The syn Ia-EGFP fusion protein and EGFP (positive control) were expressed with the expected molecular weights (~101 kDa and ~27 kDa respectively) in both transfected

HeLa (figure 6A) and NIH/3T3 cells (figure 6B). Furthermore, the single syn Ia-EGFP and EGFP bands seen on both blots indicated that the cells were not degrading the foreign proteins. There was no signal in the negative control lane (total protein from untransfected cells) on either blot. There is a lower level of expression of syn Ia-EGFP in NIH/3T3 cells (figure 6B) compared to HeLa cells (figure 6A). Fixation of transfected cells by Zamboni's precipitation-fixation method, which fixes both soluble and polymeric cellular components (Stefanini *et al.*, 1967), demonstrated that the transfection efficiency for the pSynIa-EGFP construct was significantly lower in NIH/3T3 cells than in HeLa cells (data not shown).

3.2. COMPARISON OF SYNIA-EGFP AND F-ACTIN LOCALIZATIONS IN HELA AND NIH/3T3 CELLS

3.2.1. SYN IA-EGFP COLOCALIZES WITH F-ACTIN IN HELA CELLS BUT DOES NOT ALTER F-ACTIN ORGANIZATION.

To determine whether or not synapsin Ia colocalizes with F-actin, we transiently expressed synapsin Ia-EGFP in HeLa cells and stained F-actin with rhodamine-phalloidin. The cells were fixed by a simultaneous fixation/extraction method (Falconer *et al.*, 1992) which, while fixing, extracts soluble cellular components leaving mostly insoluble components along with anything bound to these polymers. It should be noted that in all of the overlays generated by Metamorph v4.0, a yellow colour is indicative of coincidence of the green and red signals and often does not reflect colocalization. In order to

Figure 6. Fidelity of expression of syn Ia-EGFP in A) HeLa cells and B) NIH/3T3 cells as demonstrated by Western blotting. Forty hours after transfection, total protein was extracted from cells transfected with either pSynIa-EGFP or pEGFP-N1 (positive control) or from untransfected cells (negative control). Protein samples (30 µg) were separated by SDS-PAGE, and gels were blotted onto nitrocellulose. The blots were probed with anti-EGFP-HRP antibody-enzyme complex (Clontech). This complex enables direct detection of EGFP-tagged proteins using ECL.

A **B**

determine if colocalization is occurring, these overlays are used in combination with a visual comparison of EGFP fusion protein localization and cytoskeletal localization. Using conventional fluorescence microscopy, EGFP alone was found to be diffuse in the nucleus and cytoplasm (figure 7 B-B"). Synapsin Ia-EGFP was also found in the cytoplasm, especially in the perinuclear region. It was however significantly colocalized with F-actin in microspikes and membrane ruffles (figure 7 A-A").

Confocal microscopy showed the same results with more clarity (figure 8 A-A" and B-B") and also demonstrated a colocalization of synIa-EGFP with F-actin-rich spots and with the cleavage furrow of a cell undergoing cytokinesis (figure 8 B-B"). An optical section through the attached bottom of cells revealed that much of the syn Ia-EGFP which seemed diffusely localized to the perinuclear region was actually colocalized with F-actin (figure 8 A-A").

No significant colocalization of syn Ia-EGFP with F-actin in the stress fibers was noted, and there was no noticeable difference in the extent of F-actin bundling between cells expressing EGFP alone and those expressing synapsin Ia-EGFP (compare figures 7A" and 7B").

3.2.2. SYN IA-EGFP COLOCALIZES WITH F-ACTIN IN STRESS FIBERS IN NIH/3T3 CELLS BUT DOES NOT ALTER F-ACTIN ORGANIZATION.

In transfected NIH/3T3 cells, F-actin organization was again not apparently altered by syn Ia-EGFP overexpression (compare figures 9A" and 9B") and EGFP alone was diffuse in the nucleus and cytoplasm (figure 9 B-B"). However, at variance with the results obtained using HeLa cells, syn Ia-EGFP

Figure 7. Colocalization of syn Ia-EGFP with F-actin in HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing either syn Ia-EGFP (A') or EGFP (B') were stained for F-actin using rhodamine phalloidin (1:25 dilution; A'' and B'' respectively) and for DNA using Hoechst (A and B respectively). Cells were simultaneously fixed and extracted with a 4% paraformaldehyde, 0.5% Triton X-100, 0.25% glutaraldehyde solution. Images A''' and B''' are syn Ia-EGFP/F-actin and EGFP/F-actin overlays respectively. Bar, 20 μ m.

Figure 8. Colocalization of synapsin Ia-EGFP with F-actin in HeLa cells as demonstrated by confocal laser scanning microscopy. HeLa cells transiently expressing syn Ia-EGFP (A and B) were fixed and stained for F-actin (A' and B' respectively) as described in the legend for figure 7. Images A-A'' represent an optical section in the perinuclear region at the bottom of cells. Images B-B'' are a superimposition of 0.5- μ m optical sections through the z-axis of cells. Images A'' and B'' are syn Ia-EGFP/F-actin overlays. Bar, 20 μ m.

Figure 9. Colocalization of synapsin Ia-EGFP with F-actin in NIH/3T3 cells as demonstrated by conventional fluorescence microscopy. NIH/3T3 cells transiently expressing either syn Ia-EGFP (A') or EGFP (B') were fixed and stained for F-actin (A'' and B'' respectively) and for DNA (A and B respectively) as described in the legend for figure 7. Images A''' and B''' are syn Ia-EGFP/F-actin and EGFP/F-actin overlays respectively. Bar, 20 μ m.

was significantly colocalized with F-actin in stress fibers in these cells (figure 9 A-A''). As in HeLa cells, syn Ia-EGFP also colocalized with F-actin-rich spots (see overlay figure 9 A'').

3.3. COMPARISON OF SYNIA-EGFP AND MICROTUBULE LOCALIZATIONS IN HELA AND NIH/3T3 CELLS

3.3.1. SYN IA-EGFP DOES NOT COLOCALIZE WITH MICROTUBULES IN EITHER HELA OR NIH/3T3 CELLS NOR ALTER THEIR ORGANIZATION.

Staining of α -tubulin (using 5A6 mouse monoclonal anti-tubulin primary antibody and donkey anti-mouse-cy3 secondary antibody) demonstrated that syn Ia-EGFP did not colocalize with microtubules in either HeLa cells (figure 10 A-A'') nor NIH/3T3 cells (figure 11 A-A''). Overexpression of syn Ia-EGFP did not appear to alter microtubule organization nor elicit microtubule bundle formation compared to overexpression of EGFP alone (compare figure 10 A'' with 10 B'' and figure 11 A'' with 11 B''). When PBS was substituted for the primary antibody to control for non-specific secondary antibody binding, no signal was observed under the cy3 channel (figures 12 A-A'').

Figure 10. Lack of colocalization of synapsin Ia-EGFP with microtubules in HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A') and EGFP (B') were fixed as described in the legend for figure 7 and stained for microtubules with 5A6 mouse monoclonal anti- α -tubulin IgG (1:10000 dilution) and donkey anti-mouse-cy3 secondary antibody (1:400 dilution) (A'' and B'' respectively) and for DNA using Hoechst (A and B respectively). Images A''' and B''' are syn Ia-EGFP/microtubule and EGFP/microtubule overlays respectively. Bar, 20 μ m.

Figure 11. Lack of colocalization of synapsin Ia-EGFP with microtubules in NIH/3T3 cells as demonstrated by conventional fluorescence microscopy. NIH/3T3 cells transiently expressing syn Ia-EGFP (A') or EGFP (B') were fixed as described in the legend for figure 7 and stained for DNA (A and B respectively) and microtubules (A'' and B'' respectively) as described in the legend for figure 10. Images A''' and B''' are syn Ia-EGFP/microtubule and EGFP/microtubule overlays respectively. Bar, 20 μ m.

Figure 12. Control for non-specific binding by donkey anti-mouse-cy3 secondary antibody. HeLa cells transiently expressing syn Ia-EGFP (A') were fixed as described in the legend for figure 7, stained for DNA using Hoechst (A) and treated with PBS (instead of a primary antibody) followed by donkey anti-mouse cy3 antibody (1:400; A"). Bar, 20 μ m.

3.4. COMPARISON OF SYNIA-EGFP AND VIMENTIN (INTERMEDIATE FILAMENT) LOCALIZATIONS IN HELA CELLS

3.4.1. SYN IA-EGFP DOES NOT COLOCALIZE WITH VIMENTIN IN HELA CELLS NOR ALTER ITS ORGANIZATION.

HeLa cells stained for vimentin (using V9 mouse monoclonal anti-vimentin primary antibody and donkey anti-mouse-cy3 secondary antibody) demonstrated that syn Ia-EGFP did not colocalize with this intermediate filament system (figure 13 A-A"). The antibody does not cross-react with mouse cells, and so was not useable on NIH/3T3 cells. Overexpression of syn Ia-EGFP did not appear to alter vimentin organization compared to overexpression of EGFP alone (compare figures 13 A" and 13 B").

3.5. CYTOCHALASIN B TREATMENT OF HELA CELLS TRANSFECTED WITH PEGFP-SYNIA

3.5.1. SYN IA-EGFP COLOCALIZES WITH CYTOCHALASIN B-TREATED F-ACTIN.

When HeLa cells expressing syn Ia-EGFP or EGFP were treated for 45 minutes with 10 µg/ml cytochalasin B, the F-actin fibres were no longer visible and instead F-actin was concentrated in discrete spots (figures 14 B-B" and 14 C-C" respectively). This effect was specific to the drug as demonstrated by normal F-actin distribution in control syn Ia-EGFP-expressing cells treated with 0.1% DMSO (figure 14 A-A'). In cytochalasin B-treated cells, syn Ia-EGFP

Figure 13. Lack of colocalization of synapsin Ia-EGFP with vimentin in HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A') or EGFP (B') were fixed as described in the legend for figure 7 and stained for DNA using Hoechst (A and B respectively) and for vimentin (A'' and B'' respectively) using V9 mouse monoclonal anti-vimentin primary antibody (1:4000) and donkey anti-mouse cy3 secondary antibody (1:400). Images A''' and B''' are syn Ia-EGFP/vimentin and EGFP/vimentin overlays respectively. Bar, 20 μ m.

Figure 14. Colocalization of synapsin Ia-EGFP with F-actin spots in cytochalasin B-treated HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A and B) or EGFP (C) were treated with 10 μ g/ml cytochalasin B for 45 minutes (B-B'' and C-C'') or with 0.1% DMSO for 45 minutes (A-A'). Cells were fixed as described in the legend for figure 7 and F-actin was stained with rhodamine phalloidin (1:25; A', B' and C'). B'' and C'' are syn Ia-EGFP/F-actin and EGFP/F-actin overlays respectively. Bar, 20 μ m.

colocalized with F-actin in these spots (see overlay figure 14 B'') whereas EGFP alone did not (see overlay figure 14 C'').

3.5.2. SYN IA-EGFP OVEREXPRESSION IN HELA CELLS DOES NOT AFFECT REASSEMBLY OF F-ACTIN FOLLOWING CYTOCHALASIN B TREATMENT BUT COLOCALIZES WITH F-ACTIN THROUGHOUT ITS REASSEMBLY.

HeLa cells expressing syn Ia-EGFP were treated as before with cytochalasin B and then the drug was removed and the cells were simultaneously fixed and extracted at 0 minutes (figure 15 A-A''), 15 minutes (figure 15 B-B''), 60 minutes (figure 15 C-C'') and 24 hours (figure 15 D-D'') following drug removal. At each time point, syn Ia-EGFP was colocalized with F-actin in spots and in membrane ruffles (see overlays figures 15 A'', B'', C'' and D''). Stress fiber colocalization of syn Ia-EGFP in interphase cells was again not noted after reassembly was complete (see overlay figure 15 D''). The reassembly of F-actin over the time-course was not overtly affected by overexpression of syn Ia-EGFP (compare transfected and untransfected cells in figures 15 A', B', C' and D').

Figure 15. Colocalization of synapsin Ia-EGFP with F-actin and lack of effect of synapsin Ia-EGFP overexpression on F-actin reassembly over a time-course of cytochalasin B wash-off in HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A, B, C and D) were treated with 10 $\mu\text{g/ml}$ cytochalasin B for 45 minutes. Cells were fixed (as described in the legend for figure 7) at 0 minutes (A-A"), 15 minutes (B-B"), 60 minutes (C-C") or 24 hours (D-D") following removal of the drug. F-actin was stained with rhodamine-phalloidin (1:25; A', B', C' and D'). Images A", B", C" and D" are the respective syn Ia-EGFP/F-actin overlays. Bar, 20 μm .

3.6. NOCODAZOLE TREATMENT OF HeLa CELLS TRANSFECTED WITH pSYNIA-EGFP

3.6.1. SYN IA-EGFP DOES NOT COLOCALIZE WITH MICROTUBULES NOR VIMENTIN IN NOCODAZOLE-TREATED HELA CELLS, BUT DOES COLOCALIZE WITH F-ACTIN.

When HeLa cells expressing syn Ia-EGFP or EGFP were treated for 1 hour with 10 µg/ml nocodazole, the microtubule web was no longer visible and only a few “spindly” microtubules remained (figures 16 B' and 16 C' respectively). This effect was specific to the drug as demonstrated by normal microtubule distribution in control synIa-EGFP-expressing cells treated with 0.2% DMSO (figure 16 A-A'). The treatment also led to the collapse of the vimentin intermediate filament network into a mass in the perinuclear region (figure 17 B'). In nocodazole-treated cells, syn Ia-EGFP did not colocalize with the remaining microtubules (figure 16 B-B''), nor vimentin (figure 17 B-B'') nor did it become extracted during simultaneous fixation/extraction (figures 16 B, 17 A and 17 B). However, syn Ia-EGFP did colocalize with F-actin following this treatment (figure 17 A-A').

3.6.2. SYN IA-EGFP OVEREXPRESSION DOES NOT AFFECT REASSEMBLY OF MICROTUBULES FOLLOWING NOCODAZOLE TREATMENT.

HeLa cells expressing syn Ia-EGFP were treated with nocodazole as before and then the drug was removed and the cells were simultaneously fixed and extracted at 0 minutes (figure 18 A-A'), 15 minutes (figure 18 B-B'), 60

Figure 16. Localization of synapsin Ia-EGFP compared to microtubules in HeLa cells treated with nocodazole as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A and B) or EGFP (C) were treated with 10 μ g/ml nocodazole for 1 hour (B-B" and C-C") or with 0.2% DMSO for 1 hour (A-A'). Cells were fixed as described in the legend for figure 7 and were stained for microtubules (A', B' and C') as described in the legend for figure 10. B" and C" are syn Ia-EGFP/microtubule and EGFP/microtubule overlays respectively. Bar, 20 μ m.

Figure 17. Colocalization of synapsin Ia-EGFP with F-actin and lack of colocalization of synapsin Ia-EGFP with vimentin in nocodazole-treated HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A, B) were treated with 10 $\mu\text{g/ml}$ nocodazole for 1 hour. Cells were fixed as described in the legend for figure 7. F-actin was stained with rhodamine phalloidin (1:25; A') and vimentin was stained with V9 mouse monoclonal anti-vimentin primary antibody (1:4000) and donkey anti-mouse-cy3 secondary antibody (1:400; B'). Images A'' and B'' are syn Ia-EGFP/F-actin and syn Ia-EGFP/vimentin overlays respectively. Bar, 20 μm .

Figure 18. Lack of effect of synapsin Ia-EGFP overexpression on microtubule reassembly over a time-course of nocodazole wash-off in HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A, B, C and D) were treated with 10 μ g/ml nocodazole for 1 hour. Cells were fixed (as described in the legend for figure 7) at 0 minutes (A-A'), 15 minutes (B-B'), 60 minutes (C-C') or 24 hours (D-D') following removal of the drug. Microtubules were stained as described in the legend for figure 10 (A', B', C' and D'). Bar, 20 μ m.

minutes (figure 18 C-C') and 24 hours (figure 18 D-D') following drug removal. Compared to untransfected cells, overexpression of syn Ia-EGFP did not overtly affect the reassembly of microtubules over the time-course (see figures 18 A', B', C' and D').

3.7. TAXOL TREATMENT OF HeLa CELLS TRANSFECTED WITH pSYNIA-EGFP

3.7.1. SYN IA-EGFP COLOCALIZES WITH MICROTUBULE BUNDLES IN TAXOL-TREATED HeLa CELLS

When HeLa cells expressing syn Ia-EGFP or EGFP alone were treated with 5 μ M taxol for 7 hours, microtubules no longer originated from a microtubule-organizing centre, and often formed bundles (figures 19 B' and C' respectively). Control cells expressing syn Ia-EGFP treated with 0.05% DMSO did not exhibit these effects and so they were specific to the drug (figure 19 A-A'). In cells treated with taxol, syn Ia-EGFP colocalized with microtubule bundles and not as much with F-actin (figure 19 B-B''). EGFP alone colocalized with neither microtubules nor F-actin and as before was found mostly in the nucleus (figure 19 C-C'').

Figure 19. Colocalization of synapsin Ia-EGFP with microtubule bundles in taxol-treated HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A and B) or EGFP (C) were treated for 7 hours with 5 μ M taxol (B-B'' and C-C'') or 0.05% DMSO (A-A') and fixed as described in the legend for figure 7. Microtubules (A', B' and C') were stained as described in the legend for figure 10. F-actin (B'' and C'') was stained with alexa(350)-phalloidin (1:25). Images B'' and C'' are syn Ia-EGFP/microtubule and EGFP/microtubule overlays respectively. Bar, 20 μ m.

4. DISCUSSION

4.1 INTERACTION OF SYNAPSIN IA-EGFP WITH F-ACTIN IN HELA AND NIH/3T3 CELLS

In vitro studies have previously shown that dephosphorylated synapsin I binds to actin filaments and bundles them (Petrucci and Morrow, 1987; Bahler and Greengard, 1987). Our results provide evidence that synapsin I does indeed bind to F-actin *in vivo*. When HeLa (figures 7 and 8) and NIH/3T3 cells (figure 9) were simultaneously fixed and extracted with Triton X-100, synapsin Ia-EGFP was found to colocalize with F-actin. The extraction step, which removed most soluble cellular components, suggests that synapsin I was bound to F-actin, although the possibility that this interaction is indirect (i.e. through another actin-binding protein) cannot be excluded based on our results alone. Although a significant amount of EGFP remained after the extraction step (figures 7 and 9), EGFP was mainly in the nucleus and did not colocalize with F-actin, indicating that the fusion tag was not responsible for syn Ia-EGFP-F-actin colocalization. Furthermore, when Zamboni's precipitation-fixation method had been employed to fix both soluble and polymeric cellular components (Stefanini *et al.*, 1967), significantly more EGFP was present in the cytoplasm compared to following the simultaneous fixation/extraction method (data not shown). It is probable that the Triton X-100 detergent was less efficient in the extraction of EGFP from the nucleus because of delayed access to the nuclear envelope.

When HeLa cells were treated with cytochalasin B, as expected the F-actin network ceased to exist and instead F-actin was concentrated in spots (figure 14). Synapsin Ia-EGFP but not EGFP alone was also concentrated at these spots following the treatment. HeLa cells treated with the microtubule-depolymerizing drug nocodazole still showed syn Ia-EGFP-F-actin colocalization (figure 18). These results also provide *in vivo* evidence for an interaction between synapsin I and F-actin.

While syn Ia-EGFP was found at stress fibers in NIH/3T3 cells (figure 9), it did not colocalize with stress fibers in HeLa cells (figures 7 and 8). This inconsistency may be due to differences in cytoskeletal architecture or cellular environments between the two cell lines. On the other hand, rat synapsin Ia may behave differently with respect to F-actin in a rodent cell line versus a human cell line. The latter possibility seems unlikely since, at the amino acid level, synapsin I domain C which binds to F-actin is 99% identical between rat and human (Bahler *et al.*, 1989; Kao *et al.*, 1999).

Dephosphorylated synapsin I also binds to G-actin *in vitro* (Ceccaldi *et al.*, 1993) forming a F-actin nucleus which acts to accelerate the initial rate of actin polymerization (Valtorta *et al.*, 1992a; Fesce *et al.*, 1992; Valtorta *et al.*, 1992b). It is interesting that, particularly in HeLa cells (figures 7 and 8), synapsin I was especially colocalized with F-actin in microspikes and membrane ruffles and in the cleavage furrow of cells undergoing cytokinesis. These are regions where actin assembly and disassembly are most dynamic (Borisov and Svitkina, 2000 for review) and therefore the findings suggest a role

for synapsin I in actin dynamics. When cytochalasin B was removed from HeLa cells and these were fixed at various times following drug removal (figure 15), there was no overt acceleration in F-actin assembly caused by syn Ia-EGFP overexpression. However, syn Ia-EGFP colocalized with F-actin at the cell periphery throughout F-actin reassembly. In order to get an accurate depiction of the effects of syn Ia-EGFP overexpression on actin dynamics, one would need to observe the transfected cells in real-time.

Our results showing colocalization of syn Ia-EGFP with F-actin especially at the plasma membrane correlate well with observations made by Torri-Tarelli *et al.* (1990). When they applied α -latrotoxin to frog nerve-muscle preparations in the absence of extracellular Ca^{2+} , exocytosis of SVs occurred but their endocytosis was blocked. With time, synapsin I became localized at the axolamella and remained there. They hypothesized that this resulted from a synapsin I-actin interaction that persisted after SV fusion. Synapsin I is also localized at low levels on docked vesicles (Pieribone *et al.*, 1995). In addition, Hilfiker *et al.* (1998) hypothesized that domain E of synapsin I is intimately involved in the SV release process itself since its injection into the giant presynaptic terminal of squid slowed the kinetics of neurotransmitter release.

In both HeLa (figures 7 and 8) and NIH/3T3 cells (figure 9), syn Ia-EGFP colocalized with F-actin in spots. A similar localization of the actin-binding protein cortactin was recently observed in NIH/3T3 cells (Kaksonen *et al.*, 2000). These F-actin-containing spots were found to propel endosomal vesicles (Kaksonen *et al.*, 2000). Dynamic F-actin spots have also been

reported in rhodamine-actin-incorporated PtK1 fibroblasts (Schafer *et al.*, 1998). If the spots seen in our results are indeed these regions of constant actin assembly and disassembly on endosomal vesicles, then the results are interesting for two reasons. Firstly, they further implicate synapsin I in a role in actin dynamics. Secondly, they suggest that in these non-neuronal cells, synapsin I may bind simultaneously to endosomal vesicles and F-actin, not unlike its ability to bind simultaneously to synaptic vesicles and F-actin in neurons.

Bundling of actin filaments by synapsin I *in vitro* has been attributed to both its ability to self-associate (Hosaka and Sudhof, 1999) and to the possible presence of an additional actin-binding site in domain D (Bahler *et al.*, 1989; Valtorta *et al.*, 1992a). However, synapsin II, which can also bundle F-actin *in vitro* (Chilcote *et al.*, 1994; Nielander *et al.*, 1997), does not contain a domain D, suggesting that the former reasoning is more likely. However, it is not known whether or not synapsins can homo- and/or heterodimerize *in vivo*. Our results do not show any actin bundling resulting from synapsin Ia-EGFP overexpression in either HeLa or NIH/3T3 cells. The components of the *in vitro* experiments included at most dephosphorylated synapsin I, F-actin and synapsin I-depleted synaptic vesicles (Petrucci and Morrow, 1987; Bahler and Greengard, 1987; Ceccaldi *et al.*, 1995). In the living neuron, there are likely many other components to which synapsin I can bind, including spectrin (Sikorski *et al.*, 1991; Iga *et al.*, 1997), neurofilaments (Goldenring *et al.*, 1986; Steiner *et al.*, 1987) and calmodulin (Baines *et al.*, 1994) which incidentally is

also found in HeLa cells (Murray and Ward, 1984) and NIH/3T3 cells (Zippel *et al.*, 2000). If synapsin I did indeed bind to all of these cellular components *in vivo*, the probability that it will bind to itself would be greatly reduced, and therefore bundling of F-actin by synapsin I would be unlikely. In addition, we did not test the phosphorylation state of our exogenous synapsin Ia-EGFP. Finally, unlike free synapsin I, synaptic vesicle-bound synapsin I does not lead to actin bundle formation *in vitro* (Benfenati *et al.*, 1992). Taken together, these findings suggest that F-actin bundling by synapsin I does not occur *in vivo*.

4.2. INTERACTION OF SYNAPSIN IA-EGFP WITH MICROTUBULES IN HELa AND NIH/3T3 CELLS

Synapsin I also binds to microtubules *in vitro*, but it does not reduce the critical concentration for microtubule assembly nor bind to unpolymerized tubulin dimers (Baines and Bennett, 1986). At high concentrations, it induces microtubule bundle formation, possibly due to microtubule binding regions in both the head and tail domains, or alternatively to the ability of synapsin I to self-associate (Baines and Bennett, 1986; Hirokawa *et al.*, 1989; Bennett *et al.*, 1991; Bennett and Baines, 1992; Hosaka and Sudhof, 1999). We did not see any colocalization of synapsin Ia-EGFP with microtubules in either HeLa cells (Figure 11) or NIH/3T3 cells (Figure 12). In addition, when HeLa cells were treated with nocodazole, syn Ia-EGFP did not colocalize with the remaining “spindly” microtubules, nor did it become extracted during simultaneous fixation/extraction (Figure 17). When the drug was washed off and cells fixed at various times following its removal, syn Ia-EGFP overexpression had no

observable effect on microtubule reassembly. Since synapsin I does not appear to affect microtubule dynamics *in vitro* (Baines and Bennett, 1986), this may not be surprising. Again, one would need to observe the cells in real-time to obtain a more precise representation of the effects of syn Ia-EGFP on microtubule dynamics.

The results described above indicate that synapsin I does not interact *in vivo* with microtubules. However, in HeLa cells treated with taxol, syn Ia-EGFP colocalized with microtubule bundles, while the EGFP tag by itself did not (Figure 20). *In vitro* studies have shown that the affinity of synapsin I for actin is up to 5-fold higher ($K_d = 1\text{-}2\ \mu\text{M}$) than that for microtubules ($K_d = 5\ \mu\text{M}$) (Baines and Bennett, 1986; Bahler and Greengard, 1987). The taxol-induced formation of microtubule bundles simultaneously causes microtubule-bound proteins to come closer together so that they are more easily detected (Vaillant *et al.*, 1998). Therefore, although it is possible that the interaction with microtubules *in vitro* does not have physiological significance, it may be that the interaction is too weak to be seen in cells that have not been treated with the microtubule-stabilizing drug. However, one cannot exclude the possibility that syn Ia-EGFP binds weakly to the drug itself and not to microtubules.

It is interesting that syn Ia-EGFP did not colocalize with F-actin in taxol-treated cells (Figure 20). In HeLa cells, alpha-tubulin becomes acetylated in the presence of taxol (Piperno *et al.*, 1987). Acetylated alpha-tubulin is associated with increased microtubule stability, and this modification is found in many differentiated cell types, including neurons (Gunderson and Bulinski,

1986). It seems plausible that, in both HeLa cells and neurons, acetylation of alpha-tubulin increases the affinity of synapsin I for microtubules. This would result in less availability of syn Ia-EGFP for binding to F-actin in our experiments.

Since no binding to microtubules was noted, it may not be surprising that synapsin Ia-EGFP overexpression did not elicit microtubule bundling. It should be noted that the *in vitro* studies that demonstrated synapsin I-induced microtubule bundle formation used very high concentrations of synapsin I (Baines and Bennett, 1986), and these concentrations would likely not be reached in our overexpression studies, nor in neurons for that matter. Moreover, as mentioned, there are other components to which synapsin I will bind *in vivo*, and this makes microtubule bundling less likely.

4.3 INTERACTION OF SYNAPSIN IA-EGFP WITH VIMENTIN INTERMEDIATE FILAMENTS IN HeLa CELLS

There have been no *in vitro* studies of the interaction between synapsin I and vimentin. This is probably because vimentin is found in neuronal progenitor cells but not in developing or mature neurons (Ho and Liem, 1996). Nevertheless, a study of cytoskeletal interactions of synapsin I in non-neuronal cells would not be complete without including intermediate filaments.

Synapsin I binds *in vitro* with remarkable affinity ($K_d = 10$ nM) to the light neurofilament subunit (NF-L) (Steiner *et al.*, 1987) which is 65% identical to vimentin (Geisler *et al.*, 1984). No colocalization of syn Ia-EGFP with vimentin was observed in HeLa cells and no observable reorganization of these

intermediate filaments was seen to result from syn Ia-EGFP overexpression (Figure 13). When these cells were treated with the microtubule-depolymerizing drug nocodazole, the vimentin network, which is closely associated with microtubules (Ball and Singer, 1981), collapsed into the perinuclear region. Syn Ia-EGFP did not undergo a similar collapse. These results indicate that if synapsin I binds to NF-L *in vivo*, the 65% sequence identity of NF-L with vimentin is not sufficient to permit synapsin I binding *in vivo* to vimentin.

4.4 IMPLICATIONS FOR INVOLVEMENT OF SYNAPSIN I IN NEUROTRANSMISSION

Consistent with results from *in vitro* binding assays, our results provide *in vivo* evidence for an interaction between synapsin I and F-actin. Therefore, they provide support for the model of synapsin I involvement in neurotransmission, whereby dephospho-synapsin I tethers SVs to the actin-based cytoskeleton, forming a reserve pool distal to the presynaptic membrane (Hilfiker *et al.*, 1999 for review). When nerve endings are depolarized, synapsin I becomes phosphorylated by cAMP-dependent and CaM kinases, and is in turn released from SVs and F-actin. SVs are then free to enter the releasable pool, where they may be primed for exocytosis and release of neurotransmitters into synapses.

Our results also demonstrate that if synapsin I binds to microtubules *in vivo*, the interaction is likely to be one of low affinity. Microtubules splay into the growth cone of neurons (Tanaka and Kirschner, 1995) and quick-freeze

deep-etch microscopy has shown some of them to be associated with SVs (Hirokawa *et al.*, 1989). It is possible that synapsin I also tethers SVs to the microtubule-based cytoskeleton. However, the interaction between synapsin I and microtubules does not appear to be regulated in the same manner as binding to SVs (Petrucci and Morrow, 1987). While phosphorylation by cAMP-dependent and CaM kinases reduces synapsin I affinity for SVs, it actually stimulates its binding to microtubules (Petrucci and Morrow, 1987). Therefore, if the microtubule-synapsin I interaction does play a role in synaptic transmission, the model described above does not apply here.

4.5 IMPLICATIONS FOR INVOLVEMENT OF SYNAPSIN I IN NEURONAL FORMATION AND MAINTENANCE

A number of studies have implicated synapsin I in neuronal formation and maintenance. It is possible that these functions are related to its interaction with cytoskeletal components. It has been suggested that developing neurons that are deficient in synapsins fail to convert actin organization from an isotropic network to actin bundles needed for cortical rim formation along the shaft of neurites (Ferreira *et al.*, 1994). Alternatively, microtubule bundling, which is an organizational property of the neurite shaft, may be less effective in these neurons (Ferreira *et al.*, 1994). However, we did not observe F-actin nor microtubule bundling resulting from synapsin I overexpression.

Our results show that, when expressed in non-neuronal cells, synapsin I colocalizes with F-actin, often in regions where F-actin is highly dynamic. These regions include “spots”, microspikes, cleavage furrow and membrane ruffles. Therefore it is possible that synapsin I influences neuronal formation and maintenance by affecting F-actin dynamics. Alternatively, if synapsin I does indeed bind to microtubules, it could act as a cross-linker of F-actin and microtubules in the growth cone. This seems plausible, since, before synaptogenesis, synapsin I is localized to axons, especially in the distal axon and growth cone (Fletcher *et al.*, 1991). The microtubule and microfilament systems are thought to interact closely so that the directed movement of the axon is spatially and temporally regulated (Brandt, 1998).

Dephospho-synapsin I accelerates the initial rate of actin polymerization *in vitro* and in theory this promotion of F-actin polymerization should prevent axonogenesis (Mattson, 1999). However, a depletion of synapsins actually appears to either delay or hinder axonogenesis (Ferreira *et al.*, 1994; Chin *et al.*, 1995). Perhaps it is not the influence of synapsin I on actin dynamics which influences axon formation but rather its ability to link vesicles to the cytoskeleton. It has been proposed that “bulk membrane flow” plays a major role in axonogenesis (Mattson, 1999).

4.6 FUTURE PROSPECTS

Further investigation is needed to assess the effects of synla-EGFP on actin, microtubule and intermediate filament dynamics. This could be undertaken by examining the transfected cells live. By cotransfecting cells with

dsRed (Clontech)-labeled G-actin, tubulin and neurofilament constructs, the dynamics of actin, microtubules and neurofilaments respectively could be examined in real-time. Initial rates of polymerization could be measured by treating the cells with drugs to depolymerize the cytoskeletal components, and then removing the in order to follow cytoskeletal reassembly.

To establish that the synla-EGFP fusion protein is indeed binding to F-actin and microtubules and not to other cellular components that are themselves bound to the cytoskeleton, total protein extracts from transfected cells could be used in *in vitro* F-actin and microtubule binding assays. In addition, pSynla-EGFP could be co-transfected with a construct that expresses a protein that binds to either F-actin (cortactin for example) or microtubules (MAP1A for example). If a competition for colocalization is evident, then binding by synla-EGFP is occurring.

Our studies did not decipher between dephospho-synapsin I and phospho-synapsin I. Only the former should bind significantly to F-actin, while the latter may bind with greater affinity to microtubules (Petrucchi and Morrow, 1987). Antibodies specific to dephospho- and phospho-synapsins could be used to determine which form is predominantly colocalized with the cytoskeletal components.

It would also be interesting to determine the localization in non-neuronal cells of the various domains (A to E) of synapsin I. One would expect, for example, that domain C would colocalize with F-actin (Bahler *et al.*, 1989; Valtorta *et al.*, 1992a), but it is not known whether the full-length protein or that

domain alone would show more pronounced colocalization. Site-directed mutagenesis of various peptide fragments of synapsin I would allow us to determine which amino acids are necessary for F-actin and microtubule colocalization. We would also like to compare the localizations of the various synapsin isoforms (synapsins Ia, Ib, IIa, IIb, and IIIa-e). This may point to differences in their functions.

In conclusion, our results provide evidence that synapsin I binds *in vivo* to F-actin and possibly with a lower affinity to microtubules. To our knowledge this is the first *in vivo* account of these interactions. Our results also implicate it in a role in F-actin dynamics, although further investigation is needed to characterize this role. Synapsin I-cytoskeletal interactions may be significantly involved in both synaptic transmission and neuronal formation and maintenance.

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