Aus dem Institut für Integrative Neuroanatomie
Medizinische Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Synaptophysin/Synaptobrevin complex: Characterisation of the Ca^{2+}- dependent cytosolic dissociation of the complex

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To

my dearest Adamma A. and Ify Afube

"Ka Chukwu gozie unu".

CONTENTS

1. INTRODUCTION

1.1. Synaptic transmission and plasticity

Living organisms are constantly confronted by a variety of information of changing natures from their surroundings and within. The proper processing of this information ensures an adequate response by the organisms and is therefore crucial for their survival. During the process of evolution animals have developed a nervous system comprising specialised cellular circuits which perform these tasks. Circuits are formed by neurons which interact with one another, each forming a few hundred to as many as 200,000 synaptic connections, thus creating a vast network for information exchange in an organism.

The human brain is composed of a few hundred billion neurons that form about 10^{15} synapses. Sensory input from the surrounding milieu is converted to electrical impulses by specialised nerve endings and conveyed in the form of action potentials along the neurons. The transfer of these impulses to other neurons is done at specialised points of contact between neurons known as synapses. The arrival of an action potential at the synapse induces the exocytosis of specialised chemical substances known as neurotransmitters stored in synaptic vesicles (Katz et al., 1969). At the presynaptic terminal, the action potential induces the opening of Ca^{2+} channels resulting in a Ca^{2+} current which stimulates synaptic vesicular fusion with the presynaptic membrane, a process known as exocytosis. The observations of Katz about 40 years ago still form the basis of our understanding on how neurons communicate with each other today. Nonetheless, much has been done since then to elucidate the actual processes which take place from the time the action potential arrives till the neurotransmitter is released into the synaptic cleft.

Synaptic transmission represents a pivotal aspect of medical knowledge especially in the field of neurology and psychiatry. The causes of several mental diseases and neuromuscular disorders can be traced to the malfunction of synapses. Under-standing how synaptic transmission can be modulated has therefore provided a strong basis for the development of therapies and drugs for diseases and disorders which have an underlying pathology in the process of synaptic transmission.

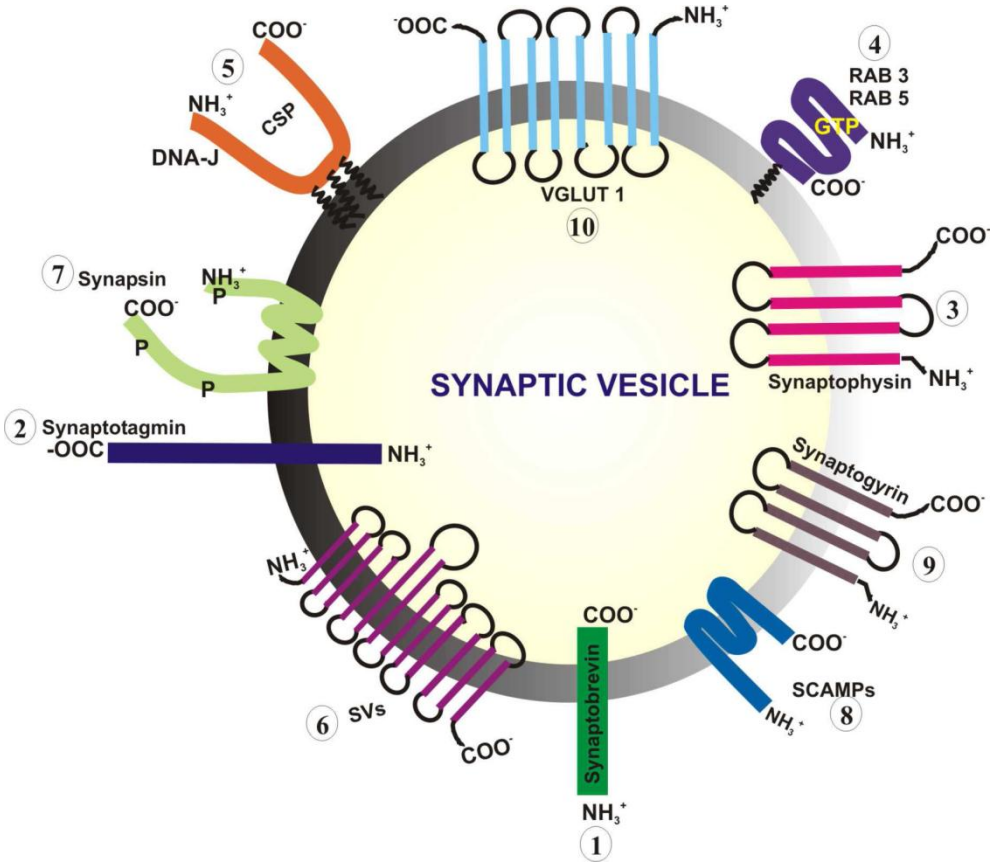
The synapse is a specialised intercellular junction between neurons that permits communication between them and thus establishes the foundation for neuronal circuitry. Each synapse is individually regulated and shows a selective action, thus presenting an advantageous site for the control of signal transmission. While one synapse may respond to and amplify even weak incoming signals, others may only respond to strong signals or sum up signals from different sources. Information transfer at the synapse is fast (fraction of a millisecond) and accurate, thus allowing the integrity of the perception and response to be preserved.

The synapse plays an outstanding role in the process of learning and memory formation. Frequent stimulation of a brain region, e.g. the hippocampus, produces a long-lasting enhancement in the effectiveness of the stimulated synapses in this region, a process known as long term potentiation (LTP). LTP is a form of synaptic plasticity that initiates long term memory formation. It increases the conductance of a synapse to signal transduction, thereby allowing a more efficient response to a given stimulus. This is achieved by two pathways; the first being an increased efficiency of the postsynaptic neurotransmitter receptors, thereby allowing stimulation by lower concentrations of the neurotransmitter. The second involves the presynaptic terminal whereby the amount of neurotransmitters released is increased either by increasing fusion competence (i.e. more vesicles are released per signal), or by increasing the amount of neurotransmitter per vesicle. Both events contribute to synaptic efficiency and are believed to be regulated, at least in part, at the level of synaptic vesicles.

1.2. Synaptic vesicles: Vehicles of synaptic transmission

All activities taking place in presynaptic terminals, directly or indirectly, involve synaptic vesicles (SVs)(Südhof, 2004). The idea of a synaptic vesicle was first suggested by Katz and his colleagues in the 1950s to explain the quantal release of acetylcholine observed at neuromuscular junctions. Today SVs represent some of the most studied and best classified organelles of living organism. Up to 300,000 SVs are concentrated in the presynaptic terminal of every neuron. The main function is the uptake and storage of neurotransmitters, with the number of transmitter molecules stored per vesicle ranging from 1000-50,000. Considering their diameter of approximately 40 nm, SVs are very small organelles with a relatively simple architecture.

Figure 1



Architecture of a glutamatergic synaptic vesicle

Representation of a synaptic vesicle with some structures of SV trafficking proteins: (1) Synaptobrevins (2) Synaptotagmin (3) Synaptophysins (4) Rabs (5) Cysteine string proteins (CSP) (6) Synaptic vesicle protein 2 (7) Synapsins (8) Secretory carrier membrane proteins (SCAMPs) (9) Synaptogyrins (10) Vesicular glutamate transporters (VGLUTs)

Synaptic vesicles contain two classes of obligatory components: 1) Transport proteins involved in neurotransmitter uptake and 2) trafficking proteins that participate in synaptic vesicle exo- and endocytosis and recycling (Südhof, 2004). The transport proteins are composed of a vacuolar-type proton pump that generates the electrochemical gradient necessary for neurotransmitter uptake and neurotransmitter transporters that mediate the actual uptake. The trafficking proteome of synaptic vesicles is complex. It includes intrinsic membrane proteins, proteins associated via post-translational lipid modifications, and peripherally bound proteins. These proteins do not share a characteristic that would make them identifiable as synaptic vesicle proteins, and little is

known about how these proteins are specifically deposited into SVs. Many of the known synaptic vesicle proteins interact with nonvesicular proteins and are linked to specific functions.

In a recent study Takamori et al. (2006) employed a combination of biophysical and proteomic technics to construct an atomic model for SVs, the first such model for any known organelle. Contrary to previous assumptions the stoichiometry of SVs is now known to be dominated by proteins with a surprising diversity of trafficking proteins. With the exception of the V-ATPase that is present in only one to two copies, SVs contain numerous copies of proteins essential for membrane traffic and neurotransmitter uptake (Takamori et al., 2006). A few of these proteins are, however, extraordinarily abundant in SVs. These include synaptobrevin2/VAMP2, with approximately 70 copies per vesicle; synaptophysin1, with 30 copies per vesicle; and synaptotagmin1, with 15 copies per vesicle (Takamori et al., 2006; Südhof, 2006).

1.3. Exocytosis

Synaptic transmission involves the fusion of synaptic vesicles with the presynaptic membrane releasing the neurotransmitter and the activation of postsynaptic receptors (Murthy & De Camilli, 2003). This fusion needs to overcome the high repulsive forces between the two negatively charged membranes in order for it to take place. To explain this process, the SNARE hypothesis of membrane fusion (synaptic vesicular membrane in particular) was formulated based on studies of membrane fusion in vitro (Söllner et al., 1993a; Rothman, 2002) and later complemented by results of genetic studies in yeast (Schekman, 2002). The hypothesis states that every transport vesicle contains a protein (or proteins) on its surface that specifies its target known as v-SNARE(s). Target membranes possess corresponding partners (t-SNAREs) which interact with v-SNAREs and lead to a subsequent membrane fusion (Pfeffer, 1996). Compelling support for this hypothesis comes from the identification of synaptobrevin/VAMP (v-SNARE), Syx and SNAP25 (t-SNAREs) as targets for the proteolytic action of clostridial neurotoxins, which are potent blockers of neurotransmitter release (Schiavo et al., 1992; Blasi et al., 1993; Montecucco, 1998). These proteins form a group of integral and associated membrane proteins known as the SNARE proteins. The SNARE proteins form a tight complex which bridges the two membranes (vesicular and plasma) and overcomes their repulsive forces. The complex formation occurs spontaneously in vitro and can be

disassembled by the ATPase-dependent protein N-ethylmaleamide-sensitive factor (NSF) in conjunction with α-SNAPs (soluble NSF attachment proteins) (Söllner et al., 1993a). Block of NSF action results in the accumulation of fusion-incompetent vesicles owing to a progressive loss of SNARE proteins available for the formation of productive trans-SNARE complexes (Söllner et al.,1993a; Hanson et al., 1997). This therefore implies that the cyclic assembly-disassembly of SNARE proteins is a crucial step in the process of exocytotic membrane fusion.

1.3.1. Ca^{2+} Triggering of exocytosis

Synapses exhibit a low but constantly present probability of release at resting potentials, causing spontaneous events of exocytosis that are reflected in electrophysiological recordings as miniature postsynaptic currents (Katz, 1969). However, Ca^{2+} influx significantly increases the probability of exocytosis by triggering at least two components of release that are probably mechanistically distinct: 1) A fast, synchronous phasic component is induced rapidly, in as little as 50 µs after a Ca^{2+} transient develops (Sabatini & Regehr, 1996) and 2) a slower asynchronous component continues for >1 s as an increase in the rate of spontaneous release after the action potential (Barrett & Stevens, 1972; Geppert et al., 1994; Goda & Stevens, 1994; Atluri & Regehr, 1998). Both components of release are strictly Ca^{2+}-dependent but change differentially upon repetitive stimulation (Hagler & Goda, 2001). Meinrenken et al. (2002) proposed a model which predicts that the only step in the signaling cascade whose speed depends on the Ca^{2+} concentration is Ca^{2+} binding to the Ca^{2+} sensor, explaining why the synaptic delay (i.e., the time between the action potential and release) is relatively independent of the Ca^{2+} concentration, whereas the magnitude of release is supralinearly dependent on the Ca^{2+} concentration. An alternative model for the Ca^{2+} dynamics during an action potential that postulates additional intrinsic heterogeneity of vesicles also explains the characteristics of release (Trommershäuser et al., 2003). A key point to take note of here is the fact that the speed with which Ca^{2+} triggers release (<400 *µ*sec) suggests that Ca^{2+} binding to the Ca^{2+} sensor only induces fusion-pore opening and does not initiate a complex enzyme reaction cascade such as is known of the Ca^{2+}/calmodulin-dependent kinase phosphorylation. The role played by such complex reactions in the modulation of synaptic exocytosis still remains to be better elucidated.

1.3.2. SNARE proteins - mediators of exocytosis

Synaptic exocytosis is mediated by three SNARE proteins: synaptobrevin (Syb) or vesicle-associated membrane protein (VAMP) located on the synaptic vesicular membrane, and syntaxin 1 (Syx1) and SNAP-25 located on the presynaptic plasma membrane (Söllner et al., 1993b). Fusion is driven by the progressive zippering of vesicle and plasma membrane SNAREs to form a four-helix bundle (Sutton et al., 1998). Although many other proteins appear to have critical roles in synaptic vesicle exocytosis, it seems likely that the SNAREs represent the minimal machinery for fusion (Weber et al., 1998). In view of these facts, the zippering of SNARE proteins still remains the most viable concept for membrane fusion. Nevertheless, a variety of other factors play critical roles in exocytosis by controlling SNARE function, as emphasized by the growing list of SNARE-interacting proteins. Two such factors, unc/munc13 and unc/munc18 (members of the Sec1 family), are particularly important, given the spectacular effect produced by their absence as seen in a complete block of synaptic vesicle exocytosis (Richmond et al., 1999; Verhage et al., 2000; Varoqueaux et al., 2002). In fact, one theory suggests that SNAREs are components of a fusion machine that includes other proteins. This has been supported by two key observations at the synapse. Firstly, deletion of synaptobrevin caused a major impairment of synaptic vesicle exocytosis but left approximately 10% of exocytosis intact (Schoch et al., 2001) with the regulated (Ca^{2+}-triggered) exocytosis more severely impaired than spontaneous exocytosis. Similar results were also obtained for SNAP25 knockouts (Washbourne et al., 2002). Thus at least at the synapse, the SNARE complex was only essential for efficient, physiologically regulated fusion, but not for fusion as such. Secondly, although the synaptobrevin deletion did not abolish exocytosis, deletion of Munc18-1 eliminated release completely (Verhage et al., 2000). This suggests a more fundamental role for Munc18-1 than synaptobrevin in membrane fusion at the synapse. Munc18-1 acts presumably by binding to the plasma membrane SNARE protein syntaxin 1 (Hata et al., 1993) and coupling it to as yet unidentified cytosolic factors to organize SNARE complex assembly (reviewed in Jahn et al., 2003).

1.4. Integral and associated proteins of the SV membrane

1.4.1. Synaptobrevin

VAMP or Syb was primarily studied in purified SVs obtained from cholinergic terminals of Torpedo (Trimble et al., 1988). Syb is a small 18 kDa integral membrane protein of SVs and is the most abundant with an average of 70 copies per vesicle. Soon after its discovery, it was cloned and sequenced in several species (Elferink et al., 1989; Südhof et al., 1989). The protein structure exhibits a very high degree of conservation in phylogenetically distant animals especially in the regions responsible for the formation of the physiologically relevant SNARE fusion complex. In addition, it forms another complex with Synaptophysin commonly known as the Synaptophysin/Synaptobrevin complex. Both complexes are mutually exclusive from one another. In a recent study it was observed that a null mutation in VAMP1/Syb is associated with neurological defects and prewean mortality in the lethal-wasting mouse mutant (Nystuen et al., 2007).

1.4.2. Synaptophysin

Synaptophysin (Syp) was the first integral membrane protein of SVs to be isolated (Wiedenmann & Franke, 1985; Jahn et al., 1985) and cloned (Leube et al., 1987; Südhof et al., 1987; Buckley et al., 1987). It is a glycoprotein of 38 kDa with four transmembrane domains spanning the SV membrane and both the NH_2- and the COOH-termini located on the cytosolic side of the membrane (Jahn et al., 1985; Buckley et al., 1987; Südhof et al., 1987). There is evidence that Syp can be phosphorylated on tyrosine rests by the tyrosine kinase c-sc which is known to be associated with SVs (Pang et al., 1988; Barnekow et al., 1990; Onofri et al., 1997), while the phosphorylation on serine residues is mediated by the Ca^{2+}/calmodulin dependent protein kinase II under physiological conditions (Pang et al., 1988; Rubenstein et al., 1993). As a major integral transmembrane protein of SVs, Syp provides a molecular marker for synapses and serves as a functional marker of the brain. In medicine, it is widely used as a marker for the determination of the density of synapses in brain disorders, as well as for the diagnosis and evaluation of neoplasms of the central nervous system and of neuroendocrine and neuroectodermal tumors (Morrison & Prayson, 2000; Wick, 2000).

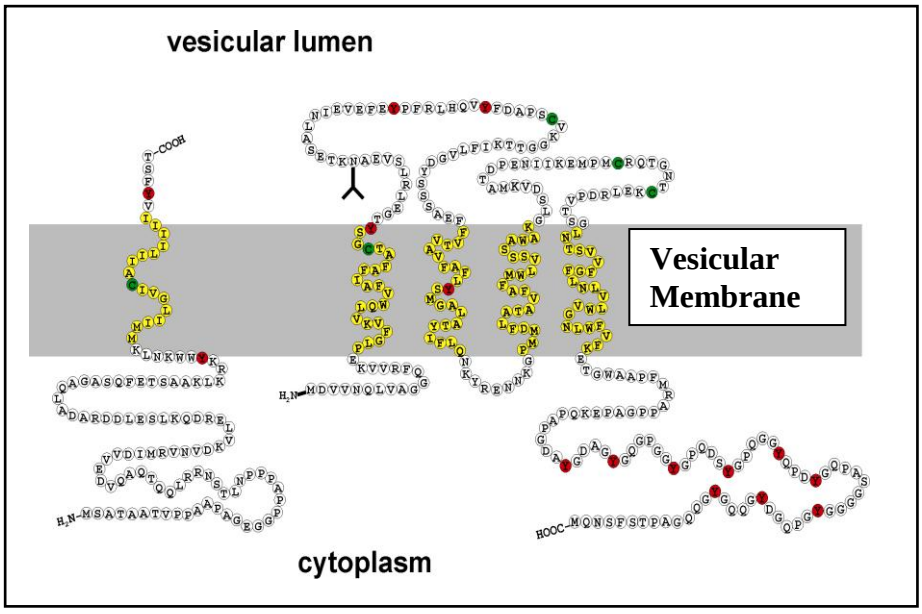
A number of interactions with other proteins have been described at the synaptic terminal including the vesicular proton pump V-ATPase (Thomas & Betz, 1990; Galli et

al., 1996; Carrión-Vásquez et al., 1998), myosin V (Prekeris & Terrian, 1997) and dynamin I (Daly et al., 2000; Daly & Ziff, 2002). The most prominent of these interactions, however, is with the v-SNARE Syb (Calakos & Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995). In spite of widespread research on Syp, the functional importance in synapses still remains to be fully understood. This has proved to be an even more difficult question than anticipated, especially as the Syp knockout mice showed no overt phenotypical changes (Eshkind & Leube, 1995; McMahon et al., 1996). Nonetheless, widespread suggestions depict Syp as a likely molecular switch that allows the association of synaptobrevin with the t-SNAREs after it dissociates from synaptophysin. Such a regulation would ensure that synaptobrevin binds only at the correct time and location to the t-SNAREs, for instance when the synapse is activated. This interaction may reflect a general mechanism for a modulation of the SNARE complex formation.

1.4.3. The Synaptophysin/Synaptobrevin-complex (Syp/Syb-complex)

It has been recently shown that the C-terminal transmembrane of Syb interacts with the Syp to form the Syp/Syb-complex (Yelamanchili et al., 2005).

Figure 2



Interaction between Syp and Syb in vesicular membrane

Although of potential functional importance, the regulation of the formation of Syp/Syb complex is largely unknown. Since it was proposed to be a mechanism for controlling the exocytotic machine (Edelmann et al., 1995), much work has been done to better understand the physiological relevance of this complex. The apparent absence of the complex in embryonic rat brain and developing neurons before synaptogenesis has led to the proposal that it represents the hallmark of SV maturation (Becher et al., 1999a). However, it still remains to be elucidated whether this developmental upregulation depends on post-translational modification such as phosphorylation, cleavage/formation of disulfide bonds or an enhancement of the cholesterol content in the SV membrane (Mitter et al., 2003).

Further studies have shown that the Syp/Syb complex is absent in various hormone-secreting neurons and neuroendocrine cells which express both Syp and Syb (Becher et al., 1999b). The complex has been increased due to persistent stimulation in kindled rat models, suggesting that it may represent a reserve pool for synaptobrevin enabling synaptic vesicles to adjust to an increased demand for synaptic efficiency (Hinz et al., 2001).

1.4.4. Syntaxins (Syxs)

Syxs are integral plasma membrane proteins that form part of the exocytotic fusion complex (SNARE complex) by functioning as target receptors for the v-SNARE Syb (Sollner et al., 1993b; Südhof et al., 1999; Lin & Scheller, 2000; Fon & Edwards, 2001). There are different isoforms that function in membrane targeting throughout the organism. Syx I is the main isoform found in the synaptic membrane of nerve terminals and will henceforth be considered. It possesses three coiled-coil domains consisting of a single C-terminal transmembrane domain, a SNARE domain and an N-terminal regulatory domain. Binding to Syb and SNAP25 is mediated by the SNARE domain. Syx I also interacts with a number of other proteins which are essential for vesicle exocytosis using its N-terminus (Südhöf, 1999; Fon & Edwards, 2001; Hay, 2001). The most notable interactions occur with munc18-1, munc13-1 and synaptotagmins (Syt), the interaction with Syt occuring in a Ca^{2+} dependent manner. Furthermore, the C-terminal transmembrane domain of Syx mediates an interaction with N- and P/Q-type voltage-gated Ca^{2+} channels. All these proteins participate in modulating the activities of Syx and ensuring a sustained role in exocytosis.

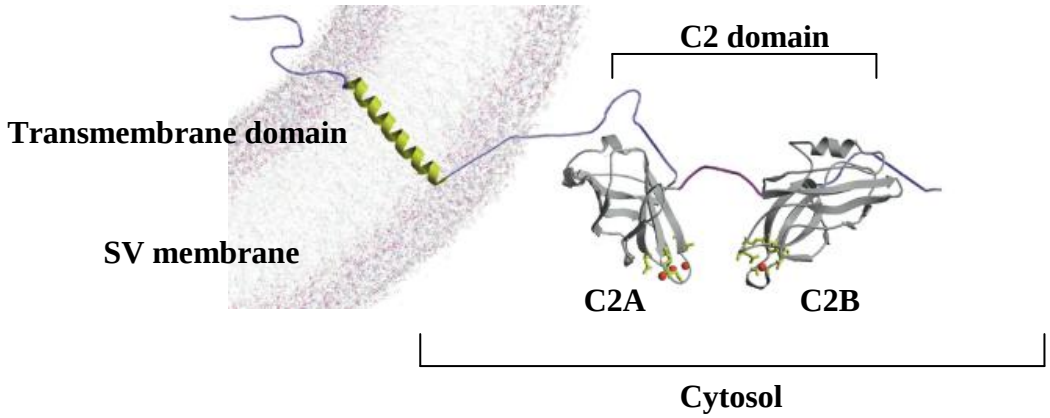
1.4.5. SNAP25 (Synaptosome-associated protein of 25 kDa)

SNAP25 assembles with Syb and Syx via two α-helices to form the SNARE fusion complex. Unlike its SNARE partners Syb and Syx, SNAP25 has no transmembrane domain, but is rather associated with the cytosolic side of the presynaptic membrane via palmytoyl side chains which are located in the middle of the molecule, probably on cysteine residues (Oyler et al., 1989; Hess et al., 1992). SNAP25 also interacts with synaptotagmins and voltage-gated Ca^{2+} channels. Loss-of-function mutation in SNAP25 has shown that a Ca^{2+}-dependent synaptotagmin binding to SNAP25 is essential for Ca^{2+} triggered exocytosis (Zhang et al., 2002). A homozygote deletion of the SNAP25 gene in mice is non-vital, while the heterozygote deletion mutants showed a phenotype similar to attention deficit hyperactivity disorder (ADHD). These observations have led to the suggestion that partial deletions in the SNAP25 gene in humans will, at least in part, predispose the individuals to ADHD (Brophy et al., 2002; Mill et al., 2002).

1.4.6. Synaptotagmins (Syts)

Synaptotagmin I is one of the most abundant (~7% of the total vesicle protein) Ca^{2+} sensing protein on the surface of SVs, and has therefore attracted considerable interest as a potential Ca^{2+} sensor that regulates SV exocytosis (Chapman & Jahn, 1994).

Figure 3



A scale model of Syt structure showing the transmembrane domain and the Ca^{2+} -binding motif – C2 domain (Adopted from Chapman, 2002)

Syts are composed of three domains: 1.) an N-terminal membrane spanning domain, 2.) a short intravesicular domain and 3.) a large cytoplasmic domain consisting of two tandem Ca^{2+} binding domains known as C2A and C2B (Perin et al., 1990; Fernandez-chacon & Südhof, 1999; Marqueze et al., 2000; Südhof, 2002).
The putative SNARE complex is indisputably the core of a conserved membrane-fusion machine. However, fusion that is mediated by purified SNAREs in vitro occurs in a long timescale (minutes) and is not regulated by Ca^{2+}, indicating that Ca^{2+} sensors are likely to function in conjunction with SNAREs to drive the fast and tightly regulated SVs fusion that is observed in the synapse. Following this perspective, the Syts (especially Syt1) have been widely studied and implicated as the main Ca^{2+} sensors in regulated exocytosis (Südhof & Rizo, 1996; Dessai et al., 2000; Littleton et al., 2001).

1.5. Vesicular neurotransmitter transporters

Specific neurotransmitter transporters occur only on subpopulations of SVs depending on the synapse type (e.g. glutamatergic or GABAergic). Transmitter uptake involves a complex and tightly regulated process; the neurotransmitters are transported into the vesicles by means of specialised proteins located in the membranes of these vesicles. So far, seven transmitter-specific vesicular neurotransmitter transporters have been identified, of which the vesicular glutamate transporters and the vesicular GABA transporter will be briefly described.

1.5.1 Vesicular glutamate transporters (VGLUTs)

Three isoforms have been cloned, of which VGLUT1 (Bellocchio et al., 2000; Takamori et al., 2000) and VGLUT2 (Fremeau et al., 2001; Takamori et al., 2001) are considered in this project. VGLUTs were initially described as brain-specific or differentiation-associated Na^{+}-dependent inorganic phosphate transporters, BNPI (Ni et al., 1994) or DNPI (Hisano et al., 2000) located in the plasma membrane. They are now known to transport glutamate with high specificity and unlike other transporters do not accept other structurally related amino acids such as aspartate. Common to VGLUT1 and 2 is a distinct and mutually exclusive distribution in the brain with VGLUT2 being dominant in the thalamic and hypothalamic regions and VGLUT1 in cortex, hippocampus and cerebellar cortex (for review see Ahnert-Hilger et al., 2003).

1.5.2 Vesicular GABA transporter (VGAT)

VGAT was cloned in parallel from Caenorhabditis elegans (McIntire et al., 1997) and from mouse (Sagné et al., 1997). Like the VGLUTs, it exhibits an even lower affinity and specificity for its substrate GABA. In addition to GABA it also transports glycine with an even lower affinity.

1.6. Perspectives

Characterisation of the Ca^{2+} dependent dissociation of the Syp/Syb complex

The Syp/Syb complex is a marker for development and activity dependent changes in the synapse irrespective of the transmitter phenotype. Regulated exocytosis at the synapse involves a complex process of protein interactions which are involved in modulating repetitive release and ensure an adequate response on arrival of a stimulus. It is in this perspective that the Syp/Syb complex was proposed as a mechanism for modulating the exocytotic machine. In a recent study, it was shown that the C-terminal transmembrane domain of Syb mediates binding to Syp (Yelamanchili et al., 2005). Nonetheless, issues addressing the physiological relevance of the complex and the nature of the bond between Syp and Syb in this complex are still far from being clarified despite extensive efforts. Previous experiments have so far suggested a cytosolic binding factor which occurs in adult cytosol and can initiate the complex in embryonal as well as neuroendocrine cells. This factor is stable to heat and of low molecular weight, possibly a lipid post-translational modification, whose dissociation from Syp is Ca^{2+} regulated (Becher et al., 1999b; Reisinger et al., 2004). The reversibility of the complex formation and its dissociation following an increase in the free Ca^{2+} concentration has also been suggested in previous studies. The Ca^{2+}-dependent dissociation, however, was suggested to require the presence of cytosol (Reisinger et al., 2004), indicating an intrinsic cytosolic factor that mediates the dissociation of the Syp/Syb-complex in the presence of Ca^{2+}.

In the present project the Syp/Syb complex is once again revisited to obtain more insight into its regulation and possible role in the process of exocytosis and synaptic plasticity. The following points were specifically examined:

1. **Establishment of the necessity of cytosol in the Ca^{2+}-dependent dissociation of the complex.**
2. **Characterisation of the Ca^{2+}-dependent cytosolic factor required for dissociation.**

2 MATERIALS

2.1 Antibodies

2.1.1 Primary antibodies

ANTIBODY NAME	PURCHASED FROM
Anti-SNAP 25 (synaptosomal attachment protein) Monoclonal, mouse, 1:10,000	Sternberger Monoclonals (Baltimore, MD, USA)
Anti-Synaptobrevin I (Clone 69.1) Monoclonal, mouse, 1:10,000	Synaptic systems (Göttingen, Germany)
Anti-Synaptophysin (Clone 7.2) Monoclonal, mouse, 1:10,000	Synaptic systems (Göttingen, Germany)
Anti-Syntaxin 1A/B (Clone HPC) Monoclonal, mouse, 1:10,000	Synaptic systems (Göttingen, Germany)
Anti-VGLUT 1 (vesicular glutamate transporter 1) Monoclonal, mouse, 1:10,000	Synaptic systems (Göttingen, Germany)
Anti-VGLUT 1 (vesicular glutamate transporter 1) Polyclonal, rabbit, 1:10,000	Synaptic systems (Göttingen, Germany)
Anti-VGLUT 2 (vesicular glutamate transporter 2) Polyclonal, rabbit, 1:10,000	Synaptic systems (Göttingen, Germany)
Anti-VGAT (vesicular GABA transporter) Monoclonal, mouse, 1:2000	Synaptic systems (Göttingen, Germany)
Anti-VGAT (vesicular GABA transporter) Polyclonal, rabbit, 1:5,000	Synaptic systems (Göttingen, Germany)

2.1.2 Secondary antibodies

ANTIBODY NAME	PURCHASED FROM
Horse anti-mouse IgG peroxidase-labelled Monoclonal, 1:10,000	Vector Laboratories (Burlingame, CA, USA)
Goat anti-rabbit IgG peroxidase labelled Polyclonal, 1:10,000	Vector Laboratories (Burlingame, CA, USA)

2.2 Buffers and Solutions

(Composition)

Antibody solution

1.5% bovine serum albumin in tris-buffered saline (TS) buffer

Blocking solution

5.0%	low fat milk powder
0.1%	Tween-20 in TS buffer

Electrophoresis buffer (10 X)

30 g	Tris
144 g	glycine
10 g	SDS

Extraction buffer for immunoprecipitations

144 mM	KCl	
20 mM	HEPES-KOH,	pH 7.4
2 mM	EDTA	
1%	Triton X-100	

Extraction buffer for binding experiments

140 mM	KCl
2 mM	EDTA
20 mM	HEPES-KOH, pH 7.4

Phosphate buffered saline (PBS) buffer

140 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
1.8 mM	KH_2PO_4, pH 7.4

Ponceau solution

0.5 % w/v	Ponceau S	
3.0 % v/v	trichloroacetic acid	

Sample buffer (3x)

12.48 ml	stacking gel buffer (4 X)	
1.50 ml	0.1 M EDTA (Na^+-salt)	pH 6.8-7.0
15 g	sucrose	
5.00 ml	1.5 M DTT	make up to 50 ml using dH_2O

Separating gel buffer (4x)

181.7 g	Tris,	pH 8.8
4.0 g	SDS	make up to 1 l using dH_2O

Sodium buffer

10.0 mM	glucose
5.0 mM	KCl
140.0 mM	NaCl
5.0 mM	$NaHCO_3$
1.0 mM	$MgCl_2$
1.2 mM	Na_2HPO_4
20.0 mM	HEPES, pH 7.4

Solution A (for BCA assay)

1.00 g	BCA-disodium	
1.70 g	Na_2CO_3	
0.16 g	Na_2-tartrate	
0.40 g	NaOH	
0.95 g	$NaHCO_3$	
100 ml	dH_2O,	pH 11.25 (NaOH).

Solution B (for BCA assay)

4 g $CuSO_4 \times 5H_2O$

in 100 ml of dH_2O

Sucrose/ Homogenization solution

0.32 M sucrose in dH_2O

Stacking gel buffer (4 X)

60.5 g	Tris pH 6.8	
4.0 g	SDS	make up to 1 l using dH_2O

TBST buffer

25 mM	Tris	
150 mM	NaCl	
3 mM	KCl	
0.05 %	Tween 20	pH 7.4

TS buffer (Tris-buffered saline) (10 X)

20 mM	Tris	
150 mM	NaCl	pH 7.5 (HCl)

Western blotting buffer (for semidry transfer)

48 mM	Tris
386 mM	glycine
3.7 g / l	10 % SDS
800 ml	H_2O
200 ml	methanol (no need to adjust pH)

2.3 Chemicals and reagents

Name of Chemical	Purchased from
40% Acrylamide/Bisacrylamide-mix (40:1)	BioRad (Hercules, CA,USA)
APS (Ammonium persulfate)	Sigma (Munich, Germany)
BCA (Sodium Bicinchoninic acid -4,4-dicarboxy-2,2-Bichinolin)	Sigma (Munich, Germany)
BSA, Fraktion V (Bovine Serum Albumin)	Roth (Karlsruhe, Germany)
DSS (Disuccinimidyl suberate)	Pierce biotechnology (Rockford, USA)
DTT (Dithiothreitol; threo-1,4-Dimercapto-2,3-butandiol)	Sigma
ECL (Enhanced Chemiluminescence)	Amersham (Buckinghamshire, UK)
EDTA (Ethylendiamine tetraacetate)	Roth (Karlsruhe, Germany)
EGTA (Ethyleneglycol bis(2-aminoethyl-tetraacetate)	Roth (Karlsruhe, Germany)
Ficoll	Pharmacia Biotech (Uppsala, Sweden)
Glycerol	Roth (Darmstadt, Germany)
Hybond C	Amersham (UK)
HEPES (N-2-Hydroxyethylpiperazin-N´-2-	Biochrom (Berlin, Germany)

ethansulfonicacid)	
LMW (Low Molecular Weight Marker)	Amersham (Buckinghamshire, UK)
Milk powder (for western blotting)	Molkerei Heideblume (Elsdorf, Germany)
Methanol	Merck (Darmstadt, Germany)
PMSF (Phenylmethylsulfonylfluoride)	Sigma (Munich, Germany
Ponceau S	Sigma (Munich, Germany)
Protease inhibitor cocktail Aprotinin 0.08 mM Leupeptin 2 mM Pepstatin A 1.5 mM PMSF (Phenylmethylsufonyl fluoride)	Sigma (Munich, Germany) Roth (Karlsruhe, Germany)
SDS (Sodium dodecylsulphate)	Sigma (Munich, Germany)
TEMED (N,N,N´,N´-tetramethylethylendiamine)	Sigma (Munich, Germany)
Triton X-100	Roth (Karlsruhe, Germany)
Tween 20	Merck (Darmstadt, Germany)
Western blot stripping buffer	Pierce (Rockford, USA)

2.4 EQUIPMENT

2.4.1 Centrifuges

Beckman L-70 ultracentrifuge
(Palo Alto, CA, USA)
Rotor Ti 70

Beckmann Optima TL ultracentrifuge
(Palo Alto, CA, USA)
Rotor: TLA-100.4

Eppendorf centrifuge 5402
(Hamburg, Germany)

Eppendorf centrifuge 5417 C
(Hamburg, Germany)

2.4.2 Centrifugal filter units

Centricon-30 (molecular weight cut-off of 30 kDa)
Millipore (Bedford, MA, USA)

2.4.3 Electrophoresis equipment

Trans-Blot SD semi-dry electrophoretic transfer cell
Bio-Rad (Hercules, CA, USA)

Power/Pac 200 power supply
Bio-Rad (Hercules, CA, USA)

Mini-PROTEAN II electrophoresis cell
Bio-Rad (Hercules, CA, USA)

2.4.4 Spectrophotometers

Dynatech MR 5000 Elisa reader

Dynatech (Ashford, UK)

3. METHODS

3.1 Preparation of synaptosomes and SVs

3.1.1 Preparation of synaptosomes from adult rat whole brain

Isolated nerve terminals (synaptosomes) were prepared from adult Wistar rat whole brains as described (Edelmann et al., 1995). In most cases, more than one rat brain was used for the experiments. The rat was anaesthetized with ether prior to decapitation. The rat skull was carefully cut open and avoiding the brain stem, the whole brain was removed quickly and placed instantly in ice-cold homogenization buffer containing protease inhibitors. All subsequent steps for the preparation were performed at 4°C. Each rat brain was homogenized in 10 ml of homogenization buffer using a dounce homogenizer (900 rpm, 9 strokes), and the homogenate (H) was subsequently centrifuged at 1,200 x g for 10 minutes in a Beckman Ti 70 rotor. The resultant pellet (mainly containing nuclei and tissue debris, P1) was stored for analysis and the supernatant (post nuclear supernatant, PNS) was centrifuged for 20 minutes at 13,000 x g to obtain the crude synaptosomal pellet (P2).

Relatively pure synaptosomes were prepared in the following manner; the crude synaptosomal pellet was reconstituted in homogenization buffer and loaded onto a Ficoll gradient made up of three layers containing 6 % (4ml), 9 % (1 ml) and 13 % (4 ml) Ficoll® 400 prepared in homogenization buffer. The gradient was then centrifuged in a Beckman SW 40 Ti rotor at 9,040 x g for 35 minutes, thereby causing the synaptosomal fraction to collect at the interface between the 9 % and 13 % Ficoll layer. This fraction was collected carefully with a glass pipette bent thermo-mechanically at the tip, diluted in sodium buffer and pelleted again at 700 x g for 3 minutes to obtain the synaptosomal pellet. As described in previous experiments (Becher et al., 1999a), both methods of synaptosomal preparation yielded similar results, so most of the experiments were carried out with crude synaptosomal extracts.

For the analysis of P3 which contains considerable amounts of SVs, the supernatant obtained after P2 was centrifuged at 350,000 x g for 30 minutes in a Beckman TLA 101.4 rotor and the resulting pellet reconstituted in the chosen buffer for the experiment.

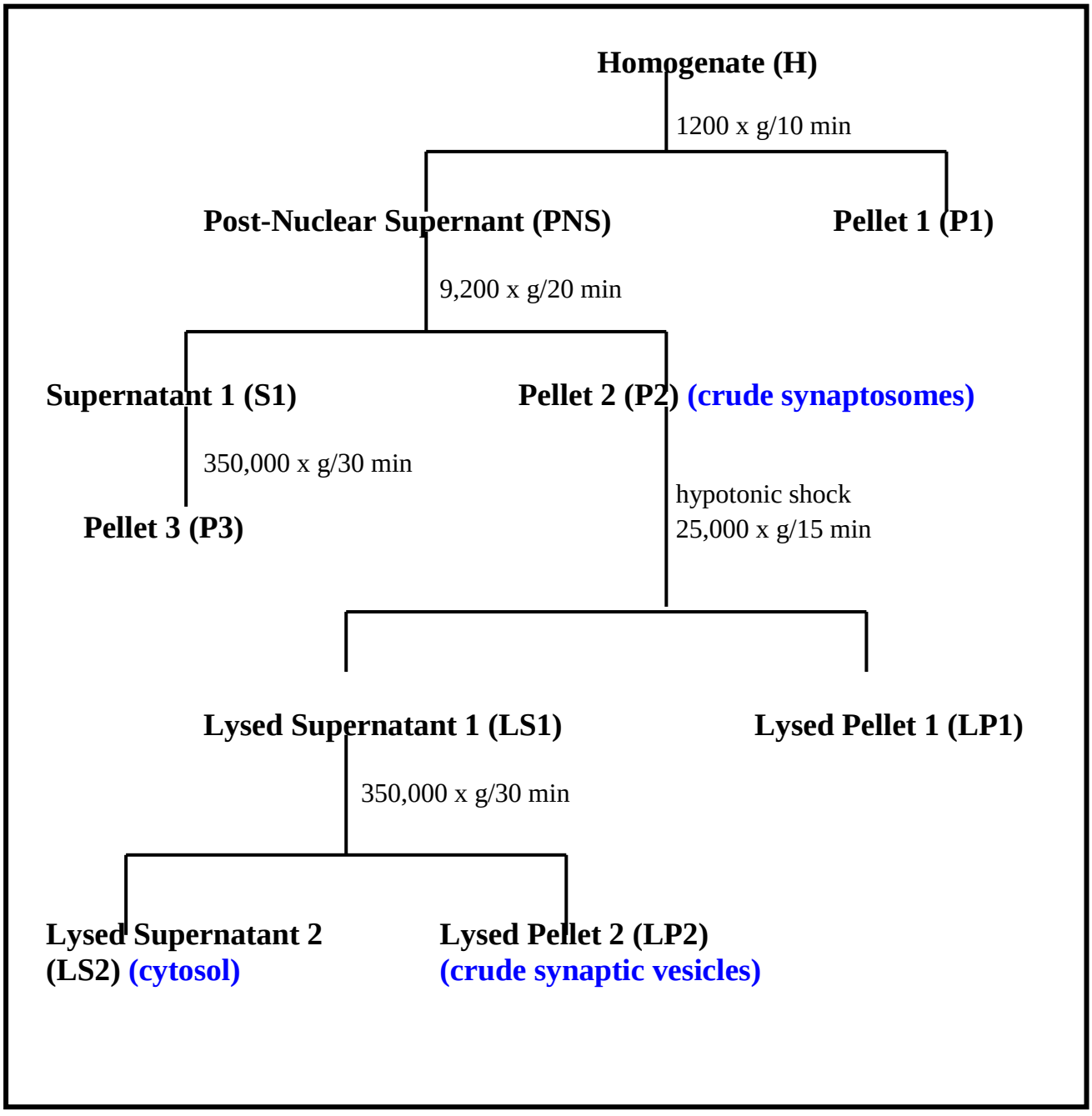
3.1.2 Isolation of crude SVs from adult rat brain

Crude SVs were prepared from adult rat brain synaptosomes following the procedure described by Huttner et al. (1983). All steps were performed on ice or at 4° C. Adult rat brain synaptosomal fraction was isolated as described above. The synaptosomal pellet was resuspended in 1ml of homogenization buffer and given a water shock (homogenization at 2000 rpm, 3 strokes) by the addition of 9 volumes of sterile water, 10 mM HEPES (pH 7.4) and protease inhibitors. The lysate was centrifuged at 25,000 x g for 20 minutes resulting in the first lysed pellet (Lysed pellet 1, LP1), which was either stored for analysis or discarded and the corresponding first lysed supernatant (LS1). The LS1 was carefully removed and centrifuged at 350,000 x g for 30 minutes in a Beckman TLA 101.4 rotor. This last centrifugation step yields a pellet (LP2) composed of crude synaptic vesicles and its corresponding diluted synaptosomal supernatant LS2. For resuspension, the SV fraction was drawn 6 times through a 23 gauge needle and subsequently 9 times through a 27 gauge needle in LS2 or buffers chosen for the preferred experimental conditions (see figure 4).

3.1.3 Calculation of free Ca^{2+} concentrations

Calculations were done using a software that permits the calculation of the amount of free divalent ions in aqueous solutions given the total amount added to the solution and vice versa. Using this software, we determined how much of Ca^{2+} ion we needed to add in experimental solutions to obtain a defined amount of free Ca^{2+} in this solution. The pH and the temperature of the solution, as well as the presence of chelators like EGTA or EDTA were taken into consideration during the calculations.

Figure 4

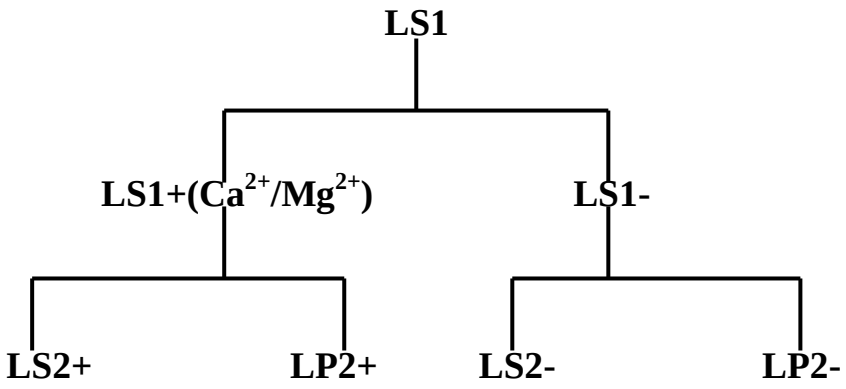


Subcellular fractionation of the rat brain; synaptic vesicle isolation

3.1.4 Preparation of SVs in the presence of calcium

Similar to 3.1.2, the P2 was resuspended in 1 ml of homogenisation buffer and given a water shock by adding a total of 9 v/v of sterile water containing 10 mM HEPES (pH 7.4), 2 mM EGTA, 2 mM EDTA and protease inhibitors. The lysate was centrifuged at 25,000 x g for 20 minutes resulting in LP1 and the supernatant LS1. A part of the LS1 is treated with a calculated amount of free Ca^{2+} ion (usually between 1 µM and 1 mM) and Mg^{2+} ion to yield LS1+ (the + denoting the presence of Ca^{2+}) before spinning down at 350,000 x g for 30 minutes to obtain SVs under Ca^{2+} conditions termed LP2+ and its corresponding supernatant LS2+ (see figure 5 below for illustration). Accordingly, LP2 obtained in this setting without Ca^{2+} treatment and its corresponding supernatant are termed LP2- and LS2-, respectively.

Figure 5



3.2 Protein determination

Protein concentrations were usually determined according to the bicinchoninic acid (BCA) method. A standard curve was prepared using six duplicate serial dilutions of bovine serum albumin (BSA) ranging from 50 µg/ml to 500 µg/ml. BSA was usually prepared in PBS or sometimes according to the buffer used for sample resuspension. Standards and appropriately diluted samples were pipetted in duplicates into the wells of a 96 well microtitre plate (20 µl/well). 200 µl of reaction solution (solution A and solution B (50:1 v/v) were added to each well, and the plate was incubated at 60°C for 30 minutes. The plate was then allowed to cool for 10 minutes before the absorbance of

the samples was measured in an ELISA reader at 550 nm. Sample protein concentrations were estimated from the BSA standard curve.

3.3 Extraction and immunoprecipitation of protein complexes

To analyse the interactions between the SNARE proteins (Syb, Syx, SNAP25) and Syp, proteins were first extracted from their lipid membranes. If membranes were present as pellets, these were suspended in extraction buffer containing Triton X-100 (1 % v/v) (protein usually at a concentration of 1mg/ml), while, if membranes were present in suspension, Triton X-100 (20 % stock solution in PBS) was added directly to the samples to a final concentration of 1 % (v/v). The extraction was carried out on ice for 1 hour on rotation. Insoluble material was pelleted at 700 x g for 5 minutes. 2 µl of ascites fluid (corresponding to about 7-10 µg of IgG) of monoclonal antibodies against Syb or Syp were added to 200 µl of extraction supernatant. The incubations were performed overnight for about 18-24 hours at 4°C under rotation. 60 µl of protein G-Sepharose suspension (diluted 3:1 in extraction buffer) was subsequently added to bind the immunoprecipitates, and the samples were incubated for a further hour at 4°C while shaking. The beads were then collected by centrifugation at 500 x g for 3 minutes. Beads were washed three times with the extraction buffer and analyzed together with their corresponding supernatants on the SDS-PAGE followed by immunoblotting.

3.4 Chemical crosslinking

Synaptic proteins were cross-linked using chemical cross-linker, DSS. LP2 (1 mg/ml) were used for cross-linking. Prior to cross-linking, the vesicles were usually resuspended in PBS or the corresponding supernatants (Lysed synaptosomal supernatant 2, LS2) as described above. DSS dissolved in DMSO was added to yield a final concentration of 1 mM. After incubation at 37°C for 45 minutes, the reaction was quenched by incubating the samples for 30 minutes at room temperature with Tris-HCl (pH 7.4) added to a final concentration of 100 mM. The reaction samples were centrifuged at 5000 x g for 3 minutes in an Eppendorf centrifuge to clear the precipitate formed due to the reaction and the samples were either directly dissolved in non-denaturating sample buffer or samples were pelleted at 350,000 x g for 30 minutes,

resuspended in non denaturating sample buffer, and analyzed by SDS-PAGE and immunoblotting.

3.5 Protein gel electrophoresis

Protein samples were electrophoresed on a denaturating sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system under discontinuous conditions according to the method of Laemmli (1970). The preferred percentage of the gel used depended on the molecular weight of the proteins to be analysed. In most cases, 10 %, 12 % or 15 % gels were used in combination with a 4 % stacking gel buffer in all cases to allow for optimal separation. A standard low molecular weight (LMW) marker was usually preferred. Protein samples to be analysed were dissolved in Laemmli buffer and heated to 90°C for 5 minutes prior to loading. The gels were prepared as follows:

Separating gel percentage	10 %	12 %
Separating gel buffer, pH 8.8 (4X stock)	2.5 ml	2.5 ml
Acrylamide/bis-acrylamide Stock (40%)	2.5 ml	3.0 ml
Water	5 ml	4.5 ml
TEMED	10 µl	10 µl
Ammonium persulfate (APS)	85 µl	85 µl

Stacking gel percentage	4 %
Stacking gel buffer, pH 6.8 4X stock	2.5 ml
Acrylamide/bis-acrylamide Stock (40 %)	1 ml
Water	6.5 ml
TEMED	10 µl
Ammonium persulfate (APS)	85 µl

Gels were electrophoresed at 80 V till the samples reached the stacking gel and then run at a constant voltage of 140 V.

3.6 Western blotting and immunodetection

Proteins were transblotted from SDS gels onto Hybond C nitrocellulose membranes (see section 2.3) at a constant ampere of 0.3 A / gel for 40 minutes in a semi-dry blot transfer chamber using Western blotting buffer (see section 2.2) for semi-dry transfer.
The nitrocellulose membranes were then immersed in Ponceau-S solution (see section 2.2) to visualize the molecular weight marker and to ensure proper protein transfer and integrity, and then destained with distilled water. Using the molecular weight marker as a reference, blots were carefully cut at appropriate heights into strips carrying the protein of interest. These strips were carefully labeled with a pencil, rinsed in TS buffer, and incubated in blocking buffer for 1 hour at room temperature with intermittent changes of blocking buffer (see section 2.2). Incubation with the primary antibody was performed at 4°C overnight or for 1-2 hours at room temperature in the antibody solution (see section 2.2). Incubation with the secondary antibody was performed for 1 hour at room temperature in antibody solution. After each incubation, blots were washed for 0.5-1 hour with the blocking solution or for 15-30 minutes with T-BST buffer. TS buffer with 0.1 % Tween-20 was preferred for stringent washes although T-BST was used sometimes. However, both methods yielded comparable results. All incubations and washes were performed on a shaker. All the immunoblots were invariably incubated

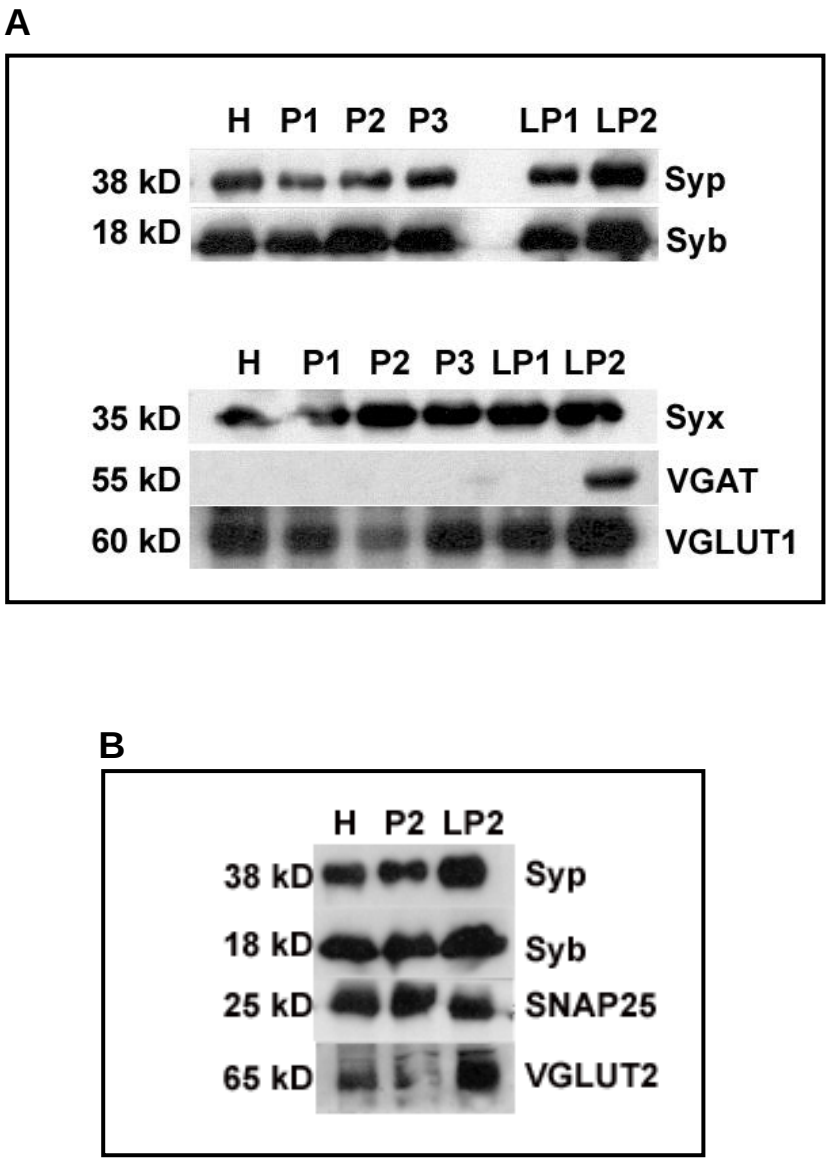
with secondary antibodies tagged with HRP (horse radish peroxidase), so the detection method used was ECL detection. The blots were bathed in ECL solution for one minute, excess solution was drained off the blots and films were exposed at different time intervals depending on the intensity of the signals. After the development, the blots were eventually dried and stored at 4°C for subsequent use.

4. RESULTS

4.1. Analysis of proteins in various sub-cellular fractions of the brain

Section 3.1 describes the preparation of synaptosomes (P2) and crude SVs (LP2).
As a first aspect, a postnuclear supernatant (PNS) and its corresponding pellet (P1) composed mainly of unlysed cells and nuclei were obtained from whole rat brain homogenate (H) after a low speed centrifugation step. Further centrifugation of the PNS yielded crude synaptosomes (P2). The P3 fraction containing vesicles of disrupted neurons and terminals was collected by high speed centrifugation of the corresponding supernatant to P2.
P2 was then subject to a hypotonic shock in 9 v/v of lysis buffer releasing the SVs. The lysed membranes were then pelleted to the first lysed pellet (LP1) and the corresponding supernatant (lysed supernatant 1, LS1) was subjected to a high speed spinning. The resulting pellet (LP2) represents crude synaptic vesicles.
Equal amounts of proteins from all the fractions (H, P1, P2, P3, LP1, LP2) were analysed via SDS-PAGE and immunoblotting for the specific synaptic vesicle proteins Syb and Syp as well as for vesicular transmitter transporters VGLUT 1 and 2 and VGAT. Additionally, the predominantly plasma membrane proteins syntaxin and SNAP25 (synapse associated protein of 25kDa) were analysed. As can be seen in Figure 6A and 6B the SV specific proteins Syb, Syp, VGLUTs and VGAT are particularly abundant in the LP2 fraction, indicating an enrichment of SVs in this fraction. On the contrary syntaxin and SNAP25 are less enriched in the LP2 fraction which further supports the preferential plasma vesicular origin of this fraction. However, during exocytosis/endocytosis cycles syntaxin and SNAP25 also sort to synaptic vesicles (Takamori et al, 2006), hence their detection in this fraction.
Considerable amounts of vesicular proteins (Syp, Syb, and VGLUT1) are also identified in the P3 pellet (Figure 6A).

Figure 6



Distribution of proteins in the sub-cellular fractions of the rat brain

A. Homogenate (H), crude synaptosomal pellet (P2) and synaptic vesicle fraction (LP2) were analysed via SDS-PAGE and immunoblotting: Equal amounts of protein were loaded for each fraction and blots were incubated with antibody against Syp, Syb, Syx, VGAT and VGLUT1.

B. Homogenate (H), crude synaptosomes (P2) and crude SVs (LP2) were separated via SDS-PAGE and blots were probed for Syp, Syb, SNAP25 and VGLUT2.

4.2. Analysis of the Syp/Syb complex via chemical crosslinking and immunoprecipitation

Two different methodical approaches were used to detect the interaction between synaptophysin and synaptobrevin. These methods are known as immunoprecipitation and chemical crosslinking. Isolated synaptic vesicles were used in all experiments.

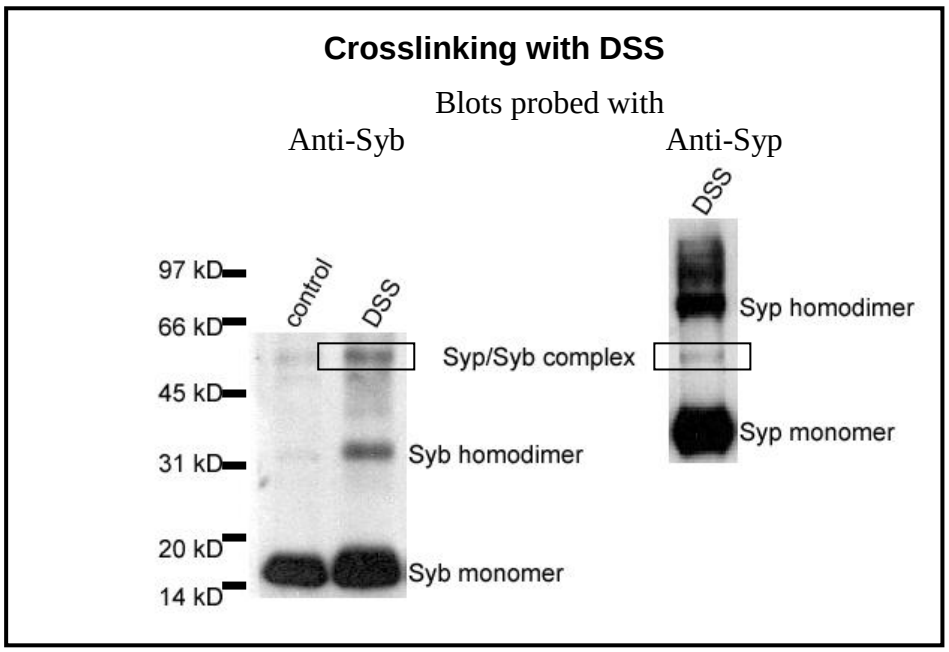
The immunoprecipitation of proteins from LP2 has previously been established by Becher et al., (1999a), adopting a similar approach as described by Edelmann et al., (1995) for purified synaptosomes. During immunoprecipitation with an antibody against Syb we expect a co-precipitation of Syp as well as syntaxin (Syx) and SNAP25 due to its interaction with these proteins at synaptic terminals to form the putative SNARE complex (Söllner et al., 1993a). Apart from the Syp/Syb complex, no interaction of Syp with the other SNARE proteins is known. Therefore we expect no co-precipitation of these proteins when anti-Syp is used for immunoprecipitation. Prior to precipitation, proteins were extracted in an extraction buffer containing 1% Triton X-100. Immunoprecipitation was carried out with an antibody against either Syp (anti-Syp) or Syb (anti-Syb) (see section 3.3). The beads (IP) and their respective supernatants (SN) were analysed via SDS-PAGE and the Western blots probed for Syp, Syb, Syx. As expected, Syx did not co-precipitate when using anti-Syp for IP (Figure 7B). However, both Syp and Syb were precipitated when either anti-Syp or anti-Syb was used, thus confirming the interaction between them. The immunoprecipitation method suffers from some shortcomings; this is mainly because a decrease in the abundance and stability of protein homo- and heterodimers in situ occurs during solubilisation of proteins in detergents such as triton (Khvotchev et al., 2004).

Chemical crosslinking has been used by several authors to study the interaction of the Syp/Syb complex in vivo (Edelmann et al., 1995; Reisinger et al., 2004; Yelamanchili et al., 2005). During this process, a covalent bond (S-S, disulfide bond) is formed between two molecules related in space. The formation of a stable bond allows the identification and study of weak or transient protein interactions which occurs in cells. DSS (Disuccinimidyl suberate) was the preferred crosslinker in this study. A final concentration of 1 mM DSS was added to the LP2 samples and incubated at 37°C for 30 minutes. The crosslinker was quenched by using 1 mM Tris-HCl and then cleared by gentle centrifugation (see section 3.4). The control was a sample with a similar setup but containing no crosslinker. Samples were analysed via SDS-PAGE followed by

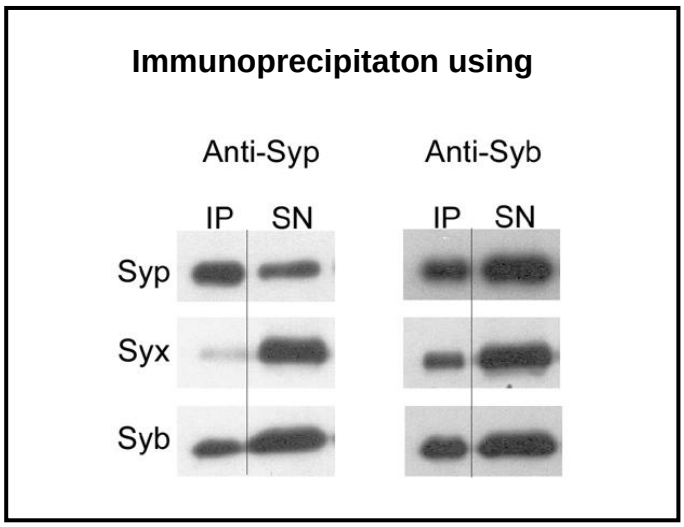
immunoblotting using antibodies against Syp and Syp. Three bands were seen in the crosslinked samples as expected (see Figure 7A, samples denoted DSS). When the membrane was probed with anti-Syb (left lanes), the first band seen just below the 20 kDa represents the Syb-monomer and is also seen in the control lane. The second band lies between 31 kDa and 43 kDa and represents the Syb-homodimer (36 kDa). The third band has a molecular weight of 56 kDa. It represents the heterodimer of Syp and Syb, otherwise known as the Syp/Syb complex. A similar band of 56 kDa is also detected when anti-Syp was used for blotting (right lane). Note that the band for the Syp/Syb complex detected with anti-Syb is more prominent than that detected with anti-Syp. This is probably due to the fact that the antigenic domain of Syp is less accessible after crosslinking. The first and third bands detected by anti-Syp at 38 kDa and 76 kDa are the monomer and the homodimer, respectively.

Figure 7

A



B



Analysis of the Syp/Syb complex via cross-linking and immunoprecipitation

A. Crude synaptic vesicles (LP2) were isolated from rat whole brain and cross-linked using DSS. Pellets were analysed via SDS-PAGE and Western blotting and probed with antibodies against Syb (Cl 69.1) or Syp (Cl 7.2).

B. SVs (LP2) were isolated from rat brain in comparable vesicles. Syp und Syb antibodies were used to immunoprecipitate synaptic proteins after extraction in 1% Triton. Immunoprecipitates (IPs) and their respective supernatants (SNs) were loaded in a relation of 1 IP : 3 SN on SDS-PAGE gels and the blots were probed with antibodies against Syb (Cl 69.1), Syp (Cl 7.2) and Syx (HPC-1).

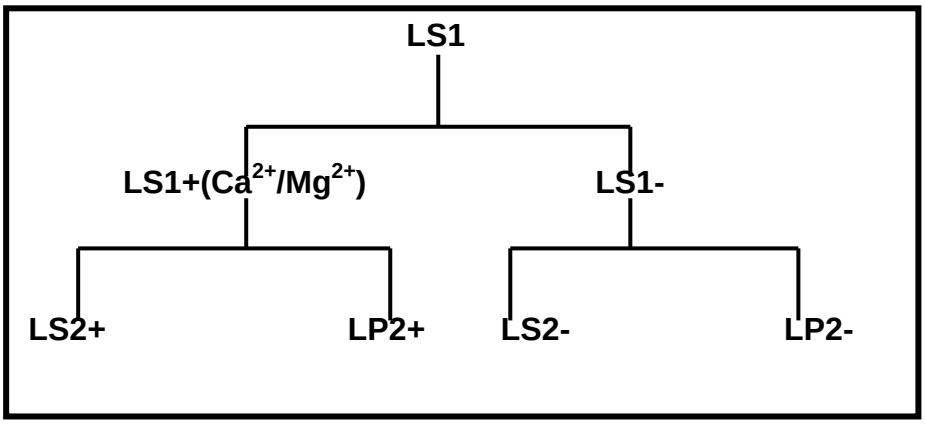
4.3. Ca^{2+}-dependent dissociation of the Syp/Syb complex

Previous studies have described a Ca^{2+}-dependent dissociation of the Syp/Syb complex in triton extracts of synaptosomes from rat brains (Prekeris & Terrian 1997; Daly & Ziff 2002). This dissociation was also observed when incubating hippocampal neurons in the presence of Ca^{2+}. However, studies to investigate the direct effect of Ca^{2+} using isolated SVs could not reproduce the previously observed dissociation of the Syp/Syb complex whether SVs were treated with Ca^{2+} alone or Ca^{2+}/Mg^{2+} as well as EGTA only (Reisinger et al., 2004). Questions therefore arose as to why the Syp/Syb complex would dissociate when synaptosomes or intact neurons were treated with Ca^{2+} but fails to dissociate at the level of isolated SVs. Synaptosomes contain cytosol including cytosolic proteins as well as SVs which are all also part of an intact neuron. All these constituents are lost in the LS2 fraction during the isolation of synaptic vesicles (LP2) by high speed centrifugation. Reisinger et al., (2004), suggested a Ca^{2+}-dependent factor rather than a direct effect of Ca^{2+} that would dissociate the Syp/Syb complex since no dissociation was observed in isolated SVs when treated with Ca^{2+}. To verify this hypothesis different sets of experiments were performed.

First, LP2 was collected after high speed centrifugation of the first lysed synaptosomal supernatant (LS1) and reconstituted in PBS buffer (section 2.2). The LP2 was then divided into four equal parts. The first sample (control) consisted of untreated LP2. The second was treated with 2 mM EGTA (EGTA is a chelator which preferentially binds Ca^{2+} and Mg^{2+} and buffers the free Ca^{2+} and Mg^{2+} ions in the concentration range below 1 µM). The last two samples were treated with 100 µM or 1 mM free Ca^{2+} both in combination with 2.5 mM Mg^{2+} (final concentrations in solution given) as calculated (see section 3.1.3). All samples were then incubated for 20 minutes at 37°C. Equal amounts of the samples were analysed via SDS-PAGE and the western blots were probed for Syb. As seen in Figure 9A the bands for the Syb monomer, homodimer and the Syp/Syb complex were clearly visible for each sample. Comparing the bands for Syp/Syb complex no difference in intensity was observed indicating the Syp/Syb complex could not be dissociated by direct Ca^{2+} influence and therefore confirms previous results (Reisinger et al., 2004).

Next the procedures were changed under which SVs were prepared in the presence of Ca^{2+}. The figure below illustrates the experimental steps:

Figure 8



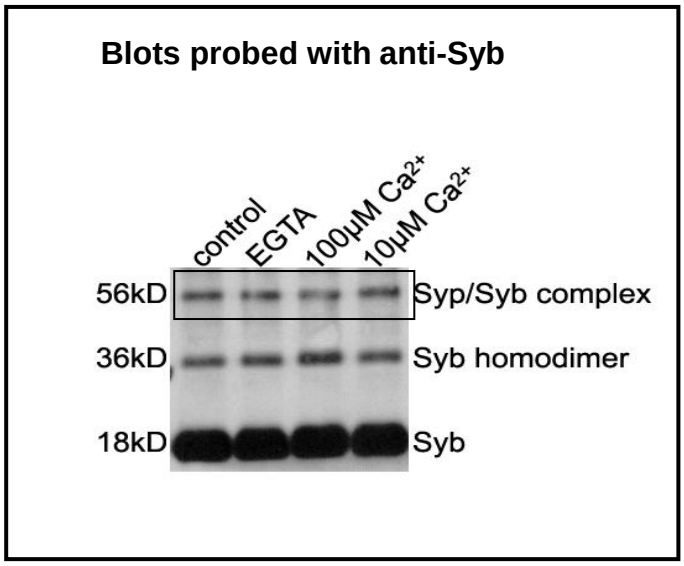
SVs isolation under Ca^{2+} condition (in accordance with Fig 5 in section 3.1.4)

Synaptosomal fractions were subjected to an osmotic shock (lysis) in the presence of 2 mM EGTA to chelate Ca^{2+} released due to lysis and HEPES to prevent drop of pH. The lysed synaptosomal suspension (LS0) was cleared of cellular debris (LP1) by a low speed centrifugation yielding an LS1 fraction. LS1 contains synaptic vesicles as well as constituents of the synaptosomal cytosol and was used for further analysis. The LS1 was divided into two samples, a control sample with no Ca^{2+} treatment and another sample treated with 100 µM free Ca^{2+}. Both samples were incubated for 20 minutes at 37°C before pelleting at 350,000 x g to obtain SVs and the corresponding LS2 (synaptosomal cytosol). Each LP2 was then reconstituted in its corresponding supernatant and subsequently subjected either to a chemical crosslinking or extracted in 1 % triton X-100 before proceeding to immunoprecipitation with anti-Syb and anti-Syp. Western blots were performed and the membranes were probed using antibodies against Syp and Syb.

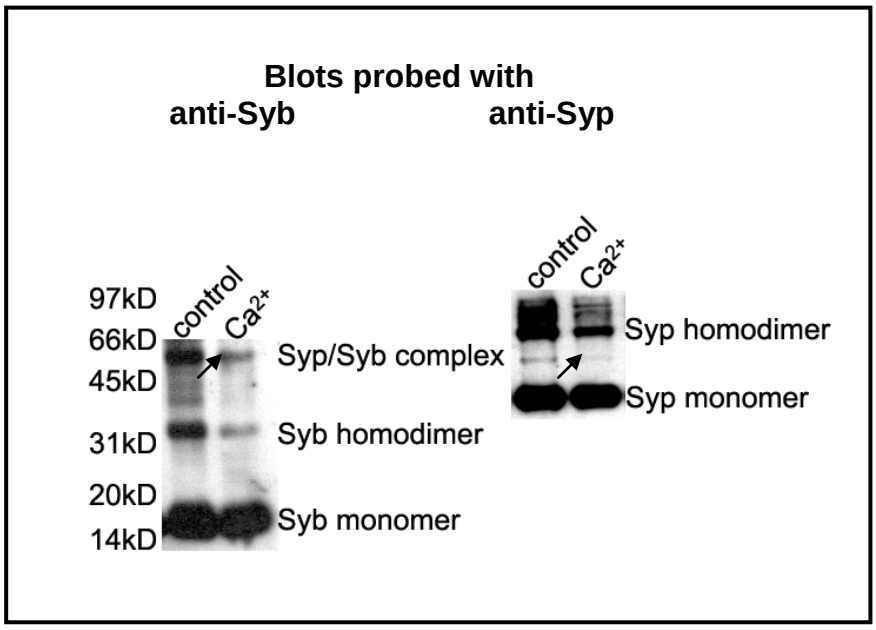
Figure 9B shows the crosslinked samples. Note that all three bands can be seen in both the control and the tested sample. However, a significant decrease in the intensity of the band for the Syp/Syb complex (56 kDa) was observed for Ca^{2+}-treated samples compared to the control irrespective of whether anti-Syb (Figure 9B, left) or anti-Syp (Figure 9B, right) was used for probing

Figure 9

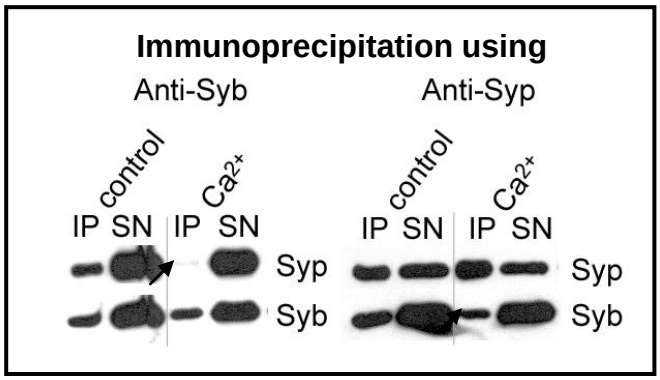
A


Blots probed with anti-Syb
control
EGTA
100μM Ca2+
10μM Ca2+
56kD
36kD
18kD
Syp/Syb complex
Syb homodimer
Syb

B


Blots probed with
anti-Syb
anti-Syp
97kD
66kD
45kD
31kD
20kD
14kD
control
Ca2+
Syp/Syb complex
Syb homodimer
Syb monomer
control
Ca2+
Syp homodimer
Syp monomer

C



Dissociation of the Syp/Syb complex under Ca^{2+} condition

A. Crude synaptic vesicles (LP2) were collected after high speed centrifugation and reconstituted in PBS buffer. Sample was divided in 4 equal portions. One sample was left native; the second was treated with 2 mM EGTA and the last two were treated with 100 µM and 1 mM Ca^{2+}, respectively, in combination with 2.5 mM Mg^{2+}. All samples were incubated for 20 minutes at 37°C and subsequently treated with DSS for cross-linking. The samples were then analysed via SDS-PAGE and Western blotting. Blot was probed using antibody against Syb. Note that there is no change in the Syp/Syb complex compared with the control samples.

B. LS1 samples were treated with or without 100 µM free Ca^{2+} and incubated at 37°C for 20 min. LP2 samples were reconstituted in their respective supernatants (LS2) and analysed via SDS-PAGE and immunoblotting using antibodies against Syp and Syb. Note a decrease in the complex compared to the control sample indicating the dissociation of the complex.

C. SVs (LP2) were prepared as described in B and 1mg/ml protein extracted in 1% Triton X-100. Immunoprecipitation was carried out with antibodies against Syp and Syb. Samples were analysed via SDS-PAGE and immunoblotting using the antibodies indicated. A decrease in the Syp/Syb complex indicates a dissociation of the complex.

No difference was seen in the bands for the Syb monomer or Syp monomer. These results imply that the Ca^{2+}-dependent dissociation has been re-established by re-introducing the cytosol.

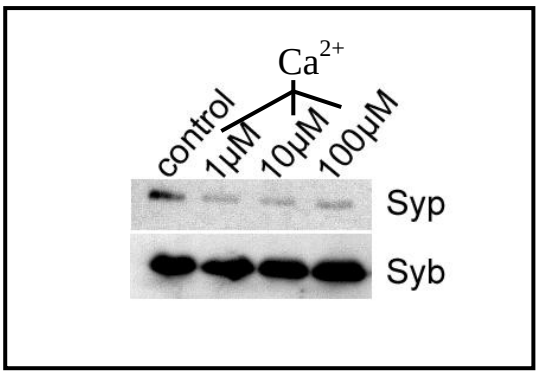
Similar results were obtained when analysing the complex using the immuno-precipitation method (see Figure 9C). Precipitation was performed using antibodies against Syp and Syb. The co-precipitation of Syp was completely abolished when precipitating with anti-Syb in the presence of Ca^{2+} as shown by the arrow (Figure 9C, left) indicating a complete dissociation of the complex. The precipitation with anti-Syb showed a significant decrease in the co-precipitated Syp (Figure 9C, right) which also represents a dissociation of the complex. Similar amounts of synaptophysin and

synaptobrevin were precipitated with the corresponding antibody irrespective of whether the samples were treated with Ca^{2+} or not.

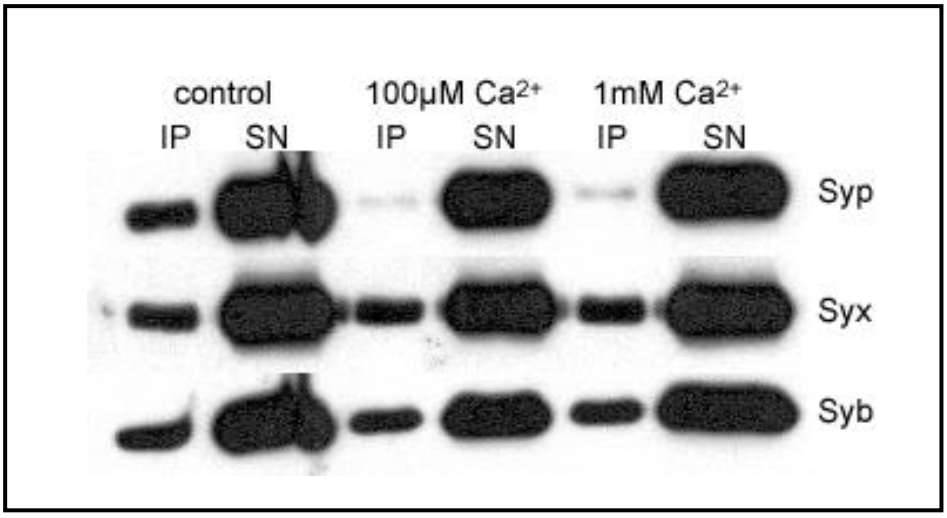
Does the dissociation of the Syp/Syb-complex depend on the concentration of Ca^{2+} used? So far we have been able to establish that the dissociation of the Syp/Syb-complex depends on the presence of Ca^{2+} ions. It was however not clear if this dissociation depends on the concentration of free Ca^{2+} used for treatment in the experimental setting. Lower dissociating concentrations of free Ca^{2+} may, however, be of physiological relevance. Therefore we performed further experiments with LP2 and LS2 prepared under conditions characterised by the absence or presence of various amounts of free Ca^{2+} to verify this question. In a first experiment LS1 was treated with either 1 µM, 10 µM or 100 µM free Ca^{2+} and in a subsequent experiment 100 µM and 1 mM free Ca^{2+} were used. The control samples for both experiments were LP2 prepared under non Ca^{2+} conditions (from untreated LS1). The LP2s were reconstituted in their corresponding LS2. All the samples were then incubated at 37°C for 20 minutes before being subjected to high speed centrifugation. Immunoprecipitation was performed with an antibody against Syb and the samples were analysed via SDS-PAGE and immunoblotting with antibodies agianst Syp and Syb. We observed a clear reduction in the co-precipitation of Syp in all Ca^{2+} treated samples (see Figure 10A and 10B). This reduction in the amount of Syp co-precipitated with anti-Syb was the same for all the Ca^{2+} concentrations used suggesting that the dissociation requires quite low physiologically relevant free concentration of Ca^{2+}. In addition the binding of Syb to Syx was analysed. Figure 10B shows that the interaction between Syb and Syx was not disturbed by Ca^{2+} treatment.

Figure 10

A



B



Concentration dependent dissociation of the Syp/Syb complex

A. Lysed synaptosomal supernatant (LS1) was treated with 1, 10 and 100µM free Ca^{2+} and incubated at 37°C for 20 min. LS1 were further pelleted to obtain crude synaptic vesicles (LP2). To prepare the samples for detergent extraction, the LP2s were resuspended in a small defined volume of their corresponding supernatants (LS2) and 1mg/ml protein was then extracted in 1% Triton X-100. Immunoprecipitation was carried out with an antibody against Syb. Samples were analysed via SDS-PAGE and immunoblotting and probed with the antibodies indicated. Note a dissociation of the Syp/Syb complex even at 1 µM Ca^{2+}.

B. Same controls were used as in gels for Fig 9C. LS1 was treated with 100µM and 1mM free Ca^{2+} and then proceeded as in 10A. Blots were probed with antibodies against Syb (69.1), Syx (HPC-1) and Syp (7.2). Note the dissociation of the complex at all Ca^{2+} concentrations.

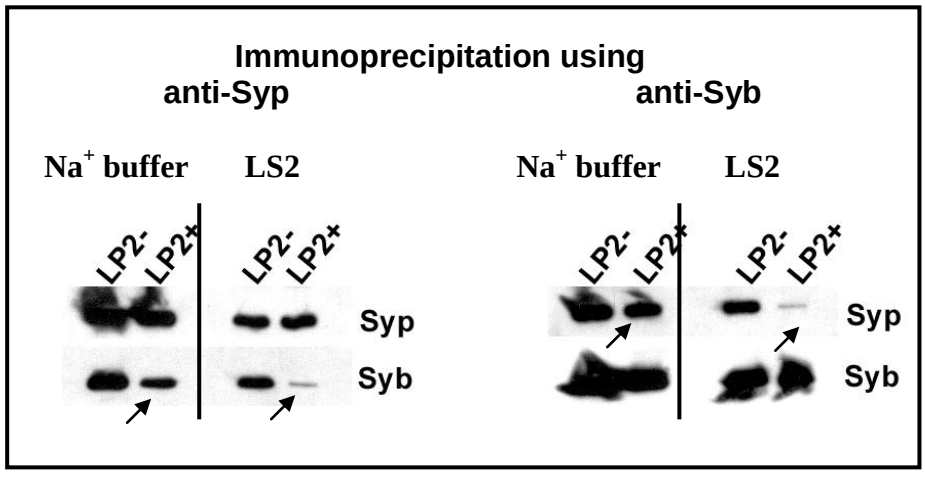
4.4. The role of a Ca^{2+} dependent cytosolic factor in the dissociation of the Syp/Syb complex

Why does the Syp/Syb complex dissociate in a Ca^{2+} dependent manner when cytosol is present? Could the dissociation be reversed in the absence of Ca^{2+} ions? Becher et al., (1999a), have shown that synaptophysin that binds to synaptobrevin is post-translationally modified. In the same study, it was observed that during development, adult synaptic cytosol acquires a certain potential to induce the formation of the Syp/Syb complex in embryonic synaptic vesicles where it was previously absent. Considering the role played by Ca^{2+} as a second messenger in cells, we suggest that an increase in intracellular Ca^{2+} concentration as is the case during neuroexocytosis may lead to the activation of a putative cytosolic factor which in turn removes the post-translational modification of synaptophysin and causes the Syp/Syb complex to dissociate. In this viewpoint, the Ca^{2+}-dependent cytosolic factor is in a dynamic equilibrium with the putative post-translational modification factor proposed by Becher et al., (1999a). At normal (low) intracellular Ca^{2+} ion concentrations the formation of the Syp/Syb complex would exceed the dissociation due to an overweight of the post-translational modification of Syp. This equilibrium is tipped over in favour of the dissociation when the intracellular Ca^{2+} concentration [Ca^{2+}] is increased due to Ca^{2+} influx during an action potential and reverses again to more formation when [Ca^{2+}] returns to normal. Proceeding along this line of thought we shall manipulate the setups to see what happens when the Ca^{2+} concentration is reduced again by resuspending LP2+ in LS2- hence reducing the concentration of Ca^{2+} or doing the opposite (i.e. LP2- in LS2+) thereby promoting Syp/Syb complex formation again.

Before proceeding we wanted to ascertain the absolute necessity of cytosol for the Ca^{2+}-dependent dissociation. For these experiments LS1 was obtained after P2 has been osmotically lysed under EGTA and HEPES conditions and the lysate cleared of cell debris by a low speed centrifugation step. High speed centrifugation of the LS1 yielded LP2 which was then arranged in two setups. In the first setup, we prepared two samples each resuspended in 2 ml of sodium buffer (see section 2.2). One sample was treated with 1 µM of free Ca^{2+} and the other was maintained untreated (control). In the second setup each sample was resuspend in 2 ml of LS2 containing either no or 1 µM of free Ca^{2+} as in the first setup. The samples were then pelleted again at 350,000 g to reobtain LP2 and resuspended in 200 µl of their corresponding supernatant each

followed by extraction with 1 % triton X-100 and immunoprecipitation with antibodies against Syb and Syp. Samples treated with Ca^{2+} were termed LP2+ and those without (control) correspondingly LP2-. Equal amounts of samples from each setup were then analysed via SDS-PAGE and immunoblotting with anti-Syp and anti-Syb. Comparing the intensity of the complex in the control and test sample for each setup, we observed that the immunoprecipitation (IP) with both anti-Syp and anti-Syb showed a significant dissociation of the complex when the Ca^{2+} treatment was carried out in the presence of cytosol (LS2) (Figure 11). Nonetheless, a slight dissociation of the complex was also observed when Ca^{2+} treatment was performed in sodium buffer in the absence of cytosol. This slight dissociation probably occurs as a result of cytosolic rests still present in the LP2 fraction after high speed centrifugation. A higher level of SV purification during experiments may clarify this situation. Nevertheless, considering the marked difference in the degree of dissociation between SVs with Ca^{2+} performed in the presence of LS2 (synaptosomal cytosol) and that performed with sodium buffer, the central role played by cytosol and/or the cytosolic factor for the dissociation of the Syp/Syb complex is quite obvious.

Figure 11



Cytosolic factor is necessary for dissociation of complex in the presence of Ca^{2+}

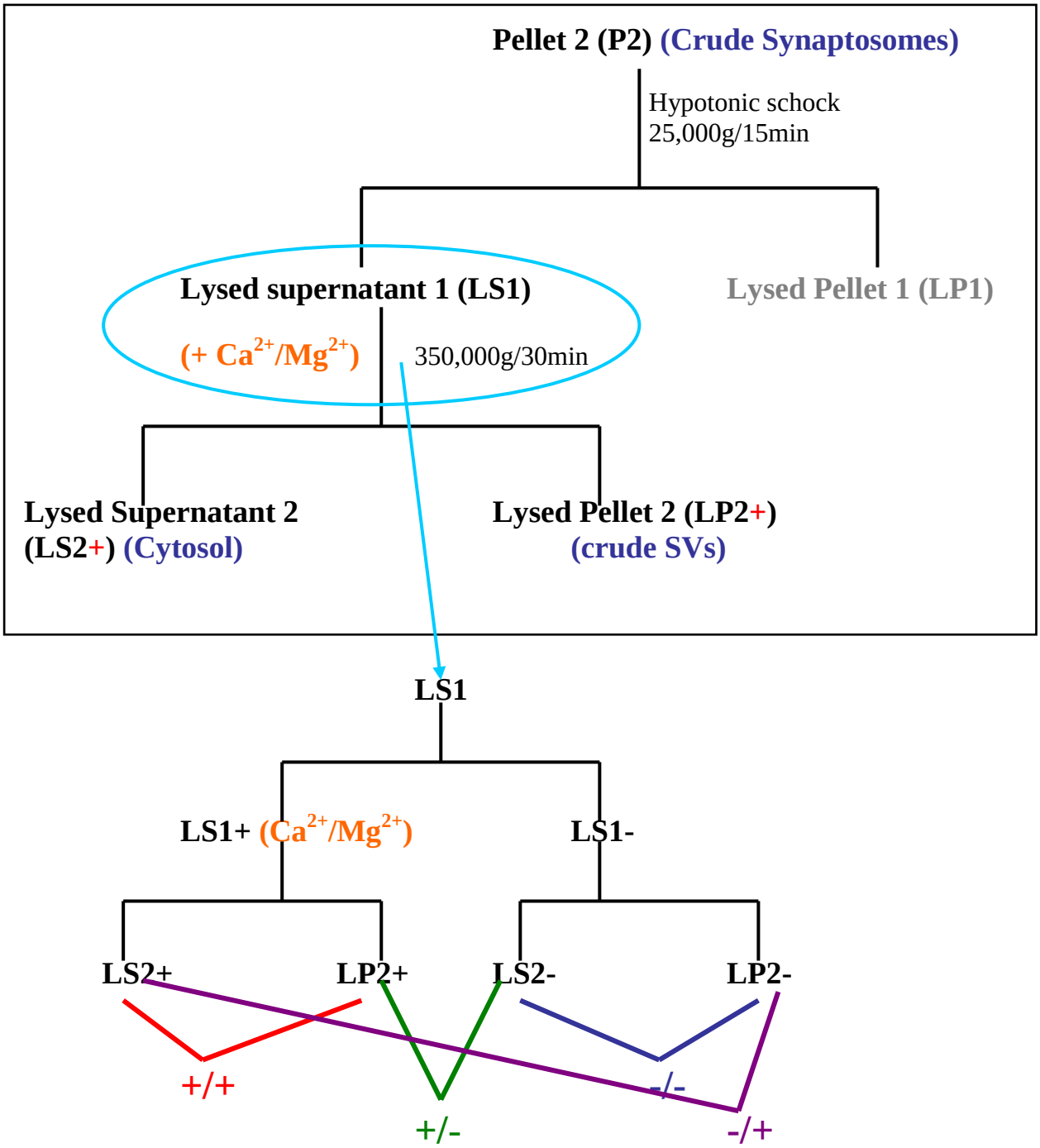
Crude SVs (LP2) were reconstituted in either 2 ml of sodium buffer (see section 2.2) or 2 ml of their respective supernatants (LS2) and treated with 1 µM free Ca^{2+} (for LP2+) or no Ca^{2+} (for LP2-) and incubated at 37°C for 20 min. Samples were then repelleted to obtain LP2 and reconstituted in their respective LS2. 1mg/ml protein was extracted in 1% Triton for each sample. Immunoprecipitation was carried out with antibodies against Syp and Syb. Samples were analysed via SDS-PAGE and immunoblotting using the antibodies indicated.

Having ultimately ascertained the necessity of cytosol for the dissociation of the Syp/Syb complex, we proceeded to manipulate the experimental set-up to get more insight into the regulation of the dissociation and re-association/formation of the complex in relation to the intracellular Ca^{2+} concentration. Two sets of LP2 were prepared as illustrated in Figure 12.

In the first set, LP2 was isolated from LS1 with no Ca^{2+} treatment. The pellet obtained was termed LP2- and its corresponding supernatant LS2- (cytosol without Ca^{2+}). In the second set, LS1 was treated with 1 µM free Ca^{2+} before high speed centrifugation to yield a pellet which was termed LP2+ and its corresponding supernatant LS2+ (cytosol + Ca^{2+}). As indicated in the diagram (Figure 12), a part of the LP2- was resuspended in LS2- yielding the sample -/- and the other part was resuspended with LS2+ yielding the sample -/+. Similarly, a part of LP2+ was resuspended with LS2+ yielding the sample +/+ and another part was resuspended with LS2- yielding the sample +/-. Samples were incubated at 37°C for 20 minutes and then repelleted by ultracentrifugation. Crosslinking or immunoprecipitation was then performed prior to analysis of the samples via SDS-PAGE and immunoblotting. The blots for the crosslinked samples were probed using antibodies against Syp and Syb and the results are shown in Figure 13A. The Syp/Syb complex was clearly detected at about 56 kDa in the sample -/- (control). All samples involving a Ca^{2+} treatment showed a dissociation of the complex. However, the +/+ sample which was prepared under absolute Ca^{2+} conditions (i.e. Ca^{2+} treatment prior to SV isolation and resuspension in LS2+) showed a higher degree of dissociation of the complex when compared with the other samples which also had Ca^{2+} treatments at one stage.

Similar results were also obtained when immunoprecipitation was performed instead of crosslinking. Immunoprecipitation with anti-Syb showed less co-precipitation of Syp in all Ca^{2+} treated samples compared to the -/- (control) sample with the least co-precipitation occurring in the sample with absolute Ca^{2+} conditions (+/+ sample). Notice the marked difference in the degree of dissociation between the +/+ sample and the +/- or -/+ sample. This could be explained from two different perspectives; 1) The less dissociation observed in the +/- samples is due to less shifting of the equilibrium to the side of Ca^{2+}-dependent dissociation factor due to a reduction in Ca^{2+} concentration in both samples. This is rather less convincing because previous experiments have shown that though the dissociation occurs when Ca^{2+} is present, no difference could be observed when various concentrations of Ca^{2+} were used (see Figure 10A & B).

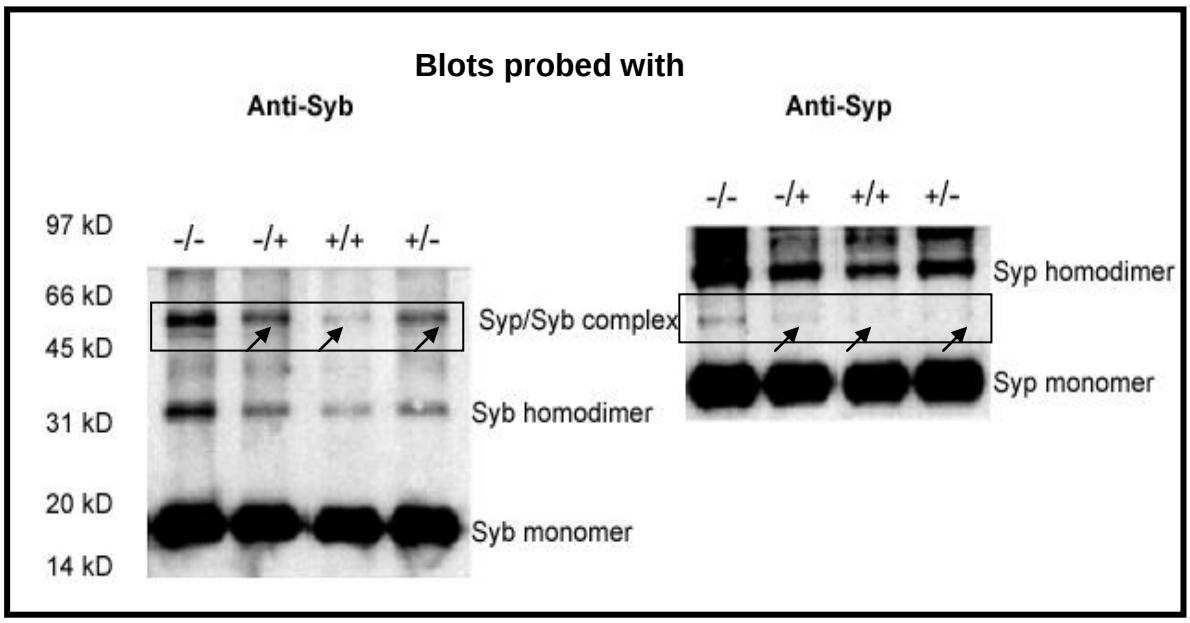
Figure 12



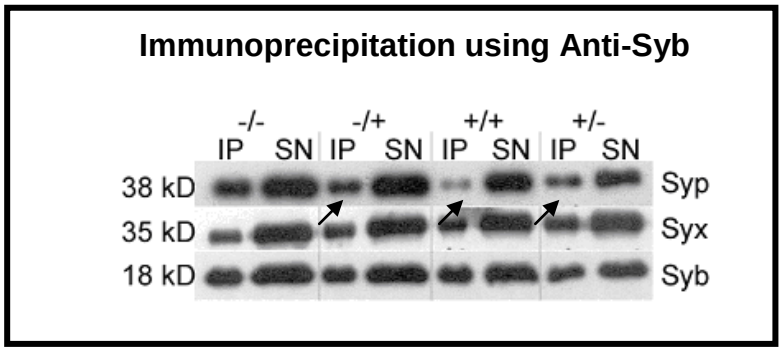
2.) Alternatively, resuspending LP2+ in LS2- will mean exposing the SVs to synaptosomal cytosol with a higher activity of the putative synaptophysin binding factor leading to formation of more of the Syp/Syb complex. Hence the complex which was previously dissociated due to Ca^{2+} treatment will also be re-associated.

Figure 13

A



B



SVs plus cytosol under various Ca^{2+} treatments; Analysis via immunoprecipitation and cross-linking

A. Lysed synaptosomal supernatant (LS1) was treated with 1 µM free Ca^{2+} and incubated at 37°C for 20 min. LP2s were reconstituted in the supernatants (LS2) as indicated in figure 12 and subsequently cross-linked using DSS. Synaptic proteins were analysed via SDS-PAGE and immunoblotting using antibodies against Syp and Syb. We observe a dissociation of the Syp/Syb complex in the samples +/+, -/+ and +/-, although less marked in the latter 2 samples which show comparable dissociation.

B. SVs (LP2) were treated as described in A and thereafter subjected to immunoprecipitation using an antibody against Syb. Synaptic proteins were analysed via SDS-PAGE and the blots probed with antibodies against Syp, Syb and Syx. Note a similar pattern of dissociation as seen in figure 13a.

4.5. Characterisation of the Ca^{2+}-dependent cytosolic factor dissociating the interaction between Synaptophysin and Synaptobrevin.

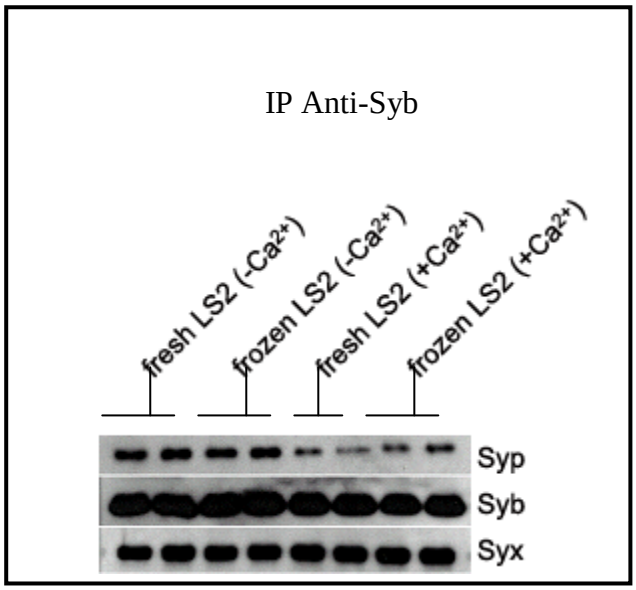
After establishing the dissociation of the Syp/Syb complex by a Ca^{2+}-dependent cytosolic factor, it was pertinent to study the properties of this factor in order to better understand its possible role for neuroexocytosis.

To be able to characterise the Ca^{2+}-dependent cytosolic factor it was necessary to isolate (concentrate) and store this factor. Considering the vast variety of possibilities, experiments were performed to verify whether freezing or high temperatures would abolish the ability of the cytosolic factor to dissociate the Syp/Syb complex. Understanding how the factor reacts to extreme temperatures would permit us to access the ease of handling the factor in larger quantities and allow better manipulations in the future.

In previous experiments surplus LS2 obtained under various Ca^{2+} conditions was usually stored in refrigerators at -80°C. Frozen LS2 was thawed slowly at room temperature and used along with freshly prepared LS2 to resuspend a set of freshly isolated SVs. Two setups were arranged. In the first set-up SVs were resuspended in 2 ml of frozen LS2 and in the second setup 2 ml of fresh LS2 was used for resuspension. One part of each setup was treated with 1 µM of free Ca^{2+} and the other part was left untreated. All samples were incubated at 37°C for 20 minutes before centrifuging again at 350,000 g for 30 minutes to recollect the SVs. Each LP2 was then reconstituted in 200 µl of its respective supernatant. Immunoprecipitation was performed with an antibody against Syb after extracting the proteins in 1 % triton to a final concentration of 1 mg/ml. Equal amounts of the samples were analysed in doubles via SDS-PAGE and immunoblotting probing for Syp, Syb and Syx. The results are shown in Figure 14.

Notwithstanding whether the LS2 was freshly prepared or had been stored frozen, a similar dissociation of the Syp/Syb complex was observed in all the samples treated with Ca^{2+}, as depicted by a reduced co-precipitation of Syp. For comparison, the complex formation with syntaxin was analysed in parallel, revealing no difference between Ca^{2+} free and Ca^{2+} treated samples.

Figure 14



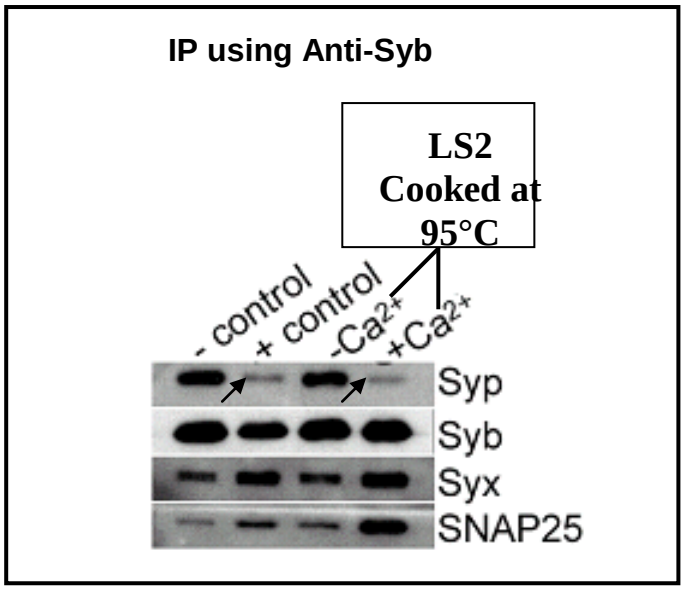
Cytosolic factor functional after freezing and storage

Crude SVs (LP2) were reconstituted in either 2 ml of previously frozen LS2 or 2 ml of their respective supernatants (LS2), treated with 1 µM free Ca^{2+} or no Ca^{2+} and incubated at 37°C for 20 min. Samples were pelleted again to re-obtain LP2s which were then reconstituted in 200 µl of their corresponding supernatants.
1 mg/ml protein was extracted in 1% Triton for each sample. Immunoprecipitation was performed using an antibody against Syb. Samples were analysed via SDS-PAGE and immunoblotting performed using antibodies against Syp, Syb and Syx. Pellets were loaded in duplicates. We observe dissociation in the LP2 irrespective of whether it was reconstituted with freshly prepared or previously frozen LS2.

In a second set of experiments, SVs were resuspended in LS2 previously cooked at 95°C for 10 minutes. One part of the sample was then treated with 1 µM free Ca^{2+} and the samples were incubated at 37°C for 20 minutes. After repelleting the SVs with a high speed centrifugation step, immunoprecipitation was performed and the samples were analysed via Western blotting along with LP2 resuspended in uncooked LS2. The results are shown in Figure 15. In both samples with a Ca^{2+} treatment, irrespective of whether the LS2 was cooked or not, the complex dissociated equally well, suggesting that the factor is stable to heat, at least within the prevailing conditions in the experiment. This result does not however exclude the fact that temperatures higher than 95°C may disrupt the factors ability to dissociate the Syp/Syb complex in a Ca^{2+}

dependent manner. Once again the interaction of Syb with its SNARE partners Syx and SNAP25 was analysed. We observe an increased interaction under the Ca^{2+} condition.

Figure 15



Cytosolic factor stable after cooking at 95°C

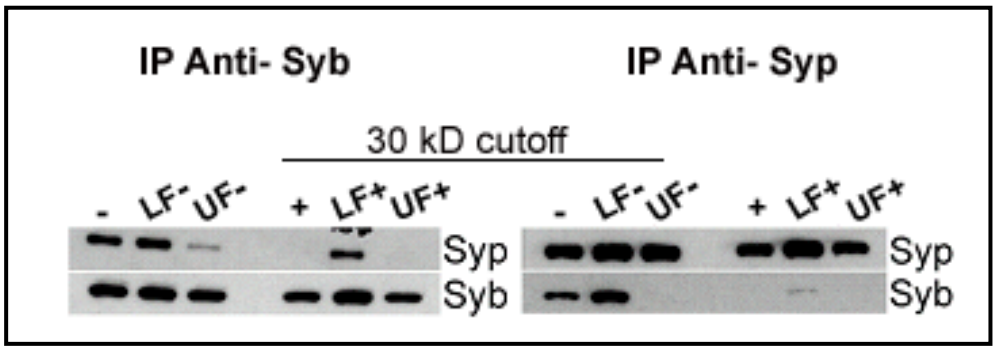
Crude SVs (LP2) were reconstituted partly in 2 ml of their corresponding LS2 that was cooked at 95°C for 10 min. Samples were either treated using 1 µM free Ca^{2+} or left with no Ca^{2+} treatment prior to incubation at 37°C for 20 min. Samples were then repelleted to obtain LP2s which were then reconstituted in 200 ml of their respective supernatants each. 1mg/ml protein was extracted in 1% Triton for each sample. Immunoprecipitation was performed using an antibody against Syb. Samples were analysed via SDS-PAGE and immunoblotting using the antibodies indicated.

We further proceeded to fractionate the synaptosomal cytosol (LS2) according to molecular weight using centricon concentrators with a molecular weight cut-off of 30 kDa (Centricon 30). The fractions were named UF (upper fraction; Molecular weight > 30 kDa) and LF (lower fraction). Detecting activity in only one of the fractions would allow us to conveniently exclude proteins of molecular weights in the other fraction. One portion of each fraction was treated with 1 µM free Ca^{2+} yielding UF+ and LF+ as well as UF- and LF- for the untreated samples. LP2 was then divided in 4 fractions prior to reconstitution with one of the fractions of LS2 mentioned above. Immunoprecipitation was performed with antibodies against Syp and Syb and the samples analysed via SDS-PAGE and immunoblotting. The Ca^{2+} dependent dissociating activity was present

in all Ca^{2+}-treated samples irrespective of the molecular weight range, although more pronounced in the UF+ (Figure 16).

Furthermore, a Ca^{2+}-independent dissociation was observed in the UF-. There also appeared to be an enhancement in the complex formation apparently when SVs were resuspended in LF-, which was even more prominent when immunoprecipitation was performed with anti-Syp.

Figure 16



Syp/Syb complex dissociation after a selective cut-off of 30 KDa

LS2 was centrifuged for 15 min using a centricon 30 to obtain an upper fraction (UF) proteins > 30 kDa and a lower fraction (LF) proteins < 30 kDa. UF+ and LF+ were treated with 1 µM Ca^{2+} while UF- and LF- had no treatment with Ca^{2+}: SVs were reconstituted in either of the supernatants and all samples incubated at 37°C for 20 min, then repelleted and reconstituted in their corresponding supernatants. 1mg/ml protein was extracted in 1% Triton for each sample. Immunoprecipitation was carried out with Syp and Syb antibodies. The respective precipitates (IP) were analysed via SDS-PAGE and the blots probed using the antibodies indicated. We observe a dissociation of the complex in all cases of reconstitution with UF as well as with LF+, although the latter´s dissociation was less marked.

In summary, the Syp/Syb interaction appears to depend on cytosolic constituents (factors) which determine the association with Syp and which are at least in part regulated by the cytosolic Ca^{2+} concentration.

5. Discussion

5.1 Synaptic transmission:- What role is played by Syp and Syb?

The proposal of the SNARE hypothesis (Söllner et al., 1993a) was a major breakthrough in the research of synaptic transmission and brought with it also a great wave of optimism in the research of synapsis. The identification of Syb, Syx and SNAP25 as the proteins responsible for the formation of the SNARE complex appeared to have finally laid the cornerstone enabling the elucidation of synaptic transmission. However, it has become more obvious that the SNARE complex, though essential for neuroexocytosis, represents only the minimum machinery required for synaptic transmission. This view has been supported by the discovery of numerous proteins which interact with the various SNARE proteins such as munc18 and msec1 (Hata et al., 1993; Pevsner et al., 1994). A number of proteins located on synaptic vesicles have also been implicated in the Ca^{2+} triggered exocytosis. One of these proteins is synaptotagmin (Syt), an abundant Ca^{2+} binding secretory vesicle protein that has been widely proposed to be a sensor for regulated exocytosis (Brose et al., 1992; Davletov & Südhof, 1993; Geppert et al., 1994; Südhof & Rizo, 1996; Desai et al., 2000; Fernandez-Chacon et al., 2001; Littleton et al., 2001). Zhang et al., (2002), demonstrated that a Ca^{2+}-dependent Syt binding to SNAP25 is essential for the Ca^{2+} triggered exocytosis. Another protein is the multifunctional Ca^{2+}/calmodulin-dependent protein kinase, an enzyme which is ubiquitously found in the cytoplasm of eukaryotic cells. It is, however, particularly abundant in the nervous system where it acounts for 0.5-1 % of the total protein, a truly high concentration for an enzyme. This enzyme is able to transfer phosphate groups from ATP to a variety of different proteins. One of the best known substrate proteins for this enzyme is synapsin 1, a vesicular membrane specific protein which is thought to be involved in the long term modulation of synaptic efficiency by playing a role in the regulated neurotransmitter release during neuroexocytosis. It is nonetheless clear that the rapid release of neurotransmitter at synaptic terminals (fraction of a millisecond) occurs too fast to be mediated by a complex enzyme reaction. The vesicular membrane protein Syp is also known to undergo phosphorylation, but this has yet to be linked to the Ca^{2+}/calmodulin-dependent protein kinase. However, it is conceivable that the phosphorylation of Syp would change

its dynamics with respect to its reactivity with other proteins such as Syb. Possibly, phosphorylated Syp would not form the Syp/Syb complex with Syb or would dissociate from an already formed complex thereby providing free Syb for the formation of the SNARE complex. The proof of this still has to be established experimentally.

Syp binds the v-SNARE Syb forming a complex which is mutually exclusive from the SNARE complex, suggesting the existence of at least two non-overlapping pools of Syb at the synapse. There are an average of 30 copies Syp per synaptic vesicle, making it second in numbers only to Syb (70 copies) in an average SV (Takamori et al., 2006). In spite of its abundance and the progress made in the understanding of its molecular mechanism of action, the exact physiological role of Syp at the synapse still remains elusive, especially given the fact that Syp knockout (KO) mice species have shown no detectable overt phenotype when compared with the wild type animals (Eshkind & Leube, 1995; McMahon et al., 1996). Obviously, the absence of an overt phenotype in KO species does not imply that Syp represents a surplus to requirements in the synapse. On the contrary, it indicates the complexity of its functions which probably need to be verified in other experimental setups. The following two hypothesis were suggested to attempt to clarify this view: 1) Syp exhibits some kind of redundancy in function, which is attributed to the presence of structural homologues that can compensate the absence of Syp. Some interesting proteins in connection with this question are the brain-specific homologue Synaptoporin (or Synaptophysin II) (Knaus et al., 1990; Fykse et al., 1993) and the synaptogyrins representing a family of paralogue SV proteins (Kedra et al., 1998). In accordance with the hypothesis of redundancy in function, a careful examination of Syp knockout mice led to the observation that retinal rod photoreceptors, which normally lack detectable levels of the Syp homologue synaptoporin and express Syp at high concentration, show several defects in cell membrane organisation and a reduced number of SVs (Spiwoks-Becker et al., 2001). Furthermore, Syp/synaptogyrin double knockout mice show clear defects in several forms of synaptic plasticity, although the basal neurotransmitter release in such mice shows no overt deficit (Janz & Südhof, 1999; Valtorta et al., 2004 review). 2) The inherent validity of the interpretation of results obtained from animal behaviour experiments is always questionable because the observable behaviour like the accomplishment of specific tasks is mostly limited to a fraction of the functions performed by these animals. The possibility of impairment of more complex cognitive functions can only be poorly accessed in such experiments making the results difficult to

interpret. Seemingly undramatic phenotypes may be dismissed as banal, although they may imply far more dramatic consequences. As an example, when neurons from Syp KO and wildtype mice were co-cultured to form heteregenotypic networks in vitro, the Syp KO neurons were impaired in their ability to form both hetero- and homosynapses, suggesting an inherent role for Syp in the formation of activity-dependent synapses (Tarsa & Goda, 2002) which is a pivotal process in synaptic plasticity and hence important for memory formation.

The reactivity of the v-SNARE Syb was initially thought to be controlled by direct interaction with accessory proteins. Using isolated synaptic vesicles, it was reported that vesicular Syb is not constitutively reactive but requires Ca^{2+} to interact with Syx and SNAP25. A regulation by a Ca^{2+} binding protein such as Syt was suggested to mediate the process (Hu et al., 2002). These data were further affirmed by observations suggesting that Syb, once inserted in liposomes, does not bind to its SNARE partners (Hu et al., 2002; Kweon et al., 2003). In a similar view, several proteins have been invoked in binding and regulating Syb. Calmodulin and Syp are prominent examples in this list. Calmodulin has been reported to bind to the C-terminal region of the SNARE motif of Syb in a Ca^{2+}-dependent manner (Quetglas et al., 2002) and possibly leads to Syb inhibition (de Haro et al., 2004). A recent study, however, has challenged the physiological role of calmodulin in regulating the activity of Syb and states that the binding of Ca^{2+}/Calmodulin to Syb does not alter its fusogenic properties (Siddiqui et al., 2007).

5.2 Regulation of the Syp/Syb complex: The association-dissociation cycle

The interaction of Syb with the vesicular membrane protein Syp is mediated primarily by the C-terminal transmembrane region of Syb (Edelmann et al., 1995; Yelamanchili et al., 2005). Considering that Syb bound to Syp cannot enter the SNARE complex, it has been suggested that the dissociation of Syb from Syp may constitute an activation step for Syb prior to the formation of the SNARE complex (Edelmann et al., 1995). While many recent works have repeatedly brought up evidence showing a connection between the dynamics of the Syp/Syb complex and neuroexocytosis, none of these has yet satisfactorily elucidated the intrinsic role this complex could play in this process. The most intriguing question is still whether the Syp/Syb interaction could act as a switch that modulates the availabilty of Syb ready to enter the SNARE fusion complex.

5.2.1 Ca^{2+} and Cytosol are required for the dissociation of the Syp/Syb complex

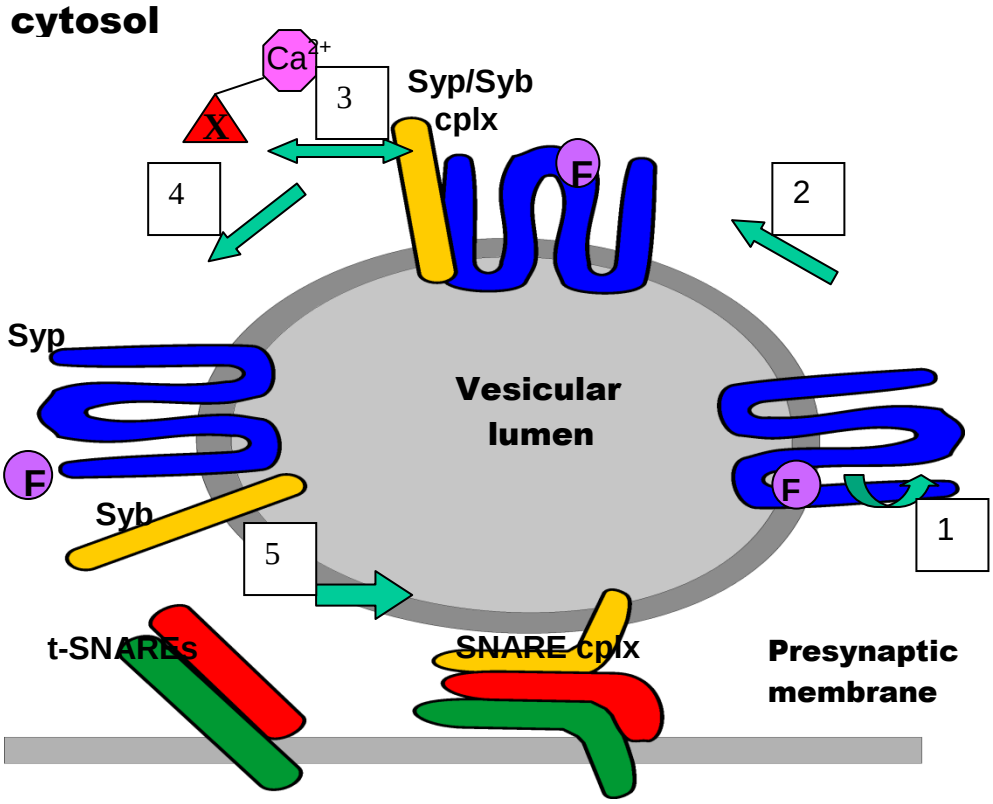
In the present project we have been able to show that the Syp/Syb complex undergoes a Ca^{2+}-dependent dissociation in its native environment. Previous studies had already indicated this dissociation, however, synaptosomes (Prekaris & Terrian, 1997; Daly & Ziff, 2002) and hippocampal neurons (Reisinger et al., 2004) were used. The first attempt in our working group failed to reproduce these results when isolated vesicles prepared from synaptosomes were used for the experiment. Using lysed synaptosomes cleared of debris we were able to obtain a dissociation of the Syp/Syb complex as described in previous studies. This observation led to the proposal that the dissociation does not entirely depend on the presence of Ca^{2+} but involves a Ca^{2+}-dependent step (Reisinger et al., 2004). A careful examination of the steps leading to the isolation of SVs from synaptosomes shows clearly that we lose the cytosol and all of its components during the last high speed centrifugation step to the supernatant LS2 (see Fig. 4). We therefore conclude that Ca^{2+} as well as cytosol is necessary for the dissociation of the Syp/Syb complex. The dissociation at physiological Ca^{2+} concentrations as well as non-physiological concentrations was comparable, indicating an intrinsic role played by Ca^{2+} rather than a mere disruptive mechanism due to a change in ionic currents. Taking these results together, we are suggesting that Ca^{2+} activates a cytosolic factor which triggers the dissociation of the Syp/Syb complex.

5.2.2 A cytosolic factor mediates the dissociation of the Syp/Syb complex

Previous findings showed that the Syp/Syb complex does not occur in embryonic brain and neuroendocrine cells although Syp and Syb could be detected in both tissues on the same vesicle. However, incubating both tissues with cytosol from adult brain led to a subsequent detection of the complex in these tissues. Subsequently, it was suggested that a post-translational modification of synaptophysin in adult brain induces the formation of the complex (Becher et al., 1999a). Further experiments to verify this hypothesis yielded results showing that unpalmitolyated Syb binds Syp (Veit et al., 2000). This ultimately implies that it is post-translationally modified Syp rather than Syb that is necessary for the upregulation of the complex. Becher et al., (1999a), suggested that a small peptide which resides in the adult cytosol on binding to Syp would change its binding behaviour to Syb. Unfortunately, attempts to show that binding Syp differs from the non-binding form using the principles of MALDI analysis failed to show any difference (Yelamanchili & Ahnert-Hilger, not published). This was attributed mainly to

the difficulties encountered in the purification of Syp during this experiment. Nevertheless, we now have experimental evidence which indicates that a cytosolic factor dissociates the Syp/Syb complex in a Ca^{2+}-dependent manner. In fact we are suggesting that a dynamic equilibrium exists between an association factor, which binds and modulates Syp (binding factor), and a dissociation factor (Ca^{2+}-dependent cytosolic factor) whose activities are modulated by Ca^{2+}. The diagram below illustrates this hypothesis:

Figure 17



Schematic model of the Syp/Syb complex cycle

1. post-translational factor (F) binds Syp and alters its binding behaviour to Syb; 2. Formation of the Syp/Syb complex; 3. Ca^{2+}-depedent cytosolic factor (X) binds the complex; 4 the post-translational factor is removed and the Syp/Syb complex dissociates; 5 Syb can now enter the SNARE complex with the t-SNAREs syntaxin and SNAP25

Both the dissociation and association factors are present in the adult brain cytosol and constitutively active during the entire life cycle of a synapse. Possibly, the post-translational modification shows a higher level of activity at resting Ca^{2+} concentrations

which accounts for the formation of more interaction between Syp and Syb, and consequently less SNARE complex being assembled. When more Ca^{2+} enters the synaptic terminal during an action potential, the equilibrium shifts in favour of the dissociation factor. The Syp/Syb interaction dissociates thereby releasing Syb for the formation of the SNARE complex. A set of experiments designed to verify this model yielded some exciting results. SVs prepared under calcium conditions and incubated with cytosol containing a calculated amount of free Ca^{2+} showed a greater degree of dissociation of the Syp/Syb complex when compared to SVs prepared under identical conditions but incubated with cytosol containing no free Ca^{2+}. Conversely, when SVs were prepared without Ca^{2+} and then subsequently incubated with cytosol containing no Ca^{2+}, the degree of dissociation was less than when SVs prepared in identical conditions were subsequently incubated in cytosol containing a calculated amount of free Ca^{2+}. These results strongly indicate that a cytosolic factor mediates the dissociation of the Syp/Syb complex. Parallel experiments performed with different amounts of free Ca^{2+} concentrations showed a similar dissociation for all concentrations used. This suggests an intrinsic activity of Ca^{2+} that becomes activated at concentrations higher than those at rest. It also strongly affirms the idea of the post-translational cytosolic factor predominantly active at resting Ca^{2+} concentrations as previously mentioned.

5.2.3 Dissociation of the Syp/Syb interaction may occur prior to SNARE complex formation

How is the dissociation of the Syp/Syb complex related to events in the synaptic terminal? Could the Syp/Syb complex really function as a switch for the SNARE complex? Clearly synaptobrevin interacts with synaptophysin in a manner that is mutually exclusive from its interaction with syntaxin and SNAP25 to form the SNARE complex. (Edelmann et al., 1995; Pennuto et al., 2002; Yelamanchili et al., 2005). Experiments to investigate the effect of excessive stimulation on the dissociation of the Syp/Syb complex using Ca^{2+}-ionophores suggested that the dissociation does not depend on the final membrane fusion but may precede the formation of the SNARE complex (Reisinger et al., 2004). The neurotoxin taipoxin was also observed to cause an increased dissociation of the Syp/Syb interaction at early stages of neurotoxicity where exocytosis is enhanced (Bonanomi et al., 2005). Though these observations strongly indicate a parallel in the synopsis of events taking place at the synapse, they

do not show a clear connection between the dissociation of the Syp/Syb complex and the enhancement of exocytosis. In a recent study, isolated SVs were incubated with the t-SNAREs syntaxin and SNAP25 and then analysed by immunoprecipitation and crosslinking (Siddiqui et al., 2007). The results obtained showed a reduction of the Syp/Syb complex detected in the analysed samples. Parallel experiments carried out in the same study showed that synaptophysin does not interact with the t-SNAREs. Taking this observation together with previous findings there is a compelling trend that suggests that Syb dissociates from Syp before binding to the t-SNAREs to form the SNARE complex. A major problem obviously posed in this study is the question of interpreting the observations made. Certainly, it is not very easy to distinguish whether the reduced amount of Syp/Syb complex detected is due to the synopsis of events assumed above or a mere competition between Syp and the t-SNAREs to bind Syb.

Considering that the dissociation of Syp/Syb complex as well as the formation of the SNARE complex are both connected by the common trigger Ca^{2+}, it is tempting to conclude that the influx of Ca^{2+} into the presynaptic terminal induces a synopsis of events which ultimately leads to the release of Syb from synaptophysin and thus facilitates the assembly of the SNARE fusion complex. Although all Syb found in SVs is not necessarily bound to Syp, it is obvious that the Syp/Syb complex constitutes a pool for Syb. Experimental evidence indicates the pool is readily releasable at the onset of exocytosis and might serve in ensuring an adequate supply of Syb for the formation of the SNARE fusion complex during synaptic transmission. The readiness with which the Syp/Syb complex is reassembled when Ca^{2+} concentration returns to normal levels at rest suggests a mechanism to regulate the temporal formation of the SNARE complex. This will prevent the unnecessary release of neurotransmitter at resting potentials.

5.3 Possible mediators of the dissociation of the Syp/Syb complex

Many proteins and macromolecules in the presynaptic terminal have been shown to interact with Syb and/or Syp as well as their heterodimer. In search of a physiological role for Syp and the Syp/Syb interaction it is important to determine how these interactions with other proteins and macromolecules influence the structural stability and the biochemical properties of the complex. A more crucial question that arises is whether any of these proteins could be responsible for the Ca^{2+}-dependent dissociation of the complex.

The Syp/Syb interaction is known to be critically dependent on cholesterol content (Mitter et al., 2003), highlighting the importance of the lipid environment in determining the stability of this complex. Interestingly, the assembly of the SNARE complex is prevented when Syb binds phospholipids on the SV membrane (Hu et al., 2002; Quetglas et al., 2002). Furthermore, Syp has been reported to bind cholesterol selectively (Thiele et al., 2000). Bearing these observations in mind, it is only logical to ask the following questions: Does a lipid modification/binding of Syp lead to a facilitation of the Syp/Syb interaction? Can a Ca^{2+}-dependent dissociation of the lipid lead to a dissociation of the complex? These questions and many more still remain to be clarified. The myosin V in nerve terminals is also known to bind to synaptic vesicles, in an interaction that is regulated by Ca^{2+} concentrations that are of physiological relevance. Crosslinking experiments indicated that myosin V specifically complexes with the synaptic vesicle proteins synaptobrevin and synaptophysin (Prekaris and Terrain, 1997). However, the brain myosin V is unlikely to play a role in regulating the Syp/Syb interaction especially considering that it is a vital component of the neuronal growth cone motility system where Syp/Syb complex is absent (Wang et al., 1996).

Another protein known to interact with synaptophysin and synaptobrevin is the V-ATPase which is found in SVs (Galli et al., 1996). This interaction is resistant to Triton-100 solubilization but sensitive to freeze-thawing. Our studies, however, show that the Syp/Syb complex would persist in both conditions making the V-ATPase an unlikely contestant for the regulation of the Syp/Syb interaction.

Ca^{2+}/calmodulin and phospholipids bind in a mutually exclusive manner to a C-terminal domain of Syb (Quetglas et al., 2002). How are these interactions relevant to the dynamics of the Syp/Syb interaction? The fact that there is still no compelling evidence that SNAREs are intrinsically sensitive to Ca^{2+} suggests that associated calcium-binding proteins confer this property in regulated exocytosis. We have not specifically evaluated the role of Ca^{2+}/calmodulin in our studies. Nonetheless, studies in permeabilised chromaffin and PC12 pheochromocytoma cells support the view that Ca^{2+}-activated calmodulin acts as a positive effector during the triggering step of regulated exocytosis although the molecular mode of action remains obscure (Chamberlain et al., 1995; kibble & Burgoyne, 1996; Chen et al., 1999a). In another study, treatment of intact PC12 cells with the irreversible calmodulin antagonist ophiobolin A prior to permeabilization totally blocked the Ca^{2+}-dependent hormone release (Quetglas et al., 2002). These experiments strongly suggest that calmodulin plays a role but does not

define the target at a molecular level. Could Syb be the target? Could calmodulin represent the Ca^{2+}-dependent cytosolic factor in nerve terminals responsible for the dissociation of the Syp/Syb complex? During this project, we observed that the putative cytosolic factor is still active after cooking at 95°C for 10-15 minutes and is likely to have a molecular weight of above 30 kDa. Although relatively weak indicators, these results point to a protein like calmodulin which in an optimal Ca^{2+} concentration would bind and dissociate synaptobrevin from Syp upstream of the process of neuroexocytosis. This would facilitate the formation of the SNARE complex and positively influence the release of neurotransmitter.

5.4 Does the interaction of Syp and Syb occur in raft-like nanodomains?

There is increasing interest as to whether the Syp/Syb complex occurs in raft-like nanodomains. Such domains have been described in literature for other SV proteins, most notably the t-SNAREs syntaxin and SNAP25 (Lang et al., 2001), involved in exocytosis. However, there has been no such description in literature addressing this concern for the Syp/Syb interaction yet. A major characteristic of these nanodomains is their cholesterol-dependency. The Syp/Syb complex has been shown to be critically dependent on cholesterol (Mitter et al., 2003). By binding to synaptobrevin, synaptophysin might generate a membrane domain on the synaptic vesicle by which an optimal alignment of reactive synaptobrevin molecules is guaranteed. Such an alignment might provide a crucial spatial and temporal proximity for synaptobrevin molecules at that site of the spherical vesicle where fusion will occur, thus promoting the efficacy of interaction with the t-SNARE partners in the plasma membrane.

5.5. Conclusion and future perspectives

Many speculations have indicated that the Syp/Syb complex is very likely to be one of several mechanisms at the presynaptic site which modulates the release of neurotransmitters. Our experiments carried out in vitro are showing a consistent link between the dissociation of Syb from the Syp/Syb complex and Ca^{2+} concentrations that prevail during neuroexocytosis. We therefore assume that Ca^{2+} concentrations that trigger neuroexocytosis would also lead to the increase of readily bindable Syb for the SNARE complex, thus enhancing the efficacy of the neuroexocytotic process. The key aspect of this project was to clarify the mechanism by which Ca^{2+} dissociates the Syp/Syb complex. It was established that the dissociation is not directly caused by Ca^{2+} but involves a process which is dependent on the presence of Ca^{2+}. Results from our experiments showed that the synaptosol (synaptic cytosol) from adult rat is the other essential factor which in combination with Ca^{2+} will lead to the above mentioned dissociation. Previous experiments showed that embryonic synaptosol could not dissociate the Syp/Syb complex in the presence of Ca^{2+}, therefore the dissociating property is acquired sometime during the development of the species. Attempts to isolate and characterise the cytosolic factor have so far been relatively unsuccessful. However, experiments performed using centricon filters with a cut-off of 30kDa showed a tendency for a factor with a molecular weight higher than 30 kDa. The integrity of the factor, seen in its ability to dissociate the Syp/Syb complex, was preserved after freezing and thawing of samples, as well as after cooking the cytosol at 95°C. Taken together, this study shows that a cytosolic factor of higher molecular weight found in adult rat brain dissociates the Syp/Syb complex in the presence of Ca^{2+} concentrations of physiological relevance. In order to establish what significance the dissociation of the Syp/Syb complex has in the regulation of neurotransmission, it will be important to further characterise and isolate the Ca^{2+}-dependent cytosolic factor responsible for this dissociation. This is proving to be very challenging so far, but may represent a key process in the modulation of neuroexocytosis.

6. Summary

Neurotransmission is highly dependent on the regulated release of neurotransmitters at the presynaptic terminal. This release is stringently regulated by numerous proteins of which several have been characterised and linked to precise functions in the regulation of neuroexocytosis. Synaptophysin (Syp) and synaptobrevin (Syb) are the most abundant proteins at the presynapse. While the role of Syb in neurotransmission is clear, that of Syp has remained widely elusive. Along with its t-SNAREs partners (Syntaxin and SNAP25), the v-SNARE (Syb) forms the SNARE fusion complex which is widely accepted to be the core component of neuroexocytosis. However, Syb also forms another complex with Syp known as the Syp/Syb complex. Both complexes are mutually exclusive. In spite of its abundance, the role of the Syp/Syb complex during neuroexocytosis is not yet clear. The present study focuses on the putative interaction between Syp and Syb and the characterisation of the Ca^{2+}-dependent dissociation of this interaction.

To study the complex we first evaluated the detection of the complex using two different methods: 1.) Crosslinking with disuccinimidyl suberate (DSS) and 2.) immunoprecipitation using antibodies against Syp or Syb. Our results showed that both methods are successful in the detection of the Syp/Syb complex. Furthermore, the results obtained for both methods are comparable.

Subsequently, the effect of Ca^{2+} on the Syp/Syb-complex was investigated. The following results were obtained: 1.) Ca^{2+} only or in combination with EGTA does not dissociate the interaction between Syp and Syb. 2.) Both Ca^{2+} and synaptic cytosol must be present for the dissociation of the Syp/Syb complex to occur. 3.) The dissociation does not depend on the amount of Ca^{2+} used but rather on its presence. The fact that the complex dissociated to the same degree with Ca^{2+} concentrations of physiological relevance (micromolar range, µM) suggests an intrinsic role for Ca^{2+} rather than a mere ionic disruption, as might be rightfully expected for higher Ca^{2+} concentrations. In a distinct setup to verify the reassociation of the complex, we observed an increase in the detection of the complex when synaptic vesicles (SVs) prepared in the presence of Ca^{2+} were reconstituted in Ca^{2+}-free cytosol as compared to reconstitution with cytosol containing Ca^{2+}. Taken together, these results suggest a

cytosolic factor whose effect on the complex is regulated by the presence or absence of Ca^{2+}.

Attempts to characterise and isolate the cytosolic factor responsible for dissociation of the Syp/Syb complex have so far stalled. However, the results obtained indicate a factor of molecular weight greater than 30kDa. Furthermore, freezing and thawing as well as temperatures as high as 95°C do not affect the integrity of the factor.

7. Zusammenfassung

Neurotransmission ist abhängig von der regulierten Freisetzung von Neurotransmittern an der Präsynapse. Diese Freisetzung wird von mehreren Proteinen reguliert. Viele dieser Proteine sind näher charakterisiert und ihre Beteiligung an der Regulation der Neuroexozytose beschrieben worden. Synaptophysin (Syp) und Synaptobrevin (Syb) sind die am häufigsten vorkommenden Proteine synaptischer Vesikel. Während die Rolle von Syb innerhalb der Neuroexozytose vergleichsweise klar ist, ist die Rolle von Syp weitgehend unklar geblieben. Syb bildet zusammen mit seinen t-SNARE Bindungspartnern Syntaxin (syx) und SNAP25 den SNARE-Komplex, der grundsätzlich als eine Kernkomponente der Neuroexozytose angesehen wird. Syb bildet einen weiteren Komplex mit Syp, den sogenannten Syp/Syb-Komplex. Beide Komplexe schließen einander aus. Trotz seiner hohen Konzentration ist die Rolle des Syp/Syb-Komplexes bei der Neuroexozytose umstritten. Die vorliegende Studie beleuchtet insbesondere die Ca^{2+}-abhängige Dissoziation der Interaktionspartner Syp und Syb. Es wurden zwei Methoden zur Detektion des Komplexes evaluiert:

1.) Quervernetzung mit Disuccinimidylsuberat (DSS) und 2.) Immunopräzipitation mit Antikörpern gegen Syp oder Syb. Die Ergebnisse zeigen, dass beide Methoden zur Detektion des Syp/Syb-Komplexes erfolgreich eingesetzt werden können und vergleichbare Resultate liefern.

In weiteren Versuchen wurde die Wirkung von Ca^{2+} auf die Stabilität des Syp/Syb-Komplexes untersucht und folgendes beobachtet: 1.) Ca^{2+} allein oder in Verbindung mit EGTA führt nicht zur Dissoziation des Syp/Syb- Komplexes. 2.) Für die Dissoziation des Komplexes werden sowohl Ca^{2+} als auch synaptisches Zytosol benötigt. 3.) Die Dissoziation durch Ca^{2+} ist konzentrationsunabhängig.

Die Tatsache, dass der Komplex bei physiologisch relevanten Ca^{2+} konzentrationen (im mikromolaren Bereich, µM) genauso gut dissoziiert, schließt eine Dissoziation aufgrund von ionischen Wechselwirkungen wie sie bei hohen Ca^{2+} konzentrationen zu erwarten wäre, aus. In weiteren Experimenten wurde die Reassoziation des Komplexes untersucht. Hierbei kann eine verstärkte Komplexbildung bei unter Ca^{2+}-bedingungen präparierten synaptischen Vesikeln (SV) beobachtet werden, die in Kalzium-freiem Zytosol resuspendiert werden im Gegensatz zu SV die unter dergleichen Bedingungen präpariert wurden und in Ca^{2+}-haltigen Zytosol resuspendiert werden.

Zusammengenommen deuten diese Ergebnisse auf einen zytosolischen Faktor hin, dessen Wirkung auf den Syp/Syb- Komplex Ca^{2+}-abhängig zu sein scheint.
Bemühungen, diesen Faktor näher zu charakterisieren und zu isolieren führten bisher zu keinem entgültigem Ergebnis. Die dabei erzielten Befunde belegen aber, dass der Faktor ein Molekulargewicht von mindestens 30kDa besitzt und dass Frier- und Auftau-Zyklen, sowie Temperaturen von bis zu 95°C keinen Einfluss auf die Integrität des Faktors haben.

8. References

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9. Abbeviations

APS	= ammonium persulfate
ATP	= adenosine triphosphate
BCA	= bicinchoninic acid-4, 4-dicarboxy-2,2-bicincholin
BNPI	= brain specific Na^+-dependent inorganic phosphate cotransporter
BSA	= bovine serum albumin
Ca^{2+}	= intracellular calcium
Cplx	= complex
C-terminal	= carboxyl terminal
Da	= Dalton
ddH_2O	= bi-distilled water
dH_2O	= distilled water
DMSO	= dimethyl sulfoxide
DNPI	= differentiation asociated Na^+-dependent inorganic phosphate cotransporter
DTT	= dithiothreitol
ECL	= enhanced chemiluminescence
EDTA	= ethylene diamine-tetra-acetic acid
EGTA	= ethylenglycolbis(2-aminoethyl-)tetraacetate
g	= gram
GABA	= Gamma-aminobutyric acid
H	= homogenate
h	= hour
HEPES	= N-2-Hydroxyethylpiperazin-N´-2-ethane-sulfonic acid
IP	= immune precipitate
LP1	= lysed pellet 1
Lp2	= lysed pellet 2 corresponding to crude SVs
LS1	= lysed supernatant 1
LS2	= lysed supernatant 2
m	= milli: prefix denoting 10^{-3}
mg	= milligram

min	= minute
ml	= milliliter
mM	= millimolar
nm	= nano meter
NMDA	= N-methyl-D-Aspartate
NSF	= N-ethylmaleimide sensitive factor
P2	= synaptosomal pellet
P3	= postsynaptosomal pellet
PAGE	= polyacrylamid gel electrophoresis
PBS	= phosphate buffered saline
PNS	= post nuclear supernatant
rpm	= rotations per minute
SDS	= sodium dodecyl sulphate
SNAREs	= synaptosomal associated protein receptors
SNAP	= soluble NSF attachment protein
SNAP25	= synaptic associated protein of 25 kDa
Syt	= synpatotagmin
SV	= synaptic vesicle
Syb	= synaptobrevin
Syp	= synaptophysin
Syp/Syb complex	= synaptophysin/synaptobrevin complex
Syx	= syntaxin
TEMED	= N,N,N´,N´- Tetramethyethylendiamine
TRIS	= tris-(hydroxymethyl)-aminomethane
t-SNAREs	= target SNAREs
V	= Volt
VAMP	= vesicle associated membrane protein
VGLUT 1/2/3	= vesicular glutamate transporters 1/2/3
VGAT	= vesicular GABA transporter
v-SNAREs	= vesicular SNAREs
v/v	= volume per volume
µg	= microgram
µl	= microliter
µmol	= micromol

°C = celcius degree centigrade

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11. Erklärung

Ich, Terence N. Afube, ekläre, dass ich die vorgelegte Dissertationsschrift mit dem Titel: „Synaptophysin/Synaptobrevin complex: Characterisation of the Ca^{2+}-dependent cytosolic dissociation of the complex“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulassige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Berlin, den

Unterschrift

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12. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht