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Synapsin I released via exosomes is an oligomannose bearing glycoprotein and an oligomannose binding lectin that promotes neurite outgrowth in *Mus musculus* (Linnaeus, 1758)

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I am a native English speaker, I have read the thesis prepared by Wang Shiwei, provided that the corrections that I have suggested are incorporated in the final thesis. I can affirm that the thesis is written in grammatically correct English that would be understandable to any native English speaker.

Sincerely,

Nathan Smith

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I. Abstract

Oligomannoses are expressed on developing and mature brains and involved in multiple important physiological and pathological processes. In order to circumvent low availability of natural saccharides for functional analysis, a phage-displayed random peptide library was screened to search for peptide mimics that interact specifically with L3 and L4 antibodies, which recognize oligomannosidic epitopes. During the panning procedures, two specific competitive elution conditions (antibodies and saccharides) were applied to increase specificity of elution. A phage clone expressing the sequence TISWWHLWPSPA emerged from all three panning protocols. The peptide was confirmed to be a peptide mimic of oligomannose by ELISA experiments. The synthetic peptide coupled to catalase for increasing solubility promoted neurite outgrowth of hippocampal neurons and reduced adhesion between cerebellar neurons and astrocytes, while a corresponding scrambled peptide showed no such activities. To identify novel oligomannose specific lectin, cross-linking, gel electrophoresis and mass spectrometry were performed and synapsin I was identified as a potential oligomannose binding protein. This was further confirmed by oligomannose dependent binding between synapsin I and adhesion molecule of glia in ELISA. In addition, synapsin I was eluted from L3 and L4 antibodies columns. When purified bovine synapsin I was treated with Endoglycosidase H and N-glycosidase F which removed high oligomannose glycans and N-linked glycans individually from glycoproteins, L3 and L4 antibodies lost their reactivities with synapsin I. Synapsin I was also recognized by some distinct lectins and the L5 antibody, which recognized Lewis^X. These results indicated complex glycosylation of synapsin I. Synapsin I was expressed and released via exosomes from cultured astrocytes and the astroglial cell line C6. Exosomes are small membrane vesicles, they are formed in multivesicular bodies by inward budding and secreted into extracellular milieu upon fusion of multivesicular bodies with the plasma membrane. Electron microscopy revealed that a preparation of exosome isolated from the cell culture medium of C6 cells showed a high degree of homogeneity of vesicles with a diameter of 80-100 nm. Incubation of astrocyte-derived exosomes in the presence of 40 mM KCl or LiCl in phosphate buffered saline released no synapsin I from exosomes, when increasing the concentrations of KCl or LiCl to 80 mM, most of synapsin I in exosomes was released into the soluble phases. Under same osmotic strength conditions (230 mM sucrose) synapsin I was not released from exosomes. C6 exosomes released

synapsin I already when incubated with PBS. Proteomic analysis of C6 exosomes showed that a number of molecules including secreted, membrane and cytosolic proteins were released from exosomes under KCl stimulation. Substrate coated synapsin I which mimics synapsin I released from exosomes to the extracellular milieu promoted neurite outgrowth of hippocampal neurons in an oligomannose dependent way. When oligomannoses were removed, synapsin I lost its promotive effects on neurite outgrowth. Synapsin I stimulates neurite outgrowth probably by interacting with the neural cell adhesion molecule NCAM, because it binds with extracellular domain of NCAM in an oligomannose dependent way and loses its promotive effects on NCAM-deficient hippocampal neurons. The release of synapsin I from astrocyte-derived exosomes under high ionic strength conditions indicates that extracellular synapsin I plays a role in modulating interactions and communication between astrocytes and neurons, in particular under conditions of high neuronal or synaptic activities.

II. Introduction

II.1 Overview of protein glycosylation

The four essential building blocks of cells are proteins, nucleic acids, lipids, and glycans. Also referred to as carbohydrates, glycans are composed of saccharides that are typically linked to lipids and proteins. After made by ribosomes, most proteins were modified during their passages through rough endoplasmic reticulum (rER) and Golgi complex by covalently adding different short chains of carbohydrates (oligosaccharides). These attached molecules vary from a few sugars to several hundred. The repertoire of sugars includes glucose, galactose, N-acetylglucosamine (GlcNAc), fucose, sialic acid, mannose (Man), N-acetylgalactosamine (GalNAc), glucuronic acid and xylose. Each monosaccharide can adopt different conformations, and the sugars can be linked to each other by different types of glycosidic bonds at different hydroxyl groups. These astonishingly varied and complex carbohydrates structures are involved in numerous biological processes, in part by regulating protein-protein and cell-cell interactions. Moreover, dysregulation of glycan synthesis is linked to the etiology for a growing number of diseases.

In glycoproteins, two main types of oligosaccharides linkages exist. When oligosaccharides were linked to a serine or threonine residue of proteins, they are called O-linked oligosaccharides, while, when attatched to an asparagine residue, they are N-linked oligosaccharides. Biosynthesis of the N-linked oligosaccharide starts on the cytosolic surface of the ER membrane by the addition of sugars, one by one, to dolichol phosphate. When two N-acetylglucosamines and five mannoses have been added, the oligosaccharide is flipped to the lumenal side of the membrane and accepts further monosaccharides from the luminal side of the ER membrane. The final oligosaccharide, with the composition $(Glc)_3(Man)_9(GlcNAc)_2$, called G-oligosaccharide, is linked to the dolichol by a high-energy pyrophosphate bond. This provides the energy for transfer of the oligosaccharide to the protein. Oligosaccharide transferase enzyme complex catalyzes the transfer of oligosaccharide to the asparagine residues within the motif Asn-X-Ser/Thr of nascent, growing polypeptide chains. The three glucoses are trimmed away by glucosidase I and II, and terminal mannoses by one or more different ER mannosidases. The ER also contains a glucosyltransferase that can reglucosylate glucose-free chains and thus establish, with glucosidase II, a deglucosylation-reglucosylation cycle. When the glycoprotein has folded and reached the Golgi complex, further mannose trimming

occurs. The addition of a GlcNAc residue is followed by trimming of two additional mannoses. During subsequent terminal glycosylation there is addition of new terminal sugars including GlcNAc, galactose, sialic acid, and fucose. The number of branches generated is variable, as are the number and identity of sugars added. Whereas the glycoforms in the ER are homogeneous, the Golgi-generated forms are highly diverse and differ widely between species (**Fig. 1**).

All N-linked oligosaccharides are based on a common pentasaccharide core structure consisting of three mannose residues and two GlcNAc residues. There are two types of N-linked oligosaccharides. In the high mannose type oligosaccharides, there is variable number of mannose residues beyond the "core structure". In the complex type oligosaccharides, there exists a variety of other sugars such as GlcNAc, galactose, sialic acid and fucose.

The synthesis of O-linked oligosaccharides occurs by the sequential addition of monosaccharide units to the newly synthesized protein as it passes through the Golgi complex. First, in the ER GlcNAc is transferred to the relevant serine (Ser) or threonine (Thr) residue of protein by GalNAc transferase, an enzyme that uses uridine diphosphate-N-acetylgalactosamine (UDP-GalNAc) as the precursor. Other monosaccharide such as galactose, GlcNAc, sialic acid, fucose are then added in the Golgi using the corresponding sugar nucleotides as precursors. The exact type and number (up to about 10) of monosaccharides added depends on the protein substrate.

In addition, several proteins are known to be attached to the plasma membrane via a specific structure that involves carbohydrate, namely a glycosyl phosphatidylinositol (GPI) anchor.

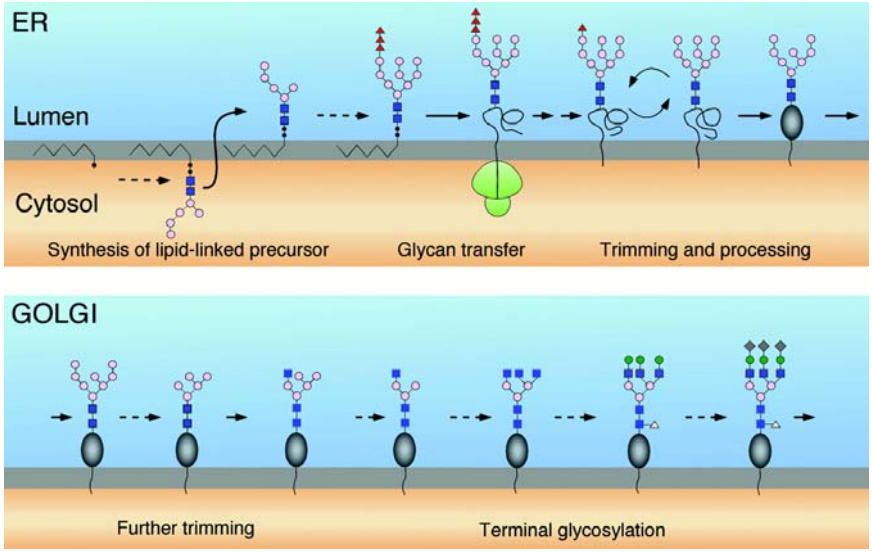

Fig. 1: Biosynthesis of N-linked oligosaccharides.

N-linked sugar chains are first assembled as a dolichol phosphate derivative (lipid linked precursor). The sugars are then transferred to the protein and followed by trimming, when the glycoproteins reach the Golgi complex, further trimming occurs. During subsequent terminal glycosylation, various sugars are added to produce different glycoforms.

II.2 Oligomannose

After the attachment of the core oligosaccharide to asparagines in the N-glycosylation consensus sequence within a protein backbone, trimming of all glucose residues without addition of further monosaccharides results in oligomannosides. The action of different mannosidases on the core oligosaccharide leads to the formation of five different N-linked oligomannosides. Formation of Man9-carrying N-glycans requires only removal of the glucose residues from the core oligosaccharide. Man8 results from additional removal of one α1,2-linked mannose from the α1,3-arm, whereas in the case of Man7, α1,2-linked mannose residues are removed from both arms. Further removal of the terminal α1,2-linked mannose from the α1,3 branch leads to Man6 and removal of two α1,2-linked mannose residues from the α1,3-branch yields Man5 (**Fig. 2**).

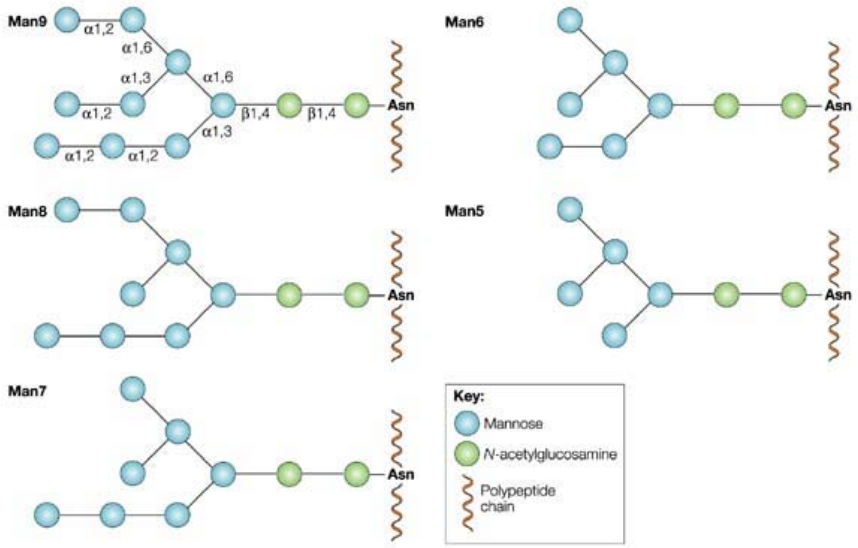

Fig. 2: Oligomannosidic glycans.

Man9 oligomannosidic glycans resulte from trimming of all glucose from a core oligosaccharide. Further trimming of mannose by different mannosidases produces a series of oligomannosidic glycans from man8 to man5. Asn, asparagine. (Kleene and Schachner, 2004)

Ordinarialy, oligomannosides are transient products during N-linked glycosylation. Unlike mature N-glycans, they are not transported to the cell surface or secreted to the

extracellular matrix (ECM) in most tissues, however, in the brain, they are expressed on some cell surface adhesion molecules such as L1, NCAM and adhesion molecule on glia (AMOG) (Kleene and Schachner, 2004). Oligomannosidic glycans comprise approximately 15% of all *N*-glycans and 50% of the neutral *N*-glycans in rodent brain. They are relatively concentrated in synapses (Matus et al., 1973), especially on the NMDA (*N*-methyl-D-aspartate) and AMPA subtypes of glutamate receptors. Around 50% of all neutral glycan chains on these two glycoproteins are oligomannosides (Clark et al., 1998). Mannosylation is also developmently regulated and coincides with the maturation of synapses. Another important N-linked glycoprotein at synapses is AMOG, it is the regulatory ß-subunit of ion pump Na^+/K^+-ATPase. An astonishing 80% of the glycans of it are oligomannosides. This unusual modification is important for transporting of the catalytically active α-subunit to the cell surface as an α2ß2 heterodimer (Gloor et al., 1990) as well as interacting with oligomannoside binding partners.

Cis-interaction between L1 and NCAM depends on oligomannosides (Horstkorte et al., 1993). The interaction was confirmed by chemical crosslinking experiments (Simon et al., 1991) and co-redistribution experiments (Thor et al., 1986). This interaction is disturbed by soluble oligomannosides and a peptide that represents the binding pocket in the fourth Ig-like domain of NCAM (Horstkorte et al., 1993). Once the *cis*-interaction between L1 and NCAM was interrupted, NCAM-assisted L1 homophilic binding in *trans*-interaction as well as neurite outgrowth and phosphorylation of L1 will be disturbed (Heiland et al., 1998). Oligomannosides can also interfere with LTP at hippocampal synapses possibly by disturbing the interaction between L1 and NCAM (Luthi et al., 1996). Basigin, another member of the Ig superfamily of recognition molecules has been recently identified as an oligomannoside receptor (Heller et al., 2003). It is involved in neuron–glia interactions and the formation and/or maintenance of the blood–brain barrier. Basigin-deficient mice show severe defects in nervous system development and spatial learning and memory, however, it is still unclear whether these phenotypes are associated with oligomannosides.

II.3 L3 and L4 antibodies

Early efforts to identify commom carbohydrate structures on neural cell adhesion molecules resulted in the production of monoclonal antibody L3 (Kucherer et al., 1987). This antibody was obtained from 4-6 week old female Sprague Dawley F1 hybrid rats

immunized with detergent lysates of crude membrane fractions from adult mouse brain purified by immunoaffinity chromatography on a monoclonal L2 antibody column, which was designated as "total L2" (Kruse et al., 1984; Rathjen and Schachner, 1984). Splenocytes of animals with high titers were chosen for fusion with the mouse myeloma clone Ag8-653 (Kearney et al., 1979). Monoclonal L3 antibody is an IgM as determined by gel filtration or SDS PAGE. 3 years later, another similar antibody L4 was reported (Fahrig et al., 1990). “Rest L2”, which remove NCAM and L1 by sequential immunoaffinities from "total L2", was used to elicit immunoreactivity (Kruse et al., 1984). The produced antibody was designated as L4 antibody and was confirmed to be IgM subclass (Fahrig et al., 1990).

For the structural analysis of the *N*-glycosidically linked glycans which are recognized by L3 and L4 antibodies, oligosaccharides of AMOG released by endoglycosidase H were used. After the treatment by this oligomannose and hybrid type glycan specific enzyme, apparent molecular weight decreases from 50 to 35 KD (Antonicek et al., 1987). Given that calculated molecular weight is 32 KD, it can be estimated that around 80% of *N*-linked oligosaccharides of AMOG are of the oligomannosidic and/or hybrid type. Both L3 and L4 lose their reactivity with endoglycosidase H treated AMOG, which imply the oligomannose or hybrid nature of these two antibodies’ epitopes. Neoglycolipids of oligomannosidic glycans prepared from oligosaccharides released from AMOG and ribonuclease B (RNase B), which is known to carry only oligomannosidic oligosaccharides (Liang et al., 1980), react with L3 antibody in enzyme-linked immunosorbent assay (ELISA). On thin-layer chromatograms, neoglycolipids derived from AMOG and RNaseB react with L3 antibody, $Man_5GlcNAc$-neoglycolipid have stronger reactivity than $Man_6GlcNAc$-neoglycolipid. At the same time, no immunoreactivity was observed with $Man_7GlcNAc$-neoglycolipid. Neoglycolipids prepared individually from oligosaccharides of known structures containing 5, 6, 7, 8 or 9 mannose (**Fig. 2**) showed decreasing reactivities with both L3 and L4 antibodies with the increasing number of mannose. It is concluded that α1,3 and α1,6-linked mannose residues exposed at the non-reducing end of the oligosaccharides are recognized by both of these antibodies. Although L3 and L4 showed similarity in most cases, there still existed some differences between them. A competition ELISA indicated that glycans recognized by L3 and L4 antibodies were distinct (Fahrig et al., 1990).

Both of these two antibodies have been showed to bind to neural cell surfaces, to inhibit cell adhesion and neurite outgrowth (Kucherer et al., 1987; Fahrig et al., 1990), and to

interfere with the interaction between L1 and NCAM (Horstkorte et al., 1993), implicates the involvement of oligomannosidic glycans in important functional roles.

II.4 Peptide mimics of saccharides and phage display library

The idea of peptide mimic of sugar came from the problem of immunology, the cell-surface polysaccharides of pathogenic bacteria and viruses and some tumors are important virulence factors and antigens and are therefore excellent targets for vaccine development. However, because of their poor immunogen nature, sugars can not induce any immune response in children < 2 years of age. They produce a T cell independent response that lacks immunological memory (Jennings, 1988). To circumvent these difficulties, carbohydrates were conjugated to proteins. These glycoconjugate vaccines elicit T cell dependent responses and may be used directly as immunogens or as priming agents for boosting with polysaccharide (Lindberg, 1999; Alexander et al., 2000). Another, and more widely accepted strategy is the use of peptide mimics of carbohydrates (Monzavi-Karbassi et al., 2002; Johnson et al., 2002; Pirofski, 2001). Although they possess unquestionable advantages as immunogens, the success of this strategy crucially depends on the ability of peptides to mimic oligosaccharide epitopes.

Commonly used parameters for selecting oligosaccharide peptide mimetics include binding to the antibodies against native sugars and the competition with native sugars for antibody binding. But sometimes, they are insufficient and perhaps not predicative of whether a peptide is a carbohydrate mimotope (Fleuridor et al., 2001). Up to now, general rules of how peptides mimic sugars, structurally or functionally, are still poorly understood.

Peptides that crossreact with carbohydrates may be identified by screening of phage-displayed peptide libraries with anti carbohydrate antibodies (Zwick et al., 1998). In 1985, George Smith demonstrated that a foreign protein sequence could be fused to the coat protein of a bacterial phage, so that it is accessible or displayed on the virus surface (Smith, 1985). Three years later, he further introduced the concept of affinity selection of phage displayed foreign target sequence from a library of displayed peptides (Scott and Smith, 1990). Since then, phage display has rapidly developed into one of the most widely used research tools and the technology has been applied to almost every discipline of biological research.

When random sequences are inserted into the phage coat proteins, it is possible to obtain a large library containing a vast number of distinct peptide sequences, each displayed on

different phages. The peptide sequence displayed on the surface, the phenotype, is physically linked to the encoding sequence in the ssDNA phage genome, the genotype. It is this link of phenotype and genotype which made it possible to select for a specific peptide, followed by subsequent amplification by re-infecting *E. coli* to obtain a virtually unlimited supply of specific peptides.

A typical experiment related phage display library includes panning; in this step, specific phage clones with specific inserted peptide sequences in the coat protein are selected with target molecules as baits in solid or solution phase. In its simplest form (**Fig. 3**), panning is carried out by incubating a library of phage-displayed peptides with a plate (or beads) coated with the target molecules, washing away the unbound phage, and eluting the specifically-bound phage. (Alternatively the phage can be reacted with the target in solution, followed by affinity capture of the phage-target complexes onto a plate or beads that specifically binds the target.) The eluted phage is then amplified and taken through additional cycles of panning and amplification to successively enrich the pool of phage in favor of the tightest binding sequences. After 3-4 rounds, individual clones are characterized by DNA sequencing and ELISA. In the past few years, many sugars' peptide mimics were reported (Murali and Kieber-Emmons, 1997; Kieber-Emmons et al., 1997; Simon-Haldi et al., 2002; Fukuda, 2006).

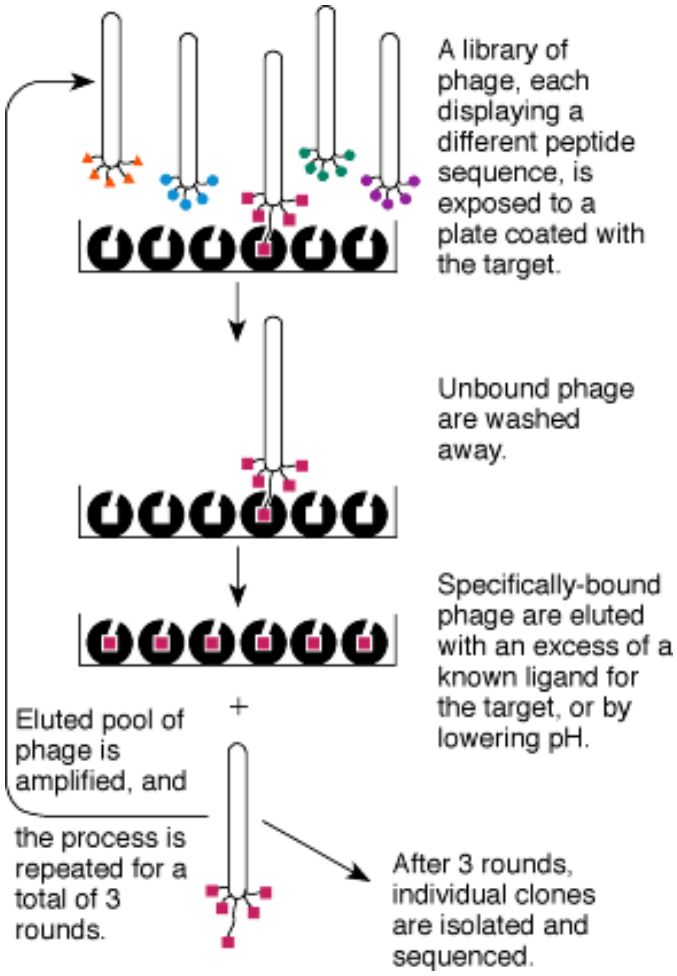

Fig. 3: Panning with a phage display peptide library.

Panning is carried out by incubating a library of phage-displayed peptides with a plate (or beads) coated with the target, washing away the unbound phage, and eluting the specifically bound phage. The eluted phage is then amplified and taken through additional binding/amplification cycles to enrich specific sequences. After 3-4 rounds, individual clones are isolated, sequenced and tested by ELISA.

II.5 Exosomes

II.5.1 Definition and distribution of exosomes

The term “exosome” was first coined by Johnstone R.M. (Johnstone et al., 1987) to name small membrane vesicles secreted from cells, as first demonstrated to occur during sheep reticular maturation (Harding et al., 1983; Pan and Johnstone, 1983). In comparison to the old definition as exfoliated membrane vesicles, “exosome” now are reserved especially as multivesicular bodies (MVBs) originate small vesicles. MVBs belongs to endosomal system which are involved in the sequestration of proteins destined for degradation in lysosomes (Gruenberg and Stenmark, 2004). An alternative fate of MVBs

is their exocytic fusion with the plasma membrane leading to the release of the 50–90 nm intraluminal vesicles (ILVs) into the extracellular milieu. The secreted ILVs are then named exosomes. In contrast to the purification of other subcellular compartments, exosomes are isolated without cell homogenization. They are harvested by differential centrifugation from the culture media.

A wide variety of cultured cell types have been reported to secrete exosomes, including dendritic cells (Thery et al., 1999; Thery et al., 2001), B lymphocytes (Wubbolts et al., 2003; Escola et al., 1998), mast cells (Skokos et al., 2003), T cells (Blanchard et al., 2002) and several epithelial cell lines (Wolfers et al., 2001). They were also reported to be released from primary culture of astrocytes (Taylor et al., 2007) and neurons (Faure et al., 2006). Many body fluids such as plasma (Caby et al., 2005), epididymal fluid (Gatti et al., 2005), semen (Utleg et al., 2003), and urine (Pisitkun et al., 2004) or maligmant effusions of cancer patients (Bard et al., 2004) were found to be exosomes positive. This term is also used to describe a complex of several exoribonucleolytic and RNA-binding proteins essential for RNA degradation and processing (Vanacova and Stefl, 2007). Here, I will focus on exosomes as small vesicles.

II.5.2 Generation and composition of exosomes

Exosomes are ILVs of MVBs once released into the extracellular milieu. The ILVs are formed by inward budding and scission of vesicles from the limiting membrane into the endosomal lumen. During this process, transmembrane and peripheral membrane proteins are incorporated into the invaginating membrane, they still maintain the same topological orientation as at the plasma membrane, at the same time the cytosolic components are also engulfed and enclosed into these vesicles. To some extent, exosomes can be regarded as a “mini-cell” and may be granted to some characteristics of whole cell.

Exosomes of different cellular origins share a common repertoire of molecules such as chaperones (Hsc70 and Hsp90); subunits of trimeric G proteins; cytoskeletal proteins (e.g., actin, tubulin, moesin); ESCRT proteins (Tsg 101, Alix); clathrin; proteins involved in transport and fusion (Rab 7, Rab 2, Annexins); and several enzymes and elongation factors (Fevrier and Raposo, 2004). Some of these common components (Tsg 101, Alix) are involved in the generation of exosomes and can then serve as markers of exosomes. On the other hand, exosomes from different cellular origins harbour cell-type specific components. Such as MHC class II and class I molecules (antigen presenting

cells), transferrin receptor (TfR) (reticulocytes), A33 antigen (IEC), CD3 (T cells), and the GluR2/3 subunits of glutamate receptors (neurons).

Besides proteins, message RNA and micro RNA are also enriched in exosomes. The exosomes derived from a human (HMC-1) and mouse (MC/9) mast cell lines were found to transport RNA to neighboring mast cells, which was then translated indicating that the transferred RNA was biologically active. The RNA transferred through exosomes can confer new functions to the target cells (Valadi et al., 2007).

Various protein composition of exosomes are sorted at the endosomal level into ILVs, this may involve several participants such as ESCRT components, lipids and/or tetraspanins-enriched microdomains. ESCRT-independent mechanisms are also likely to operate for sorting of transmembrane cargo proteins in ILVs of MVBs (Wolfers et al., 2001). In relation to GPI-anchored proteins and other ‘‘raft’’-associated proteins such as flotillin, stomatin or lyn, their sequestration in exosomes reflects the presence of lipid raft-like domains in exosomal membranes. The lipid microdomains and their components could be themselves involved in the generation of the ILVs (De Gassart et al., 2003) or in concert with other proteins with affinity for ‘‘raft-like domains’’ such as tetraspanins (Hemler, 2003; Wubbolts et al., 2003).

II.5.3 Functions of exosomes

Exosome functions depend on the cell type from which they were derived and the composition of exosomes in terms of lipids, carbohydrates and proteins.

How soluble proteins that lack a signal sequence are released from cells has been the focus of considerable research, and some mechanisms have recently been defined. One method is through association with exosomes, such as release of translationally controlled tumor protein (TCTP) (Amzallag et al., 2004). Another function attributed to exosomes includes the constitutive extracellular release of tumor necrosis factor (TNF) receptor 1 (Zhang et al., 2006). The cytokine interleukin (IL)-1b and possibly the chemokine regulated activation normal T cell expressed and secreted (RANTES) may also be secreted by exosomes (Chen et al., 2006). Other studies have defined a role for exosomes in ectodomain shedding and consequently a vehicle for the cellular export of soluble molecules like L1 (CD171) and CD44 in ovarian carcinoma cells (Stoeck et al., 2006).

Exosomes are also involved in antigen presentation of immune cells. Exosomes secreted by EBV-transformed B cells can stimulate human $CD4^+$ T-cell clones in an antigen-

specific manner (Raposo et al., 1996). Exosomes produced by mouse DCs pulsed with tumor peptides induced the rejection of established tumors in a T-cell-dependent fashion (Zitvogel et al., 1998). Exosomes can also transfer antigens from tumor cells to DCs (Wolfers et al., 2001) and therefore functions in antigen cross-presentation. Exosomes from tumor cells carry MHC molecules along with tumor antigens like melan-A/MART1 (melanoma tumor), which can be recognized by T cells (Mears et al., 2004).

HIV-1 and other retroviruses can exploit the machinery of MVBs generation and can be released into the extracellular milieu upon fusion of ‘‘hijacked’’ MVBs with the cell surface (Pelchen-Matthews et al., 2004).

Tumor cell-derived exosomes containing tumor antigens plus MHC class I molecules can transfer tumor antigens to DCs to induce a $CD8^+$ T-cell dependent anti-tumor immune response (Hao et al., 2006). Interestingly, tumor derived exosomes may have broader activity than previously believed as one study showed that exosomes isolated from different tumors inhibited not only syngeneic but also allogenic tumor growth, indicating that tumor-derived exosomes may harbor some common tumor antigens (Wolfers et al., 2001).

Some special molecules in exosomes can be detected from biological fluids, these molecules then may be served as biomarkers for early diagnosis. Composition of exosomes may show a dynamic patter during the courses of some diseases, which offers a convenient method to monitor the progress of disease.

II.5.4 Exosomes in the nervous system

Faure *et al.* have demonstrated that rat and mouse cortical neurons secrete exosomes in culture that have the typical features (size, density and saponin sensitivity) seen in other cell types (Faure et al., 2006). Using proteomic methods, they found that neuronal exosomes largely resemble those of non-neural cell types, e.g. expressing Alix, Tsg101, tubulin, 14-3-3 proteins, annexins, clathrin heavy chain, HSC70, GAPDH, etc. (Valadi et al., 2007; Wubbolts et al., 2003; Thery et al., 2001; Mears et al., 2004). In addition, the exosomes contained neuron-specific components. For example, AMPA receptor subunit GluR2/3 was detected within purified neuronal exosomes, but in contrast, NMDA receptor subunit NR1 and PSD-95 were not detectable (Faure et al., 2006). Neuronal-specific cell adhesion molecule L1 was detected, as was cellular prion protein. The secretion of exosomes was markedly stimulated by K^+ depolarization. Exosomes have also been shown to be secreted by cultured astrocytes, because they carry HSP70 which

has neuroprotective effects upon neurons, exosomes may contribute to glial-neuronal communication (Taylor et al., 2007; Tytell, 2005).

It has recently reported that proteins associated with the neurodegenerative disorders, such as prion protein (PrP^C) (associated with prion disease) and the amyloid precursor protein (APP) (associated with Alzheimers disease), can be incorporated into ILVs of MVBs and subsequently released into the extracellular milieu, which may answer how prion protein spread and how amyloid protein deposite in extracellular space.

Prion diseases are a group of invariably fatal neurodegenerative diseases that affect both humans and animals. According to the prion protein hypothesis, an abnormal isoform of the host encoded PrP^C, referred to as PrP^{Sc}, is the major component of the infectious agent causing transmissible spongiform encephalopathies (Prusiner, 1982). *Ecroyd et al* examined the characteristics of PrP^C isoforms present in the male reproductive tract and identified the majority bound to membrane vesicles which they referred to as epididymosomes (Ecroyd et al., 2004).These epididymosomes are presumed to be exosomes (Sullivan et al., 2005). Subsequently, Robertson et al. found PrP^C on platelet-derived exosomes (Robertson et al., 2006).

It has been recently demonstrated that both PrP^C and PrP^{Sc} are released in association with exosomes from a neuronal cell line (GT1-7) that endogenously expresses PrP (Vella et al., 2007). It provides evidence on the transmission of exosome-associated PrP^{Sc} to heterologous cell types in addition to homologous cell types. Exosomes from the prion-infected neuronal cell line were found to be very efficient initiators of prion propagation in uninfected recipient cells and also to non-neuronal cells. Moreover, the neuronal cell line was highly susceptible to infection by non-neuronal cell-derived exosome PrP^{Sc} suggesting that exosomes are very effective in transferring prion infectivity. The finding that exosomes released from infected cells can initiate conversion in cells originating from different tissues is significant for prion pathogenesis, as it may mimic the spread of infectivity around the lymphoreticular system and to the central nervous system. These data are supported by those of Schatzl et al. (Schatzl et al., 1997), who showed the transfer of infectivity from infected GT1-7 cell media to N2A cells.

Alzheimers disease is the most common form of dementia in humans and is characterised pathologically by the extracellular deposition of insoluble amyloid fibrils as amyloid plaques in the brain. The main component of amyloid is polymerized β-amyloid peptide (Aβ), a 39–43 amino acid residue peptide produced by proteolytic cleavage from the amyloid precursor protein (APP) (Cai et al., 1993). The link between Aβ and its

association with exosomes was made recently by Rajendran *et al.* They proposed that intracellular accumulated Aβ in MVBs is incorporated into exosomes and upon fusion of the MVBs with the plasma membrane, Aβ is released into the extracellular environment in association with exosomes, other exosomal proteins such as Alix and flotillin have also been found to accumulate in the plaques of AD patient brains (Rajendran et al., 2006).

II.6 Synapsin I

II.6.1 Background

In 1969, a cAMP-dependent protein kinase was discovered in the nervous system (Kuo and Greengard, 1969). 3 years later, one substrate protein was identified and called Protein I (Johnson et al., 1972; Ueda et al., 1973). It was the first identified substrate molecule for endogenous cAMP-dependent phosphorylation in the nervous system. Protein I was found to be a nerve terminal-specific, synaptic vesicle-associated protein (De Camilli et al., 1983a; Huttner et al., 1983). Because of its specific localization at synapses, it was renamed as synapsin I.

Synapsin I belongs to synapsins family which, up to now, have five distinct isoforms (Yang-Feng et al., 1986; Huang et al., 1982; Kao et al., 1998; Kao et al., 1999; Hosaka and Sudhof, 1998). This is one of the most abundant families of synaptic proteins, comprising around 1% of the total protein in the brain (Ueda and Greengard, 1977; Goelz et al., 1981; Browning et al., 1987), they are expressed in neurons and are specifically localized in the presynaptic compartment of the synapses (De Camilli et al., 1983a; Finger et al., 1990). The structure of the synapsins includes both conserved and variable domains which form synapsin I, II and III by various combinations. There exist eight domains from A to H (Sudhof et al., 1989). The most conserved protein domains are A and C, which are present on all members of synapsins, also conserved is the domain E, which is shared by synapsin Ia, synapsin IIa and synapsin IIIa isoform. Here I will focus on the synapsin I.

II.6.2 Structure and physical/chemical properties of synapsin I

Synapsin I gene was found in the X chromosome at band XP11 in humans and at band XA1-A4 in mice (Yang-Feng et al., 1986). Synapsin Ia and synapsin Ib isoforms derive from differential splicing of the primary transcripts (Sudhof et al., 1989). The molar ratio of Ia/Ib is around 1:2 (Ueda and Greengard, 1977). They represent approximately 0.4%

of the total protein in mammalian brain (Goelz et al., 1981). They migrate in SDS-polyacrylamide gels with apparent molecular weights of 86,000 and 80,000 for the Ia and Ib forms, respectively. Both isofroms contain same domains from A to D, difference between two isoforms lies in the C-terminal domains, synapsin Ia involves domain E, while, synapsin Ib involves domain F. Domain D is only present in synapsin I, it is rich in basic residues and also abundant in glycine and proline residues. Domains A-C is referred as the head region of the molecule, while domain D-E/F is referred as tail region. Synapsin I has an extremely basic isoelectric point (pI greater than 10.5) and bears a strong positive charge at physiologic pH. The tail region appears to be responsible for the basicity of the protein, since fragments corresponding to the head region exhibit isoelectric points close to neutrality. Synapsin I is a surface active molecule, actually, it was shown that synapsin I has one of the highest surfactant activities ever measured for protein molecules. The surface activity is mostly contributed by the head region. This can be explained by the unusual amino acid composition of the highly conserved domain C of synapsin I, which is both hydrophobic (39% hydrophobic residues) and highly charged (27% charged residues) and has sequences with a high potential for forming amphiphilic α-helices and β-strands (Sudhof et al., 1989).These observations suggest that synapsin I, and in particular its head region, has a high potential for forming ordered structures when exposed to an amphiphilic environment like a membrane surface and may involved in the high affinity anchoring to the synaptic vesicles (De Camilli et al., 1990).

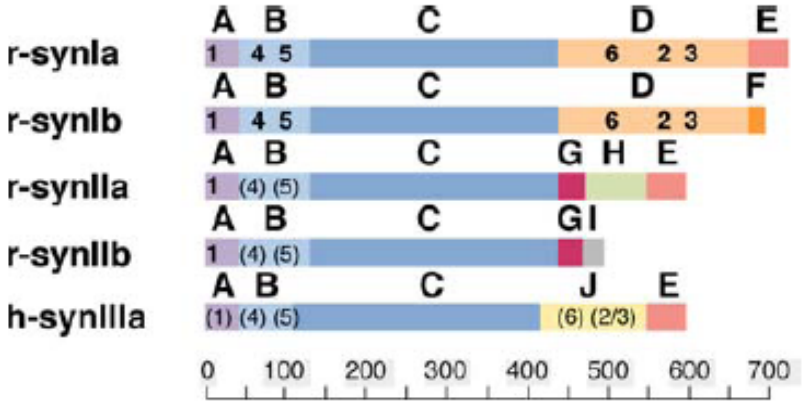

Fig. 4: Domain model of the vertebrate synapsin family.

Rat synapsins Ia (r-synIa) and Ib (r-synIb), rat synapsins IIa (r-synIIa) and IIb (r-synIIb) and human synapsin III (h-synIIIa). Phosphorylation sites (sites 1–6) are indicated in bold, with putative phosphorylation based on the presence of consensus phosphorylation sites in brackets. The distinct sites are subject to phosphorylation by the different kinases (Fdez and Hilfiker, 2006).

II.6.3 Distribution of synapsin I

Synapsin I has a widespread distribution in the central and peripheral nervous system. It was also reported in the nervous tissue of a variety of vertebrate and invertebrate species (De Camilli et al., 1983a; Sorensen and Babitch, 1984; Bixby and Reichardt, 1985; Goelz et al., 1985; Volknandt et al., 1987; Valtorta et al., 1988; Mitschulat, 1989). Within neurons the synapsin I is concentrated in nerve terminals. It was found at all axon terminals of vertebrate neurons, including typical presynaptic nerve terminals, varicose nerve endings of sympathetic neurons, sensory endings, and nerve terminals of the neurohypophysis (De Camilli et al., 1983b; De Camilli et al., 1988; Scarfone et al., 1988; Navone et al., 1989; Finger et al., 1990). Electron microscopic immunocytochemistry demonstrated that, within intact nerve terminals, the synapsin I is selectly concentrated in the region occupied by synaptic vesicles (De Camilli et al., 1983b). In mature neurons only very low levels of diffuse synapsin I could be detected in region of the cell other than nerve terminals. At early developmental stage 1-2 of cultured hippocampal neurons (Dotti et al., 1988), the staining of synapsin I antibodies was somewhat granular in appearance and could be detected in the cell body and in the minor processes. During stage 3, an stage of axonal outgrowth, synapsin I was already markedly concentrated in the distal portion of the axon and its growth cone, while in cell bodies, the level of synapsin I remained quite elevated. During stage 4, large puncta of synapsin I immunofluorescence first appeared, and as in more mature cultures, they were selectively localizes to sites of contact between axons and somata or dendrites. At this stage, only faint levels of synapsin I were visible in cell bodies (Fletcher et al., 1991). Recently, it is also found to be expressed in epithelial cells (Bustos et al., 2001), ß cells of pancreatic islets in vivo (Krueger et al., 1999), cultured astrocytes (Maienschein et al., 1999) and several cultured cell lines of neural and endocrine origin such as PC12 (Romano et al., 1987), AtT-20 (Tooze et al., 1989) and MIN6 insulinoma (Matsumoto et al., 1995).

II.6.4 Phosphorylation of synapsin I and synaptic vesicle mobility

After discovered as the first identified substrate protein of cAMP-dependent protein kinase (Johnson et al., 1972; Ueda et al., 1973), synapsin I was found to be phosphorylated by a variety of protein kinases and phosphorylation served as an important functional regulatory switch involved in synaptic vesicle mobilization and neurotransmitters release. 6 distinct sites on different domains are subject to phosphorylation by a variety of protein kinases **(Fig. 4)**. Site 1 on the A domain can be

phosphorylated by cAMP-dependent protein kinase A (PKA) and $Ca2^{+}$/calmodulin-dependent protein kinase I (CaM kinase I). Site 2 and 3 on the domain D are targets for phosphorylation by $Ca2^{+}$/calmodulin-dependent protein kinase II (CaM kinase II) (Czernik et al., 1987). Site 4 and 5 on B domain and site 6 on D domain are phosphorylated by mitogenactivated protein kinase (MAP kinase). Site 6 on D domain can also be phosphorylated by cyclin-dependent protein kinase (cdk5) (Jovanovic et al., 1996).

The phosphorylation mediated changes in the ability of synapsins to bind to synaptic vesicles and actin are well documented. For example, phosphorylation of synapsins by PKA/CaM kinase I or by CaM kinase II drastically decreases their affinity for synaptic vesicles (Schiebler et al., 1986; Hosaka et al., 1999; Menegon et al., 2006), whilst phosphorylation by MAP kinase has no effect on the affinity of synapsins for synaptic vesicles, but decreases their affinity action (Jovanovic et al., 1996). Such *in vitro* findings are paralleled by experimental evidence *in vivo*. For example, phosphorylation at site 1 or sites 2-3, or calcium-dependent dephosphorylation at sites 4-6, leads to the reversible dissociation of synapsins from vesicle clusters and their concomitant dispersion into the axon (Chi et al., 2001; Chi et al., 2003). Phosphorylation of synapsin I regulates neurotransmittcr release by modulating the as/dissociation the molecule from synaptic vesicles and/or from F-actin (Jovanovic et al., 1996; Matsubara et al., 1996; Bahler and Greengard, 1987; Valtorta et al., 1992; Hosaka et al., 1999; Chi et al., 2001). Dephospho-form of synapsin I binds synaptic vesicles, binds and bundles F-actin, and promotes G-actin polymerization **(Fig. 5)**. Phosphorylation of synapsin I at either site generally reduces G-actin nucleating activity and F-actin-bundling activity, which seems to be related to the flexibility of the presynaptic actin network organization. Phosphorylation of synapsin I at sites 2 and 3 and at site 1 reduces its binding to synaptic vesicles, which seems to be related to synaptic vesicle mobilization for neurotransmitter release in the presynaptic terminal. Experiments using synaptosomes indicated the importance of CaMKII-dependent phosphorylation and MAPK dependent phosphorylation of synapsin I for the regulation of neurotransmitter release (Greengard et al., 1993; Nichols et al., 1992; Jovanovic et al., 2000).

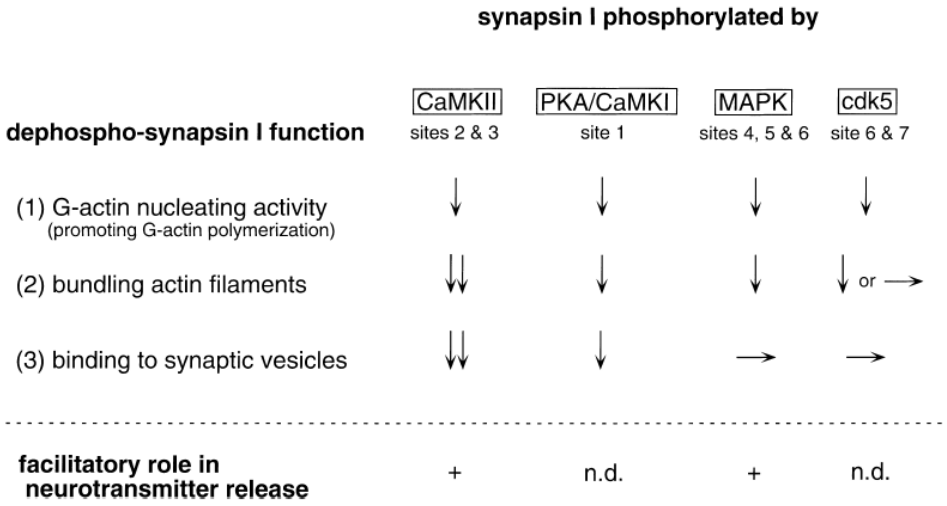

Fig. 5: Summary of the effects of phosphorylation on synapsin I function.

Dephospho-form of synapsin I binds synaptic vesicles, binds and bundles F-actin and promotes G-actin polymerization. These functions are modified by phosphorylation of synapsin I at multiple sites by various protein kinases. Phosphorylation of synapsin I at different sitse generally reduces G-actin nucleating activity, F-actin-bundling activity and binding to synaptic vesicles. PKA, Cyclic AMP-dependent protein kinase; CaMKI, $Ca2^{+}$/calmodulin-dependent protein kinase I; CaMKII, $Ca2^{+}$/calmodulin-dependent protein kinase II; MAPK, mitogen-activated protein kinase. n.d., not determined (Yamagata, 2003).

II.6.5 Glycosylation of synapsin I

Up to now, there exists very limited information about glycosylation of synapsin I. *Cole et al* identified seven O-GlcNAcylation sites (Ser^{55}, Thr^{56}, Thr^{87}, Ser^{516}, Thr^{524}, Thr^{562}, and Ser^{576}) on synapsin I by analysing of HPLC-purified digests of rat brain synapsin I. These glycosylation sites are clustered in domain B and D and are flanked by five phosphorylation sites. The presence of an O-GlcNAc at sites Thr^{562} and Ser^{576} resulted in a 66% increase in the K_m of calcium/calmodulin-dependent protein kinase II phosphorylation of site Ser^{566} with no effect on its V_{max}. They concluded that O-GlcNAcylation likely plays a more direct role in synapsin I interactions, possibly influencing synapsin I's ATPase activity or its association with c-Src tyrosine kinase, than simply modulating the protein's phosphorylation (Cole and Hart, 1999). *Murrey et al* found that synapsin Ia and Ib are the major fucose-α (1-2)-galactose [Fucα(1-2)Gal] glycoproteins in mature cultured neurons and the adult rat hippocampus. Inhibition of synapsin I's fucosylation significantly decrease its cellular half-life. Fucosylation of synapsin I can also protects synapsin I from degradation by the calcium-activated protease calpain. Defucosylation of synapsin I has critical consequences for neuronal

growth and morphology, leading to stunted neurite outgrowth and delayed synapse formation (Murrey et al., 2006).

II.7 Roles of NCAM in neurite outgrowth

Neural cell adhesion molecule (NCAM) belongs to the Ig superfamily of cell adhesion molecules (CAMs). NCAM consists of five membrane-distal Ig modules (termed Ig I – V) and two membrane-proximal FN3 modules (termed FN3, I – II), it is encoded by NCAM1 gene, and alternative splicing of a number of exons gives rise to three isoforms, NCAM-120, NCAM-140, and NCAM-180. These isoforms are named according to their apparent molecular weight. All isoforms of NCAM have similar ectodomains, but NCAM-120 lacks a transmembrane domain and is linked to the membrane via a glycosylphosphatidylinositol (GPI) anchor. NCAM-140 and NCAM-180 are transmembrane forms and differ in the length of their intracellular domain, with NCAM-140 being shorter than NCAM-180 (Horstkorte et al., 1993).

Application of NCAM antibodies reduced neuronal neurite outgrowth of retinal neurons grown on astrocytes (Neugebauer et al., 1988), dorsal root ganglion neurons grown on Schwann cells and fibroblast monolayers (Seilheimer and Schachner, 1988). These studies indicated that NCAM's adhesive properties may be involved in stimulating axonal growth. However, NCAM can induce axonal growth even as soluble fragments (Meiri et al., 1998).

Both homophilic (Doherty et al., 1990; Doherty et al., 1991) as well as heterophilic (Williams et al., 1994a) interactions of NCAM lead to the induction of intracellular signaling pathways promoting neurite outgrowth. It is well known that NCAM induces intracellular signaling via an IgSF receptor tyrosine kinase, the fibroblast growth factor receptor (FGFR). All three isoforms of NCAM interact with FGFR (Kiselyov et al., 2003; Sanchez-Heras et al., 2006). To activate FGFR, NCAM interactions between molecules on opposing surfaces (*trans* homophilic interactions) are indispensable (Kiselyov et al., 2005). These interactions accelerate the formation of zipper structures that cluster NCAM which in turn may cluster bound FGFR molecules at the plasma membrane. Such clustering results in the dimerization of FGFR which in turn activate itself via *trans* autophosphorylation. Its tyrosine kinase activity thereby leads to the phosphorylation of regulatory tyrosine residues (Schlessinger, 2000). Interacting partners such as phospholipase C_γ domain, (PLC_γ) which binds via its SH2 domain can now bind to the

phosphorylated residues in the cytoplasmic FGFR and gets subsequently activated (Saffell et al., 1997). Activated PLC$_\gamma$ cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These downstream second messengers trigger calcium influx from intracellular stores and extracellular space into the cytoplasm (Doherty et al., 1991; Williams et al., 1994b; Kiryushko et al., 2006).

The increase in calcium concentration in response to NCAM signaling via FGFR strengthens the complex between NCAM140 and RPTPα via spectrin. Upon NCAM activation and palmitoylation the complex redistributes into lipid rafts (Niethammer et al., 2002; Bodrikov et al., 2005). This transformation triggers the activation of downstream signaling pathways, e.g. the activation of the focal adhesion kinase (FAK) (Niethammer et al., 2002) which in turn leads to the activation of the mitogen activated protein kinase (MAPK) pathway (Schmid et al., 1999). MAPKs are serine/threonine specific protein kinases that regulate the activity of several transcription factors such as c-fos, NFκB and CREB. The kinases translocate to the nucleus and alter the transcription of their target genes (Pawson and Scott, 1997) which will ultimately lead to the elogation of neurites.

III. Aims

Oligomannosidic glycans are widely expressed on adhesion molecules in the nervous system and are involved in many important physiological and pathological procedures (Kleene and Schachner, 2004). However, because of their limited natural sources and difficulty in chemical synthesis, it is meaningful to explore surrogates, like peptides that structurally and functionally mimic the oligosaccharides (Zwick et al., 1998). The first aim of present study is to explore oligomannose mimicking peptide by screening phage display library. The structural and functional mimicking properties of the selected peptide will be tested.

Peptide mimicry is widely used to produce carbohydrate surrogates which elicit efficient immunoreactivity, especially for developing vaccines (Kieber-Emmons et al., 1997), however, few biochemical studies utilize sugar mimicking peptides (Simon-Haldi et al., 2002). Present study will apply the oligomannose mimicking peptide in cross-link experiment to search for oligomannose binding proteins from mouse brain homogenates.

Oligomannose bearing glycoproteins are involved in many important biological processes (Kleene and Schachner, 2004), it is meaningful to explore novel oligomannose bearing glycoproteins. The present work will use immunoaffinity chromatography to explore novel oligomannose bearing glycoproteins. The effect on neurite outgrowth of the oligomannose bearing glycoprotein (synapsin I) will also be investigated. To elucidate the underlying mechanism by which synapsin I exerts effect on neurite outgrowth, involved binding partner of synapsin I will also be identified.

Since synapsin I is a N-linked oligomannosidic glycoprotein, it may be a secreted protein that is released from the cell and act as an extracellular protein. It is expressed in cultured astrocytes and is associated with cytosolic organelles (Maienschein et al., 1999). I then check whether it is expressed on exosomes. Exosomes are bioactive vesicles originate for multivesicle bodies, they are released into extracellular milieu upon fusion of the multivesicle bodies with plasm membrane, it serves as an important intercellular messenger (Taylor et al., 2007; Tytell, 2005). The mechanisim of how the exosomes release their cargo proteins will be investigated in the present study.

IV. Materials

IV.1 Chemicals

All chemicals were purchased from the following companies: Amersham Pharmacia Biotech (Freiburg, Germany), Bio-Rad (Hercules, CA, USA), Carl Roth (Karlsruhe, Germany), GibcoBRL (Life technologies, Karlsruhe, Germany), Invitrogen (Karlsruhe, Germany), Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich (St. Louis, MO, USA). The Phage display library kit was obtained from New England Biolabs (Frankfurt am Main, Germany). Dynabeads M-270 Epoxy was obtained from Dynal Biotech (Oslo, Norway). MBS, sulfo-SBED, sulfo-link coupling gel and MagnaBindTM Streptavidin beads were obtained from Pierce Biotechnology (Rockford, Illinois, USA). Endoglycosidase H and PNGase F were obtained from Roche Diagnostics (Mannheim, Germany). Peptides and scrambled peptides were commercially synthesized by Schafer-N (Copenhagen, Denmark). PD-10 columns were purchased from GE Healthcare (Uppsala, Sweden). Blocking solution and Low cross buffer were obtained from Candor Bioscience (Weissensberg, Germany). Cell culture materials were ordered from Nunc (Roskilde, Denmark) or Life Technologies.

IV.2 Solutions and buffers

Phosphate buffered saline (PBS)

10 mM Na_2HPO_4
2.5 mM NaH_2PO_4
150 mM NaCl
3 mM KCl
pH 7.4

PBS Tween (PBST)

PBS
0.05% Tween-20

Tris buffered saline (TBS)

25 mM Tris
150 mM NaCl
2 mM KCl
pH 7.4

Tris buffered saline Tween (TBST)

TBS

0.1% Tween-20

0.1 M sodium phosphate buffer (pH 7.4)

20 mM NaH_2PO_4

80 mM Na_2HPO_4

pH 7.4

3 M ammonium sulfate

3 M $(NH4)_2SO_4$

0.1 M sodium phosphate buffer (pH 7.4)

Polyethylene glycol-8000/sodium chloride (PEG/NaCl)

2.5 M NaCl

20%(W/V) polyethylene glycol-8000

Conjugation buffer:

PBS (pH 7.4)

2 mM EDTA

DTNB solution:

50 mM sodium acetate in 1ml H_2O

2 mM DTNB

Elleman's solution

50 µL DTNB solution

100 µL 1 M Tris (pH 8.0)

840 µL H_2O

Homogenization buffer

50 mM Tris-HCl (pH 7.4?)

2 mM EDTA

Protease inhibitor cocktail (1:50)

Coupling buffer:

50 mM Tris (pH 8.5)

5 mM EDTA

Quenching solution

10 mM L-cysteine-HCl

Swelling solution	
	1 mM HCl
Coupling solution	
	100 mM $NaHCO_3$
	500 mM NaCl
	pH 8.3
Blocking solution	
	200 mM glycine
	500 mM NaCl
	pH 8.0
Washing buffer A	
	100 mM Na-Acetate
	500 mM NaCl
	pH 4.0
Washing buffer B	
	100 mM $NaHCO_3$
	500 mM NaCl
	pH 8.3
Washing buffer C	
	PBS (pH 7.4)
Washing buffer D	
	25 mM Tris
	150 mM NaCl
	5 mM EDTA
	pH 7.4
Washing buffer E	
	25 mM Tris
	150 mM NaCl
	1% Triton X-100
	0.02% NaN_3
	pH 7.4
Washing buffer F	
	25 mM Tris

500 mM NaCl
0.1% Triton X-100
0.02% NaN_3
pH 7.4

Elution buffer

50 mM ethanolamine
150 mM NaCl
0.2% CHAPS
pH 11.5

5X reaction buffer for Endo H

250 mM sodium acetate
pH 5.5

20 X denaturing buffer for Endo H

2% SDS
10% ß-mercaptoethanol

5X reaction buffer for PNGase F

0.5 M sodium phosphate
pH 7.4

10 X denaturing buffer for PNGase F

2% SDS
10% ß-mercaptoethanol

Buffer 1 for lectin staining

1 mM $MgCl_2$
1 mM $MnCl_2$
1 mM $CaCl_2$
TBS (pH 7.5)

Buffer 2 for lectin staining

0.1 M Tris-HCl
0.1 M NaCl
0.05 M $MgCl_2$
pH 9.5

SDS polyacrylamide gel electrophoresis:

Sample buffer (2x)	125 mM Tris-HCl (pH 6.8)
	4% SDS
	20% Glycerol
	10% β-mercaptoethanol
	0.00625% Bromphenol blue
Running buffer	25 mM Tris
	192 mM Glycin
	0.1% (w/v) SDS
Stacking gel 4%	3.68 ml deionized water
	0.625 ml of 1 M Tris-HCl (pH 6.8)
	0.05 ml of 10% SDS
	0.665 ml of 30% Acrylamide-Bisacrylamide 37:1
	25 µl of 10% APS
	5 µl TEMED
Separating gel 10%	3.45 ml deionized water
	4.65 ml of 1 M Tris-HCl (pH 8.8)
	0.125 ml of 10% SDS
	4.17 ml of 30% Acrylamide-Bisacrylamide 37:1
	62.5 µl of 10% APS
	6.25 µl TEMED

Coomassie blue staining:

Staining solution	1% coomassie blue
	45% methanol
	10% acetic acid
Destaining solution	45% methanol
	10% acetic acid

Western blot analysis:

Blocking buffer	5% (w/v) skim milk powder in PBS (pH 7.4)
Blotting buffer	25 mM Tris

	192 mM Glycin
	0.001% (w/v) SDS
Stripping buffer	0.5 M NaCl
	0.5 M acetic acid

IV.3 Media

IV.3.1 Bacterial media

Tetracycline stock	20 mg/ml Tetracycline in ethanol
IPTG/Xgal stock	1 g Xgal in 25ml dimethyl formamide
	1.25 g IPTG
LB medium	10 g/l bacto-tryptone (pH 7.4)
	5 g/l NaCl
	5 g/l yeast extract
Agarose top	10 g/l bacto-tryptone
	5 g/l NaCl
	5 g/l yeast extract
	7 g/l agarose
	1g/l $MgCl_2$ x $6H_2O$
LB/IPTG/Xgal plate	15 g/l agar in LB medium
	1 ml IPTG/Xgal stock
LB/Tet plate	15 g/l agar in LB medium
	1 ml tetracycline stock

IV.3.2 Cell culture media

Dissection solution for hippocampal neuron culture

	Hanks balanced salt solution (HBSS)
	4.16 mM $NaHCO_3$
	10 mM HEPES
	34 mM D-glucose
	12 mM $MgSO_4$
	2.88 mg/ml BSA
	5 µg/ml Gentamycin
	pH 7.4

Digestion solution for hippocampal neuron culture

5 mg/ml Trypsin
1 mg/ml DNase I
135 mM NaCl
5 mM KCl
7 mM Na_2HPO_4
25 mM HEPES
4.16 mM $NaHCO_3$
pH 7.4

Dissociation solution for hippocampal neuron culture

1 mg/ml DNAse I
Dissection solution

Serum free culture medium for hippocampal neuron

Neurobasal A
2 mM L-glutamine
2% B27
Gentamycin 5µg/ml

Astrocyte culture

Culture medium	Dulbecco's modified Eagle's medium (DMEM)
	50 U/ml penicilline/streptomycine
	2 mM glutamine
	10% fetal calf serum (FCS)

C6 cell culture

Culture medium	Dulbecco's modified Eagle's medium (DMEM)
	50 U/ml penicilline/streptomycine
	2 mM glutamine
	10% fetal calf serum (FCS)

IV.4 Molecular weight standard

Precision plus proteinTM dual color standards — 6 µl of the protein ladder (BioRad) were loaded into the slots of a SDS-PAGE gel

Band No.	Apparent molecular weight (kDa)
1	250
2	150
3	100
4	75*
5	50
6	37
7	25*
8	20
9	15
10	10

* orientation band (pink in color)

IV.5 Antibodies

IV.5.1 Primary antibodies

Synapsin Ⅰ — Mouse monoclonal antibody against C terminal region of synapsin Ia and Ib. The antibody was purchased from Synaptic Systems (Göttingen, Germany).
WB: 1:10,000; ELISA: 1:2,000

L3 — Immuno-affinity purified rat monoclonal antibody (IgM) against oligomannosides (reactivity: Mann5 > Mann 6 > Mann 7 >> Mann 8 >> Mann 9)
WB: 5 µg/ml; ELISA: 10 µg/ml

L4 — Immuno-affinity purified rat monoclonal antibody (IgM) against oligomannosides (reactivity: Mann5 > Mann 6 > Mann 7 >> Mann 8 >> Mann 9)
WB: 5 µg/ml; ELISA: 10 µg/ml

HNK1 — Mouse monoclonal antibody (IgM) recognizing only the

	sulfated HNK1 epitope WB: 1:1,000
412	Rat monoclonal antibody (IgG) recognizing the HNK1 epitope with and without sulfate WB: 1:1,000
735	Mouse monoclonal antibody (IgG) against polysialic acid (PSA) kind gift of Rita-Gerardy-Schahn (Zelluläre Chemie, Medizinische Universität Hannover, Germany) WB: 1:5,000
L5	Rat monoclonal antibody (IgM) against $Lewis^X$ WB: 1:1,000
HSC70	Goat polyclonal antibody against a peptide mapping at the carboxy terminus of Hsc70 of human origin (identical to corresponding mouse sequence) Santa Cruz Biotechnology WB: 1:1,000
Actin	Rabbit polyclonal antibody against beta actin Sigma WB: 1:1,000
GADPH	Mouse monoclonal antibody against Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH) Santa Cruz Biotechnology WB: 1:2,000
Alix (AIP1)	Mouse monoclonal antibody against ALG-2 interacting protein X (Alix or AIP1) Becton-Dickinson WB: 1:1,000

IV.5.2 Secondary antibodies

Horseradish peroxidase-coupled (HRP) secondary anti-M13 antibodies were included in phage display library kit. All HRP coupled anti-mouse, rat, rabbit and goat secondary antibodies were purchased from Dianova (Hamburg, Germany) and were used in dilutions between 1:10,000 and 1:20,000 for Western blot and 1:5,000 in ELISA assays. HRP coupled neutravidin was purchased from Pierce.

V. Methods

V.1 Phage display

A phage display library (Ph.D.-12™ Phage Display Peptide Library Kit; New England Biolabs, Inc.) containing linear random peptide 12-mers fused to the N-terminus of a minor coat protein (PIII) of M13 phage was used for screening for oligomannose mimicking peptides. The library includes 2.7 x 10^9 independent phage clones.

For screening, monoclonal antibodies recognizing oligomannosidic glycans (L3 and L4; 10 µg each) were immobilized on 0.5 mg magnetic epoxy beads (Dynabeads M270; Dynal Biotech, Oslo, Norway), followed by blocking with 0.5% BSA in 0.1 M sodium phosphate buffer (pH 7.4). Antibody coupled beads were incubated with the phage display library and three rounds of panning were carried out as described (Simon-Haldi et al., 2002). Briefly, 4 x 10^{10} phages (10 µl of the original library) were allowed to bind with L3 or L4 antibody coated beads. After incubation for 30 min at room temperature, beads were washed 10 times with 1 ml Tris-buffered saline (TBS, pH 7.4) with 0.1% (v/v) Tween 20 (in the second and third round of panning, the concentration of Tween 20 was increased to 0.3% and 0.5% to enrich phage clones that interact specifically with the antibodies). After washing, bound phages were eluted using three different elution protocols. For L3 antibody coated beads, L3 antibody (10 µg in 100 µl TBS) and oligomannoses isolated from RNase B (RB) (5 µg in 100 µl TBS) were used to elute individual phages; for L4 antibody coated beads, L4 antibody (10 µg in 100 µl TBS) was used to elute phages. Eluted phages were amplified for further rounds of panning; in the following rounds of panning 2 x 10^{11} amplified phages were used as input. After three rounds of panning, individual phage clones were isolated. The single-stranded DNA of the phages was purified and used for DNA sequencing (sequencing was performed at the sequencing facility of the ZMNH). DNA sequences were translated into amino acids and peptides were synthesized (Schafer-N, Copenhagen, Denmark) according to their sequences.

V.2 Conjugation of synthetic peptides to catalase

Because of low solubility of synthesized peptides, they could not be used for cell culture or interaction studies. Furthermore, carbohydrates are normally presented to interaction partners by the proteins to which they are attached and are not found free on the cell surface or within the extracellular matrix. To gain more soluble compounds and to

present the peptides like the natural oligomannoses, they were coupled to catalase (Sigma-Aldrich, Inc. USA) using the cross-linker *m*-maleimido-benzoyl-*N*-hydroxy-succinimidyl ester (MBS; Perbio Science, Bonn, Germany). In order to do this, one cysteine was added to the C-terminus of the peptide to allow the reaction with the maleimide group on MBS. Briefly, catalase (final concentration: 0.1 mM) in 0.9 ml conjugation buffer (5 mM EDTA in PBS, pH 7.2) was mixed with MBS (final concentration: 1 mM) dissolved in 0.1 ml dimethyl sulfoxide (DMSO). The molar ratio of MBS to catalase in this mixture is 10:1, which guarantees saturated conjugation of *N*-hydroxy-succinimide (NHS) esters on MBS to the amine groups on catalase. After 30 minutes incubation at room temperature, unbound MBS was removed by running the mixture through a PD-10 column (GE Healthcare, Uppsala, Sweden). The flow through was collected and incubated with peptides (final concentration: 1 mM) freshly dissolved in 0.2 ml 20% (v/v) DMSO in PBS. This mixture was then incubated at room temperature for 30 minutes and dialysed (Slide-A-Lyzer dialysis cassetts 30 kD MWCO; Pierce) against 4 L TBS at 4°C overnight to remove uncoupled peptides. The mixture was then concentrated by using Vivaspin centrifugation tubes (Vivaspin 20, Vivascience, Göttingen, Germany) with a 30 kD membrane cut off. To quantify the efficiency of conjugation, an Ellman's test (Bulaj et al., 1998) was applied to determine free sulfhydryl groups. In this paper, concentrations of peptides imply the coupled peptides to catalase such as catalase-MBS-P1 (CP1), catalase-MBS-scrambled peptide of P1 (CSP1), catalase-MBS (C) alone was used as carrier protein control.

V.3 Enzyme-Linked ImmunoSorbent Assay (ELISA)

In order to evaluate the binding of L3 and L4 antibodies to selected phage clones, ninety-six wells (Nunc-Immuno Modules Maxisorp Loose, Roskilde, Denmark) were coated with 50 µl L3 or L4 antibody (10 µg/ml) in phosphate buffered saline (PBS) per well and incubated overnight at 4°C. Wells were blocked with 100 µl blocking solution (2% BSA in PBS, pH 7.4) for 1 hour at room temperature. Then, 5 x 10^9 purified phages in 50 µl blocking buffer were added to each well and incubated for 1 hour at room temperature. Plates were washed with 100 µl PBST (0.03% Tween 20 in PBS, pH 7.4) for 5 times. Subsequently, to detect phages that bound to L3 and L4 antibodies anti-M13 antibodies conjugated with horseradish peroxidase (HRP) diluted 1:5,000 in 50 µl blocking solution were added and wells were incubated for 1 hour at room temperature. The plates were then washed for 5 times with 100 µl PBST. After the last washing step,

0.5% o-phenylenediamine dihydrochloride (OPD; Perbio Science, Bonn, Germany) solution was added as a substrate for the HRP. The absorbance was read at 490 nm in a microtiter plate reader (µQuant, BioTek, Bad Friedrichshall, Germany).

To monitor binding between catalase coupled peptides and L3 and L4 antibodies and to evaluate competition between oligomannoses on RNase B and oligomannose mimicking peptides for binding to these antibodies, two additional ELISAs were carried out. Considering that L3 and L4 are rat antibodies belonging to the IgM subclass, which may produce unspecific signals and high background, two special buffers were used in these ELISA experiments. Wells of 96-well microtiter plates were coated with serial dilutions of catalase coupled peptides in 50 µl blocking solution (CANDOR Bioscience GmbH, Weißensberg, Germany) overnight at 4°C and then wells were blocked with 100 µl blocking solution for 1 hour at room temperature. L3 and L4 antibodies (10 µg/ml) in 50 µl low cross buffer (CANDOR Bioscience GmbH) were added to the wells and incubated for 1 hour at room temperature. After washing with PBST for 5 times, 50 µl HRP-conjugated anti-rat secondary antibodies diluted 1:5,000 in low cross buffer were added and wells were incubated for 1 hour at room temperature. The wells were then washed for 5 times with PBST. After the last washing step, the HRP activity was monitored by using 0.5% OPD solution as a substrate. The absorbance was read at 490 nm.

In case of competitive ELISAs, wells were coated with constant concentrations of catalase coupled peptides (40 µg/ml) in 50 µl blocking solution. Before adding L3 and L4 antibodies to the wells, they were pre-incubated for 30 minutes in 50 µl low cross buffer containing different concentrations of RNase B or RNase B treated with endoglycosidase H (RB(-)), which removes oligomannoses from the protein backbone. RB(-) then served as negative control. The mixtures were then added to the wells and wells were incubated for 1.5 hour at room temperature. The following procedures were the same as the conditions described for the binding test above.

To identify the oligomannose specific interaction between AMOG and synapsin I, AMOG and AMOG treated with Endoglycosidase H (AM (-)) were coated (10 pmol in 50 µl PBS) overnight at 4°C. After blocking with 100 µl 1% BSA for 1 hour, synapsin I alone (6.7 pmol in 50 µl 1% BSA) or synapsin I pre-incubated with sugars isolated from AMOG for 30 min (50 µg/ml) were incubated with the substrate coated AMOG for 1 hour at room temperature. After washing, bound synapsin I was detected by addition of 1:2,000 diluted anti-synapsin I antibodies and corresponding secondary antibodies.

In order to evaluate the oligomannose dependent interaction between synapsin I and NCAM, purified bovine synapsin I (SYN; 6.7 pmol in 50 µl PBS) and the same amount of synapsin I treated with Endo H (SYN(-)) were immobilized on a microtiter plate at 4°C overnight. After blocking with 1% BSA at room temperature for 1 hour, 6.5 pmol of NCAN-Fc fusion proteins in 50 µl 1% BSA were put on top of coated synapsin I substrate. In parallel, the same amount of NCAM-Fc in 50 µl 1% BSA was pre-incubated with AMOG and AMOG(-) (40 pmol) at room temperature for 30 minutes before putting on top of the coated substrate. Plates were incubated at 4°C overnight. The wells were washed with TBST (0.03% Tween 20 in PBS, pH 7.4) and incubated with an anti-human Fc antibody conjugated to HPR (1:5,000) at room temperature for 1 h. The wells were washed with TBST and developed with 0.5% OPD solution as substrate.

V.4 Neurite outgrowth

Dissociated hippocampal neurons obtained from C57/BL6J wild type mice and NCAM deficient mice of postnatal day zero (P0) were used in neurite outgrowth experiments. Briefly, isolated hippocampi from mouse brains were placed in ice cooled dissection solution (4.16 mM $NaHCO_3$, 10 mM HEPES, 34 mM D-glucose, 12 mM $MgSO_4$, 2.88 mg/ml BSA, 5 µg/ml Gentamycin in HBSS) and then digested in digestion solution (5 mg/ml Trypsin and 1 mg/ml DNase I with 135 mM NaCI, 5 mM KCI, 7 mM Na_2HPO_4, 25 mM HEPES and 4.16 mM $NaHCO_3$ in H_2O) for 15 minutes at room temperature. Tissues were then washed three times with ice cooled dissection solution to remove remaining trypsin and DNase I and dissociated in dissociation solution (1mg/ml DNase I in dissection solution) with fire polished Pasteur pipettes. The dissociated cells were then re-suspended in 10 ml dissection solution and centrifuged at 800 g for 10 minutes at 4°C. The cells were then re-suspend in culture medium (Neurobasal A supplied with 2 mM L-glutamine, 2% B27 and 5 µg/ml gentamycin). 2.5 x 10^4 cells were plated onto each coverslip in 24-well plates. Cells were cultured for 24 hours in a humidified chamber at 37°C with 5% CO_2. Cultures were fixed with 2.5% glutaraldehyde and stained with toluidine blue. The length of neurites was determined for neurons that were not in contact with other cells and that had at least one process that was as long as the diameter of the cell body. Measurements were performed with Axiovision software V4.6 (Carl Zeiss Microimaging, Göttingen, Germany).

A panel of molecules was substrate coated. Class coverslips were first coated with 0.01% poly-L-lysine (PLL), followed by coating with individual test substances. To investigate the functional roles of the oligomannose mimicking peptides catalase coupled peptide 1 (CP1), catalase coupled scrambled peptide 1 (CSP1) and catalase (C) (100 µg/ml), RB and RB(-) (50 µg/ml) were coated overnight at 4°C. To determine the oligomannose dependent promotive effects of synapsin I on neurite outgrowth, native synapsin I (10 µg/ml), denaturated synapsin I (10 µg/ml) and synapsin I treated with Endo H and PNGF (10 µg/ml) were coated. All test molecules were diluted in 100 µl HBSS for every coverslip and coated overnight at 4°C. PLL coated coverslips were also coated with 100 µl HBSS without addition of any molecule as control. Before plating, these coverslips were washed three times with ice-cooled HBSS. For further experiments, RB and RB(-) serving as oligomannose donor and corresponding control were heated at 60°C for 10 minutes to deactivate their enzymatic activities; they were diluted in pre-warmed culture medium and added into the culture medium 1 hour after plating of cells.

V.5 Cell adhesion

Astrocytes were obtained from the cortex of newborn C57/BL6J wild type mice by seeding single-cell suspensions into Petri dishes (5×10^5 cells/ml) in DMEM with 10% fetal calf serum (FCS), 2 mM L-Glutamine and 50 U/ml penicillin/streptomycin. On the third day, the culture medium was changed to serum free medium. After 7 days in culture, freshly dissociated cerebella neurons from GFP mice (Okabe et al., 1997) were prepared as described (Chen et al., 1999; Loers et al., 2005) and suspended in HBSS and adjusted to a cell density of 2×10^5 cells /ml. Both astrocytes and neurons were independently pre-incubated with CP1 (200 µg/ml), CSP1 (200 µg/ml) and C in Ca^{2+}-Mg^{2+} free Hank´s buffered salt solution (CMF-HBSS) for 5 min on ice. Pre-incubation with no additives served as control. Afterwards, neurons were added onto culture wells with astrocytes and incubated for 10 min at room temperature in a reciprocal shaker at 40 cycles per min. Not bound neurons were removed from the astrocyte monolayer by four gentle washings with CMF-HBSS. Adhering neurons were counted under fluorescence light. Aggregated plaques of green cells were neglected. Adhering neurons were scored by counting of cells in at least 50 microscopic fields at 100 x magnification with an inverted fluorescence microscope (Carl Zeiss Microimaging, Göttingen, Germany). The adhesion of untreated neurons to untreated astrocytes was set to 100%.

V.6 Identification of oligomannose receptors by cross-linking

In order to identify potential oligomannose specific receptors, a cross-linking experiment was performed. Sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azido-benzamido) hexanoamido] ethyl-1,3'-dithiopropionate (Sulfo-SBED) (Perbio Science, Bonn, Germany), a trifunctional cross-linking reagent containing a biotin, a sulfonated N-hydroxysuccinimide (Sulfo-NHS) active ester and a photoactivatable aryl azide was used. NHS esters react with primary amines of catalase coupled peptides and form covalent amide bonds; these peptides can then serve as bait molecules to fish potential interacting proteins out of brain homogenates. A protein interacting with the oligomannose mimetic is captured by the photoreactive aryl azide moiety. The interacting complex is then isolated and the disulfide bond subsequently reduced. Upon reduction of the disulfide bond, the biotin "label" is "transferred" to the interacting proteins and makes it possible to detect the interacting proteins. In this experiment, CP1, CSP1 and C were used as bait molecules. In each reaction, 0.25 mg sulfo-SBED pre-dissolved in 6 µl DMSO were incubated with 200 µg catalase coupled peptides in 200 µl PBS in the dark for 30 minutes under constant stirring at room temperature. Non-reacted cross-linker was removed by using Vivaspin concentrators with 10 kD molecular weight cut-off. The cross-linker coupled CP1, CSP1 and C were then incubated with crude brain homogenate. Because expression levels of glycans reach a peak during the second week of development, 2 weeks old wild-type mouse brains were used for preparing brain homogenates. Briefly, brains were isolated and homogenized immediately in ice-cooled homogenization buffer (50 mM Tris-HCI, 2 mM EDTA and 1:25 diluted EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)). Homogenates were then centrifuged at 1,000 g and 4°C for 10 minutes. The supernatants were collected and solubilized with 50 mM N-octyl ß-D-glucopyranoside overnight. Protein concentrations were determined and 1 mg protein in 1 ml homogenization buffer was incubated with conjugated peptides overnight at 4°C. All above procedures were carried out in the dark. Aryl azide groups in above mixtures were then photo-activated under UV light (365 nm) for 15 minutes on ice. To enrich aryl azide captured and biotin coupled molecules, 50 µl of magnetic MagnaBind™ Streptavidin beads (Perbio Science, Bonn, Germany) were incubated with the photo-activated mixtures for 1 hour at room temperature with mild rotation. Beads then were washed 5 times with 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS, Sigma-Aldrich, Taufkirchen, Germany) in PBS. Beads were

then boiled in 2x SDS sample buffer and resolved by SDS gel electrophoresis. Biotinylated proteins were detected by Western blot analysis using horseradish peroxidase conjugated streptavidin.

V.7 Mass spectrometry

After SDS-PAGE the gel was stained with colloidal Coomassie-blue and protein bands were cut out. After successive treatment with dithiothreitol and iodacetamide, in-gel digestion of proteins by 5 ng trypsin/µl (Promega, Mannheim, Germany) in 50 mM NH_4HCO_3 was carried out overnight at 37°C. Gel pieces were then repeatedly extracted with 50% acetonitrile/5% formic acid/45% distilled water, and the combined extracts were dried down in a vacuum concentrator, re-dissolved in 5% methanol/5% formic acid in water, desalted on a C18 µZipTip (Millipore), eluted with 1 µl 60% methanol/5% formic acid/35% water and analyzed by nano-electrospray mass spectrometry in a QTOF II instrument (Micromass, Manchester, UK). The MS/MS spectra obtained by collision-induced fragmentation of the peptides were evaluated both manually and by the Mascot MS/MS ion search algorithm (Matrix Sciences, London, UK).

V.8 Pull-down experiment

In order to confirm that the free oligomannose mimicking peptide 1 can also bind to synapsin Ⅰ, a pull-down experiment was performed to compare the binding patterns of the oligomannose mimicking peptide 1 and its scrambled peptide. 0.4 mg peptides were dissolved in 1 ml coupling buffer (50mM Tris/HCI, 5mM EDTA, pH 8.5) and incubated with 1 ml Sulfo-link coupling gel (Perbio Sciene, Bonn, Germany) for 15 minutes with mild agitation and then for further 30 minutes at room temperature without agitation for maximal coupling efficiency. Afterwards, the gel was washed three times with coupling buffer. Remaining active iodoacetyl groups were blocked by addition of 1 ml quenching buffer (10 mM L-cysteine-HCI) and the mixture was incubated for 15 minutes at room temperature. The probes were washed for three times with coupling buffer and 5 µg purified synapsin Ⅰ in PBS were added to the pepide-coupled gels and probes were incubated overnight at 4°C. After the overnight incubation, the probes were washed three times with PBS and the gels were boiled with 50 µl SDS sample buffer. Samples were then resolved by SDS gel electrophoresis and Western blotting.

V.9 Immunoaffinity chromatography with L3 and L4 antibodies

120 mg freeze dried powder of CNBr-activated sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) was incubated with 5 ml swelling solution (1 mM HCl) for 15 min at 4°C. The resin was washed three times with swelling solution and two times with 5 ml coupling solution (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3) for 15 min at 4°C and then pelleted by centrifugation at 200 g for 5 min. 200 mg L3 or L4 antibodies were incubated with pre-treated resin in coupling solution overnight at 4°C with end over end rotation. The resin was washed with 10 ml coupling buffer and remaining active groups were blocked by incubation with 5 ml blocking solution (200 mM glycine, 500 mM NaCl, pH 8.0) at 4°C with end over end rotation. After three circles of washing the resin with buffer A (100 mM sodium acetate, 500 mM NaCl, pH 4.0), buffer B (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3) and buffer C (PBS, pH 7.4) the resin was packed into a column. The column was washed with 20 ml buffer D (25 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4) and brain homogenate was applied to the column. After overnight incubation at 4°C with end over end rotation the column was washed with 10 ml buffer E (25 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.02% NaN_3, pH 7.4) and followed by 10 ml buffer F (25 mM Tris, 500 mM NaCl, 0.1% Triton X-100, 0.02% NaN_3, pH 7.4). Proteins that bound to L3 or L4 antibodies were eluted with elution buffer (50 mM ethanolamine, 150 mM NaCl, 0.2% CHAPS, pH 11.5). The eluate was dialyzed against TBS and concentrated with Vivaspin concentrators with 5 kD molecular weight cut-off.

V.10 Deglycosylation of RNaseB, AMOG and synapsin I

In order to remove oligomannose or N-glycans from different glycoproteins, two deglycosidases were used: Endo-β-N-acetylglucosaminidase H (Endo H; Roche Applied Science) which cleaves asparagine-linked mannose-rich oligosaccharides, while peptidyl-N glycosidase F (PNGF; Roche Applied Science) cleaves all N-linked glycans from glycoproteins. For the Endo H treatment, 10 µg glycoprotein, 10 µl of 5x reaction buffer (250 mM sodium acetate, pH 5.5), 2.5 µl 20x denaturing buffer (2% SDS, 10% *β*-mercaptoethanol) were incubated in a total reaction volume of 45 µl at 100°C for 10 min. 5 µl Endo H (25 mU) were added and probes were incubated at 37°C for 3 h. For treatment with PNGF 10 µg glycoprotein, 10 µl 5x reaction buffer (0.5 M sodium phosphate, pH 7.4), 5 µl 10x denaturing buffer (2% SDS, 10% *β*-mercaptoethanol) were incubated in a total reaction volume of 45 µl at 100°C for 10 min. 2.5 µl 10% Triton X-

100 and 2.5 µl PNGase F (2.5 U) were added to the probes and the mixture was incubated at 37°C for 3 h.

V.11 Lectin staining

Glycans on synapsin I were detected with the DIG glycan differentiation kit (Roche Applied Science). 0.5 µg synapsin I was resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were blocked for 30 minutes in blocking solution, washed twice for 10 minutes with TBS and once with buffer 1 (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ in TBS pH 7.5) and incubated with different lectin solutions in buffer 1 for 60 minutes. Afterwards, membranes were washed 3 times for 10 minutes in TBS, incubated with anti-Digoxigenin-AP for 60 minutes, washed three times for 10 minutes in TBS and stained with staining solution (200 µl NTB/BCIP solution in 10 ml buffer 2 (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). After staining, the membranes were rinsed 5 times with double distilled water and then dried on paper towels before bands were examined.

V.12 Isolation of exosomes from astrocytes and C6 cells

In order to increase the production of exosomes of C6 cells, a two-compartment bioreactor (CELLine; IBS Integra Biosciences, Chur, Switzerland) was used. This bioreactor consists of one chamber for holding large volumes of medium (up to 1,000 ml) and another compartment for holding cells. Between these two compartment a 10 kDa semi-permeable membrane allows small molecules to diffuse from one compartment to another, whereas vesicles secreted by proliferating cells are retained within the cell compartment. The culture medium in the medium compartment consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin, while in the cell chamber, serum was omitted from medium to avoid potential contamination of exosomes from serum. The bioreactor was maintained at 37°C in 5% CO_2. 7 days after C6 cells were inoculated, the medium from the cell compartment was collected to purify exosomes. By changing medium in the medium chamber cells were maintained in the proliferating state and exosomes could be collected several times. Medium from the cell compartment was collected every 7 days and centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 10,000 g for 30 min followed by ultracentrifugation of

the resulting supernatant at 100,000 g for 3 h. The resulting pellet containing the exosomes was collected.

Astrocytes were cultured for 2 weeks with serum-containing DMEM medium and then in serum-free DMEM medium for 12 h. Cell culture supernatants were serially centrifuged as described for C6 cells. Exosomes were resuspended in PBS plus 40 mM KCl or LiCl, 80 mM KCl or LiCl for 5 min at 37° C. In parallel, exosomes were resuspended in 190 mM or 230 mM sucrose buffered with 5 mM HEPES and incubated for 5 min at 37°C. The samples were centrifuged at 100,000 g for 1 h. After centrifugation, pellets were resuspended in sample buffer and supernatants were subjected to protein precipitation by trichloroacetic acid (TCA)/aceton. Briefly, 250 µl TCA was added to 1 ml supernatant and the mixture was incubated for 10 min at 4° C. Probes were centrifuged at 20,000 g for 5 min, the supernatant was removed leaving the protein pellet intact. The protein pellet was washed two times with 200 µl cold acetone, centrifuged at 20,000 g for 5 min and dried by heating the tube to 95° C for 5 min to drive off the acetone. The resulting protein pellets were resuspended in 2x SDS sample buffer.

V.13 Electron microscopy

Exosome pellets were fixed with fixing solution (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate buffer) for 60 min at room temperature. After washing with PBS for 10 min, exosomes were embedded in 2% agarose and postfixed in 1% OsO_4 in 0.1 M PBS for 30 min on ice. After fixing with OsO_4 exosomes were dehydrated by incubation with increasing concentrations of ethanol (30%, 50%, 70%, 80% and 90%) for 15 min and then washed two times with 100% ethanol and once with propylenoxid for 15 min. Specimens were then treated with propylenoxid/epoxy resin (1:1) for 90 min and propylenoxid/epoxy resin (1:2) for 2 h followed by embedding in epoxy resin. Ultrathin sections were cut on a Vibratome and examined using an electronmicroscope Zeiss 902.

VI. Results

VI.1 Identification of an oligomannose mimicking peptide by screening of a phage display library

VI.1.1 Consensus sequences of eluted phages

I attempted to identify novel oligomannose recognizing receptors in mouse brain. Because oligomannosidic glycans from natural sources are only available in limited amounts and also difficult to synthesize chemically, I had to use a surrogate. Peptides have shown to structurally and functionally mimic oligosaccharides and thus are potential surrogates for carbohydrates (Zwick et al., 1998). Therefore, an oligomannose mimicking peptide had to be identified. The oligomannose-recognizing antibodies L3 and L4 were applied to screen a commercially available phage display library. The library contains linear random peptide 12-mers fused to the N-terminus of a minor coat protein (PIII) of M13 phage. Phages that bind to L3 and L4 antibodies were eluted and the inserted peptides on PIII of these phages were considered to be peptide mimics of the carbohydrate epitope (oligomannose).

After three rounds of panning and elution of specifically bound phages with antibody/sugar elution, 20 individual clones from each elution were randomly selected and DNA sequences of phage inserts were determined. Consensus sequences of peptides that inserted are shown in Table 1. The peptide H-TISWWHLWPSPA-OH was both found with antibody (L3 and L4) and mannose elution (designated as P1) and the peptide H-FYNPFFWGSPSM-OH was found with L3/mannose and L4 elution (designated as P2). The peptides H-SFPYWWTLTEPM-OH (P_S) and H-NHPFADQWYPTE-OH (P_N) were found with L3 antibody elution. These four peptides contain a high frequency of prolines (1:6), which is much higher than a random frequency (1:19). Additionally, a high ratio of aromatic amino acids (phenylalanine, F;tyrosine, Y; tryptophan, W), which constitute around 30% of all amino acids (15:48) can be found. Third, amino acids with cyclic structure (proline, P; histidine, H and aromatic amino acids) make up almost half of sequences (25 of 48) found.

Table 1: Consensus sequences and frequency of peptides isolated from a 12-mer peptide library with oligomannose-specific antibodies L3 and L4 with mannose elution.

Clone	Amino acid sequence	Bait/Elution	Frequency
P_1	TISWWHLWPSPA	L3/L3	2/20
		L4/L4	2/20
		L3/Mannose	2/20
P_2	FYNPFFWGSPSM	L4/L4	4/20
		L3/Mannose	3/20
P_S	SFPYWWTLTEPM	L3/L3	2/20
P_N	NHPFADQWYPTE	L3/L3	7/20

VI.1.2 Binding between phage clones with consensus sequences and L3/L4 antibodies

In order to verify the binding between selected phage clones and L3 and L4 anibodies, phages with concensus sequences, which were regarded as candidates because of their higher emerging frequency, were amplified and titrated. Same amounts of phages were applied in ELISA. As control, a wild type phage clone without any insertion designated as P_W was also amplified and tested. The experiment showed that all selected phage clones were recognized by L3 and L4 antibodies except for P_W, while phage clone P1 showed the highest reactivity with both L3 and L4 antibodies (**Fig. 6**).

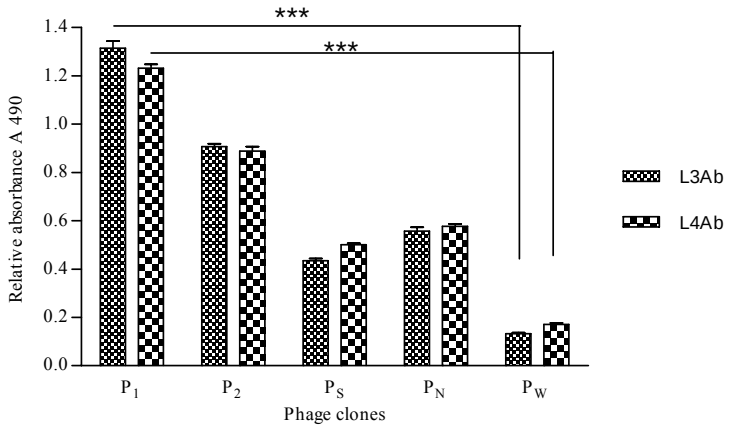

Fig. 6: Binding between selected phage clones and L3 and L4 antibodies.

L3 or L4 antibodies (10 µg/ml; 50 µl/well) were substrate coated in microtiter plates. After blocking with 2% BSA, phage clones (1 x 10^8/µl; 50 µl/well) were incubated with the coated substrata. After one hour incubation and wash, bound phages were detected with anti-M13 antibody conjugated with HRP (1:5,000) and 0.5% OPD as substrate. Mean values ± SEM from triplicate experiments. ***, $p < 0.0001$. (Unpaired student *t* test)

VI.2 Characterization of oligomannose mimicking properties of synthetic peptides

From the binding phage clones, insert sequences were determined and corresponding peptides synthesized. Because of the low solubility of the synthetic peptides in neutral aqueous buffers, they can not be applied to *in vivo* experiments. In order to improve solubility, peptides were conjugated to a soluble protein, catalase. Because P1 can be recognized by both, L3 and L4 antibodies and showed the highest reactivity with these antibodies, it was chosen as "prototype" oligomannose mimicking peptide for the coupling experiments. The mimicking properties of catalase coupled peptide P1 were then tested by binding and competition ELISA. In binding tests, different concentrations of peptide P1 coupled to catalase were substrate coated and incubated with both oligomannose-specific antibodies (**Fig. 7A-B**). The binding was concentration dependent and displayed a typical saturation curve. The scrambled peptide of P1 with the amino acid sequence H-HPLWSWASWIPT-OH showed a weak, probably, unspecific interaction with L4 antibody (**Fig. 7B**), but no interaction with the L3 antibody (**Fig. 7A**). At the same time, activated catalase, which served as a protein carrier control, and BSA as additional coat control showed no interaction with L3 and L4 antibodies (**Fig. 7A-B**).

One widely accepted criterion for carbohydrate mimicry is competition between natural sugars and sugar mimicking reagents. In this experiment, competition between natral oligomannose and oligomannose mimicking peptides for binding to the L3 and L4 antibodies was evaluated. RNaseB (RB), a protein that bears oligomannose residues, was used as donor of natural oligomannose. At the same time, RNaseB digested with endoglycosidase H to remove the attached oligomannoses (RB(-)), served as protein backbone control. Plates were substrate coated with a constant concentration of catalase coupled peptides or catalase alone. Before adding L3 and L4 antibodies to the wells, they were pre-incubated with different concentrations of RB and RB(-) for 30 min at room temperature. The experiment showed that, with increasing amounts of RNase B, signals of binding between antibodies and catalase coupled peptide 1 decreased in a concentration dependent manner, while, in presence of RB(-), which was devoid of carbohydrates, there was no binding (**Fig. 7C-D**). This result can be interpreted as following: oligomannoses on RNaseB compete with the oligomannose mimicking peptides for binding to the antibodies L3 and L4. When the binding sites of the antibodies are saturated by binding to the oligomannoses on RNaseB, they are not free to interact with the oligomannose mimicking peptide any more and can be removed in

the washing of the ELISA. As expected, the protein backbone of RNaseB is not able to disturbe the interaction between the antibodies and the oligomannose mimicking peptide. Interestingly, addition of 2 mg/ml RNaseB is able to fully block the interaction between L4 antibody and catalase coupled peptide P1, but inhibits the interaction of the peptide and L3 antibody only to 50% (**Fig. 7C**). This indicates a different sensitivity towards oligomannose and for the P1 peptide of the L3 and L4 antibodies. These results confirm that the peptide is indeed an oligomannose mimicking peptide.

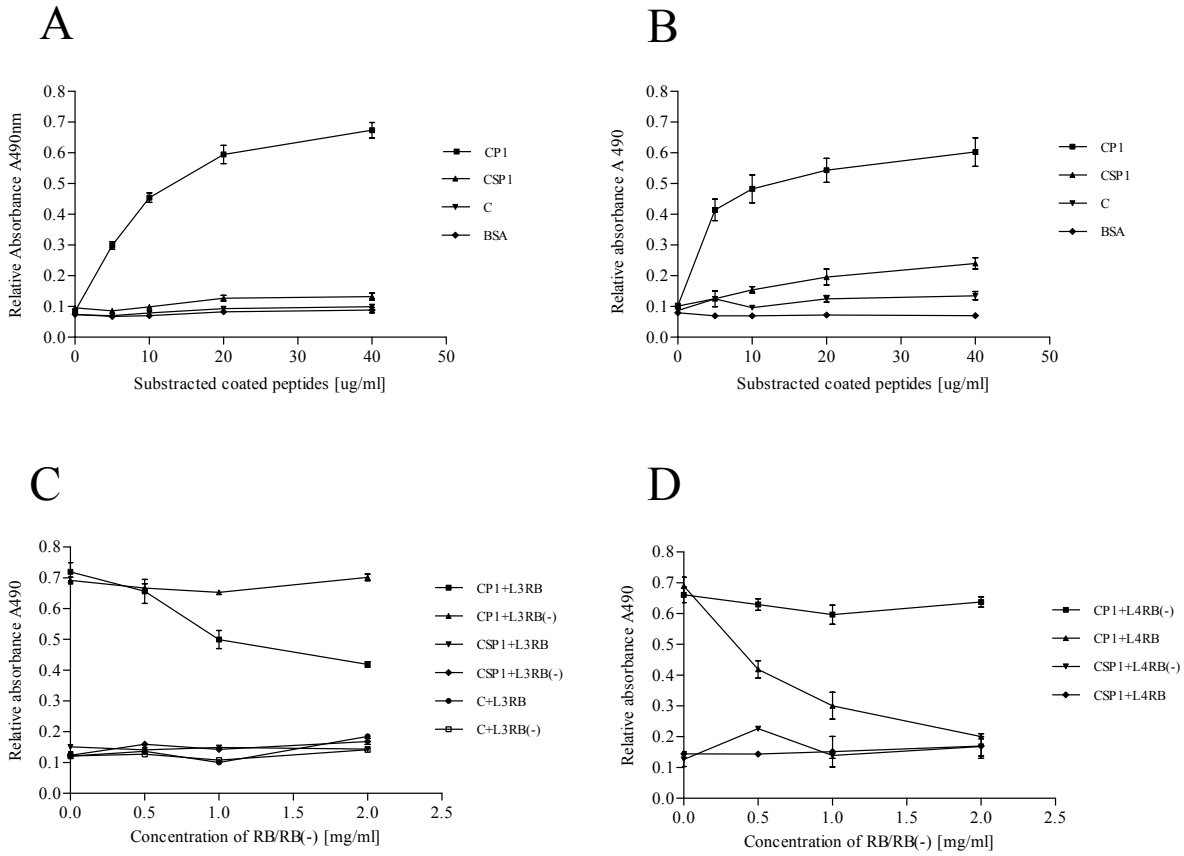

Fig. 7: Characterizations of a synthetic oligomannose mimicking peptide.

Binding of L3 **(A)** and L4 **(B)** antibodies to different amounts of substrate coated catalase (C), catalase coupled oligomannose mimicking peptide P1 (CP1) or catalase coupled scrambled peptide (CSP1) was determined by ELISA. Competition between the oligomannose mimicking peptide P1 and RNaseB bind with antibodies L3 and L4 was determined by ELISA. L3 **(C)** and L4 **(D)** antibodies were pre-incubated with different amounts of untreated RNase B (RB) or Endo H treated de-glycosylated RNase B (RB(-)) and added to the substrate coated peptide. Mean values ± SEM from three independent experiments were shown.

VI.3 Oligomannose mimicking peptide 1 stimulates neurite outgrowth and disturbs adhesion between neurons and astrocytes

Oligomannoses have been reported to promote neurite outgrowth (Chandrasekaran et al., 1994a; Chandrasekaran et al., 1994b; Tanzer et al., 1993), I tested whether oligomannose mimicking peptide 1 was functionally active and can also triggered neurite outgrowth as natural oligosaccharides. Hippocampal neurons were used to study the influence of catalase coupled peptides. RNase B (RB) as natural oligomannose donor and peptides were pre-coated on poly-L-lysin (PLL) treated coverslips. PLL alone served as coat control. Outgrowth of hippocampal neurons was promoted in the presence of oligomannose mimicking peptide (146.7±12.42) and RB (138.8±6.326), while the scrambled peptide, catalase and RB(-) showed no effects (**Fig. 8A**). These results confirm that oligomannoses presented on a protein backbone can stimulate neurite outgrowth and P1 can functionally mimic oligomannose residues.

Since glycans are involved in a wide rage of interaction between different cell types, oligomannose binding antibodies L3 and L4 interfere with the adhesion between neurons and astrocytes (Fahrig et al., 1990). I then tested whether the oligomannose mimicking peptide P1 could also affect the interaction between neurons and astrocytes by a short term adhesion assay. Homogenous populations of astrocytes and granule neurons from early postnatal mouse cerebellum were tested as target and probe cells individually under Ca^{2+}-independent conditions in the presence of oligomannose mimicking peptide or scrambled peptide and catalase. Under the assay conditions used, oligomannose mimicking peptide decreased the adhesion between neurons and astrocytes to 50%. The scrambled peptide showed a weak effect but statistically not significant (**Fig. 8B**). These results confirm that the oligomannose mimicking peptide is functionally active in stimulating neurite outgrowth and impeding adhesion between neurons and astrocytes.

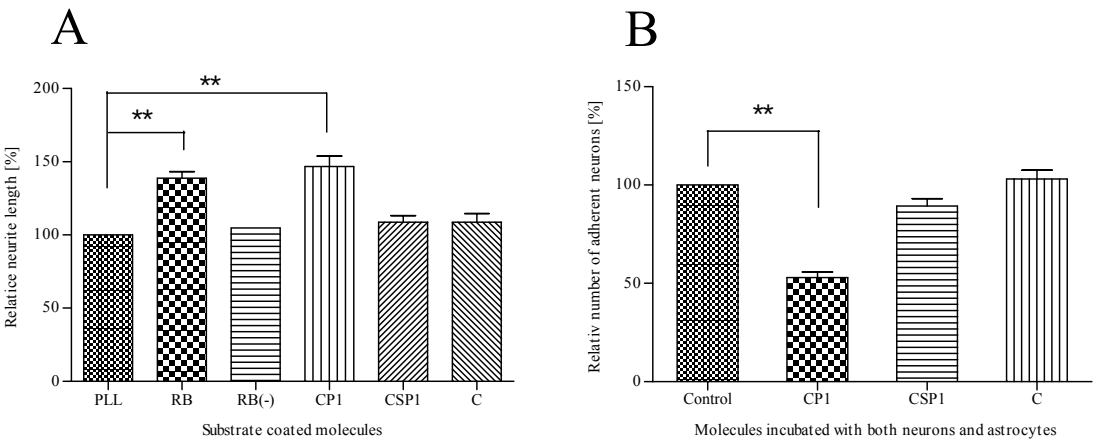

Fig. 8: The oligomannose mimicking peptide P1 promotes neurite outgrowth of neurons and disturbs adhesion between neurons and astrocytes.

(**A)** Hippocampal neurons were grown on substrate coated PLL alone or with RNaseB (RB), de-glycosylated RNaseB (RB(-)), catalase coupled oligmannose mimicking peptide (CP1), catalase coupled scrambled peptide (CSP1) and catalase (C). After 24 hours in culture, cells were fixed and the length of the longest neurite per cell was determined. Mean length of the longest neurites obtained from PLL treatment was set to as 100%. (**B)** Freshly prepared GFP-expressing cerebellar granule neurons were pre-incubated without (Control) or with catalase coupled oligomannose mimicking peptide (CP1), catalase coupled scrambled peptide (CSP1) and catalase (C) and were added to a monolayer culture of astrocytes. After removing non-adherent cells GFP-positive cells attached to the astrocyte monolayer were quantified. Mean number of adherent cells in the control was set to 100%. Mean values ± SEM of three independent experiments are shown (** indicates $p < 0.01$ obtained by one-way ANOVA test with Dunnett's multiple comparison).

VI.4 Identification of synapsin I as oligomannose binding lectin

Next, I applied the oligomannose mimicking peptide in a cross-linking approach to identify novel oligomannose binding proteins in mouse brain. Oligomannose mimicking peptide, scrambled peptide and catalase were individually conjugated to the trifunctional crosslinker sulfo-SBED and these conjugates were used as baits to isolate bound target proteins from crude brain homogenate. By UV crosslinking, target proteins were covalently attached to the bait proteins to which they bound and were isolated via the biotin moiety present on the cross-linker. Reducing conditions during gel electrophoresis led to transfer of the biotin label from the bait to the bound target protein(s). Western blot analysis with streptavidin to detect biotinylated proteins revealed a very intense staining of a protein band with an apparent molecular weight of 75 kDa and a weak staining of several other protein bands, when Oligomannose

mimicking peptide was used as bait, whereas no biotinylated protein was detectable when scrambled peptide or catalase were used as baits (**Fig. 9B**). Protein bands showing an apparent molecular weight of around 75 kDa were cut out from a Coomassie blue stained gel (**Fig. 9A**) and mass spectrometry of these bands identified synapsin I. To confirm that synapsin I was identical to the 75 kDa protein that predominantly bound to the oligomannose mimicking peptide, Western blot analysis using synapsin I specific antibodies was carried out. A strong synapsin I signal around 75 kDa was observed when the oligomannose mimicking peptide was used as bait, whereas no signal or only a weak signal was seen when scrambled peptide or catalase were used as bait, respectively (**Fig. 9C**).

Above results show interaction between catalase coupled peptides and synapsin I, in order to test that free peptide can also interact with synapsin I, free peptides coupled to sulfo-link agrose were used as bait molecules. Purified bovine synapsin I was used as input. The eluates were resolved by SDS-PAGE and analyzed by Western-blotting. The analysis showed that the oligomannose mimicking free peptide can also bind to synapsin I (**Fig. 9D**). Under the conditions used, also the scrambled free peptide could bind to synapsin I, however, much weaker than oligomannose mimicking free peptide. These results indicat synapsin I is probably an oligomannose binding protein.

To further confirm that synapsin I acts as an oligomannose specific lectin, the binding of purified synapsin I or synapsin I pre-incubated with AMOG-derived oligomannoses to substrate coated oligomannose bearing AMOG or to Endo H treated AMOG was determined in ELISA. Synapsin I showed binding to untreated oligomannose carrying AMOG (AM+SYN), but a reduced binding to Endo H treated oligomannose-lacking AMOG (AM(-)+SYN). The binding of synapsin I to AMOG was also reduced when synapsin I was pre-incubated with AMOG-derived oligomannose (AM+(Sugar+SYN)) (**Fig. 9E**). These results indicate that synapsin I is an oligomannose binding lectin-like protein.

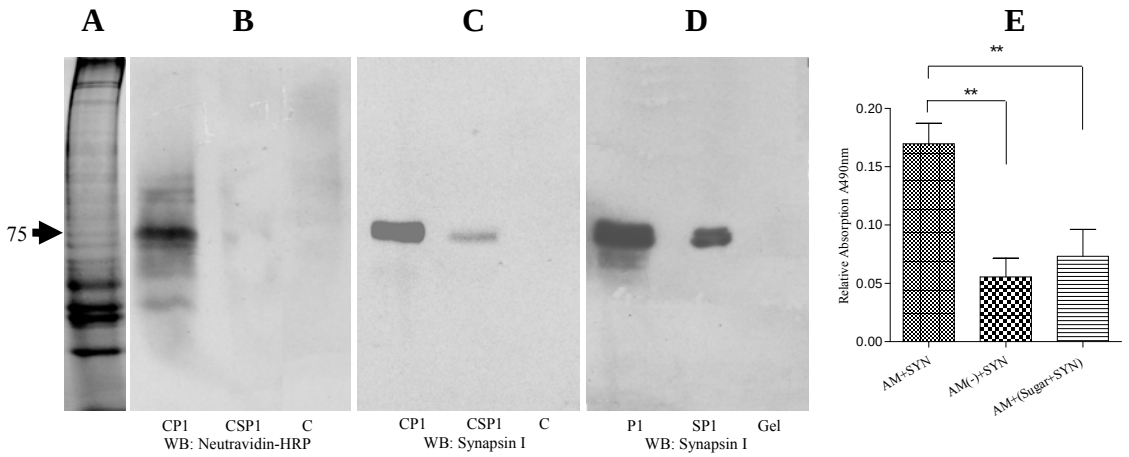

Fig. 9: Synapsin I is a oligomannose specific lectin.

Catalase coupled oligomannose mimicking peptide (CP1), catalase coupled scrambled peptide (CSP1) and catalase (C) were conjugated to the crosslinker sulfo-SBED. The conjugates were incubated with detergent extracts of crude brain homogenates. After UV crosslinking, proteins bound to the conjugates were isolated, separated by gel electrophoresis and subjected to Western blot analysis using Coomassie blue staining **(A),** strepavidin **(B)**, or Western blot analysis with synapsin I antibody **(C)**. Eluates of free peptide coupled column were also resolved by Western blot analysis with synapsin I antibody **(D)**. **(E)**The binding of synapsin I to substrate coated AMOG was determined by ELISA. Purified synapsin I was added on top of coated AMOG (AM+SYN) or Endo H treated deglycosylated AMOG (AM(-)+SYN). Synapsin I was also pre-incubated with AMOG-derived oligomannose and then added on top of substrate coated AMOG (AM+(sugar+SYN)). Mean values ± SEM of three independent experiments are indicated (** indicates $p < 0.01$ obtained by one-way ANOVA test with Dunnett's multiple comparison).

VI.5 Synapsin I is an oligomannose bearing glycoprotein

In order to search for oligomannose interacting lectins in mouse brain, L3 and L4 antibodies which specificly recognize oligomannose glycans were conjugated to the CNBr-activated sepharose 4B. This pre-activated medium allows a convenient way to immobilize ligands containing primary amines by the cyanogen bromide method. In parallel, same amount of rat serum was also coupled as control. After coupling, beads were incubated with brain homogenates, followed by washing and elution steps. Samples were then resolved by gel electrophoresis and analysed by Western blotting. When probed with L3 (**Fig. 10A**) and L4 antibodies (**Fig. 10B**), the most prominent band emerged slightly above the 75 kDa protein marker. This band also showed positive signal when probed with synapsin I antibodies (**Fig. 10C**). In the rat serum control

column, there was no visible band when probed with both L3, L4 antibodies and synapsin I antibody (**Fig. 10A-C**). Above experiments showed that synapsin I was recognized by L3 and L4 antibodies and this interaction resulted from the oligomannosidic glycans on synapsin I. To further confirm this with direct evidence, purified bovine synapsin I was digested with Endo H and PNGF individually, the former enzyme cleavages high oligomannose glycans from glycoproteins, while the latter one releases N-linked glycans. After digestion, synapsin I lost its reactivity with L3 and L4 antibodies, while the reaction to synapsin I antibodies was largely preserved (**Fig. 10D**). These results indicate that there are oligomannoses on synapsin I and these oligosaccharides are N-linked glycans.

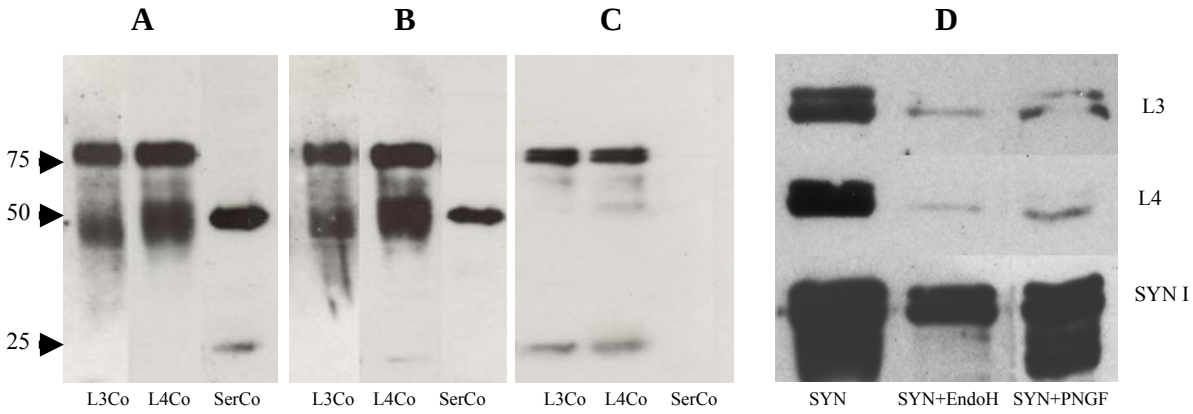

Fig. 10: Synapsin I is a oligomannose bearing glycoprotein.

Synapsin I was eluted from L3 and L4 antibody columns **(A-C)**: Eluates from L3 antibodies coupled column (L3Co), L4 antibodies coupled column (L4Co) and rat serum coupled column (SerCo) were probed with L3 antibodies **(A)**, L4 antibodies **(B)** and synapsin I antibodies **(C)**. The most prominent band emerge slightly above 75 kDa **(L3Co, L4Co of A-C)**, while eluate of rat serum coupled column shows no bands **(SerCo of A-C)**. Bands around 50 and 25 kDa probably represent heavy and light chains of antibodies leaking from the columns. Purified bovine synapsin I was treated with enzymes to remove high-oligomannose glycans from it **(D)**. Samples after these treatments were resolved by SDS-PAGE and immunoblot with L3, L4 and synapsin I antibodies. Enzymes treatments significantly decrease signals when probed with L3 and L4 antibodies, while change slightly when probed with synapsin I antibody.

VI.6 Other glycans on synapsin I

Glycosylation is a general post-translational modification mechanism that modulates the structure and function of most proteins. Synapsin I has been reported to have O-linked N-acetylglucosamine (O-GlcNAc) (Luthi et al., 1991) and fucose-alpha(1-2)-galactose [Fucalpha(1-2)Gal] (Murrey et al., 2006) carbohydrates modifications. Besides these, there exists very little knowledge about the glycosylations of synapsin I. I stained synapsin I with different anti-saccharide antibodies, anti-HNK1 and Ab412 which recognize HNK-1 epitope showed no reactivity, Ab735 which recognizes PSA also showed no signal, while L5 antibody which recognizes $Lewis^X$ interacted with synapsin I **(Fig. 11A**). When synapsin I was treated with PNGF, there was no change of signal of $Lewis^X$ (**Fig. 11C**), which means that $Lewis^X$ on synapsin I is probably an O-linked glycan. Bovine purified synapsin I were then stained with a batch of lectins to explore other putative saccharide epitopes. Galanthus nivalis agglutinin (GNA) recongnizes terminal oligomannose. Datura stramonium agglutinin (DSA) recognizes Gal-(1–4) GlcNAc in complex and hybrid N-glycans, in O-glycans and GlcNAc in O-glycans. Peanut agglutinin (PNA) recognizes the core disaccharide galactose (1–3) Nacetylgalactosamine of O-glycosidically linked carbohydrate chains. Sambucus nigra agglutinin (SNA) recognizes sialic acid linked (2–6) to galactose of complex sialylated N-glycan and correspondingly linked sialic acids in O-glycan. Maackia amurensis agglutinin (MAA) recognizes sialic acid linked (2–3) to galactose. All of them interacted with synapsin I (**Fig. 11B**). These results suggest a complex pattern of synapsin I's glycosylation.

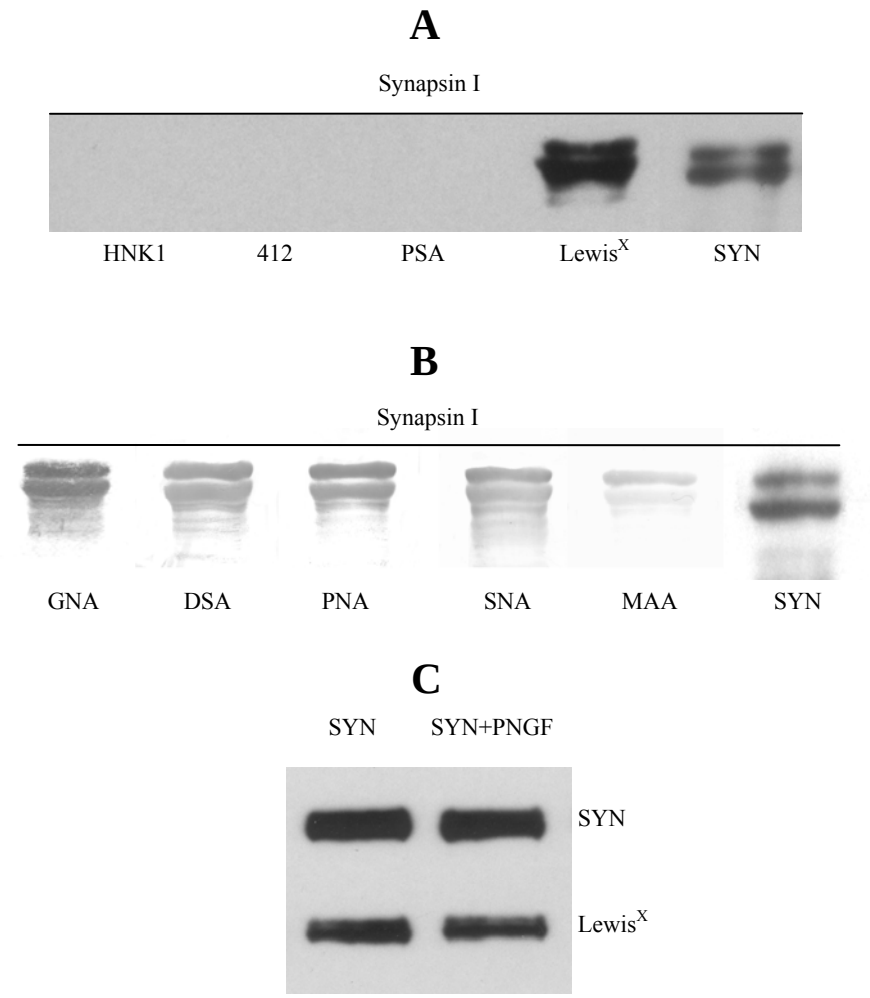

Fig. 11: Absence of HNK-1 and PSA, presence of Lewi ^{X} and other putative saccharide epitopes on synapsin I.

Purified bovine synapsin I was probed with different antibodies against HNK1 (a-HNK1 and Ab412), PSA (Ab735) and Lewis^x (L5Ab). **(A)** Only L5 antibodies show a band which suggests that Lewis^x epitopes on synapsin I. **(C)** When synapsin I was digested with PNGF, which remove N-linked glycans from protein, there show no reduction of signals of L5Ab, which suggests that Lewis^x on synapsin I is not a N-linked glycan. **(B)** Different lectins staining of synapsin I. GNA: Galanthus nivalis agglutinin; DSA: Datura stramonium agglutinin; PNA: Peanut agglutinin; SNA: Sambucus nigra agglutinin; MAA: Maackia amurensis agglutinin.

VI.7 Synapsin I is present in exosomes of cultured mouse astrocytes and C6 cells

Since synapsin I is a glycoprotein and a glycan binding protein, it is likely that synapsin I is a secreted protein that is released from the cell and acts as an extracellular protein. So far, synapsin I is known as neuronal-specific protein which is present on synaptic vesicles and modulates neuronal transmitter release via synaptic vesicles. However, it has been reported that synapsin I was expressed in other cell types, such as primary

cultured astrocytes (Maienschein et al., 1999). Therefore, I first used cultured neurons or astrocytes for cell surface biotinylation and immunocytochemical staining to check whether synapsin I is detectable as extracellular protein at the cell surface of these cells. Although neurons and astrocytes expressed synapsin I, no indications for extracellular synapsin I were obtained by these methods. Therefore, I tested the possibility that synapsin I is released from cells via exosomes which mediate the transport of cytosolic proteins from one to another cell or to the extracellular milieu. Exosomes are small intralumenal vesicles formed in multivesicular bodies and released to the extracellular space upon fusion of multivesicular bodies (MVBs) with the plasma membrane. I used cultured astrocytes to isolate exosomes from cell culture supernatants by serial centrifugation. Significant amount of exosomes were isolated from the cell culture supernatants of cultured astrocytes. Western blot analysis showed that the exosomal fraction obtained from cultured astrocytes contained the exosomal marker proteins Alix and hsc70 and synapsin I (**Fig. 12**). These results imply that synapsin I is released from astrocytes via exosomes. Similar results were also showed by exosomes from C6 cells. The analysis of the exosomal fraction by electron microscopy showed a high degree of homogeneity: around 95% of the particles are vesicles having a diameter between 80-100 nm (**Fig. 13**), which fits well with the reported size of exosomes

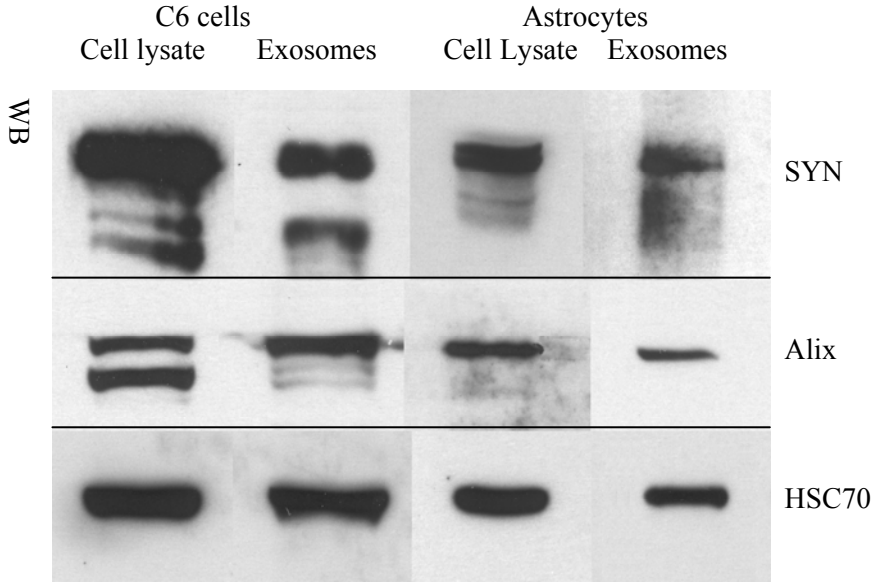

Fig. 12: Synapsin I is present in exosomes of C6 cells and cultured mouse astrocytes.

Lysates of cultured mouse astrocytes and C6 cells and exosomal fractions isolated from the cell culture supernatant by serial centrifugation were probed in Western blot analysis with synapsin I, ALIX and hsc70 antibodies.

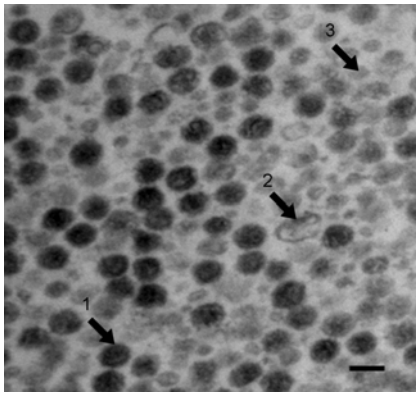

Fig. 13: Exosomes of C6 cells analysed by electron microscopy.

The 100,000g pellets were fixed and analysed under the electron microscope. Arrow 1 represents a typical exosome vesicle with a diameter around 90 nm, arrow 2 indicates an un-identified vesicle, arrow 3 indicates membrane fragments. Scale bar, 100 nm.

VI.8 Synapsin I is released from exosomes of cultured mouse astrocytes and C6 cells under high ionic strength

Next, I tested whether synapsin I is released from exosomes when exosomes are exposed to conditions which prevail at high synaptic or neuronal activity, namely elevated K^+ concentration. Exosomes were prepared from astrocyte, resuspended in PBS and incubated in the absence or presence of either 40 mM or 80 mM KCl. Incubation with 40 mM or 80 mM LiCl served as control. After ultracentrifugation, the pelleted exosomes and the supernatant fractions containing proteins released from exosomes were subjected to Western blot analysis. After incubation in PBS or in the presence of 40 mM KCl or LiCl, synapsin I was exclusively found in the pellet fraction, but not in the supernatant (**Fig. 14B-C**), indicating that it was not released from the exosomes. However, in the presence of 80 mM KCl and, unexpectedly, of 80 mM LiCl, synapsin I was nearly exclusively detectable in the supernatant and only very small amounts were seen in the pellet fraction **(Fig. 14D**), indicating that nearly almost all synapsin I was released from the exosomes. Actin which was present in exosomes was not released from the exosomes under these conditions (**Fig. 14A-D**). To exclude that the release of synapsin I was due to an osmotic effect, isolated exosomes were incubated in 190 mM and 230 mM sucrose which molarities corresponded to that of 40 mM KCl or LiCl and 80 mM KCl or LiCl in PBS. After ultracentrifugation, synapsin I was found only in the pellet fractions showing that it was not released from exosomes in the presence of 190 or 230 mM sucrose (**Fig. 14C-D**). By contrast, when exosomes of

C6 cells were incubated with PBS on presence of 20 mM KCl, most of synapsin I was released to the soluble phase (**Fig. 14A**).

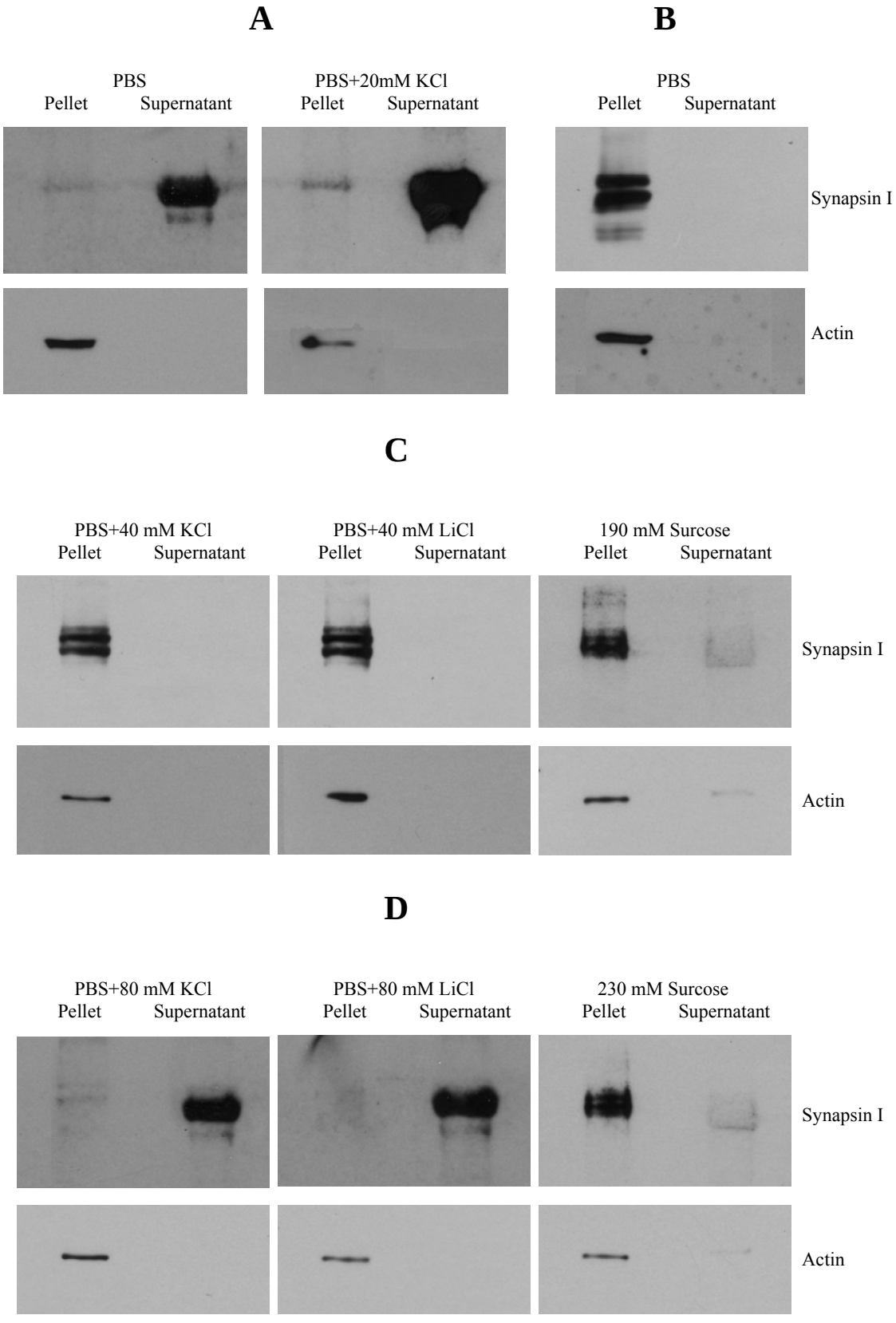

Fig. 14: Synapsin I is released from exosomes of C6 cells and cultured mouse astrocytes under high ionic strength.

Exosomes of C6 cells **(A)** and astrocytes **(B-D)** were incubated with PBS or PBS plus different concentrations of KCl or LiCl or same osmotic strength offered by sucrose buffered by 5 mM HEPES. After incubated in water bath at 37℃ for 5 minutes, the samples were centrifuged again at 100,000 g for 1 hour to isolate membrane fractions (pellet) and soluble fractions (supernatant) which was subjected to TCA/Aceton precipitation to concentrate the proteins. Both samples were resolved by SDS-PAGE and immunobloting.

VI.9 Proteomic analysis of pellet and soluble fractions of C6 cell exosomes treated under high ionic strength conditions

Since synapsin I released from exosomes, I want to identify other proteins that are released. A proteomic analysis was applied to identify releasable and unreleasable proteins of C6 exosomes under high ionic strength challenge. As predicted, exosomes of C6 cells harbor proteins commonly expressed in different cellular origin, for example, proteins that associate with endosomal trafficing such as programmed cell death 6-interacting protein (Pdcd6ip/Alix) and tsg101, which are widely used as marker proteins of exosomes; molecules involved in cell structure and motility such as actins, moesin, radixin, heat shock proteins such as heat shock 70 kDa protein which serves as exosome maker in the present work, signaling proteins such as 14-3-3, guanine nucleotide-binding protein, proteins associated with transcription and synthesis such as histones and ubiquitin. Interestingly, NCAM and Basigin were also presented in exosomes; these adhesion molecules are oligomannose binding proteins and may participate in the adhering activities of exosomes (Appendix Table 2).

Under the experimental conditions used, a number of molecules were released by KCl stimulation. Most of them are soluble and/or secreted proteins, such as glia-derived nexin which is involved in neurogenesis, collagen alpha chain and EMILIN-1 which are associated with cell adhesion. Interestingly, some typical cytosolic proteins such as 14-3-3 were also released under high ionic strength which indicates that these proteins could play a role in the extracellular matrix (Appendix Table 3).

VI.10 Synapsin I promotes neurite outgrowth in an oligomannose dependent manner

So far, synapsin I has been considered as a cytosolic protein, but I showed that cultured astrocytes release synapsin I via exosomes and that synapsin I was released from astrocyte-derived exosomes by elevated ion strength. To elucidate the role of extracellular synapsin I as oligomannose carrying and binding protein, I investigated whether exogenous substrate coated synapsin which should mimic the functions of endogenous extracellular synapsin I affected neurite outgrowth of hippocampal neurons in an oligomannose-dependent manner.

I substrate coat native synapsin I (SYN) (**Fig. 15A**), and synapsin I denatured (dSYN) and treated with Endo H (dSYN(-)) which remove oligomannose from glycoprotein (**Fig. 15B**). The experiment showed that, native synapsin I and denatured synapsin I promoted neurite outgrowth to a very similar degree, while when oligomannose was absent, synapsin I lost its promotive effects. In order to further confirm the oligomannose dependent promotive effects, RNaseB, as a donor of oligomannose, was added to the medium to saturate oligomannose receptors on the cell surface, the analysis showed that it decreased the degree of promotion, while, when oligomannose on RNaseB was removed, the promotive effect was rescued. These results suggest that exogenous synapsin I may promote neurite outgrowth largely in an oligomannose dependent manner. Peptide mimics of oligomannose (CP1) also showed its mimetic characters as oligomannose on RNaseB (**Fig. 15A**).

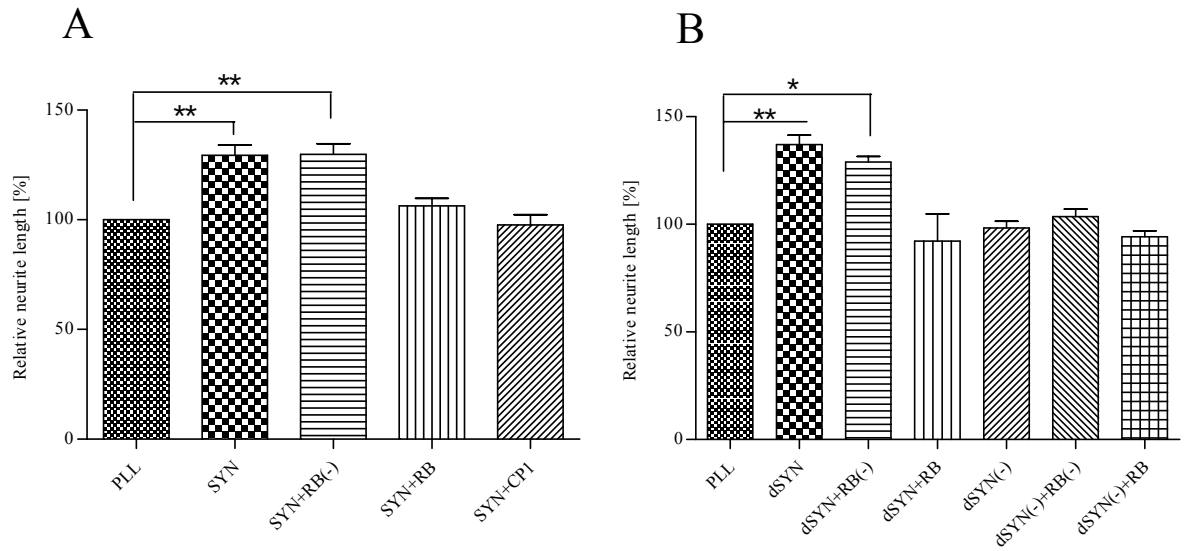

Fig. 15: Synapsin I promotes neurite outgrowth in an oligomannose dependent manner.

Native synapsin I **(A)**, denatured synapsin I and denatured synapsin I treated with Endo H **(B) were** substrate coated on coverslips. 1 hour after plating the hippocampal neurons, RB, RB(-) or CP1 were added to culture medium to saturate the potential oligomannose receptors on neurons and then compete with oligomannose on substrate coated synapsin I. After 24 hours in culture cells were fixed and the length of the longest neurite per cell was quantified. Mean length of the longest neurites obtained from PLL treatment was taken as 100% in every experiment. The means ± SEM of two **(A)** or three **(B)** dependent

experiments are shown. *, $p < 0.05$ compared with PLL. (One-way ANOVA with Dunnett's multiple comparison test.)

VI.11 Synapsin I binds with extracellular domain of NCAM in an oligomannose denpendent way

Astrocytes are involved in functional modulation of neurons, such as neurite outgrowth. Since synapsin I is release from astrocytes via exosomes and promotes neurite outgrowth in an oligomannose dependent manner, it is likely that the recognition of the extracellular astrocyte-derived oligomannose bearing synapsin I by neuronal oligomannose binding cell surface receptors may trigger neurite outgrowth. NCAM is an oligomannose binding neuronal protein involved in neurite outgrowth (Horstkorte et al., 1993) and thus, a prime candidate to interact with synapsin I in an oligomannose dependent manner. Therefore, I performed an ELISA using untreated or Endo H treated (SYN(E)) and PNGF (SYN(P)) synapsin I and the extracellular domain of NCAM conjugated to human Fc fragment to analyze whether NCAM binds to oligomannoses on synapsin I. NCAM showed a concentration dependent and saturable binding to substrate coated synasin I, while, the close homologue of L1(CHL1), a adhesion molecule here served as a control, showed no specific binding to NCAM (**Fig. 16A**). NCAM-Fc bound to synapsin I (SYN+NCAM), but not to deglycosylated synapsin I (SYN(E)+NCAM or SYN(P)+NCAM). When NCAM-Fc was pre-incubated with oligomannose bearing AMOG (SYN+(NCAM+AM)), binding of NCAM-Fc to synapsin I decreased to that obtained with deglycosylated synapsin I. In contrast, preincubation with Endo H deglycosylated AMOG (SYN+(NCAM+AM(-))) had no effect on binding of NCAM-Fc to synapsin I (**Fig. 16B-C**). These results clearly showed an oligomannose dependent binding of NCAM to synapsin I.

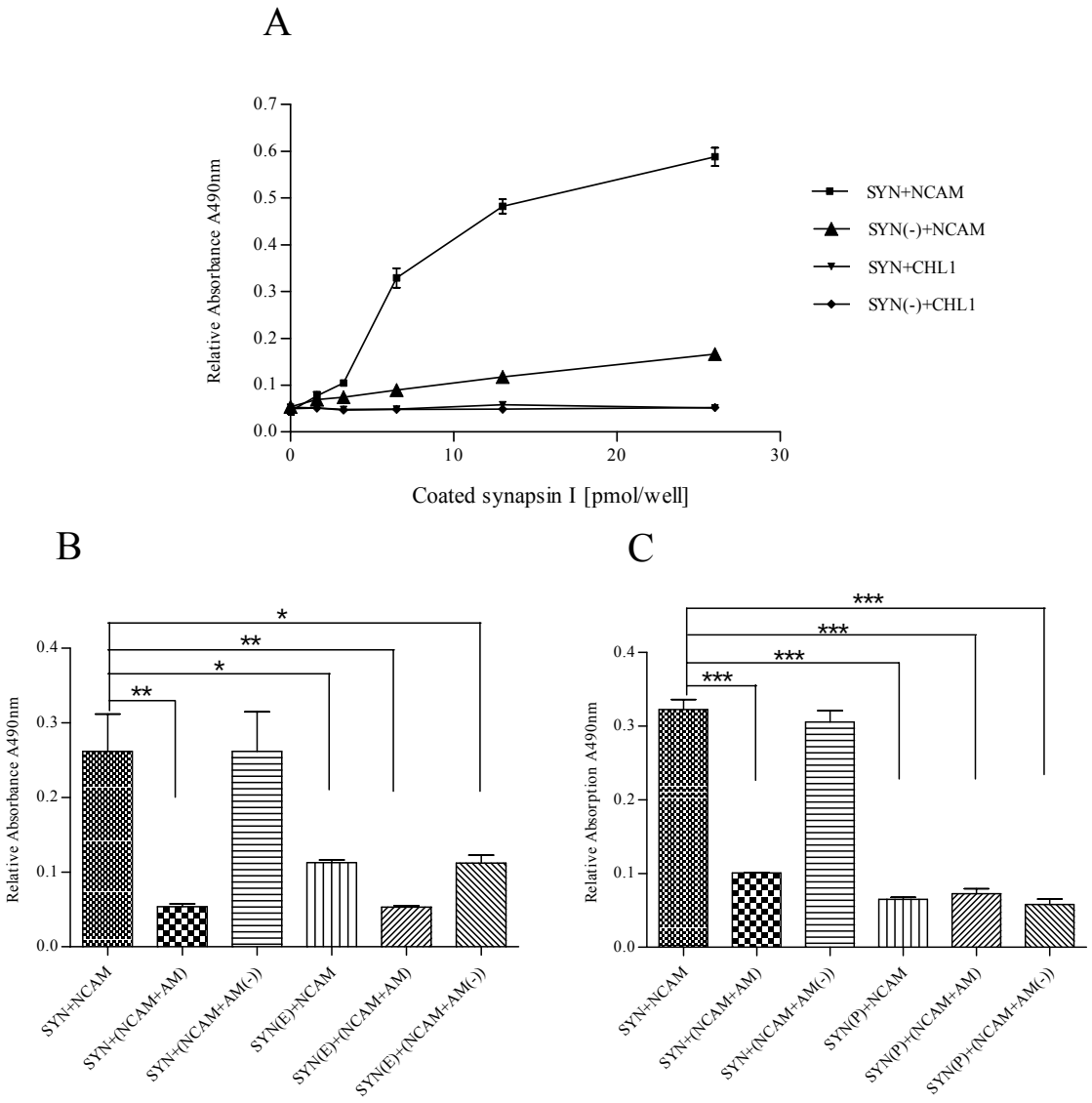

Fig. 16: Synapsin I binds with extracellular domain of NCAM in an oligomannose denpendent way.

Synapsin I (SYN), synapsin I treated with Endo H (SYN(E)) and synapsin I treated with PNGF (SYN(P)) were substrate coated. After blocking, NCAM-Fc, NCAM-Fc preincubate with AMOG (NCAM+AM), NCAM-Fc preincubate with AMOG without oligomannose ((NCAM+AM(-)) were incubated with substrates, after washing, binding of NCAM-Fc was monitored with anti-human Fc fragment antibodies. AMOG served as oligomannose donor. The means ± SEM of three dependent experiments are shown. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with PLL. (One-way ANOVA with Dunnett's multiple comparison test.)

VI.12 Promotion of neurite outgrowth by synapsin I is NCAM dependent

Since synapsin I promote neurite outgrowth and interact with NCAM in an oligomannose dependent way, it is reasonable to speculate that the promotive effects of neurite outgrowth is mediated by interacting with NCAM. To test this assumption I compared neurite outgrowth of NCAM deficient hippocampal neurons and wild type neurons. Converslips were substrated

with synapsin I (SYN) and synapsin I treated with Endoglycosidase H (SYN(-)). The experiment showed that Synapsin I did not enhance neurite outgrowth of NCAM-deficient neurons (**Fig. 17**). This suggests that promotion of neurite outgrowth by extracellular synapsin I is mediated via its oligomannose dependent interaction with NCAM at the cell surface of neurons.

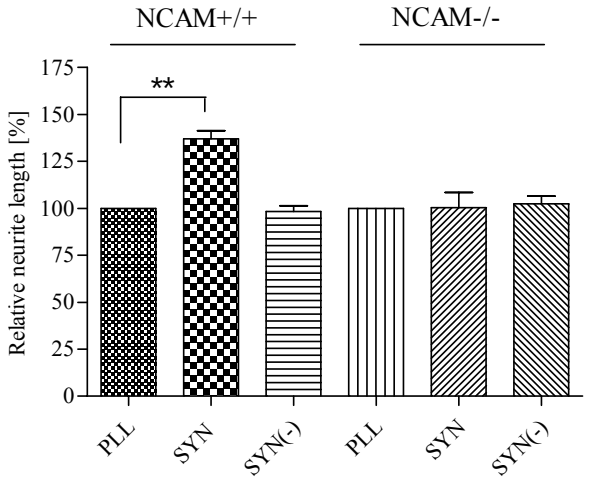

Fig. 17: Synapsin I does not promote neurite outgrowth in hippocampal neurons of NCAM deficient mice.

Hippocampal neurons of NCAM-deficient (NCAM-/-) and wild-type (NCAM+/+) mice were grown on substrate coated synapsin I (SYN) or heat-denatured synapsin I treated with Endo H (SYN(-)). After 24 hours in culture cells were fixed, stained and the length of the longest neurite per cell was determined. Mean length of the longest neurites obtained on PLL was set to 100%. Mean values ± SEM of two independent experiments are shown (** indicates $p < 0.01$ obtained by one-way ANOVA test with Dunnett's multiple comparison).

VII. Discussion

The first aim of the present work was searching for peptide mimics of oligomannosidic glycans. This was accomplished by screening a phage display library with two monoclonal antibodies L3 and L4, which recognize oligomannose (Kucherer et al., 1987; Fahrig et al., 1990) and the isolation of one clone which is a structural and functional mimic of oligomannose.

When L3 antibody served as both target and competitor, out of 20 selected clones only two showed the P1 sequence. Clone P_N had a high frequency (7 out of 20) (Table 1), however, showed low reactivity with both antibodies (**Fig. 6**). This indicates that high frequency does not imply high specificity. Another interesting phenomenon is the high ratio of aromatic amino acids (phenylalanine, F; tyrosine, Y; tryptophan, W) in the discovered clones, which constitute around 30% of all amino acids of selected sequences (15 out of 48). Because the size, cylic shape and hydrogen-bonding potential of aromatic acids resemble those of sugars, there is obviously a preference for aromatic acids in mimicking (Hoess et al., 1993). However, peptide mimics of carbohydrates without aromatic acids were also reported (Gevorkian et al., 2005). Up to now, the principal roles of peptide mimicking of saccharides are still unclear.

VII.1 Functional properties of oligomannose mimicking peptide 1

Oligomannoses have been proved to initiate cell spreading of laminin-adherent murine melanoma cells (Chandrasekaran et al., 1994b), high mannose glycoforms of laminin were the most effective ones for restoring cell spreading to B16 cells and neurite outgrowth to PC12 cells (Chandrasekaran et al., 1991). Specific oligomannosides such as Man6 and Man9 promoted cell spreading, while Man3 had virtually no effect (Chandrasekaran et al., 1994a). In the present experiments, oligomannose mimicking peptide increases around 50% of neurite outgrowth than that of scrambled peptide (Fig. 8A). Since NCAM and Basigin showed mannose lectin-like properties (Horstkorte et al., 1993; Heller et al., 2003; Sherblom et al., 1989), NCAM was showed in this experiment to interact with oligomannose on synapsin I, it is reasonable to conject that P1 also bind with NCAM and this interaction may partly underlie its promotive effects on neurite outgrowth.

Astrocyte and glial-neuron interactions play critical roles in brain development, which is partially mediated by glycoproteins, including adhesion molecules and growth factors

(Tomas et al., 2002). In the present study, adhesion between cerebellar neurons and astrocytes was significantly reduced by addition of catalase-coupled oligomannose mimicking peptide P1 but not by the scrambled peptide or the peptide carrier catalase (**Fig. 8B**). This observation indicated that oligomannoses can modulate the interaction between neurons and astroglia. A variety of glycoproteins especially adhesion molecules on neuron and astrocyte carry oligomannosides (Kleene and Schachner, 2004; Sakisaka and Takai, 2005) and a number of oligomannose receptors also exist on both neurons and astrocytes (Regnier-Vigouroux, 2003; Zanetta, 2003). They may be involved in the interaction between these two cells. P1, which mimic oligomannosides, was supposed to bind with oligomannose receptors in pre-incubation and disturbed the native interaction between ligands and receptors. However, detailed molecular mechanisms of this interaction still deserve further study.

VII.2 Synapsin I as both oligomannose lectin and oligomannose bearing glycoprotein

Peptide mimic emerged originally as surrogate of saccharide for developing antibody and vaccine or as possible drug to inhibit adhering of pathogens to host. Little was reported about its application in biochemistry research. Here, I used oligomannose mimicking peptide 1 as bait molecule to fish potential oligomannose receptor and got synapsin I, The interaction between P1 and synapsin I was confirmed by cross-link experiment and pull-down experiment (**Fig. 9A-D**). In an ELISA assay, synapsin I interacted with AMOG in an oligomannose dependent way (**Fig. 9E**), which further confirmed the oligomannose specific lectin nature of synapsin I. Comparison of the amino acid sequences of synapsin I with some lectins showed that it shares some sequence homologies with galectin 3 in domanin A, mannose binding protein and surfactant protein D in domain C, collectin and surfactant protein D in domain D. Although similarity in sequences between synapsin I and surfactant protein D may reflect their common physio-chemical characteristic as cell-suface active molecules, the common sequences shared with galectin 3, mannose binding protein and collectin add structural evidence for synapsin I as mannose specific lectin.

I also demonstrated that synapsin I was a N- and O-glycosylated glycoprotein. Since synapsin I possess one N-glycosylation site on its D domain (Murrey et al., 2006), this site is likely to carry the oligomannose. I found that synapsin I was eluted from L3 and L4 antibody columns and was proved to be oligomannose bearing glycoprotein (**Fig.**

10). Besides oligomannose, there are also O-linked Lewis^X and some unidentified saccharide epitopes on synapsin I (**Fig. 11**). Synapsin I as both oligomannose glycans and oligomannose lectins may naturally inspire the idea of homophilic interaction. Because it's strong surface activity and tendency to self-aggregation, the glycan dependent homo interaction on synaptic vesicles, normally a weak power, may be overwhelmed. However, on the plasma membrane, by interacting with other molecules, no matter as lectins or as glycoproteins, synapsin I may also play important roles.

VII.3 Astrocytes produce synapsin I via exosomes

In this study I demonstrate that cultured astrocytes express synapsin I and release it via exosome (**Fig. 12**). This data contrasts most early reports which described it as a "neuron-specific" protein (Johnson et al., 1972; Ueda et al., 1973; De Camilli et al., 1983a; De Camilli and Greengard, 1986; Ueda et al., 1979). In these prilimary studies, they use morphological methods to exam the distribution of synapsin I immunoactivity in the central and peripheral nervous systems. In the section of cerebellum, a patchy fluroresence was present in the granule cell layer where synapses were clustered in glomeruli, while in molecular layer where synapses were very abundant, an almost continuous flurosence was visible. In cerebral cortex, tightly apposed flurosence dots were visible throughout the neuropile of the cerebral cortex, nonneuronal cells do not show any specific fluorescence (De Camilli et al., 1983a). Because resolution of immunostaining depends on specificity and sensitivity of antibodies, quantity and accessibility of antigens and many other experimental constraints, one cannot exclude the possibility that synapsin I is also expressed *in vivo* in non-neuronal cells, like astrocytes. The first indication that astrocytes also produced synapsin I was obtained by (Maienschein et al., 1999). In this study, synapsin I was found to be associated with organelles of varying size and astrocytes were activated by lipopolysaccharides, indicating that expression of synapsin I occurs especially under pathophysiological conditions, such as inflammation. Although our cultured astrocytes may be partially activated, the results obtained from our cultured astrocytes are more akin to the physiological conditions.

Up to now, there lake evidence that under *in vivo* circumstance astrocytes can produce synapsin I, but it is reasonable to speculate that under some special conditions, for example, pathological stimulations, astrocytes may produce synapsin I. Since astrocytes have long been considered to be nutritive and supportive to neurons, they may work by

releasing some bioactive molecules which modulate the function of neurons.

VII.4 Exosome release synapsin I under high ionic strength

For gial-neuron communication, not only direct cell-to-cell contact but also exchange of soluble factors is important. Exosome, which has been discovered to be formed during reticulocyte maturation and to discard transferring receptor (Johnstone et al., 1987) is recently regarded to be involved in intercellular communications.

Exosomes transfer tumor antigens from tumor cells to dendritic cells and induce anti-tumor reponse (Hao et al., 2006; Muntasell et al., 2007) and are involved in cell- to-cell spread of infectious agents, like HIV (Gould et al., 2003; Fang et al., 2007) and pathogenic form of Prion protein (PrP^{Sc}) (Robertson et al., 2006). PrP^{Sc} is the major component of the infectious agent causing transmissible spongiform encephalopathies (Prusiner, 1982). Exosomes from the prion-infected neuronal cell line were initiators of prion propagation in uninfected recipient cells and also to non-neuronal cells (Schatzl et al., 1997). Exosomes also play a role in pathogenesis of Alzheimer´s disease which is the most common form of dementia in humans and characterized by the extracellular deposition of insoluble amyloid plaques in the brain. The main component of the amyloid plaques, the β-amyloid peptide, which is produced by proteolytic cleavage from the amyloid precursor protein APP (Cai et al., 1993) is released into the extracellular environment in association with exosomes (Rajendran et al., 2006). I found here that exosomes from astrocytes released synapsin I in the presence of high ionic strength, while other cargo proteins, like actin was not released, indicating the intact states of exosomes **(Fig 14)**. The selective release of synapsin I from astrocytes under distinct conditions suggests a regulated releasing mechanism for synapsin I.

During neuronal activity, potassium ions are transferred from the cytoplasm to the extracellular space. Under pathological conditions extracellular potassium reaches up to 12 mM (Heinemann and Lux, 1977) or 25 mM during seizure (Hansen, 1985). An extreme case are spreading depression waves which result in transient elevations of 30 ± 80 mM in the intact nervous tissue (Irwin and Walz, 1999). Elevated ionic strength may trigger *in vivo* exosomes to release distinct cargo proteins into extracellular milieu and these released molecules may modulate the cell interactions and communications.

Astrocytes showed intimate contact with neurons throughout the nervous system (Grosche et al., 2002; Grosche et al., 1999). Astrocytes typically extend five to eight major processes, each of which ramifies into fine and essentially uniformly distributed

leaflet-like appendages (Bushong et al., 2002) that can enwrap neuronal structures. These structural characteristics offer an optimal environment for the interactions between neurons and astrocytes.

It is then reasonable to speculate, exosomes from astrocytes which stand adjacently with neurons can free their cargo proteins under potassium originates from neurons' activity, these bioactive molecules in turn modulate the functional states of neurons. As showed by present experiment, a batch of proteins was released from KCl challenged exosomes of C6 cells **(Table 3)**. They are then considered to play multiple functional roles. Ionic strength modulated releasing of exosome cargo molecules may represent a novel mode of intercellular communication.

VII.5 Synapsin I promotes neurite outgrowth by interacting with NCAM in an oligomannose dependent way

Astrocytes, the predominant glial cell type in the central nervous system, were originally thought to play passive nutritive and metabolic roles for neurons. But recently, more and more lines of evidence showed that, they play more active and complex roles for neurons (Nedergaard et al., 2003), for example, neuronal migration (Rakic, 1978; Rakic, 1972; Ma et al., 2005; Gierdalski and Juliano, 2002; Gierdalski and Juliano, 2002; Ma et al., 2005), neurite outgrowth (Tomaselli et al., 1988; Tardy, 2002; Oh et al., 2008), and axon guidance (Silver et al., 1982; Johansson and Stromberg, 2002; Hung and Colicos, 2008).

In 1980s, it is observed that when neurons were cultured on top of monolayers of glial cells (astrocytes and Schwann cells), they have more robust processes elongation compared with that on nonglial cells (e.g., fibroblasts) (Fallon, 1985). Astrocytes have long been regarded to promote neurite outgrowth by releasing soluable neurotrophic factors (Muller and Seifert, 1982; Walicke et al., 1986; Hatten et al., 1988; Mizuno et al., 1998; Martinez and Gomes, 2005; Deumens et al., 2006; Johansson and Stromberg, 2002; Oh et al., 2008).

In this work, I could show that synapsin I was released from exosomes under high ionic strength. When substrate coated, which mimics the presence of extracellular synapsin I, it promoted neurite outgrowth in an oligomannose-dependent way (**Fig. 15**). In addition, synapsin I also bound to the extracellular domain of NCAM in an oligomannose-dependent way (**Fig. 16**). When NCAM was absent on the neuronal cell surface, synapsin I failed to promote neurite outgrowth (**Fig. 17**).

NCAM has long been regarded to be involved in neuronal outgrowth (Neugebauer et al., 1988; Horstkorte et al., 1993; Heiland et al., 1998; Anderson et al., 2005; Hansen et al., 2007). The fourth immunoglobulin-like domain of NCAM shows sequence homology to the carbohydrate recognition domains of animal C-type lectins and plant lectins, and can bind with oligomannosidic carbohydrates. Neurite outgrowth can be retained by oligomannosidic glycans and glcopeptides, glycoproteins and neoglycolipids containing oligomannosidic glycans (Horstkorte et al., 1993). These facts indicate that extracellular molecules especially those bearing oligomannosidic glycans can promote neurite outgrowth by interacting with NCAM. As discussed above, synapsin I is a novel NCAM interacting and neurite outgrowth promotive molecule.

VIII. References

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IX. APPENDIX

IX.1 Abbreviations

Ab	Antibody
AMOG	Adhesion molecule on glia
AMPA	α-**a**mino-3-hydroxy-5-**m**ethylisoxazole-4- **p**ropionic **a**cid receptor
APP	Amyloid precursor protein
APS	Ammonium peroxosulfate
BME	Basal Medium Eagle
BSA	Bovine serum albumine
CAMs	Cell adhesion molecules
CCR5	Chemokine receptor 5
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio] -1-propanesulfonate
CM	Catalase-MBS
CMP1	Catalase coupled P1
CMSP1	Catalase coupled scrambled peptide of P1
CNS	Central nervous system
CSL	Cerebellar soluable lectin
DMEM	Dulbecco's modified Eagle medium
CMF-HBSS	Ca^{2+}-Mg^{2+} free Hank´s buffered salt solution
CTLs	Cytotoxic T lymphocytes
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EM	Electron Microscope
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
Endo H	Endoglycosidase H

ELISA	Enzyme-linked immunosorbent assay
F	Phenylalanine
FCS	Fetal calf serum
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GPI	Glycosyl phosphatidylinositol
GP 120	Glycoprotein 120
GP 41	Glycoprotein 41
GFP	Green fluorescent protein
H	Histidine
HBSS	Hank´s buffered salt solution
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ILVs	intraluminal vesicles
IPTG	Isopropyl-D-thiogalactopyranoside
LB	Lysogeny broth
LTP	Long term potentiation
Man	Mannose
MALDI-TOF MS	Matrix assisted laser desorption ionisation time-of-flight mass spectrometry
min	Minute
MWCO	Molecular weight cutoff
MVBs	Multivesicular bodies
NaAc	Sodium acetate
Nabs	Neutralizing antibodies
NCAM	Neural cell adhesion molecule
NMDA	*N*-methyl-D-aspartate
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
P	Proline
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PEG/NaCl	Polyethylene glycol/sodium chloride
PLL	Poly-L-lysine
PrP^C	Prion protein
RER	Rough endoplasmic reticulum
RB	Ribonuclease B
RT	Room temperature
Ser	Serine
SDS	Sodium dodecyl sulfate
SP1	Scrambled peptide of P1
ssDNA	Single-stranded DNA
Sulfo-NHS	Sulfonated N-hydroxysuccinimide
Sulfo-SBED	Sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-Hexanoamido]ethyl-1,3-di-thiopropionate
TCA	Trichloroacetic acid
TEMED	N,N,N',N',-tetramethylethylene diamine
Tet	Tetracycline
TCTP	Translationally controlled tumor protein
Thr	Threonine
TNF	Tumor necrosis factor
Tris	Trimethylolaminomethane
UDP-GalNAc	Uridine diphosphate-N-acetylgalactosamine
v/v	Volume per volume
W	Tryptophan
w/v	Weight per volume
x-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
Y	Tyrosine

IX.2

Table 2: Proteomic analysis of proteins present in the pellet fraction derived from C6 cell exosomes

Exosomes of C6 cells were incubated with 80 mM KCl in PBS followed by 100,000g ultracentrifugation, pellet and supernatant parts were collected individually and subjected to proteomic analysis. Percent coverage (% Seq. Cov.) and peptide matched (Peptide Count) are indicated. Molecular function, biological process and location are derived from the Swiss-prot protein knowledgebase.

Protein name	Gene symbol	% Seq. Cov.	Peptide Count	Molecular function	Biological process	location
Fibronectin	Fn1	23.1	81	Heparin binding, protease activator activity, protein binding	Acute-phase response, cell-matrix adhesion, cell-substrate junction assembly, transmembrane receptor protein tyrosine kinase signaling pathway, wound healing	Secreted
Hspa8 protein	Hspa8	54.7	63	ATP binding	Chaperone cofactor-dependent protein folding	Cytoplasm
Programmed cell death 6-interacting protein	Pdcd6ip (Alix)	33.6	58	Protein binding	Protein transport, apoptosis	Cytoplasm
Isoform 1 of Chondroitin sulfate proteoglycan 4	Cspg4	12	38	Developmental protein, Transducer	Angiogenesis, differentiation, tissue remodeling	Membrane
Sodium/potassium-transporting ATPase subunit alpha-1	Atp1a1	24.1	33	Sodium:potassium-exchanging ATPase activity	Potassium ion transport, regulation of cellular pH, sodium ion transport	Membrane
Membrane-organizing extension spike protein	Mosin	31.5	38	Cytoskeletal protein binding		Membrane
Actin, cytoplasmic 1	Actb	38.9	31	ATP binding, protein binding	Cytoskeleton	Cytoplasm
Heat shock protein 2	Hspa2	22.6	24	ATP binding, unfolded protein binding	Stress response	Membrane, nucleus
Milk fat globule-EGF factor 8 protein isoform 1	Mfge8	19.7	22	Integrin binding phosphatidylethanolamine binding	Cell adhesion, phagocytosis, engulfment	Membrane
Elongation factor 1-alpha 1	Eef1a1	26.8	23	Elongation factor	Protein biosynthesis	Cytoplasm
Envelope protein	Mela	16.1	24	Structural molecule activity	viral infectious cycle	Envelope protein
EMILIN-1	Emilin1	16.8	24	Extracellular matrix constituent conferring elasticity	Cell adhesion	Secreted
Polyadenylate-binding protein 1	Pabpc1	25	25	Nucleotide binding	mRNA processing and splicing	Cytoplasm
Ceruloplasmin isoform a	Cp	9.5	17	Copper ion binding, ferroxidase activity	Aging, oxidation reduction, response to nutrient	Secreted
Alpha-2-macroglobulin-P	A2m	3.3	14	Protein binding, wide-spectrum protease inhibitor activity	Female pregnancy	Secreted

Ubiquitin	Rps27a	80.5	14	Protein binding	Protein modifier	Membrane, nucleus
Complement component 3	C3	7.7	21	Endopeptidase inhibitor activity, protein binding	Complement alternate pathway Complement pathway	Secreted
Radixin	Rdx	12.2	15	Actin binding	Apical protein localization, arbed-end actin filament capping	Cytoplasm
Phosphoglycerate kinase 1	Pgk1	23.5	17	ATP binding, hosphoglycerate kinase activity	Glycolysis, phosphorylation	Cytoplasm
Glia-derived nexin	Serpine2	18.6	15	Heparin binding, serine-type endopeptidase inhibitor activity	Differentiation, neurogenesis	Secreted
Beta-actin-like protein 2	Actbl2	21.3	17	ATP binding, protein binding	Cytoskeleton	Cytoplasm cytoskeleton
Ezrin	Ezr	9	14	Actin filament binding, cell adhesion molecule binding, protein self-associatio	Actin filament bundle formation, establishment or maintenance of apical/basal cell polarity, regulation of cell shape	Membrane
ATPase, Na+/K+ transporting, alpha 3 polypeptide	Atp1a3 ATPase	10.4	14	Potassium ion binding, sodium ion binding, sodium /potassium-exchanging ATPase activity	Ion transport, potassium transport, sodium transport, sodium/potassium transport	Membrane
Integrin beta-1	Itgb1	7.1	18	Integrin receptor	Cell adhesion	Membrane
Alpha-enolase	Eno1	23.7	15	Magnesium ion binding, phosphopyruvate hydratase activity, serine-type endopeptidase activity	Glycolysis	Membrane
Integrin alpha 6	Itga6	8.1	13	Calcium ion binding, integrin binding, receptor activity	Cell adhesion mediated by integrin, cell-matrix adhesion, cellular response to extracellular stimulus, filopodium formation, integrin-mediated signaling pathway, leukocyte migration, odontogenesis of dentine-containing tooth, positive regulation of cell-cell adhesion	Membrane, cytoplasm
Annexin A2	Anxa2	24.2	15	Calcium ion binding, calcium-dependent phospholipid binding, cytoskeletal protein binding	Angiogenesis, collagen fibril organization, fibrinolysis	Secreted
Immunoglobulin superfamily member 8	Igsf8	16.9	14	Protein binding		Membrane
5'-nucleotidase	Nt5e	14.8	13	5'-nucleotidase activity, nucleotide binding, zinc ion binding	Nucleotide catabolic process	Membrane
Isoform 9 of CD44 antigen	Cd44	7.6	12	Hyaluronic acid binding, protein binding, receptor activity	Cell adhesion	Membrane
Poly(A) binding protein, cytoplasmic 3	Pabpc3	12	14	RNA binding, nucleotide binding		
Charged multivesicular body protein 4b	Chmp4b	30.4	12		Protein transport	Cytoplasm
Heat shock 70 kDa protein 1L	Hspa1a	7.6	10	Chaperone	DNA repair, response to heat. telomere maintenance	Cytoplasm
Guanine nucleotide-binding protein G(i), alpha-2 subunit	Gnai2	16.6	10	GTP binding, GTPase activity, protein binding, signal transducer activity	Inhibition of adenylate cyclase activity by G-protein signaling, muscarinic acetylcholine receptor signaling pathway	

Isoform 1 of Tripartite motif-containing protein 47	Trim47	11.7	10	Zinc ion binding		Cytoplasma
Tumor susceptibility gene 101 protein	Tsg101	17.4	11	Small conjugating protein ligase activity, transcription corepressor activity	Growth regulation, Protein transport	Cytoplasm Endosome Membrane Nucleus
Glypican-1	Gpc1	8.8	9			Membrane
Heat shock 70 kDa protein 1B	Hspa1b	8.9	12	Chaperone	DNA repair, response to heat. telomere maintenance	Cytoplasm
Clathrin heavy chain 1	Cltc	3.9	11	Protein binding	Intracellular protein transport, vesicle-mediated transport	Cytoplasmic vesicle, membrane
Biglycan	Bgn	17.3	10	Protein binding		Secreted
14-3-3 zeta		28.4	6	Protein domain specific binding, protein kinase C inhibitor activity	Differentiation, neurogenesis	Cytoplasm
Serine protease HTRA1	Htra1	10	10	Insulin-like growth factor binding, serine-type endopeptidase activity	Negative regulation of transforming growth factor beta receptor signaling pathway, proteolysis, regulation of cell growth	Secreted
Heat shock protein HSP 90-beta	Hsp90ab1	9.9	6	Chaperone	Stress response	Cytoplasm
Syndecan binding protein isoform 2	Sdcbp	15.4	9	Protein C-terminus binding, protein heterodimerization activity, protein homodimerization activity		Cytoplasm
EGF-like repeats and discoidin I-like domains 3 isoform b	Edil3	12.1	9	Calcium ion binding, protein binding	Cell adhesion, multicellular organismal development	Secreted
Histone H2A type 2-C	Hist2h2ab	20.9	7	DNA binding	Nucleosome assembly	Nucleosome, nucleus
Potassium-transporting ATPase alpha chain 1	Atp4a	2.7	8	ATP binding, hydrogen:potassium-exchanging ATPase activity, magnesium ion binding, potassium ion binding protein heterodimerization activity	Metabolic process, pH reduction, potassium ion transport	Membrane
Glypican-4	Gpc4	9.9	9			Membrane
Isoform 8 of Neuronal membrane glycoprotein M6-b	Gpm6b	14.2	7			Membrane
ADP-ribosylation factor 3	Arf3	19.9	8	GTP binding	Protein transport ,small GTPase mediated signal transduction, vesicle-mediated transport	Golgi apparatus
1a4 Sodium/potassium-transporting ATPase Subunit alpha-4	Atp1a4	2.7	9	ATP binding, magnesium ion binding monovalent inorganic cation transmembrane transporter activity, potassium ion bindin	Metabolic process, potassium ion transport, regulation of cellular pH, sodium ion transport, sperm motility	Membrane

Hist1h2ac;Hist1h2ab;Hist1h2ai;Hist1h2ad; Hist1h2an	Hist1h2ae	20.8	7	DNA binding	Nucleosome assembly	Nucleosome, nucleus
L-lactate dehydrogenase A chain	Ldha	10.2	8	L-lactate dehydrogenase activity, protein binding	Anaerobic glycolysis, cellular response to extracellular stimulus, oxidation reduction	Cytoplasm
Histone H2A	Hist1h2aa	20.9	6	DNA binding	Nucleosome assembly	Nucleosome, nucleus
Murinoglobulin-1	Mug1	2.4	6	Protein binding, serine-type endopeptidase inhibitor activity, wide-spectrum protease inhibitor activity		Secreted
Endophilin-A2	Sh3gl1	14.7	6	Lipid binding, protein binding	Endocytosis	Early endosome membrane
Collagen alpha-1(VI) chain	Col6a1	6.8	7	Protein binding, structural molecule activity	Cell adhesion	Secreted
Alpha-2-macroglobulin	Pzp	2.7	7	Protein binding, serine-type endopeptidase inhibitor activity, wide-spectrum protease inhibitor activity		Secreted
Flotillin-1	Flot1	8.6	6			Membrane
Galectin-3-binding protein	Lgals3bp	5.2	7	Protein binding, scavenger receptor activity	Cell adhesion	Secreted
Isoform 2 of Histone H2B type 1-P	Hist1h2bp	35.5	7	DNA binding	Nucleosome assembly	Nucleosome, nucleus
CD 81 antigen	Cd81	13.1	5	Protein binding	Activation of MAPK activity, phosphatidylinositol biosynthetic process, positive regulation of 1-phosphatidylinositol 4-kinase activity, positive regulation of B cell proliferation, positive regulation of cell growth, positive regulation of peptidyl-tyrosine phosphorylation, protein localization	Membrane
Ras-related protein Rap-1A	Rap1a	18.5	6	GTP binding， GTPase activity， Ras GTPase binding	Negative regulation of cell cycle， small GTPase mediated signal transduction	Membrane
ADP-ribosylation factor 6	Arf6	26.9	6	GTP binding, protein binding	Apoptosis, liver development, protein transport, vesicle-mediated transport	Golgi apparatus Endosome, plasma membrane
Plasma membrane calcium ATPase 1	Atp2b1	3.2	9	ATP binding, ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	ATP biosynthetic process, calcium ion transport	Membrane
Calpain 5, isoform CRA_a	Capn5	10.3	7	Calcium-dependent cysteine-type endopeptidase activity	Proteolysis	Cytoplasm
Annexin A1	Anxa1	9.8	5	Calcium ion binding, calcium-dependent phospholipid	Arachidonic acid secretion, cell cycle, regulation of cell proliferation,	Cytoplasma,

				binding, phospholipase A2 inhibitor activity	signal transduction	nucleus
CD9 antigen	Cd9	5.3	5	Protein binding	Cell adhesion, cell motion, fusion of sperm to egg plasma membrane, negative regulation of cell proliferation, paranodal junction assembly	Membrane
Histone cluster 2, H3c1 isoform 1	Hist1h3e	24.3	7	DNA binding	Nucleosome assembly	Nucleosome, nucleus
Isoform Alpha-3A of Integrin alpha-3	Itga3	4.1	7	Protein binding, receptor activity	Cell adhesion, integrin-mediated signaling pathway, memory, neuron migration	Basolateral plasma membrane integrin complex synaptosom
Isoform 2 of Rab GDP dissociation inhibitor beta	Gdi2	8.6	4	Rab GDP-dissociation inhibitor activity, rab GTPase activator activity	Protein transport, regulation of GTPase activity, small GTPase mediated signal transduction	Golgi apparatus, membrane
Murinoglubulin-4	Mug4	1.9	6	Protein binding, serine-type endopeptidase inhibitor activity, wide-spectrum protease inhibitor activity		Secreted
Murinoglobulin-2	Mug2	1.9	6	Protein binding, serine-type endopeptidase inhibitor activity, wide-spectrum protease inhibitor activity		Secreted
Isoform 1 of Protein sidekick-2	Sdk2	1.4	8	Potein binding	Cell adhesion	Membrane
Voltage-dependent calcium channel subunit alpha-2/delta-1	Cacna2d1	4.4	5	Calcium channel activity, calcium ion binding, protein binding, voltage-gated ion channel activity	Regulation of calcium ion transport	Membrane
MCG5546, isoform CRA_c	Pabpc4	2.3	8	RNA binding, nucleotide binding		
Charged multivesicular body protein 2a	Chmp2a	14.4	5		Protein transport	Cytoplasm, late endosome membrane
H2-gs10 protein	H2-gs10	7.8	6		Antigen processing and presentation	Membrane
Ras-related protein	Rab-5C	17.1	4	GTP binding, GTPase activity	Endosome organization, protein transport, regulation of endocytosis, small GTPase mediated signal transduction	Early endosome membrane, endocytic vesicle, melanosome, plasma membrane
Annexin A6 isoform b	Anxa6	4	5	Calcium ion binding, calcium-dependent phospholipid binding	Calcium ion transport, regulation of muscle contraction	Cytoplasm
Annexin A11	Anxa11	7.2	4	Calcium ion binding, calcium-dependent phospholipid binding, phosphatidylethanolamine binding, protein binding		Melanosome, nucleus

Annexin A7	Anxa7	3.5	5	Calcium ion binding, calcium-dependent phospholipid binding, protein binding	Cell proliferation, cellular calcium ion homeostasis, cellular water homeostasis, hemostasis, regulation of cell shape, response to salt stress	Cytoplasm, nuclear envelope, plasma membrane, vesicle
Protein S100-A4	S100a4	17.8	3	Calcium ion binding, identical protein binding		
Isoform 1 of Fibroblast growth factor receptor-like 1	Fgfrl1	7.2	5	Protein binding, receptor activity	Ngative regulation of cell proliferation	Membrane
CD82 antigen	Cd82	7.9	4			Membrane
Glyceraldehyde-3-phosphate dehydrogenase	GAP DH	7.9	7	NAD binding, glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity	Glucose metabolic process	Cytoplasm
Aldo-keto reductase family 1, member B10	AKR1B10	10.1	5	Aldo-keto reductase activity, protein binding	Cellular aldehyde metabolic process, digestion, oxidation reduction, steroid metabolic process	Cytoplasm
Aldose reductase-related protein 2	Akr1b8	10.1	5	Aldehyde reductase activity	Oxidation reduction	Cytoplasm
Integrin alpha-1	Itga1	2.8	5	Calcium ion binding, magnesium ion binding, protein binding, receptor activity	Cell adhesion, cellular extravasation, integrin-mediated signaling pathway, neutrophil chemotaxis	Membrane
Ferritin heavy chain	Fth1	15.9	5	Ferric iron binding, ferroxidase activity	Cellular iron ion homeostasis, immune response, iron ion transport, negative regulation of cell proliferation, oxidation reduction	
H-2 class I histocompatibility antigen, Q7 alpha chain	H2-Q7	7.8	5	MHC class I protein complex, integral to membrane	Antigen processing and presentation of peptide antigen via MHC class I, immune response	Membrane
Isoform 2 of Cytosol aminopeptidase	Lap3	8.2	4	Aminopeptidase activity, magnesium ion binding, manganese ion binding, metalloexopeptidase activity, zinc ion binding	Proteolysis	Mitochondrion
14-3-3 protein epsilon	Ywhae	8.2	4	Protein domain specific binding	Cerebral cortex development , hippocampus development, negative regulation of protein amino acid dephosphorylation, neuron migration , protein targeting	Cytoplasm
Ephrin-B1	Efnb1	14.2	5	Ephrin receptor binding	Axon guidance, embryonic pattern specification, neural crest cell migration, positive regulation of T cell proliferation	Membrane
Isoform 1 of STE20-like serine/threonine-protein kinase	Slk	1.5	7	ATP binding, DNA binding, nuclease activity, protein serine/threonine kinase activity	Apoptosis, nucleotide-excision repair, protein amino acid phosphorylation	Cytoplasm
Keratin, type II cytoskeletal 1b	Krt77 Keratin	4.2	3	Structural molecule activity	Cytoskeleton	Keratin filament
Guanine nucleotide-binding protein G(I) /G(S)/G(T)	Gnb3	5	4	GTPase activity, signal transducer activity	G-protein coupled receptor protein signaling pathway	

subunit beta-3						
Syndecan-4	Sdc4	22.7	4	Cytoskeletal protein binding	Signal transmembrane	Membrane
Ms10t;0610037M15Rik 37 kDa protein	H2-Q6	6	5	MHC class I protein complex	Antigen processing and presentation	Membrane
Vacuolar protein sorting-associating protein 4A	Vps4a	8.2	4	ATP binding, nucleoside-triphosphatase activity	Protein transport	Endosome membrane, perinuclear region of cytoplasm
Vacuolar protein sorting-associating protein 4B	Vps4b	7	4	ATP binding, nucleoside-triphosphatase activity	Endosome organization, potassium ion transport, protein transport	Endosome membrane, perinuclear region of cytoplasm
ADP-ribosylation factor 4	Arf4	6.1	5	GTP binding	Protein transport, small GTPase mediated signal transduction, vesicle-mediated transport	Golgi apparatus, plasma membrane
G Ras-related protein Rab-35	Rab35	16.9	4	GTP binding	Protein transport, small GTPase mediated signal transduction	Melanosome
Proteasome subunit alpha type-7	Psma7	10.5	4	Threonine-type endopeptidase activity	Ubiquitin-dependent protein catabolic process	Cytoplasm, nucleus, proteasome core complex
Ras GTPase-activating-like protein IQGAP1	Iqgap1	1.4	6	Ras GTPase activator activity, calmodulin binding	Regulation of small GTPase mediated signal transduction	Cytoplasm, neuron projection, plasma membrane
Keratin, type II cytoskeletal 1	Krt1	3.8	3	Structural molecule activity		Membrane, intermediate filament
Chloride intracellular channel protein 1	Clic1	12.4	3	Chloride ion binding, voltage-gated chloride channel activity	Chloride transport	Membrane, nucleus
Vacuolar protein sorting-associated protein 37B	Vps37b	9.8	4		Protein transport	Endosome membrane
Profilin-1	Pfn1	22.1	5	Actin binding	Neural tube closure, regulation of transcription from RNA polymerase	Membrane,

					II promoter, sequestering of actin monomers	nucleus
Alpha-actinin-4	Actn4	3	3	Actin filament binding， calcium ion bindin， protein homodimerization activity	Actin filament bundle formation, negative regulation of cell motion, positive regulation of cell motion, positive regulation of pinocytosis	Cytoplasm
Aldose reductase	Akr1b3	8.5	5	Aldehyde reductase activity	Oxidation reduction	Cytoplasm
Isoform 1 of BRO1 domain-containing protein BROX	Brox	5.4	5			Membrane
Isoform 2 of Basigin	Bsg	6.6	2	Mannose binding		Membrane
Keratin, type II cytoskeletal 73	Krt73	2.2	4	Structural molecule activity	Cytoskeleton	Cytoplasm
MHC classIb T5	EG667977	4.2	5		Antigen processing and presentation of peptide antigen via MHC class I, immune response	MHC class I protein complex, integral to membrane
Isoform 2 of Poly(rC)-binding protein 2	Pcbp2	7.3	3	DNA binding, RNA binding		Nucleus, ribonucleo protein complex
Plasma membrane Ca++ transporting ATPase 4 splice variant a	Atp2b4	1.5	5	ATP binding, calcium-transporting ATPase activity	ATP biosynthetic process, calcium ion transport	Membrane
Ras-related protein Rab-5A	Rab5a	11.6	2	GTP binding, GTPase activity	Endocytosis, protein transport, small GTPase mediated signal transduction	Membrane
Neural cell adhesion molecule 1 isoform 2	Ncam1	1.7	5	Heparin binding, protein binding	Cell surface receptor linked signal transduction, homotypic cell-cell adhesion, positive regulation of calcium-mediated signaling	Membrane
Phospholipid scramblase 3	Plscr3	6.1	4	SH3 domain binding, calcium ion binding	Cholesterol homeostasis, glucose homeostasis	Membrane
THO complex subunit 2	Thoc2	0.3	7	RNA binding, protein binding	RNA splicing, mRNA processing, mRNA transport	Nucleus
Isoform 1 of Calmodulin-regulated spectrin-Associated protein 1	Camsap1	1.2	5			
Isoform M2 of Pyruvate kinase isozymes M1/M2	Pkm2	3.2	5	Magnesium ion binding, potassium ion binding, protein binding, pyruvate kinase activity	Glycolysis	Flagellum, mitochondrion
Actin-related protein 2/3 complex subunit 4	Arpc4	13.1	2	Actin binding	Actin filament polymerization	Cytoplasm, cell projection
Proteasome subunit beta type-4	Psmb4	9.5	2	Threonine-type endopeptidase activity	Ubiquitin-dependent protein catabolic process	Cytoplasm, nucleus
Keratin, type II cytoskeletal 71	Krt71	2.3	3	Structural molecule activity	Cytoskeleton	Cytoplasm
guanine nucleotide binding protein, alpha stimulating,	Gnal	2.5	4	GTP binding, signal transducer activity	Activation of adenylate cyclase activity, activation of adenylate cyclase activity by dopamine receptor signaling pathway, sensory	

olfactory type isoform 2					perception of smell	
Isoform 2 of Alpha-crystallin A chain	Cryaa	12.1	2	Structural constituent of eye lens	M phase specific microtubule process, camera-type eye development, response to heat	Cytoplasm
Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas	Gnas	1	4	GTP binding, GTPase activity, signal transducer activity	Activation of adenylate cyclase activity by dopamine receptor signaling pathway, cartilage development, embryonic cranial skeleton morphogenesis, embryonic hindlimb morphogenesis	Membrane
Glypican-6	Gpc6	3.2	2			Membrane
Actin-related protein 2	Actr2	7.3	3	ATP binding, actin binding	Cytoskeleton	Cytoplasm,
Fructose-bisphosphate aldolase	Aldoa	6	4	Fructose-bisphosphate aldolase activity	Glycolysis	
Aquaporin-1	Aqp1	7.1	2	Water channel activity	Water transport	Membrane
Tubulin beta-5 chain	Tubb5	6.8	3	GTP binding, GTPase activity, structural constituent of cytoskeleton	Microtubule-based movement, protein polymerization, spindle assembly	Cytoplasm,
Ras-related protein Rab-11A	Rab11a	9.7	2	GTP binding	Protein transport, small GTPase mediated signal transduction	Membrane
Isoform 2 of Intercellular adhesion molecule 1	Icam1	4.5	2	Protein binding	T cell antigen processing and presentation, cell adhesion mediated by integrin, leukocyte adhesion, regulation of cell adhesion	Membrane
keratin complex 1, acidic, gene 10	Krt10	3.7	2	Structural molecule activity	Cytoskeleton	Cytoplasm
Charged multivesicular body protein 3	Vps24	7.1	4		Protein transport	Cytoplasm, membrane
Dihydropyrimidinase-related protein 2	Dpysl2	5.1	4	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	Cell differentiation, nervous system development	Cytoplasm
Sperm associated antigen 17	Spag17	1.3	5	Protein binding		Cytoplasm
Nuclease-sensitive element-binding protein 1	Ybx1	5.9	2	RNA binding, protein binding, single-stranded DNA binding	RNA splicing, embryonic development, mRNA processing, DNA-dependent regulation of transcription	Cytoplasm, nucleus
Isoform 1 of Neuroplastin	Nptn	9.6	2	Cell adhesion molecule binding	Homophilic cell adhesion, positive regulation of long-term neuronal synaptic plasticity	Membrane
flotillin 2 isoform 1	Flot2	3.3	4	Protein binding	Cell adhesion	Membrane
Kinesin-like protein KIF15	Kif15	0.3	6	ATP binding, microtubule motor activity	Microtubule-based movement	Cytoplasm
Isoform 1 of Ectonucleotide pyrophosphatase /phosphodiesterase family member 1	Enpp1	1.7	3	Endonuclease activity, metal ion binding, nucleic acid binding, nucleotide diphosphatase activity, phosphodiesterase I activity	Metabolic process, negative regulation of fat cell differentiation, negative regulation of ossification	Membrane
RAB5B, member RAS oncogene family, isoform CRA_b	Rab5b	14.9	2	GTP binding, GTP-dependent protein binding, GTPase activity	Endosome organization, protein transport, regulation of endocytosis, small GTPase mediated signal transduction	Cytoplasm
Tetraspanin-7	Tspan7	6.4	3			Membrane
Tropomyosin 3, gamma	Tpm3	14.7	6		Brain development	Cytoplasm

IX.3

Table 3: Proteomic analysis of proteins present in the soluble fraction derived from C6 cell exosomes .

Protein Name	Gene symbol	% Seq. Cov.	Peptide Count	Molecular function	Biological process	location
Fibronectin	Fn1	30.4	125	Heparin binding, protease activator activity, protein binding	Acute-phase response, cell-matrix adhesion, cell-substrate junction assembly, transmembrane receptor protein tyrosine kinase signaling pathway, wound healing	Secreted
Isoform Long of Complement C3 (Fragment)	C3	18.2	62	Endopeptidase inhibitor activity, protein binding	Complement alternate pathway Complement pathway	Secreted
Actin, cytoplasmic 1	Actb	29.1	31	ATP binding, protein binding	Cytoskeleton	Cytoplasm cytoskeleton
Glia-derived nexin	Serpine2	19.1	23	Heparin binding, serine-type endopeptidase inhibitor activity	Differentiation, neurogenesis	Secreted
Alpha-actinin-4	Actn4	13.9	21	Actin filament binding， calcium ion bindin， protein homodimerization activity	Actin filament bundle formation, negative regulation of cell motion, positive regulation of cell motion, positive regulation of pinocytosis	Cytoplasm
Clusterin	Clu	18.1	18			Secreted
Alpha-2-macroglobulin-P	A2m	3.5	16	Protein binding, serine-type endopeptidase inhibitor activity, wide-spectrum protease inhibitor activity		Secreted
Collagen alpha-1(VI) chain	Col6a1	10.6	21	Protein binding, structural molecule activity	Cell adhesion	Secreted
Inter-alpha-trypsin inhibitor heavy chain H2	Itih2	7.4	17	Serine-type endopeptidase inhibitor activity	Hyaluronan metabolic process	Secreted
Ceruloplasmin	Cp	11.6	19	Copper ion binding, ferroxidase activity	Aging, oxidation reduction, response to nutrient	Secreted
Smooth muscle gamma-actin	Actg2	14.5	22	ATP binding, protein binding	Cytoskeleton	Cytoplasm
L-lactate dehydrogenase A chain	Ldha	19.6	14	L-lactate dehydrogenase activity, protein binding	Anaerobic glycolysis, cellular response to extracellular stimulus, oxidation reduction	Cytoplasm
Serotransferrin	Trf	6.6	19	Ferric iron binding	Cellular iron ion homeostasis	Secreted
Alpha-2-macroglobulin	Pzp	2.7	12	Protein binding, serine-type endopeptidase inhibitor activity, wide-spectrum protease inhibitor activity		Secreted

Gag-Pol polyprotein	Mela	2.7	12	DNA biding	DNA integration	
EMILIN-1	Emilin1	6.3	13	Extracellular matrix constituent conferring elasticity	Cell adhesion	Secreted
30 kDa protein	Ldha	12.5	9	L-lactate dehydrogenase activity, protein binding	Anaerobic glycolysis, cellular response to extracellular stimulus, oxidation reduction	Cytoplasm
Nucleobindin-1	Nucb1	11.1	10	DNA binding, calcium ion binding		Membrane
Biglycan	Bgn	15.4	9	Protein binding		Secreted
Apolipoprotein A-I	Apoa1	7.2	7	Cholesterol transporter activity, high-density lipoprotein binding, identical protein binding, lipid binding	Adrenal gland development, blood vessel endothelial cell migration, cholesterol biosynthetic process, cholesterol efflux, endothelial cell proliferation, glucocorticoid metabolic process, lipoprotein biosynthetic process, negative regulation of hydrolase activity, phospholipid metabolic process, positive regulation of transferase activity, regulation of cholesterol absorption, regulation of protein amino acid phosphorylation, sequestering of lipid	Secreted
Proteasome subunit alpha type-1	Psma1	13.7	10	Threonine-type endopeptidase activity	Ubiquitin-dependent protein catabolic process	Cytoplasm, nucleus, proteasome core complex
Keratin, type II cytoskeletal 1	Krt1	5.5	9	Structural molecule activity		Membrane,
Alpha-actinin-1	Actn1	3	12	Actin filament binding，calcium ion bindin，protein homodimerization activity	Actin filament bundle formation, negative regulation of cell motion, positive regulation of cell motion, positive regulation of pinocytosis	Cytoplasm
Putative uncharacterized protein	Itih4	2.9	8	Serine-type endopeptidase inhibitor activity	Hyaluronan metabolic process	Secreted
Murinoglobulin-1	Mug1	1.8	12	Protein binding, serine-type endopeptidase inhibitor activity, wide-spectrum protease inhibitor activity		Secreted
Moesin	Msn	6.1	13	Cytoskeletal protein binding, structural molecule activity	Apical plasma membrane, basolateral plasma membrane, extrinsic to membrane	Membrane
Inter-alpha-trypsin inhibitor heavy chain H3	Itih3	3.1	14	Serine-type endopeptidase inhibitor activity	Hyaluronan metabolic process	Secreted
14-3-3 protein zeta/delta	Ywhaz	21.2	9	Protein domain specific binding	Cerebral cortex development , hippocampus development, negative regulation of protein amino acid dephosphorylation, neuron migration , protein targeting	Cytoplasm
Keratin, type II cytoskeletal 1b	Krt77	4.2	7	Structural molecule activity	Cytoskeleton	Cytoplasm

Pigment epithelium-derived factor	Serpinf1	12.5	8	Heparin binding, serine-type endopeptidase inhibitor activity	Differentiation, neurogenesis	Secreted
Antithrombin-III	Serpinc1	8.6	9	Heparin binding, serine-type endopeptidase inhibitor activity	Differentiation, neurogenesis	Secreted
Murinoglubulin-4	Mug4	1.3	13	Protein binding, serine-type endopeptidase inhibitor activity, wide-spectrum protease inhibitor activity		Secreted
Serum albumin	Alb	3.8	7	DNA binding, copper ion binding, drug binding, lipid binding, protein binding, pyridoxal phosphate binding, toxin binding	Cellular response to starvation, hemolysis by symbiont of host red blood cells, maintenance of mitochondrion location, negative regulation of apoptosis	Secreted
Milk fat globule-EGF factor 8 protein isoform1	Mfge8	10.2	7	Integrin binding phosphatidylethanolamine binding	Cell adhesion phagocytosis, engulfment	Membrane
Isoform 1 of Chondroitin sulfate proteoglycan4	Cspg4	2.4	5	Developmental protein, Transducer	Angiogenesis, Differentiation, Tissue remodeling	Membrane
Hba-a2 Hemoglobin subunit alpha	Hba-a2 alpha	23.9	8	Heme binding, iron ion binding, oxygen binding, oxygen transporter activity	Oxygen transport	

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