The Role of Fucose in Learning and Memory: The Identification of a Fucosylprotein, Synapsin I, in the Rat Brain and the Characterization of its Fucosylation.

Thesis by

Wen-I Luo

In Partial Fulfillment of the Requirements
for the Degree of
Master of Science

California Institute of Technology
Pasadena, California

2004
(Submitted March 31, 2004)

The Role of Fucose in Learning and Memory: The Identification of a Fucosylprotein, Synapsin I, in the Rat Brain and the Characterization of Its Fucosylation

Abstract

Previous studies have shown that the glycoproteins containing the fucose moiety are involved in neuronal communication phenomena such as long-term potentiation and memory formation. These results imply that fucose containing glycoproteins might play an important role in learning and memory. To understand the role of fucose in neuronal communication, and the mechanisms by which fucose may be involved in information storage, the identification of fucosylproteins is essential. This report describes the identification and characterization of fucosylproteins in the brain, which will provide new insights into the role of the fucose involved molecular interactions.

Introduction

1. Fucose in Learning and Memory

Glycoconjugates mediate a variety of biological events including fertilization, immune defense, viral replication, parasite infection, cell-cell adhesion, degradation of blood clots and inflammation.[1-7] It has been demonstrated since the 1960s that various carbohydrates and glycoconjugates are found in the central and peripheral nervous system. There is a growing body of evidences to suggest that carbohydrates are important in the formation, maturation, and functional modulation of synapses, which is the fundamental

unit of the nervous system.[8, 9] Carbohydrates are also implicated in the regulation of synaptic plasticity, which is the permanent functional transformation that arises in a particular system of neurons as a result of appropriate stimuli or their combination.[10] Glycoconjugates are one of the major constituents of synaptic membranes, where they impart specificity to the synapse and allow the communication between pre- and post-synaptic neurons via the recognition and adhesion properties of their carbohydrate moiety.[11-12]

Experiments on the sugar L-fucose (Fuc) (Fig. 1) in the mammalian brain suggest that it might play a pivotal role in the neuronal processes of learning and memory. Fucosylated glycoproteins are enriched in the central nervous system and may account for as much as 85% of the protein-bound sugars in the synaptic plasma membrane.[13, 14] Formation of memory is accompanied by fucose incorporation into proteins.[11] Long-term potentiation (LTP), which is an important electrophysiological model for long-lasting information storage, can be induced in the hippocampal CA1 region of rat brain or chick brain after fucose administration. Increases in [^{3}H]-fucose incorporation into glycoproteins and enhanced fucosyltransferase activity have been detected after LTP induction in the CA1

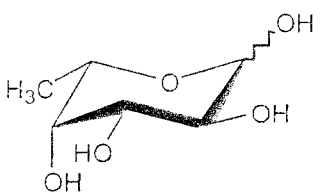

Figure 1. L-Fucose (L-Fuc)

region. The incorporation of L-fucose or the trisaccharide 2'-fucosyllactose [Fucα(1-2)Galα(1-4)GlcNAc] has been shown to enhance LTP maintenance both *in vivo* and *in vitro* and to influence neurotransmitter release.[15-17]

Studies have also implicated that the disaccharide Fucα(1-2)Gal (Figure 2) is involved in memory formation.[11, 16, 18] Day-old chicks, having spontaneously pecked at a small bright bead coated with a bitter-tasting substance, learn to avoid similar looking, but dry beads subsequently. Injection of 2-deoxy-2-D-galactose (2-dGal) to chicks 2 hours prior to or 2 hours after training causes reversible amnesia for this passive avoidance task in chicks tested 24 hour after training.[11] 2-dGal has been shown to inhibit the fucosylation of proteins in the brain by competing with galactose for formation of the α(1-2) linkage (Figure 3).[11] In addition to inducing amnesia in animals, 2-dGal has also been shown to modulate communication on a cellular level. For instance, the specific inhibition of fucosylation at galactose residues with 2-dGal also prevents LTP maintenance in the CA1 region of rat hippocampal slices and in the dentate gyrus of freely moving rats.[12, 16, 17] In addition, experiments conducted in our lab have shown

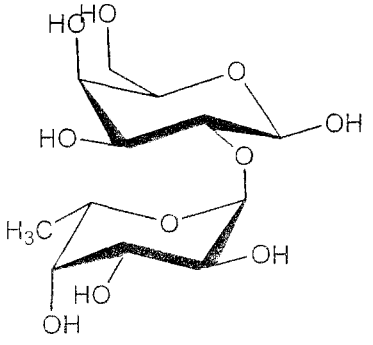

Figure 2. Fucoseα(1-2)galactose [Fucα(1-2)Gal]

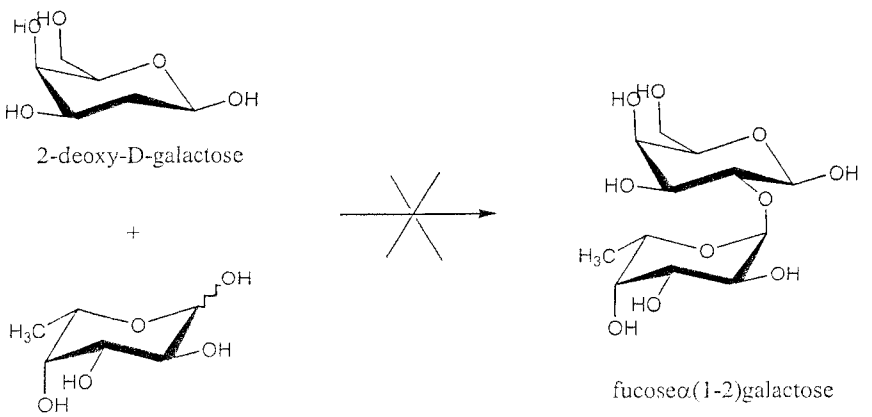

Figure 3. The lack of a C-2 hydroxyl group of 2-deoxy-D-galactose (2-dGal) prevents the α(1-2) glycosidic linkage formation with fucose

that treatment of hippocampal neurons with 2-dGal inhibits neurite outgrowth (Gama and Hsieh-Wilson, unpublished results).

These experimental results show that fucose might play a crucial role in learning and memory; however, the detailed mechanism of how fucose affects learning and memory process is unknown and remains a great challenge.

2. Structural Patterns of Fucosylated Glycans

L-Fucose (6-deoxy-L-galactose) is an important monosaccharide component of many *N*- and *O*-linked glycans produced in a wide variety of organisms. Fucose is characteristic in two structural features: the lack of a hydroxyl group on the carbon at the 6-position and the L-configuration.[19] Through the improving methods in glycan

analysis, various fucose containing glycans have been analyzed and the knowledge about the structural features of fucosylated glycans has increased.

Two features that often vary for fucosylated glycans are the number of fucose moieties and the type of linkage of fucose. Fucose is frequently found as a terminal modification of glycan structures. Normally, fucose is attached by an alpha linkage to galactose (Gal) or to *N*-acetylglucosamine (GlcNAc) (Table 1);[20] however, non-terminal fucose epitopes have also been shown. For instance, in *Schistosoma*, a human parasite

Table 1. Typical structures of fucosylated glycans

Name	Structure
Lewis^x; Le^x; CD15	Galβ(1-4)GlcNAc- | Fucα(1-3)
Lewis^y; Le^y	Fucα(1-2)Galβ(1-4)GlcNAc- | Fucα(1-3)
Lewis^a; Le^a	Galβ(1-3)GlcNAc- | Fucα(1-4)
Lewis^b; Le^b	Fucα(1-2)Galβ(1-3)GlcNAc- | Fucα(1-4)

that can generate many kinds of fucosylated glycans, several fucosylated glycans with multifucosylated sequence such as Fucα(1-2)Fucα(1-3) or Fucα(1-2)Fucα(1-2)Fucα(1-3) have been found, and a unique multifucosylated sequence: Fucα(1-2)Fucα(1-[3GalNAcβ(1-4)Fucα(1-2)Fucα(1-2)Fucα(1-3)GlcNAcβ(1-3)Galα(1-] has been identified on their *O*-glycans.[21-23] Unusual *O*-linked fucosylation, in which fucose is linked directly to the hydroxyl groups of serines or thereonines, has also been known.[24] This kind of fucosylation has been shown to occur in epidermal growth factor (EGF)-like module on a few proteins, such as tPA, plaminogen activators and blood clotting factors. Most of this kind of modification occurs to a specific consensus sequence C-X-X-G-G-T/S-C (X: any amino acid). But, there are some cases showing that *O*-fucosylation does not match this sequence.[25, 26] For instance, the Notch receptor is a transmembrane signaling receptor that can initiate *fringe* gene transcription by extracellular stimuli. This receptor is modified by *O*-fucosyltransferase (OFUT1), so that fucose is attached to a

Table 2. Acceptors of fucosyltransferases

Name	Structure
Type 1	Galβ(1-3)GlcNAc
Type 2	Galβ(1-4)GlcNAc
Type 3	**Galβ(1-3)GalNAc**

serine or threonine within EGF domain, and it is found that the *O*-linked fucose is required for Notch signaling process.[27]

Fucosylated glycans are synthesized by fucosyltransferases. Complete and partial sequences of many fucosyltransferases originating from prokaryotes and eukaryotes, have been determined. At this time, thirteen different fucosyltransferase genes have been identified in the human genome.[28, 29] For instance, human α(1-2)-fucosyltransferases are responsible for the transfer of fucose to the α(1-2)-linkage of a terminal residue. The H-type α(1-2)-fucosyltransferases, an enzyme that is expressed from the gene *FUT1*, is found to prefer type 2 substrates (Table 2), while the Se-type α(1-2)-fucosyltransferases, another enzyme that is expressed from the gene *FUT2*, is found to prefer type 1 and type 3 substrates.[30, 31]

3. Approaches toward the Identification of Fucosylproteins in the Rat Hippocampus and the Characterization of Its fucosylation

Little is known about how fucose participates in neuronal communication and synaptic plasticity. Based on the known mechanism of other signaling pathways and preliminary results from our group, we have postulated three models for the mechanism of fucose mediated neuronal communications (Figure 4).

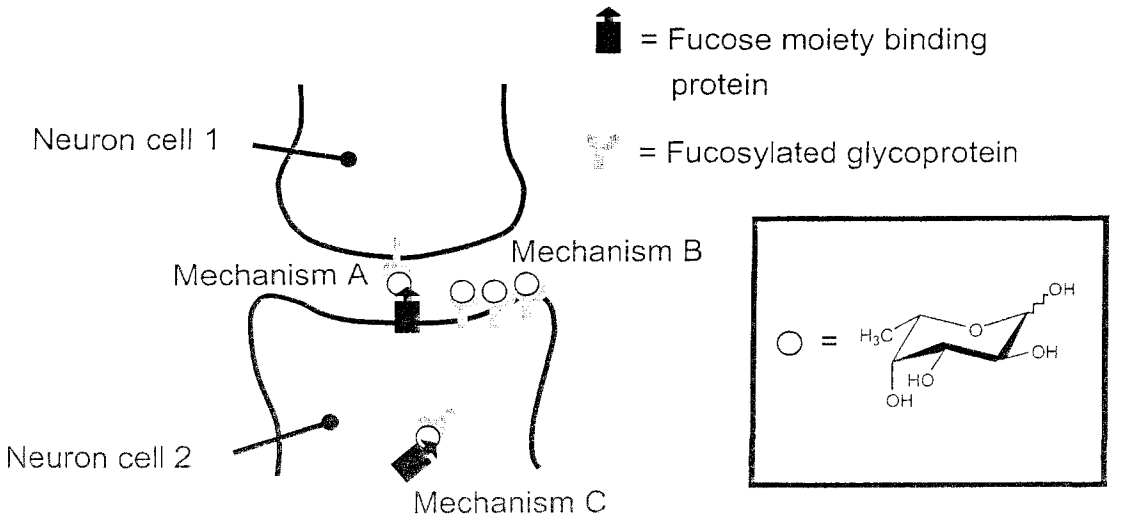

Figure 4. Proposed models for the mechanisms of fucose involvement in cell connections. Mechanism A: fucose moiety functions as a recognition element and mediates protein-protein interactions. Mechanism B: fucose moiety serves as a targeting element, regulating the trafficking of a glycoprotein to the synapse. Mechanism C: fucose moiety mediates the neuronal connections intracellularly.

In the first model (mechanism A), the fucose moiety serves as a "recognition element" and can be recognized by a carbohydrate binding protein, known as a lectin. Through this protein- protein interaction at the cell surface, certain intracellular signaling cascades might be triggered, and neuronal communication can be achieved. In the second model (mechanism B), the fucose moiety serves as a "targeting element" that signals can be directed from fucosylproteins to the synapse by certain cellular events such as protein folding or trafficking, and the epitope may perform a functional role in neuronal communication. Due to the complexity of the brain, it is possible that both models exist simultaneously. Finally, it is also possible that the fucosylproteins mediate

the modulation of neuronal connections from the inside of the cell, instead of at the cell surface.

This research report describes our efforts toward the identification and characterization of fucose bearing glycoproteins in the rat hippocampus. In this pursuit, several approaches have been employed, including one- and two-dimensional gel electrophoresis, mass spectrometry, Western blotting, immunoprecipitation, chemical deglycosylation, enzymatic deglycosylation, lectin binding and radiolabeling. Our preliminary studies suggest that synapsin I, a protein that is important for neurotransmitter release as well as neurite outgrowth, is a fucosyl glycoprotein.

Synapsin I is the first member of the synapsin family, which is known to be one of the most abundant synaptic proteins.[32] We have demonstrated that synapsin I can be recognized by fucose-specific antibody and lectins. We anticipate that identification of fucosyl glycoproteins will help elucidate the role of the fucose moiety in neurons. Moreover, these studies may provide new insights into the molecular mechanisms that regulate neuronal communication and memory formation.

Results and Discussion

1. Fucose Specific Antibody, A46-/B10

Monoclonal antibody, A46-B/B10, which is specific for Fucα(1-2)Galβ(1-4)GlcNAc and/or Fucα(1-2)Galβ(1-4)GalNAc, was obtained as a generous gift from Dr. Uwe Karsten (Max-Delbruck Centre for Molecular Medicine). As described before, studies had shown that this antibody can be used as a probe for Fucα(1-2)Gal bearing proteins, and can cause amnesia in rats.[33, 34] The competition test in our group shows that this antibody also can recognize L-fucαOEt and L-fucβOEt, but not the D-fucose derivatives (Kalovidouris and Hsieh-Wilson, unpublished results). We obtained ascites fluid of this antibody from Cocalico Biologicals (Reamstown, PA), then purified the IgM antibody by using mannan-binding protein immobilized column.

2. Identification of Fucosylproteins in the Rat Hippocampus

Previous work in our group investigated the expression of proteins containing fucose epitope in embryonic (E18) and adult rat hippocampus by using the antibody A46-B/B10 to determine if the fucose epitope is present at synapse. The hippocampus region of E18 and adult rat brain was dissected, dissolved and lysed. The lysate was resolved on 1-dimensional (1-D) SDS-PAGE gels. All proteins on the gel were transferred onto PVDF membrane, and immunoblotted with the primary antibody A46-B/B10. The

results indicate that several glycoproteins can be recognized by this antibody. In the adult rat hippocampus, two major fucosylproteins of approximately 70kDa and 75kDa were observed, as well as minor fucosylproteins of approximately 40kDa and 60kDa. In the embryonic rat hippocampus, by using the antibody A46-B/B10, different fucosylproteins, including two major fucosylproteins from the embryonic brain are approximately 55kDa and 60kD, were detected. These results suggest that the expression of the fucose epitope and/or the expression of the fucose containing glycoproteins is developmentally regulated.

To identify fucosylproteins in the rat hippocampus, we used a proteomic approaches. The hippocampal region from the adult rat brain was dissected, dissolved and lysed in a buffer containing protease inhibitors. The cellular lysates were resolved by 1-D or 2-D SDS-PAGE gel. For the 1-D gels, proteins were separated by molecule size under denaturing conditions in the presence of SDS. For the 2-D gels, proteins were separated by isoelectric focusing, in the first dimension, and by molecule size under denaturing conditions in the second dimension. Proteins resolved on the gels were stained using Coomassie Brilliant Blue, or were Western blotted by transfer onto PVDF membrane, followed by probing with the antibody A46-B/B10. Then, we compared the Coomassie Blue stained gel with the corresponding Western blot. Several bands on the Coomassie

Blue stained gel corresponded to the bands on the Western blot, and were excised from the gel. After this treatment, the excised bands were digested with trypsin, a protease which cleaves the peptide bond at either lysine or arginine residues. The resulting tryptic peptide mixtures were then analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).[35-38]

Using this procedure, we identified the major bands of approximately 70kDa and 75kDa from the Coomassie Blue stained gels as synapsin Ia and Ib. 11 peptides had masses within 50 ppm of the predicted masses for trypsin-digested synapsin I from the NCBI database (Table 3). These matching peptides provide 11.2% sequence coverage

Measured Masses	Calculated Masses	Residues	Amino Acid Sequences
771.4974	771.4841	169-177	RNGVKVVRS
816.4343	816.4402	413-421	KMTQALPRQ
832.4416	832.4351	413-421 Met-Ox	KMTQALPRQ
878.4217	878.4372	328-337	RTSVSGNWKT
908.4891	908.4590	554-564 pyroGlu	RQAGPPQATRQ
946.4320	946.4205	186-195 pyroGluMet-Ox	RQHAFSMARN
951.4920	951.4648	554-564 pyroGlu	RQASQAGPGPRT
963.4618	963.4471	186-195 Met-Ox	RQHAFSMARN
968.4832	968.4914	600-611	RQASQAGPGPRT
1001.4694	1001.4944	695-704	RKSFASLFSD
1187.6637	1187.7152	176-187	RSLKPDFVLIRQ

Table 3. Synapsin I peptides identified from the MALDI-TOF peptide mass map of the synapsin I sequence. The peptides correspond to the correct species (rat), and the protein has the correct molecular weight (74kDa for synapsin Ia and 70kDa for synapsin Ib), and pI value (9.8). Importantly, these results meet nearly all of the criteria established by Jensen *et al*, for the positive identification of proteins by MALDI-TOF

analysis.[39]

In addition, we observed a third protein that migrated close to synapsin Ia and Ib of approximately 80kDa, called *N*-ethylmaleimide-sensitive factor (NSF). 24 peptides had masses within 50 ppm of the predicted masses for trypsin-digested NSF from the NCBI database (Table 4). These matching peptides provide 29.7% sequence coverage of the

Measured Masses	Calculated Masses	Residues	Amino Acid Sequences
573.3811	573.3724	266-272	KTLLARQ
677.3547	677.3405	4-11	RTMQAARC
715.4395	715.4507	702-709	KVWIGIKK
750.4602	750.4336	728-735	KFLALMRE
766.4367	766.4286	728-735 Met-Ox	KFLALMRE
779.4186	779.3908	581-588	KCQAMKKI
789.4878	789.5238	143-151	KLFGLLVKD
812.4583	812.4670	44-51	KYIFTLRT
830.4079	830.4161	509-517	KWGDPVTRV
871.4451	871.4202	587-595	KIFDDAYKS
894.4840	894.5235	727-735 Met-Ox	RKFLALMRE
903.4636	903.4900	469-478	KAESLQVTRG
913.4401	913.4420	225-233	KEFSDIFRR
973.5584	973.6046	639-649	KLLIIGTTSRK
999.4333	999.4821	572-582 Met-Ox	KMIGFSETAKC
1013.5810	1013.5566	271-281 PyroGlu	RQIGKMLNARE
1137.5060	1137.5540	293-304	KYVGESEANIRK
1142.6107	1142.6574	607-618	RLLDYVPIGPRF
1145.5678	1145.6166	187-199	KAENSSLNLIGKA
1207.5369	1207.5595	304-315	KLFADAEEEQRR
1231.5816	1231.6244	150-162	KDIEAMDPSILKG
1291.6136	1291.6646	434-447	KNFSGAELEGLVRA
1294.6103	1294.6683	555-567	KIAEESNFPFIKI
1335.5678	1335.6116	27-39	KDYQSGQHVMVRT

Table 4. NSF peptides identified from the MALDI-TOF peptide mass map

NSF sequence. The peptides correspond to the correct species (rat), molecular weight (83kDa), and pI value (6.4). NSF is involved in synaptic vesicle exocytosis. It is an ATPase that requires SNAP in order to bind to the SNARE complex. Upon ATP hydrolysis by NSF, it helps the dissociation of the SNARE core complex.[40]

To further investigate whether synapsin Ia and Ib were fucosylated, the two proteins were isolated from rat hippocampal lysates by immunoprecipitation[41] and were probed with the antibody A46-B/B10 and an anti-synapsin I antibody by Western blotting. The Western blots show that synapsin Ia and Ib can be successfully immunoprecipitated, and are recognized by the fucose-specific antibody A46-B/B10 (Figure 5). This data strongly suggests that synapsin Ia and Ib are fucosylated.

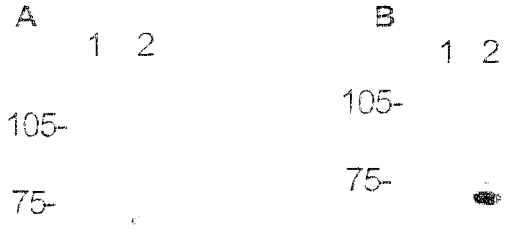

Figure 5. Immunoprecipitation (IP) of synapsin I from adult rat hippocampal lysates. (A) Western blot of immunoprecipitated proteins probed with anti-synapsin I. (B) Western blot of immunoprecipitated proteins probed with antibody A46-B/B10. Upper band is synapsin Ia, lower band is synapsin Ib
Lane 1: control beads (IP without anti-synapsin I).
Lane 2: IP beads (IP with anti-synapsin I).

To examine the fucosylation of NSF, the protein was isolated from rat hippocampal lysates by immunoprecipitation and was probed with the antibody A46-B/B10 and an anti-NSF antibody by Western blotting. The Western blots show that NSF can be successfully immunoprecipitated, but cannot be recognized by the fucose-specific

antibody, A46-B/B10, confirming that NSF is not fucosylated (Figure 6).

Figure 6. Immunoprecipitation (IP) of NSF from adult rat hippocampal lysates. (A) Western blot of immunoprecipitated proteins probed with anti-NSF. (B) Western blot of immunoprecipitated proteins probed with antibody A46-B/B10. Lane 1: control beads (IP without anti-NSF). Lane 2: IP beads (IP with anti-NSF)

3. Physiological Role and the Function of Synapsin I

Synapsin I, the first member of the synapsin family, was discovered in 1972. This family of proteins is one of the most abundant of synaptic proteins, and comprised about 1% of the total protein content in the brain. The synapsins are major substrates for cyclic AMP (cAMP)-dependent protein kinase and Ca^{+2}/calmodulin-dependent protein kinase. In addition, they are expressed only in neurons and are specifically located in the presynaptic terminal of the synapses. The family is encoded by three distinct genes, synapsin I, II and III, which, due to different mRNA splicing forms, generate five known synapsins, Ia, Ib, IIa, IIb and IIIa.[35] The difference between the a type and the b type isoforms of the synapsins is restricted to the C-terminal region. The expression of the

synapsin isoforms among neurons varies in different regions of the brain. However, distinct functions for the isoforms have not been determined at this point. The primary structure of the

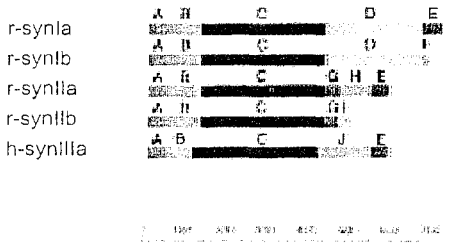

Figure 7. Domain model of the synapsin family, including rat synapsin Ia (r-synIa) and Ib (r-synIb), rat synapsin IIa (r-synIIa) and IIb (r-synIIb), and human synapsin IIIa (h-synIIIa). Scale bar, amino acid residues.

synapsins can be divided into several domains (Figure 7), the most conserved domains of which are the A and C domains. The A domain has been shown to control the interaction of synapsin with vesicles via phosphorylation and de-phosphorylation, such that nearly all phosphorylated synapsin I is found in the cytosol and non-phosphorylated synapsin I is associated with the vesicle membrane. The C domain has been suggested to have enzymatic capabilities due to its structural similarity to some ATP-dependent synthetases, but this has not been confirmed. The E domain has been shown to be a certain component for the a isoform of synapsins at the C-terminal region.[42-44]

The synapsin family is believed to be involved in neurotransmitter release and neuronal development. Synapsins are able to interact in a phosphorylation-dependent manner with various cytoskeletal proteins, including actin (Figure 8). Although the

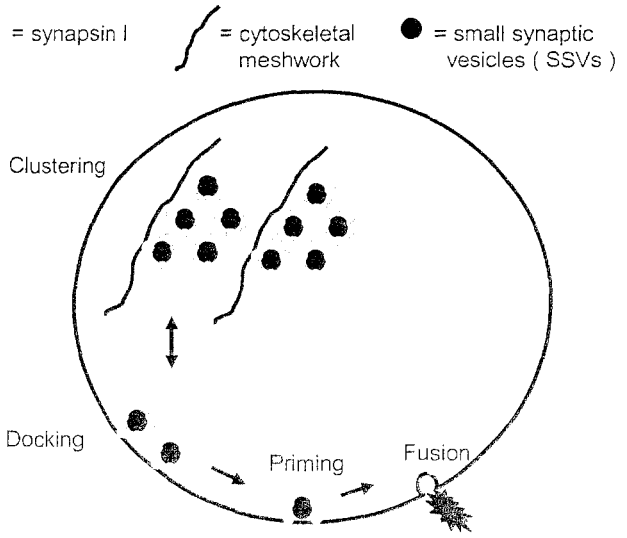

Figure 8. The model for synapsins serving as a regulator of neurotransmitter release. Synapsins are believed to tether synaptic vescles and cytoskeletal meshwork together in the nerve terminal

underlying mechanisms of synaptic vesicle cluster formation are not completely understood, previous studies have revealed that synapsins maintain the reserved pool of vesicles by tethering synaptic vesicles to each other and to the cytoskeletal meshwork.[32, 45-46] In addition, the synapsins are also crucial for the formation of synaptic contacts, neurite outgrowth and synaptogenesis.[32, 46] Abnormal synapse formation has been observed in synapsin I knock-out mice, and retardation of synapse formation during the initial phases of development has been shown in synapsin I, II, and I/II deficient cultured hippocampal neurons. It is known that each synapsin has a distinct role in the stages of the elongation and differentiation of axons and dendrites, while the mechanisms underlying synapsins-regulated neurite outgrowth remain unclear. Experimental data indicates that synapsin I and II have similar functions during the process of neurotransmitter release and the formation of synaptic contacts, while synapsin III seems

to have a different functional role in both processes.[32, 45-47]

4. Characterization of the Fucosylation of Synapsin I

To establish that synapsin I is indeed fucosylated and identify the specific sites of fucosylation on the synapsins, several approaches, including chemical and enzymatic cleavage of the carbohydrate moiety, and radiolabeled fucose incorporation into mammalian cell expressed synapsin Ia, were tried.

4.1 Enzymatic *N*-Deglycosylation

To investigate whether the fucose moiety is *N*- or *O*- linked to synapsin I, we tried *N*-deglycosylation by using both PNGaseF and EndoH_f, which are enzymes that cleave carbohydrate moiety attached to asparagines via a *N*-linkage. The hippocampal region of adult rat brain was dissected, dissolved and lysed in lysis buffer containing protease inhibitor. The samples were treated with either PNGaseF or EndoH_f. The results from both enzymatic cleavages indicate that fucosyl glycans are not cleaved by PNGaseF or EndoH_f (Figure 9). Although the resistance of PNGaseF deglycosylation of some fucosylated glycoconjugates has been reported,[48] it is interesting to note that the amino acid sequence analysis of synapsin Ia and Ib reveals only one consensus site for *N*-linked

glycosylation. These results imply that the fucose moiety is *O*-linked to the synapsin I and further investigation has to be done.

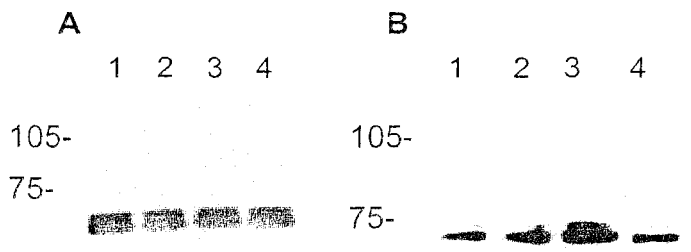

Figure 9. Enzymatic *N*-deglycosylation of synapsin I from adult rat hippocampal lysate. (A) Western blot of proteins from lysate probed with anti-synapsin I. (B) Western blot of protein from lysate probed with antibody A46-B/B10. Upper band is synapsin Ia and lower band is synapsin Ib.
Lane 1: experiment of PNGaseF *N*-deglycosylation (with PNGaseF).
Lane 2: control of PNGaseF *N*-deglycosylation (without PNGaseF).
Lane 3: experiment of Endo H_f *N*-deglycosylation (with Endo H_f).
Lane 4: control of Endo H_f *N*-deglycosylation (without Endo H_f).

4.2 Fucose-specific Lectin Binding Assay

Three fucose specific lectins were tested for the recognition of synapsin I: TP, UEA-I and AAA. TP is known to most strongly binding to Lewis^y epitope, UEA-I binds favorably to H type 2 fucosylated glycan and AAA is binds favorably to Lewis^b or Fucα(1-6) terminal residue.[49] The lectin binding test on 4 different types of proteins was performed using the three different lectins. Synapsin I, glycosylated proteins

Lewis^{x}-BSA (MW 66) as a positive control, and non-fucose containing protein CREB (MW 43) and non-glycosylated protein BSA (MW 65) as negative controls. The positive control Lewis^{x}-BSA can be recognized by TP, but not by UEA-I and AAA. The negative controls BSA and CREB can not be recognized by any of the three lectins. Synapsin I can be recognized by TP and UEA-I, but not by AAA, the reason might be due to that the fucosylated glycan pattern of synapsin I can not be recognized by AAA. These results provide evidence that synapsin I is a fucosylated protein (Figure 10).

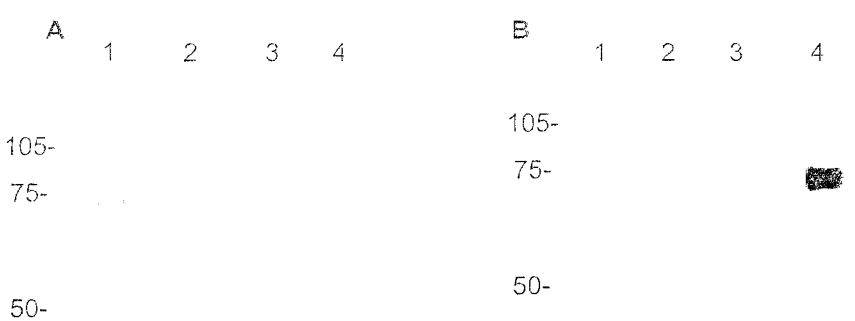

Figure 10. Western blot probed with lectins.
(A) Western blot probed with UEA-I. (B)Western blot probed with TP.
Lane 1: synapsin I
Lane 2: BSA (MW 65)
Lane 3: CREB (MW 43)
Lane 4: Lewis^{x}-BSA (MW 66)

4.3 Radiolabeled Fucose Incorporation

To examine whether the synapsin I is fucosylated, radiolabeled fucose incorporation

was utilized in this pursuit.

Antibody G143, which can recognize synapsin Ia, Ib, IIa and IIb, and antibody G304, which can recognize synapsin Ia, IIa and IIIa, were tested for the optimum concentration of Western blotting and the specificities of synapsin isoforms. Adult and embryonic rat hippocampal lysates were used as the sample sources. The test shows that these two antibodies are very specific to their antigens (Figure 11).

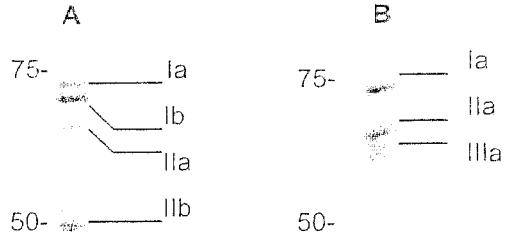

Figure 11. (A)Western blot of synapsins from adult hippocampal lysate probed with G143; (B)Western blot of synapsins from embryo hippocampal lysate probed with G304.

Different mammalian cell systems, including HEK293, HeLa and CHO cells, and different transfection methods, including calcium phosphate precipitation method and lipid-mediated transfection method, were performed to test the expression of synapsin Ia. For the calcium phosphate precipitation method, HEK 293 cells were first used to test the optimum pH value of HEPES-buffered saline solution (HeBS) for calcium phosphate precipitation since the optimum pH range for this method is extremely narrow (between

7.05 and 7.12).[50] Transfected cells were lysed in lysis buffer and resolved on SDS-PAGE gel. Proteins on the gel were then transferred onto PVDF membrane, and the membrane was probed with A46-B/B10 or an anti-synapsin I antibody (Chemicon).

In order to confirm that the expressed protein is synapsin Ia, immunoprecipitation was conducted after successful transfection of synapsin Ia in HEK293 cells.[41] Synapsin Ia was expressed in HEK 293 cells through calcium phosphate precipitation, and immunoprecipitated by anti-synapsin I (Chemicon). The Western blot of immunoprecipitation probed with anti-synapsin I shows that synapsin Ia from HEK 293 can be immunoprecipitated by anti-synapsin I, and the Western blot probed with A46-B/B10 shows that the expressed synapsin Ia from HEK 293 is fucosylated (Figure 12).

Figure 12. Immunoprecipitation (IP) of synapsin Ia from expressed from HEK 293 cells, which were transfected by using calcium phosphate precipitation method. Synapsin Ia was immunoprecipitated with anti-synapsin I.
(A) Western blot of immunoprecipitated protein probed with antibody anti-synapsin I.
(B)Western blot of immunoprecipitated protein probed with A46-B/B10.
Lane 1: IP beads (IP with anti-synapsin I).
Lane 2: control beads (IP without anti-synapsin I).

After the confirmation that synapsin Ia can be expressed in mammalian cell system, we conducted radiolabeled fucose incorporation experiments.[51-54] Cells were incubated with L-[5,6-^{3}H]fucose followed by transfection and immunoprecipitation. Although the radiolabeled fucose has been incorporated into the cell system, the autoradiography revealed only low level of radiolabeled fucose incorporation, which suggests that the signal from radiolabeled synapsin Ia might be too weak to be detected. There are at least two possible reasons for the unsuccessful incorporation. The first reason might be due to the lack of appropriate fucosyltransferase in HEK 293 cell system. To test this possibility, we (1) used different mammalian cell lines, including HeLa and CHO cells; and (2) co-transfected synapsin Ia with fucosyltransferase plasmids. The calcium phosphate precipitation method did not work well in HeLa and CHO cell system. The ineffective transfection might be due to the unmodified transfection conditions. Calcium phosphate precipitation method is finicky since this method is sensitive to many parameters including pH and cell line type.[50] In order to use these two different cell lines, we had to use a different transfection method. So, lipid-mediated transfection was chosen for the expression in HeLa and CHO cell system (Figure 13). Fucosyltransferase plasmids, *FUT1* and *FUT2* (kindly provided by Dr. Lowe),[30, 31] were also co-transfected to increase the extent of fucosylation of synapsin Ia (Figure 14). The blot probed with

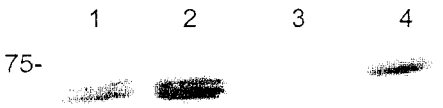

Figure 13. Synapsin Ia expressed by using Lipofectamine 2000 (Invitrogen) mediated transfection method. The Western blot is probed with TP.
Lane 1: CHO, transfected with plasmid (4 μg synapsin Ia + 4 μg pcDNA3.1).
Lane 2: CHO, transfected with plasmid (8 μg synapsin Ia).
Lane 3: HeLa, transfected with plasmid (4 μg synapsin Ia + 4 μg pcDNA3.1).
Lane 4: HeLa, transfected with plasmid (8 μg synapsin Ia).

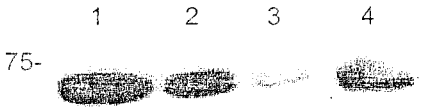

Figure 14. Synapsin Ia expressed by using Lipofectamine 2000 (Invitrogen) mediated transfection method. The Western blot is probed with TP.
Lane 1: HEK 293, transfected with plasmid (4 μg synapsin Ia + 4 μg pcDNA3.1).
Lane 2: HEK 293, transfected with plasmid (4 μg synapsin Ia + 4 μg *FUT* 1).
Lane 3: HEK 293, transfected with plasmid (4 μg synapsin Ia + 4 μg *FUT* 2).
Lane 4: HEK 293, transfected with plasmid (8 μg synapsin Ia).

lectin TP shows that the expressed synapsin Ia is fucosylated; however, the blot probed with G304 indicates that lipid-mediated transfection efficiency is quite low. Another test of the blot probed with G143 shows much stronger signals of the expressed synapsin Ia (Figure 15); however, the molecular weight of the expressed synapsin Ia is lower than the correct molecular weight of synapsin Ia. A possible implication of this result is that the expressed synapsin Ia might be truncated in the end since G304 can recognize the

epitope closed to C-terminal domain, while G143 can recognize the epitope closed to the N-terminal domain, or the expressed synapsin Ia was degraded due to unclear reasons. We believe that HeLa and CHO cells are both possible alternative for transfection system. We can further pursue the radiolabeling studies using these cells.

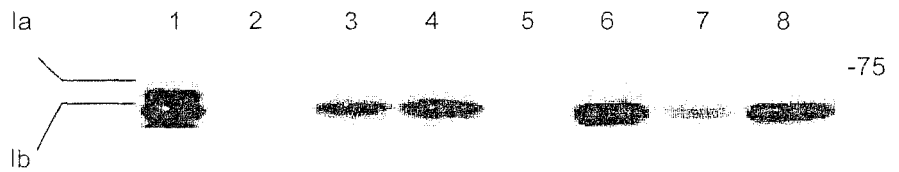

Figure 15. Synapsin Ia expressed by CHO cells using Lipofectamine 2000 mediated transfection method and by HEK293 cells using calcium phosphate precipitation method. Western blot was probed with G143.
Lane 1: synapsin I from adult hippocample cell lysate. The upper band is synapsin Ia and the lower band is synapsin Ib.
Lane 2: CHO, transfected with plasmid (16 μg pcDNA3.1).
Lane 3: CHO, transfected with plasmid (8 μg synapsin Ia + 8μg pcDNA3.1)
Lane 4: CHO, transfected with plasmid (8 μg synapsin Ia + 8μg *FUT* 2)
Lane 5: HEK 293, transfected with plasmid (10 μg pcDNA3.1).
Lane 6: HEK 293, transfected with plasmid (5 μg synapsin Ia + 5μg pcDNA3.1)
Lane 7: HEK 293, transfected with plasmid (5 μg synapsin Ia + 5μg *FUT* 1)
Lane 8: HEK 293, transfected with plasmid (5 μg synapsin Ia + 5μg *FUT* 2)

A second possible reason for this unsuccessful incorporation might be due to the nature of fucose metabolism pathway. Two pathways have been demonstrated for the synthesis of GDP-fucose in the cytosol of all mammalian cells: the *de novo* pathway and the salvage pathway. The *de novo* pathway transforms GDP-mannose to GDP-fucose

via enzymatic reactions, and the salvage pathway synthesizes GDP-fucose from extracellular or lysosomal free fucose. Quantitative studies of fucose metabolism in HeLa cells indicate that greater than 90% of GDP-fucose is derived from the *de novo* pathway, even in cells incubated with radiolabeled fucose.[55, 56] These results imply that the incorporation of fucose in the media should be quite low. To resolve this problem, the suppression of the *de novo* pathway can be conducted.

4.4 Chemical Deglycosyaltion

Trifluoromethanesulfonic acid (TFMS) was employed for chemical deglycosylations, but unsuccessful because of serious degradation of the proteins of lysate samples.[57-59] We also attempted deglycosylation of lysate samples using the ECL glycoprotein detection kit, which uses sodium metaperiodate to oxidize carbohydrate to form aldehydes, which are selectively labeled with biotin-conjugated hydrazine. Our preliminary results of periodate cleavage were promising, and thus this method can be pursued further.

4.5 Enzymatic Deglycosyaltion by Fucosidase

Attempted deglycosylation of lysate samples with α(1-2) fucosidase (Calbiochem)

was performed, but was also unsuccessful, and the reason might be due to other factors existed in lysate sample that can cause the degradation of the enzyme, or the fucosidase is inaccessible to cleave the glycan on the intact protein because of the wrapping of glycans by the folding synapsin I.[60-63] To circumvent this potential problem, mass analysis of the fucosidase treatment of purified synapsin I resolved on SDS-PAGE gel was conducted. Protein bands were excised. The in-gel trypsin digestion of excised protein samples was conducted and the tryptic digestion peptides were further treated with α(1-2, 3, 4, 6)-fucosidase. Samples, including before fucosidase treatment and after fucosidase treatment, were then analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The control mass spectrum of synapsin Ib trypsin digests treated without fucosidase is given in Figure 16A, and the mass spectrum of synapsin Ib trypsin digests treated with fucosidase is given in Figure 16B. The spectrum was submitted to the database, and the protein was confirmed by searching databases with lists of known tryptic peptide masses generated by mass spectra. The results from 5 independent experiments show that there is no evident missing peak or newly generated peak after fucosidase treatment. This indicates the cleavage of fucose from synapsin I was not successful.

There are at least two possibilities. First, it is possible that this type of fucosidase,

which is more specific to α(1-6) linkage, might not work well in our system; second, the abundance of fucosylation of synapsin I might be too low to be detected; third, the fucosylated peptide fragments may not be detectable due to ion suppression effects or the size of the fragmentation. Other types of fucosidase can be tried, and the future efforts can shift toward the analysis of fucosylated synapsin I fragment instead of using synapsin I. To test the first possibility, we can use different fucosidases to perform this experiment. To test the second possibility, the level of fucosylated synapsin I can be increased using methods such as lectin affinity column, to pull down the fucosylated

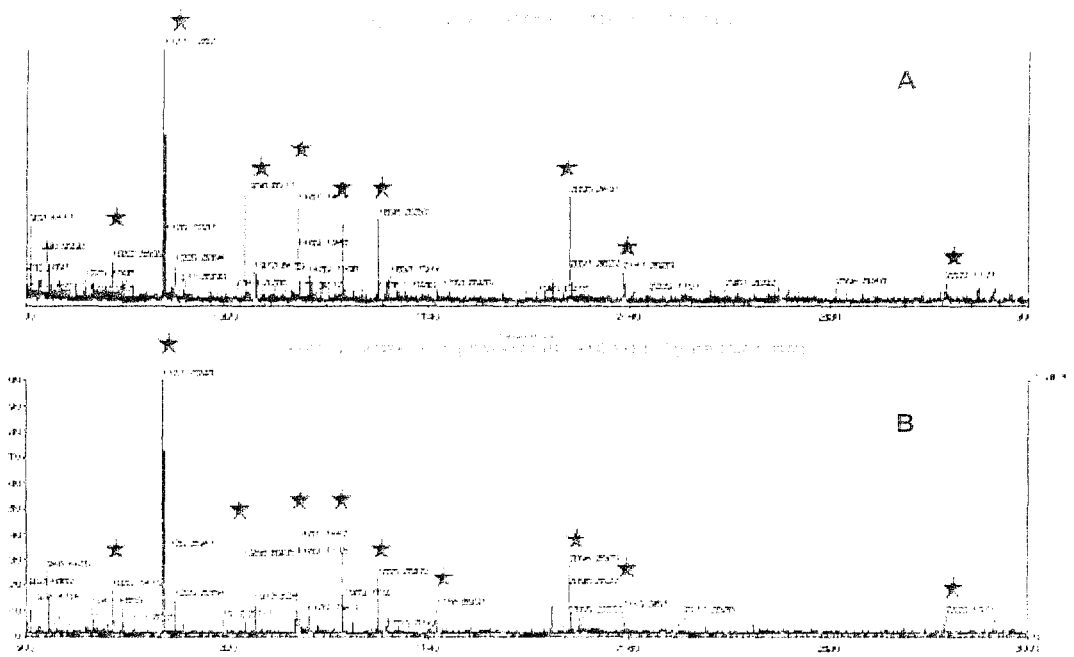

Figure 16. MALDI-TOF mass spectrum of peptides of obtained from trypsin digested synapsin Ib, major synapsin Ib peaks labeled with asterisk on the top of the peak.
(A) Trypsin digest of synapsin Ib treated without α(1-2, 3, 4, 6)-fucosidase.
(B) Trypsin digest of synapsin Ib treated with α(1-2, 3, 4, 6)-fucosidase.

synapsin I protein or peptides, and then perform MALDI-TOF experiment.

Experimental Section

General. Unless otherwise stated, chemicals were purchased from commercially available sources; molecular biology reagents were purchased from Fisher (Fairlawn, NJ), and were used as received. Protease inhibitors were purchased from Roche (Indianapolis, IN) and Aldrich (ST.Louis, MO). Absorbance spectra were performed on a Uvikon Spectrophotometer 930. Purified synapsin I from bovine brain and plasmid of rat synapsin Ia were a generous gift from Dr. Porton (Laboratory of Molecular and Cellular Neuroscience, The Rockfeller University, New York, NY). Antibody A46-B/B10, which is specific for fucose residue, was a generous gift from Dr. Uwe Karsten (Max-Delbruck Centre for Molecular Medicine, Germany). Antibody anti-synapsin I was purchased from Chemicon (Temecula, CA). Antibody anti-synapsin G143, which can recognize synapsin Ia, Ib, IIa and IIb, and anti-synapsin G304, which can recognize synapsin Ia, IIa and IIIa, were generous gifts from Dr. Porton (Laboratory of Molecular and Cellular Neuroscience, The Rockfeller University, New York, NY). The expression plasmid of synapsin Ia was kindly provided by Dr. Porton (Laboratory of Molecular and Cellular Neuroscience, The Rockfeller University, New York, NY). The expression plasmid of the human H-type blood group α(1,2)-fucosyltransferase (*FUT1*) and human Se-type blood group α(1,2)-fucosyltransferase (*FUT2*) were kindly provided by Dr. Lowe (Howard Hughes Medical Institute, University of Michigan, Ann Habor, MI). The plasmid pcDNA3.1 was used for mock transfection.

Preparation of Antibody A46-B/B10. A46-B/B10 containing ascites fluid was obtained from Cocalico Biologicals (Reamstown, PA), and purified the IgM antibody by using mannan-binding protein (MBP) immobilized column (Pierce) according to the instructions from manufacturer.

Adult Rat Hippocampal Dissection. 100g male Sprague-Dawley rats were purchased from Charles River Laboratories (Kingston, Mass) and housed at the California Institute of Technology Laboratory Animal Facilities. Rats were anesthesize with carbon dioxide for 5 minutes and immediately euthanized by decapitation with a guillotine (Kent Scientific Co.) All veterinary care, treatment, and euthanasia procedures were conducted according to guidelines set forth by Caltech's Institution Animal Care and Use Committee (IACUC). The brain was promptly removed and placed on ice. The hippocampus was quickly dissected, lysed in lysis buffer [0.2% sodium deoxycholate;

150 mM NaCl; 50 mM Tris; 1% NP-40 (pH 8.0)] supplemented with protease inhibitors (aprotinin, 20 μg/ml; leupeptin, 20 μg/ml; antipain, 20 μg/ml; chymostatin, 5 μg/ml; pepstatin, 5 μg/ml; PMSF, 1 mmol), homogenized with glass Dounce homogenizer, and sonicated. The homogenized solution was then centrifuged at 12,000 rpm for 10 min, the supernatant was removed and considered hippocampal lysates. Protein concentration of hippocampal lysates was determined using the BCA Protein Assay (Pierce).

SDS-PAGE Electrophoresis. For one-dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE), loading dye containing reducing agent dithiothreitol (DTT) was added to each sample. Each sample solution was boiled for 2 min, and then cooled to room temperature. One-dimensional SDS-PAGE was performed using standard methods on the Amersham system. 9% acrylamide gels were used in all experiments. Gels were stained with 0.2% Coomassie Brilliant Blue R250 in 50% methanol in water containing 5% acetic acid for 30 min, then destained overnight with the same solvent, excluding the dye. For two-dimension SDS-PAGE, each sample was mixed with 2D-gel solution (7 M urea; 2 M thiourea; 4% CHAPS; 0.5% ampholyte). Each sample was loaded into isoelectric focusing (IEF) gel-strip (Amersham) and the electrophoresis was performed using IPGphor IEF system (Amersham). Gel-strip was then loaded on the top of the second dimension SDS gel, and proteins were separated using standard methods on the Amersham system as for 1-D gel electrophoresis.

Western Blotting. Proteins on SDS gels were transferred onto PVDF membranes (Millipore) for at least 12 hr in transfer buffer [20 mM Tris-HCl; 120 mM glycine; 20% methanol (pH 8.6)] using standard methods on Amersham blot transfer system. The blotted membranes were reacted with the fucose specific antibody, A46-B/B10, using the following protocol: first, membranes were blocked for 1hr with sodium periodate treated BSA and rinsed with TBST [50 mM Tris-HCl; 150 mM NaCl; 0.1% Tween-20 (pH 7.4)], and then incubated with 0.5 μg/ml antibody A46-B/B10 generated from ascites fluids in TBST overnight at 4°C with rocking. Immunoreactivity was visualized by incubation with a horseradish peroxidase conjugated goat anti-mouse antibody (Pierce, Rockford, IL). Western blot membranes probed with antibody anti-synapsin I (Chemicon), G143 and G304 were blocked with 5% non-fat milk in TBST and rinsed with 0.25% non-fat milk in TBST. Membranes were incubated with antibody anti-synapsin I, G143 or G304 in TBST for 1 hr at room temperature with rocking. Immunoreactivity was visualized by incubation with a horseradish peroxidase conjugated goat anti-rabbit

antibody (Pierce, Rockford, IL).

Lectin TP, *Tetragonolobus purpureas* or *Lotus tetragonolobus*, labeled with horseradish peroxidase, was purchased from Sigma (St.Louis, MO). AAA, *Anguila anguila* agglutinin, and UEA-I, *Ulex europaeus*, labeled with horseradish peroxidase, were purchased from EY Laboratories (San Mateo, CA). For TP blots, membranes were blocked with 5% non-fat milk in TBST and rinsed with 0.25% non-fat milk in TBST, and then incubated with 1.0 μg/ml lectin in TP lectin solution, which was prepared according to the manufacturer (Sigma), overnight at 4℃ with rocking. For AAA and UEA-I, membranes were blocked for 1hr with sodium periodate treated BSA and rinsed with TBST, and then incubated with 1.0 μg/ml lectin in TBST overnight at 4℃ with rocking. Blots were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) on ECL film (Amersham).

In-Gel Digestion of Proteins. Protein bands (for 1-D gel electrophoresis) or spots (for 2-D gel electrophoresis) were excised from the gel, destained and shrunk by dehydration in acetonitrile. Gel pieces were reduced in 10 mM dithiothcritol solution, alkylated with 50 mM iodoacetamide solution, and incubated with 12.5 μg/ml trypsin in 50 mM ammonium bicarbonate solution at least 12 hr at 37℃. The trypsin digested peptide mixture was extracted using 5% formic acid in 50% acetonitrile, dried down to the volume less than 30 μl, and then purified through C18 reverse phase chromatography.

MALDI-TOF MS. Peptide samples were mixed with the matrix, 2,5-dihydroxybenzoic acid (DHB), and deposited onto the surface of stainless steel sample plate by fast evaporation of the solvent. MALDI mass spectra were performed on a Voyager DE-PRO matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Applied Biosystems). Ion signals produced by trypsin autodigestion peptides, which were present in all peptide mass spectra, were used for internal mass calibration standard. Peptide mass errors were within 50 ppm. Proteins were identified by searching databases with lists of known tryptic peptide masses generated by mass spectra. The software, Protein Prospector, was used to query protein nonredundant sequence databases maintained and updated by National Center for Biotechnology Information (NCBI, Bethesda, MD).

Immunoprecipitation. Cell lysate samples in lysis buffer were precleared with a 50% protein A-agarose bead slurry in lysis buffer for 2 hr with rocking at 4℃. The

anti-synapsin G304, which is specific for synapsin Ia, IIa and IIIa, or the rabbit polycolonal anti-synapsin I from Chemicon (Temecula, CA), which is specific for synapsin I (mixture of synapsin Ia and Ib), was pre-incubated with 50% protein A-agarose bead slurry in lysis buffer [0.2% deoxycholate, 50 mM Tris, 150 mM NaCl, 1% NP-40 (pH 8.0), supplemented with protease inhibitors] with rocking at 4°C for 2 hr. Then the antibody-immobilized protein A-agarose beads were gathered by centrifugating at 7,000 x g for 5 min. Precleared lysates were mixed with the antibody-immobilized protein A-agarose beads and incubated with rocking at 4°C for another 3 hr. After incubation, synapsin on the antibody-immobilized protein A-agarose bead was isolated by centrifugation at 7,000 x g for 5 min, washed three times with lysis buffer, and then once with 10 mM Tris buffer (pH 7.4), then dissolved in SDS gel loading dye and boiled for 2 min. Proteins were resolved by 9% SDS gel. The proteins on the gel were transferred onto PVDF membrane, and then probed with the primary antibody A46-B/B10 or the anti-synapsin.

Chemical Deglycosylation. For trifluoromethanesulfonic acid (TFMS) deglycosylation, proteins from hippocampal lysates were precipitated by mixing with a 15% trichloroacetic acid (TCA) solution, then centrifugation, and the pellet was isolated. The pellet was transferred into a reaction vial, and TFMS was added to the reaction vial under nitrogen at 0°C. The reaction was incubated for 2 hr with stirring. After incubation, the reaction mixture was cool down to -20°C in a dry ice-ethanol bath, and then was neutralized by the gradual addition of 60% pyridine in water. The neutralized solution was dialyzed in 0.01% ammonium bicarbonate (pH 7.3) at 4°C and lyophilized. Dried sample was dissolved in SDS gel loading dye and boiled for 2 min.

For sodium metaperiodate deglycosylation, the lysate samples were diluted in 200 mM acetate buffer (pH 5.5), and all procedures were performed according to the instructions of the ECL glycoprotein detection kit (Amersham).

Enzymatic Deglycosylation. For PNGaseF deglycosylation, lysate samples were lysed in denaturing solution (0.5% SDS; 1% β-mercaptoethanol), boiled for 10min. and then cooled to room temperature. Deglycosylation was performed according to the instructions of the manufacturer (New England Biolabs). PNGaseF was added, and the reaction mixture was incubated at 37°C for 18 hr. The reaction was terminated by adding SDS gel loading dye.

For Endo H_f deglycosylation, lysate samples were lysed in denaturing solution (0.5% SDS; 1% β-mercaptoethanol), boiled for 10 min, then cooled to room temperature. Deglycosylation was performed according to the instructions of the manufacturer (New England Biolabs). Endo H_f was added, and the reaction mixture was incubated at 37°C for 18 hr. The reaction was terminated by adding SDS gel loading dye.

For α(1-2) fucosidase deglycosylation experiment, lysate samples were lysed in denaturing buffer [0.4% SDS; 0.2 M β-mercaptoethanol; 200 mM sodium phosphate (pH 5.0)], boiled for 5 min, then cooled to room temperature. Hydrolysis of fucose was performed according to the instructions of the manufacturer (Calbiochem). 5 mU α(1-2) Fucosidase from *Xanthomonas* sp. was added, and the reaction mixture was incubated at 37°C for 18 hr. The reaction was terminated by adding SDS gel loading dye.

For α(1-2, 3, 4, 6) fucosidase deglycosylation experiment, lysate samples or trypsin digested peptide samples, were incubated with 20 mU α(1-2, 3, 4, 6) fucosidase from bovine kidney (Prozyme, San Leandro, CA) at 37°C for 24 hr. After fucosidase treatment, trypsin digested samples were further purified through C18 reverse phase chromatography, dried down, and dissolved in 0.1% TFA solution. Samples were purified through C18 reverse phase chromatography again.

Cell Culture. HEK293 and HeLa cells were cultured and maintained in Dulbecco's modified Eagle media (DMEM) purchased from Gibco (Carlsbad, CA), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin-streptomycin (Gibco, Carlsbad, CA). CHO cells were grown in Ham's F12 media purchased from Fisher (Fairlawn, NJ), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell cultures were incubated in either 60 mm or 100mm dishes, at 37°C in a humidified atmosphere (95%) and 5% CO_2 incubator for 2 or 3 days.

Transfection. Cells were transiently transfected with plasmids using different transfection methods including calcium phosphate precipitation method and lipid-mediated transfection methods. For the calcium phosphate precipitation transfection method, cells were grown to 70% confluence. The expression plasmid was resuspended in $CaCl_2$ solution and then transferred dropwise to HEPES-buffered saline solution (HeBS, pH 7.06) to form a calcium phosphate precipitate. The calcium phosphate precipitate was layered directly onto cells and the cells were incubated at 37°C. After 6 hr incubation, the cell media was replaced by fresh media. Cells were incubated

and harvested at the desired confluence point. For the lipid-mediated transfection method, cells were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were incubated without antibiotics to 90% confluence. The lipid reagent and the expression plasmid were diluted in media without serum and antibiotics respectively. The two parts were then combined and incubated at room temperature for several minutes to allow the plasmid and lipid reagent complex form. The complex was layered directly onto cells. Cells were then incubated at 37°C to the 6 hr, and the cell media was removed and was replaced by fresh media devoid of antibiotics. Cells were incubated and harvested at the desired confluence point.

Radiolabeling. Cells were incubated to the 50% confluence in 10 mm dishes, and transfected with the expression plasmid. After 6 hr incubation at 37°C, the cell media was removed and replaced with 7 ml fresh media containing 20 μCi/ml of L-[5,6-^{3}H]fucose (specific activity 60 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO). The cells were incubated at 37°C and harvested at the desired time point.

Preparation of Cell Lysates. Cell lysates were prepared from confluent cells. Cells were collected and washed with media and then suspended in lysis buffer [0.2% deoxycholate, 50 mM Tris, 150 mM NaCl, 1% NP-40 (pH 8.0), supplemented with protease inhibitors] with the volume 2.5 times the cell pellet weight. The lysed cells were sonicated and then centrifuged at 12,000 rpm for 10 min at 4°C to remove the nuclei and cell debris. The supernatant was then separated. The protein concentration was assayed using the BCA Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin standards.

Autoradiography. Protein gels were stained with Coomassie blue dye and then destained. The destained gels were incubated in Amplify (Amersham, Piscataway, NJ) for 30 min and washed with 2% glycerol solution for 5 min. Autoradiography was performed using Kodak MP films. Protein gels were placed under the film and incubated at -80°C.

Reference

(1) Dwek, R. A. *Chemical Reviews* **1996**, *96*, 683-720.

(2) Feizi, T.; Mulloy, B. *Current Opinion in Structural Biology* **2001**, *11*, 585-586.

(3) Gagneux, P.; Varki, A. *Glycobiology* **1999**, *9*, 747-755.

(4) Helenius, A.; Aebi, M. *Science* **2001**, *291*, 2364-2369.

(5) Kobata, A. *Glycoconjugate Journal* **2000**, *17*, 443-464.

(6) Rademacher, T. W. *Current Opinion in Biotechnology* **1998**, *9*, 74-79.

(7) Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A. *Science* **2001**, *291*, 2370-2376.

(8) Yamaguchi, Y. *Biochimica Et Biophysica Acta-General Subjects* **2002**, *1573*, 369-376.

(9) Martin, P. T. *Glycobiology* **2002**, *12*, 1R-7R.

(10) Cowan, W. M.; Sudhof, T. C.; Stevens, C. F., Eds. *Synapses*; The John Hopkins University Press: Baltimore, 2001.

(11) Rose, S. P. R. *Behavioural Brain Research* **1995**, *66*, 73-78.

(12) Matthies, H.; Kretlow, J.; Smalla, K. H.; Staak, S.; Krug, M. *Neuroscience* **1999**, *91*, 175-183.

(13) Krusius, T.; Finne, J. *European Journal of Biochemistry* **1977**, *78*, 369-379.

(14) Zanetta, J. P.; Reeber, A.; Vincendon, G.; Gombos, G. *Brain Research* **1977**, *138*, 317-328.

(15) Matthies, H.; Schroeder, H.; Smalla, K. H.; Krug, M. *Learning & Memory* **2000**, *7*, 227-234.

(16) Krug, M.; Wagner, M.; Staak, S.; Smalla, K. H. *Brain Research* **1994**, *643*, 130-135.

(17) Matthies, H.; Staak, S.; Krug, M. *Brain Research* **1996**, *725*, 276-280.

(18) Lorenzini, C. G. A.; Baldi, E.; Bucherelli, C.; Sacchetti, B.; Tassoni, G. *Neurobiology of Learning and Memory* **1997**, *68*, 317-324.

(19) Taylor, M. E.; Drickamer, K. *Introduction to Glycobiology*; Oxford University Press: New York, 2003.

(20) Staudacher, E.; Altmann, F.; Wilson, I. B. H.; Marz, L. *Biochimica Et Biophysica Acta-General Subjects* **1999**, *1473*, 216-236.

(21) Xu, X. F.; Stack, R. J.; Rao, N.; Caulfield, J. P. *Experimental Parasitology* **1994**, *79*, 399-409.

(22) Wuhrer, M.; Kantelhardt, S. R.; Dennis, R. D.; Doenhoff, M. J.; Lochnit, G.; Geyer, R. *European Journal of Biochemistry* **2002**, *269*, 481-493.

(23) Huang, H. H.; Tsai, P. L.; Khoo, K. H. *Glycobiology* **2001**, *11*, 395-406.
(24) Harris, R. J.; Leonard, C. K.; Guzzetta, A. W.; Spellman, M. W. *Biochemistry* **1991**, *30*, 2311-2314.
(25) Harris, R. J.; Spellman, M. W. *Glycobiology* **1993**, *3*, 219-224.
(26) Panin, V. M.; Shao, L.; Lei, L.; Moloney, D. J.; Irvine, K. D.; Haltiwanger, R. S. *Journal of Biological Chemistry* **2002**, *277*, 29945-29952.
(27) Okajima, T.; Irvine, K. D. *Cell* **2002**, *111*, 893-904.
(28) de Vries, T.; Knegtel, R. M. A.; Holmes, E. H.; Macher, B. A. *Glycobiology* **2001**, *11*, 119R-128R.
(29) Becker, D. J.; Lowe, J. B. *Glycobiology* **2003**, *13*, 41R-53R.
(30) Larsen, R. D.; Ernst, L. K.; Nair, R. P.; Lowe, J. B. *Proceedings of the National Academy of Sciences of the United States of America* **1990**, *87*, 6674-6678.
(31) Kelly, R. J.; Rouquier, S.; Giorgi, D.; Lennon, G. G.; Lowe, J. B. *Journal of Biological Chemistry* **1995**, *270*, 4640-4649.
(32) Ferreira, A.; Rapoport, M. *Cellular and Molecular Life Sciences* **2002**, *59*, 589-595.
(33) Karsten, U.; Pilgrim, G.; Hanisch, F.-G.; Uhlenbruck, G.; Kasper, M.; Stosiek, P.; Papsdorf, G.; Pasternak, G. *Br. J. Cancer* **1988**, *58*, 176-181.
(34) Zuschratter, W.; Seidel, B.; Smalla, K. H.; Karsten, U.; Scheich, H.; Jork, R. *Neuroreport* **1991**, *2*, 445-448.
(35) Aebersold, R.; Mann, M. *Nature* **2003**, *422*, 198-207.
(36) Parker, K. C.; Garrels, J. I.; Hines, W.; Butler, E. M.; McKee, A. H. Z.; Patterson, D.; Martin, S. *Electrophoresis* **1998**, *19*, 1920-1932.
(37) Walikonis, R. S.; Jensen, O. N.; Mann, M.; Provance, D. W.; Mercer, J. A.; Kennedy, M. B. *Journal of Neuroscience* **2000**, *20*, 4069-4080.
(38) Harvey, D. J. *International Journal of Mass Spectrometry* **2003**, *226*, 1-35.
(39) Jensen, O. N.; Podtelejnikov, A. V.; Mann, M. *Analytical Chemistry* **1997**, *69*, 4741-4750.
(40) Clary, D. O.; Griff, I. C.; Rothman, J. E. *Cell* **1990**, *61*, 709-721.
(41) Daly, C.; Ziff, E. B. *Journal of Neuroscience* **1997**, *17*, 2365-2375.
(42) Hilfiker, S.; Pieribone, V. A.; Czernik, A. J.; Kao, H. T.; Augustine, G. J.; Greengard, P. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences.* **1999**, *354*, 269-279.
(43) Sudhof, T. C. *Nature* **1995**, *375*, 645-653.
(44) Valtorta, E.; Benfenati, F.; Basudev, H. *Biochemical Society Transactions.* **1996**, *24*, 645-653.

(45) Rosahl, T. W.; Spillane, D.; Missler, M.; Herz, J.; Selig, D. K.; Wolff, J. R.; Hammer, R. E.; Malenka, R. C.; Sudhof, T. C. *Nature* **1995**, *375*, 488-493.
(46) Ferreira, A.; Li, L.; Chin, L. S.; Greengard, P.; Kosik, K. S. *Molecular and Cellular Neuroscience* **1996**, *8*, 286-299.
(47) Feng, J.; Chi, P.; Blanpied, T. A.; Xu, Y.; Magarinos, A. M.; Ferreira, A.; Takahashi, R. H.; Kao, H. T.; McEwen, B. S.; Ryan, T. A.; Augustine, G. J.; Greengard, P. *Journal of Neuroscience.* **2002**, *22*, 4372-4380.
(48) Tretter, V.; Altmann, F.; Marz, L. *European Journal of Biochemistry* **1991**, *199*, 647-652.
(49) http://plab.ku.dk/tcbh/Lectins12/DiVirgilio/paper.htm/
(50) http://www.mrw2.interscience.wiley.com/eponline/
(51) Etchison, J. R.; Summers, D. F.; Georgopoulos, C. *Journal of Biological Chemistry* **1981**, *256*, 3366-3369.
(52) Mayliepfenninger, M. F. *Archives of Biochemistry and Biophysics* **1994**, *311*, 469-479.
(53) Wiese, T. J.; Dunlap, J. A.; Yorek, M. A. *Journal of Biological Chemistry* **1994**, *269*, 22705-22711.
(54) Wiese, T. J.; Dunlap, J. A.; Yorek, M. A. *Biochimica Et Biophysica Acta-General Subjects* **1997**, *1335*, 61-72.
(55) Yurchenco, P. D.; Atkinson, P. H. *Biochemistry* **1977**, *16*, 944-953.
(56) Codogno, P.; Bauvy, C.; Seve, A. P.; Hubert, M.; Ogierdenis, E.; Aubery, M.; Hubert, J. *Journal of Cellular Biochemistry* **1992**, *50*, 93-102.
(57) Horvath, E.; Edwards, A. M.; Bell, J. C.; Braun, P. E. *Journal of Neuroscience Research* **1989**, *24*, 398-401.
(58) Sojar, H. T.; Bahl, O. P. *Archives of Biochemistry and Biophysics* **1987**, *259*, 52-57.
(59) Edge, A. S. B.; Faltynek, C. R.; Hof, L.; Reichert, L. E.; Weber, P. *Analytical Biochemistry* **1981**, *118*, 131-137.
(60) Matthews, R. T.; Kelly, G. M.; Zerillo, C. A.; Gray, G.; Tiemeyer, M.; Hockfield, S. *Journal of Neuroscience* **2002**, *22*, 7536-7547.
(61) Lammert, A.; Brockmann, G.; Renne, U.; Kiess, W.; Bottner, A.; Thiery, J.; Kratzsch, J. *Biochemical and Biophysical Research Communications* **2002**, *298*, 798-804.
(62) Goldberg, M.; Peshkovsky, C.; Shifteh, A.; Al-Awqati, Q. *Journal of Biological Chemistry* **2000**, *275*, 24622-24629.
(63) Aspberg, A.; Binkert, C.; Ruoslahti, E. *Proceedings of the National Academy of*

Sciences of the United States of America **1995**, *92*, 10590-10594.