Appendix 2

Early Efforts for Identification of the Fucα(1-2)Gal Glycoproteome

A2.1. Background

Identification of Fucα(1-2)Gal glycoproteins has been technically challenging due to their low abundance in neuronal tissues and the difficulty of isolation by antibody and lectin affinity chromatography. Here we report our early efforts to identify the Fucα(1-2)Gal proteome using an antibody affinity column with antibody A46-B/B10. This monoclonal antibody has been shown to be highly specific in binding the blood group H type II and type IV trisaccharides (Fucα(1-2)Galβ(1-4)GlcNAc and Fucα(1-2)β(1-3)GalNAc respectively).(1) This antibody interferes with a brightness disrimination task in rats when intrahippocampally injected pre- and post-training. The animals have less retention for the learning paradigm and exhibit amnesia for the event, suggesting that the Fucα(1-2)Gal epitope is important for the underlying molecular mechansisms of memory formation in mammals.(2)

We tried multiple approaches to identify and purify Fucα(1-2)Gal glycoproteins such as 1D and 2D-gel electrophoresis. Our studies were hampered by the low binding affinity of the antibody to Fucα(1-2)Gal disaccharides, and our attempts to identify the proteome led to the isolation of mostly cytosolic proteins. We next employed lectin affinity chromatography using the *Lotus tetragonobolus* lectin (LTL) and were able to isolate cytosolic proteins such as CRMP-2 and Rab-GDI, however, we were never able to confirm the presence of a Fucα(1-2)Gal moiety on either protein. We developed a successful method for the isolation of Fucα(1-2)Gal glycoproteins using UEAI lectin

affinity chromatography. Through this approach, we identify tenascin C as a prominent Fucα(1-2)Gal glycoprotein present in rat cortex and confirm is fucosylation by immunoprecipitation experiments. In addition we find the neural cell adhesion molecule NCAM, the Na^+/K^+ ATPase, and multiple heterogeneous ribonucleoproteins.

A2.2. Results

A2.3. Preliminary Attempts to Identify the Fucα(1-2)Gal Proteome by Antibody A46-B/B10 Enrichment and Lectin Affinity Chromatography

To identify Fucα(1-2)Gal glycoproteins from the brain, we first sought to use antibody A46-B/B10 which has previously been shown to recognize physiologically relevant epitopes.(2, 3) Antibody A46-B/B10 was covalently cross-linked with dimethylpimelimidate to protein A-sepharose beads. Coupling efficiency was monitored by dot blot analysis. After the column was created, embryonic rat forebrain lysates were fractionated by standard procedures and purified over the anti-Fucα(1-2)Gal column. A test of different wash conditions suggests that TBST works best to wash away non-specific proteins as opposed to lysis buffer, and we observed the enrichment of many Fucα(1-2)Gal glycoproteins in column eluates as detected by Western blotting with antibody A46-B/B10 (Figure A2.1, A and B). In particular, we observe enrichment of bands at approximately 200, 180, 120, 105, 75, 55, 40, and 35 kDa from TBST washed columns. We next attempted to isolate and identify proteins from the antibody column by running a Coomassie-stained gel in parallel with the Western blot using antibody A46-B/B10 and overlaying the images. While bands were still present in the fourth wash as indicated by Coomassie staining, further washing of the columns led to loss of

enrichment of fucosylated bands, thus we maintained these mild wash conditions in hopes that we could still identify putative Fucα(1-2)Gal glycoproteins. However, eluate lanes from Coomassie-stained gels contained the same enriched bands that were in the wash (Figure A2.1 C), suggesting that Fucα(1-2)Gal enriched bands were too weak to be detected by Coomassie staining. Despite this issue, we attempted to isolate proteins from bands of interest that overlayed with the Western blot in hopes that we could detect them by mass spectrometry. These bands were excised from the Coomassie-stained gel, even if no protein was detected, digested with trypsin, and subjected to MALDI-TOF analysis. The peptides were searched against the nonredundant NCBI protein database. Through this method, we identified pyruvate kinase, the heterogeneous ribonucleoprotein (hnRNP), phosphoenolpyruvate carboxylase (PEPCase), the protein kinase Raf 1, heat shock protein 70, and a mannosidase. We were surprised to isolate mostly cytosolic proteins, suggesting that this methodology was not working properly, and Fucα(1-2)Gal glycoproteins are not enriched in sufficient quantities for MALDI-TOF analysis.

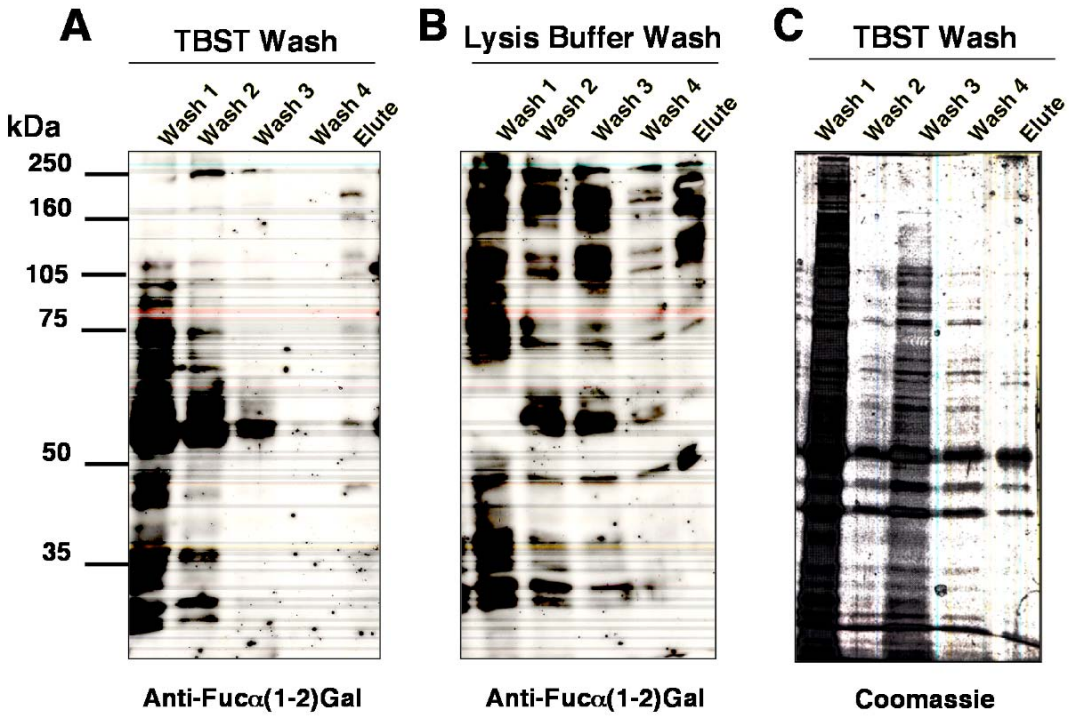

Figure A2.1. Optimization of antibody A46-B/B10 affinity column wash conditions. Western blot using antibody A46-B/B10 of wash conditions with TBST (A), or Lysis buffer wash (B). Coomassie stain of TBST wash conditions (C). Washes 1-4 are 10% (100 μL) of the total wash and are from sequential washes collected. The elution is 20% of the total eluate from the antibody column in (A) and (B), and 80% in (C).

We next attempted to run 2D gel electrophoresis in an attempt to better separate the proteins for MALDI-TOF analysis. However, we were never able to purify enough Fucα(1-2)Gal glycoproteins to detect them on the Coomassie-stained gels nor by silver stain. We also tried lysates before purification. Here, we were able to detect more proteins and overlayed the gels with a western using antibody A46-B/B10 to excise bands of interest (Figure A2.2). Again, we saw similar proteins in the MALDI-TOF analysis, suggesting that this methodology was not working properly either.

Joshua Klein, a rotation student in the laboratory, exploited the use of a known fucose-binding lectin LTL, to run in parallel with the antibody A46-B/B10 column.

Column eluates were immunoblotted with antibody A46-B/B10 and he isolated a larger number of proteins from the LTL column, suggesting that this route was more desirable

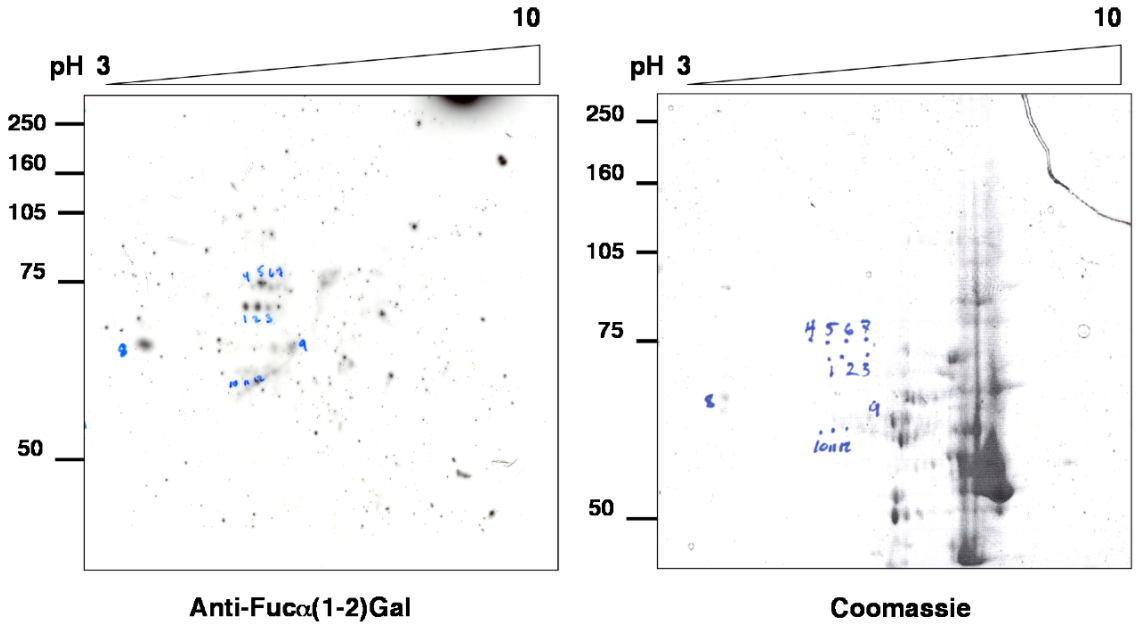

Figure A2.2. 2-Dimensional gel electrophoresis of embryonic rat forebrain lysates. Immunoblotted with antibody A46-B/B10 (left panel) or stained with Coomassie (right panel). Bands excised are indicated above for samples 1-12.

to isolate to Fucα(1-2)Gal glycoproteins (data not shown). However, many of the proteins eluted from the LTL column were not specifically detected by antibody A46-B/B10, which may suggest the isolation of fucosylated glycoconjugates with other linkages. He was able to isolate a number of proteins including pyruvate kinase, the collapsin-response mediator protein CRMP-2, and Rab-guanine dissociation inhibitor (Rab-GDI). We next tried to confirm some of these proteins by immunoprecipitation studies. CRMP-2, which is 64 kDa protein involved in neuronal development, growth cone collapse and generating neuronal polarity,(4-6) was immunoprecipitated from embryonic rat forebrain, however we were not able to detect fucose in the

immunoprecipitate lane, suggesting that this protein is non-specifically present in the sample (data not shown). We next examined whether we could detect fucose on Rab-GDI, a protein involved in vesicular transport.(7, 8) We immunoprecpitated Rab-GDI from h embryonic and adult rat forebrain. Analysis of the results suggests that Rab-GDI may be glycosylated (data not shown). The bands run very similar, but are too close the antibody band at 50 kDa to be conclusive and the molecular weights are slightly shifted, suggesting that the protein is most likely not fucosylated. Since we had some success with the LTL column in purifying Fucα(1-2)Gal glycoproteins, we next made attempts to develop a procedure to isolate these glycoproteins with UEAI lectin. UEAI is highly specific for interaction with terminal Fucα(1-2)Gal disaccharides on glycoconjugates. While LTL also binds fucose, this lectin can be promiscuous in recognizing fucose present in other linkages. Therefore, we developed a novel protocol using UEAI lectin and proteins purified from rat forebrain (containing the cortex, hippocampus, and striatum). Through much experimentation, we developed and optimized a binding protocol specific for UEAI lectin. In addition, we found that protein lysates bound better at room temperature incubations, rather than the standard 4 °C incubation, thus enhancing our capture of Fucα(1-2)Gal glycoproteins. We also moved towards using LC/MS instead of MALDI-TOP analysis to better separate the peptides in each sample and to enhance sensitivity. We observed that the quantity of glycoproteins eluted from a 100 μL column was too small to detect by MS analysis when eluted with α-L-fucose. Therefore, we scaled up our columns and created a larger 1 mL lectin column to capture more Fucα(1-2)Gal glycoproteins. In addition, we concentrated the 10 mL elution down to 100 μL, so that we could load all proteins isolated onto one lane of an SDS-PAGE gel.

Using this approach, we were successfully able to detect proteins specifically eluted from UEAI columns. In addition, we used a protein-A-agarose column as a control to detect non-specific binding (data not shown). We did observe a number of proteins bound in

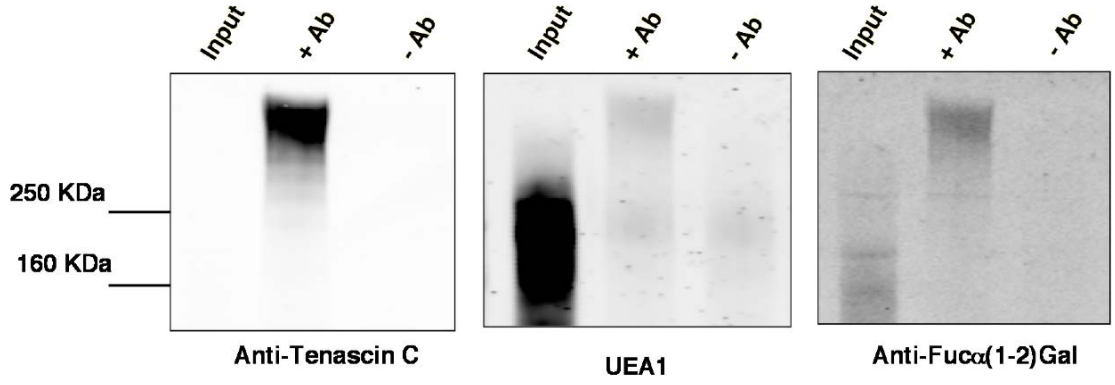

Figure A2.3. Immunoprecipitation confirms that tenascin C is a Fucα(1-2)Gal glycoprotein. Adult rat forebrain was immunoprecipitated with anti-tenascin C (lanes 2) or no antibody (control, lanes 3) and resolved by SDS-PAGE followed by immunoblotting with anti-tenascin C (left panel) UEAI (middle panel) or antibody A46-B/B10 (right panel). The input is 10% (100 μg) of the total IP. 20% of the eluated was loaded for the anti-tenascin C blot, and 40% each was loaded for the UEAI and antibody A46-B/B10 blots.

the control columns, confirming the importance of using a control. We next excised 22 bands from each lane of the silver-stained gel, and subjected them to LC-MS/MS for identification in collaboration with Dr. Scott Ficarro and Dr. Eric Peters at the Genomics Institute of the Novartis Foundation (GNF). The data was searched against the NCBI non-redundant database, and each sample was analyzed in Bioworks. We were able to isolate a number of proteins from each sample. Any protein identified in the control column was considered non-specific (Table A2.1). We identified a number of proteins including tenascin C, NCAM, the Na^+/K^+ ATPase and a number of heterogeneous ribonucleoproteins. Our biggest hit was for the proteins tenascin C. Tenascin C is an extracellular matrix protein involved in central synaptic differentiation.(9) We next

confirmed the presence of tenascin C as a Fucα(1-2)Gal glycoprotein by immunoprecipitation from rat cortex. We were able to specifically detect fucosylation of tenascin C with both antibody A46-B/B10 and UEA1 lectin, further suggesting that tenascin C is a Fucα(1-2)Gal glycoprotein (Figure A2.3). However, despite our identification of tenascin C, we still identified a lot of soluble cytosolic proteins in the proteome, which either suggests that a lot of Fucα(1-2)Gal glycoproteins exist in the cytosol contrary to previous findings, or that these proteins are non-specifically isolated in the UEAI lectin column eluates. Due to the extensive difficulty we had in identification of the Fucα(1-2)Gal proteome and potential isolation of non-specific proteins, we sought to development a new method to increase our signal-to-noise ration for identifying the Fucα(1-2)Gal proteome.

MW Range	Protein	MW (predict)	Function
160+ KDa	Tenascin C	222 KDa	Extracellular matrix protein, cortical defects in KO
	Ubiquitin-protein ligase e3 component n-recognition	193 KDa	ubiquitin ligase (*Skp1*)
	DEAD/H box polypeptide 9	141 KDa	RNA helicase
	NCA2 NCAM	80 KDa	Cell adhesion molecule, neurite fasciculation, outgrowth
	NCA1 NCAM	94 KDa	Cell adhesion molecule, neurite fasciculation, outgrowth
130-160 KDa	Na+/K+ transporting ATPase	112 KDa	membrane polarization create electrochemical gradient neurc
	DEAD/H box polypeptide 9	141 KDa	RNA helicase
	Tenascin	222 KDa	Extracellular matrix protein, cortical defects in KO
	Splicing factor 3b, subunit 3 (130 Kda)	139 KDa	spliceosome
95-130 KDa	Na+/K+ transporting ATPase	112 KDa	membrane polarization create electrochemical gradient neurc
	hnRNP U	88 KDa	scaffold attachment factor A/RNA binding
	eukaryotic translation elongation factor 2	94 KDa	protein synthesizing machinery
	hnRNP R	70 KDa	complexes polyA-mRNA in neurons
80-95 KDa	doublecortin and CaM kinase-like l	80 KDa	kinase, regulates m-tube org.
	DCK1 ser/thr protein kinase	80 KDa	controls neuronal migration in developing brain
	spermatid perinuclear RNA-binding protein	71.3 KDa	KO has neurologic and sperm effects
	Gry-rbp	68.5 KDa	RNA-binding protein
	hn RNP R	70 KDa	complexes polyA-mRNA in neurons
	polyA-binding protein, cytoplasmic 1	70 KDa	mRNA stabilization
70-80 KDa	78 KD Glucose-Regulated Protein	71.8 KDa	ATP-binding, in ER
	DEAD/H box polypeptide 5	67.5 KDa	RNA helicase (ATP-binding), transcriptional co-activator
	hnRNP L	61.4 KDa	packaging/rpocessing RNA
	Siah binding protein 1	59.5 KDa	RNA metabolism
60-70 KDa	DEAD/H box polypeptide 5	67.5 KDa	RNA helicase (ATP-binding), transcriptional co-activator
	U2 small nuclear RNA auxiliary factor	52.3 KDa	RNA Splicing
	hnRNP ROK	50.9 KDa	polyC binding K protein
50-60 KDa	hnRNP H1	49.4 KDa	polyA-mRNA binding
35-50 KDa	neuronal pentraxin I precursor	47.3 KDa	similar to bp for snake venom toxin taipoxin
	hnRNP A1 beta	35.2 KDa	RNA helix destablizing protein
	hnRNP A/B	31.4 KDa	Transcription regulator in developing brain
33-35 KDa	ROA2 hnRNP	38.8 KDa	pre-RNA processing
30-33 KDa	ROA2 heterogeneous nuclear ribonucleoprotein	38.8 KDa	pre-RNA processing
	B-cell Receptor-associated protein 37	32.8 KDa	modulates estrogen receptor
	protein kinase, protein activator interferon-inducible RNA	34.4 KDa	protein kinase regulates translation
25-30 KDa	Cleavage and Polyadenylation specific factor 5	24.9 KDa	3' RNA cleavage

Table A2.1 Summary of proteins identified by LC-MS/MS of rat cortical neuronal lysates listed by apparent molecular weights.

A2.4 Discussion

Identification of the Fucα(1-2)Gal proteome has suffered from many complications that hinder the isolation of proteins for mass spectrometry. Low abundance of Fucα(1-2)Gal glycoproteins and the low affinities of antibody- and lectin-carbohydrate interactions often precluded the ability to identify these proteins. We attempted to identify Fucα(1-2)Gal glycoproteins by a variety of techniques, such as antibody affinity chromatography with antibody A46-B/B10, lectin affinity

chromatography with LTL and UEAI lectins, and 1D- and 2D-gel electrophoresis. The only method that yielded successful identification of Fucα(1-2)Gal glycoproteins was the isolation of glycoproteins with UEAI lectin.

In adult forebrain, the most predominant Fucα(1-2)Gal glycoprotein identified was the extracellular matrix glycoprotein tenascin C. Tenascin C is present during neuronal development and regulates wound healing and neural regeneration in adult tissues.(10) The tenascins interact with cell adhesion molecules, other Fucα(1-2)Gal glycoproteins identified by our studies. An important role in nervous system development is the interaction between tenascins and CAMs. These interactions lead to neuronal outgrowth, migration, and pathfinding. Tenascin C knock-out mice display deficits in hippocampal synaptic plasticity.(11) and NCAM is involved in develop and plasticity of the nervous system,(12) which suggests an important role for fucosylated glycoproteins in the molecular events that may underlie synaptic remodeling and plasticity.

In addition to tenascin C, the largest class of proteins identified was the family of heterogeneous ribonucleoproteins (hnRNPs). These molecules are involved in the trafficking of mRNA between the nucleus and the ctyosol. Interestingly, nuclear proteins associated with chromatin are reported to be fucosylated and are specifically recognized by UEAI lectin,(13) suggesting that this may represent a novel class of cytosolic Fucα(1-2)Gal glycoproteins.

Furthermore, we identify the protein doublecortin, which is enriched in the leading process of growth cones in neurons. Defects in doublecortin lead to lissencephaly in humans, which is characterized by defects in neuronal migration that

lead to epilepsy and mental retardation.(14) Finally, we identify neuronal pentraxin I (NPI) as a putative Fucα(1-2)Gal glycoprotein. NPI is reported to play a role in excitatory synapse remodeling.(15) It has also been shown to mediate neuronal death in the pathogenesis of Alzheimer's disease.(15) Cumulatively, these proteins suggest important roles for Fucα(1-2)Gal glycoproteins in the molecular mechanisms that may underlie development and synaptic plasticity.

A2.5 Materials and Methods

Animals, Tissue Isolation and Homogenization

C57BL/6 wild-type animals and Sprague-Dawly rats were maintained in accordance with proper IACUC procedures. Adult male mice ages 3-4 months were anesthetized with CO_2 and dissected to remove the forebrain. For Western blotting, dissected tissues were cut into small pieces and placed immediately on ice, then lysed in boiling 1% SDS with sonication until homogeneous (5V:W). For lectin affinity chromatography, the adult rat forebrain was isolated and homogenized in lectin binding buffer (100 mM Tris pH 7.5/ 150 mM NaCl/ 1mM $CaCl_2$/ 1 mM $MgCl_2$/ 0.5% NP-40/ 0.2% Na deoxycholate plus protease inhibitors) by passing through a 26G needle 5 times, then sonicated to homogeneity. Samples were clarified by centrifugation at 12,000G x 10 min. Lysates were between 6 to 10 mg/mL total protein concentration as determined by the BCA protein assay (Pierce) for lectin affinity chromatography.

Creation of Antibody-A46-B/B10 Protein A-Sepharose Column

100 μL of goat IgG anti-μ-specific IgM at 13.6 mg/mL (1.36 mg total IgG) was dialyzed into 100 mM $NaHCO_3$ pH 8.0 / 500 mM NaCl. Equilibrate protein A-sepharose beads (100 μL beads) into dialysis buffer. Rotate beads plus goat IgG at 4 °C for 2 days. Spin down bead and wash 3 × dialysis buffer. Buffer exhange Antibody A46-B/B10 IgM into 0.1 M borate buffer pH 8.2 (concentration 0.78 mg/mL). Wash protein A beads with 0.1 M borate buffer pH 8.2 3 × 500 μL. Resuspend in 20 volumes of 100 mM dimethylpimelimidate (Pierce) in 0.2 M triethanolamine and rotate at RT for 45 min. Quench reaction by centrifuging beads, removing supernatant, and resuspending in 100 μL of 100 mM ethanolamine pH 8.2. Incubate 5 min with rotating at RT. Wash beads with 3 × 500 μL 0.1 M borate buffer pH 8.2. Store beads in 0.1 M borate buffer pH 8.2 / 0.02% NaN_3.

Purification of Fucα(1-2)Gal by the Antibody Column

Embryonic rat forebrain was homogenized in lysis buffer (50 mM Tris pH 8.2 / 150 mM NaCl / 0.2% Na deoxycholate / 1% NP-40) supplemented with protease inhibitors. The tissue was passed through a 26G needle 5 times, then sonicated on ice 8 x 5 s puleses. Tissue was spun at 12,000 × G for 10 min and the supernatant was removed for experiments. Equilibrate goat IgG anti μ-specific IgM protein A-sepharose in lysis buffer. Bind proteins for 3 h in batch at 4 °C with rotating. Wash column with 4 CV of TBST. Elute proteins with 50 mM diethylamine pH 11.5 / 0.5% Na deoxycholate. Proteins were resolved by SDS-PAGE as described previously.(16)

2D Gel Electrophoresis

Embryonic forebrain was lysated in 1.25 × Urea buffer (1.3 × Urea/thiourea / 5% CHAPS ; 0.625% Ampholy resins pH 3-10 (Amersham). Homogenize through 22-G needle 10×, then sonicate on ice 5 × 5 s pulses. Pass through 22-G needle 5×. Spin at 15,000 × G for 10 min. Remove supernatant for 2D gel. Take 200 μL of protein lysate, and add 2 μL of 0.2% bromophenol blue / 10 μL of reducing agent (Amersham) / 38 μL of H_2O. Set up 2D gel using pH 3-10 strips (Amersham). Run isoelectric focusing overnight for ~22 h. Make 15 mL per isoelectric focusing strip of SDS equilibration buffer (50 mM 1.5 M Tris pH 8.8 / 6 M Urea / 30% glycerol / 0.001% bromophenol blue). Split solution in half and add 1% DTT to one half, and 2.5% iodoacetamide to the other half. Equilibrate isoelectric focusing strip in SDS equilibration buffer with DTT for 15 min at RT, followed by SDS equilibration buffer with iodoacetamide for 15 min at RT. After strips have equilibrated, run 10% SDS-PAGE by place strip on the top of the gel and seal with 0.5% low melting agarose in SDS running buffer. Run gels at 0.15 mA/gel until the solution enters the gel, then run at 30 mA per gel for ~ 5 h or until dye front reaches the bottom. Parallel equiperiments were run to stain one gel with Coomassie brilliant blue and the second gel was transferred for western blotting with antibody A46-B/B10.

Lectin Affinity Chromatography and SDS-PAGE

One mL bed volume of *Ulex europaeus* agglutinin I (UEAI) conjugated to agarose (Vector Labs,) and control Protein A conjugated to agarose (Vector Labs,) columns were packed ~333 μL into 3 minicolumns run in parallel (BioRad). The resin

was equilibrated with 10 column volumes (CV) lectin binding buffer. 3 mL of olfactory bulb lysate at 6-10 mg/mL was bound in batch at RT for 4 h. Columns were repacked and the flowthrough was passed 3 additional times over the column. Columns were washed with 40 CV of lectin binding buffer, followed by 10 CV of lectin binding buffer lacking detergent (NP-40 and Na deoxycholate). Proteins were eluted in 10 CV of lectin binding buffer lacking detergent supplemented with 200 mM α-L-Fuc and protease inhibitors.

Protein eluates were concentrated in 10,000 molecular weight cut-off (MWCO) centricons (Millipore) followed by 10,000 MWCO microcons (Millipore) to 100 μL. Following concentration, samples were boiled with 35 μL of 4× SDS loading dye and loaded onto 10% SDS gels for electrophoresis as described previously.(16)

Coomassie staining, Peptide Extraction, and In-Gel Tryptic Digests

Coomassie-stained gels were incubated in 300 mL of 1 g Coomassie brilliant blue/ 50% MeOH/ 10% HOAc for 1 h, then destained in the same solution without Coomassie brilliant blue with multiple changes until bands were visible. Peptide extraction and in-gel tryptic digests were as reported in Chapter 2.

LTQ LC-MS Analysis

Approximately 50% of gel extractions were loaded onto a 360 μm O.D. × 75 μm precolumn packed with 4 cm of 5 μm Monitor C18 particles (Column Engineering) as described previously.(17)

Immunoprecipitation

CRMP was immunoprecipitated from rat embryonic forebrain homogenized in 25 mM sodium phosphate, pH 6.0/ 10 mM 2-mercaptoethanol/ 10 mM tetrasodium pyrophosphate/ 1mM EGTA plus protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride/ 1 μg/mL leupeptin/ 1 μg/mL pepstatin/ 1 μg/mL antipain). Proteins were immunoprecipitated with 4 μg of C4G antibody (courtesy of Ihara) at 4°C for two hours. 50 μL of protein A sepharose was added and the immunoprecipitates incubated 4 °C for another two h. Immunoprecipitates were washed 3 × 500 μL lysis buffer, boiled in 1X SDS-PAGE loading dye and subjected to SDS-PAGE for blotting with C4G (1:2000) or antibody A46-B/B10 at 5 μg/mL.

Rab GDI and tenascin C was homogenized in 1% SDS and neutralized with Neutralization buffer. Rab GDI was immunopreciptated with 4 μg of antibody (Synaptic Systems) and tenascin C was immunoprecipitated with 4 μg of antibody (Santa Cruz) for 4 hr at 4 °C, followed by 30 μL of Protein-A-sepharose for 2 more h.

A2.6. References

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