3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Primary antibodies

Names	Dilution	Sources	Clones
Synaptotagmin 1(mAb)	IF (1:200)	Synaptic System	604.4
Synaptotagmin 1(mAb)	IF (1:1000) IB (1:2000)	Synaptic System	41.1
Synaptophysin (pAb)	IF (1:500)	Synaptic System	
Synaptophysin (mAb)	IF (1:500)	Synaptic System	7.2
Synaptobrevin 2 (pAb)	IF (1:500)	Synaptic System	
Synaptobrevin 2 (mAb)	IF (1:500)	Synaptic System	69.1
Proton pump (pAb)	IF (1:1000)	Synaptic System	
CHC (mAb)	IF (1:1000)	Affinity Bioreagents	X22
CHC (mAb)	IB (1:10)	De Camilli's lab	TD1
AP-2-β1/β2 (mAb)	IB (1:1000)	BD Biosciences	
AP-2-β1/β2 (mAb)	IF (1:1000)	Kirchhausen T	
AP-2 α-adaptin (mAb)	IB (1:1000)	Affinity Bioreagents	
FLAG (mAb)	IF (1:400) IB (1:1000)	Sigma	M2
Transferrin receptor (mAb)	IF, IB (1:250)	Zymed Laboratories	H68.4
EEA1 (pAb)	IF (1:100)	Zerial M	07JF3
PIP5KIγ (mAb)	IB (1:250)	BD Transduction lab	12
SV2 (mAb)	IF (1:250)	De Camilli's lab	C10H44

3.1.2 Secondary antibodies

Names	Dilution	Sources	Species	Clones, batches
Alexa 488 GoatαMouse	IF (1:200)	Molecular Probes	Goat	HRP conjuagted
Alexa 488 GoatαRabbit	IF (1:200)	Molecular Probes	Goat	HRP conjugated
Alexa 594 GoatαMouse	IF (1:200)	Molecular Probes	Goat	HRP conjugated
Alexa 594 GoatαRabbit	IF (1:200)	Molecular Probes	Goat	HRP conjuagted
GoatαMouse	IB (1:5000)	Jackson Immunores	Goat	HRP conjugated
GoatαRabbit	IB (1:10000)	Jackson Immunores	Goat	HRP conjugated
GoatαMouse	IB (1:2000)	Jackson Immunores	Goat	AP conjugated
GoatαRabbit	IB (1:2000)	Jackson Immunores	Goat	AP conjugated
GoatαMouse	IF (1:10)	Jackson Immunores	Goat	Serum, unlabelled
RabbitαMouse	IB (1.1000)	Jackson Immunores	Rabbit	Unlabelled

3.1.3 Reagents, chemicals and consumables

$H_2{}^{18}O$ was purchased from Euriso-top GmbH, Saarbrücken, Germany (95% ^{18}O). Methyl-β-cyclodextrin (MβCD) and cholesterol were bought from Aldrich. All lipids were purchased from Sigma, including Folch Fraction I from bovine brain (B1502), L-α-Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) (P9763), L-α-Phosphatidylinositol 4-monophosphate (PI(4)P) (P9638), L-α-Phosphatidyl-D-*myo*-inositol 3-monophosphate (PI(3)P) (P3953), L-α-Phosphatidylcholine (PC) (P3841), 3-sn-Phosphatidylethanolamine (PE) (P7693), 3-*sn*-

Phosphatidic acid (PA) (P9511), L-α-Phosphatidylinositol (PI) (P8443), 1,2-Diacyl-*sn*-glycero-3-phospho-L-serine (PS) (P7769). Guanosine-5'-diphosphate (GDP), Guanosine-5'-triphosphate (GTP) and Guanosine-5'-O-(3-thiotriphosphate) GTPγS were purchased from Sigma. 100 nm, 200 nm, 400 nm polycarbonate membranes (Diameter 19mm) were bought from AVESTIN, Inc. All other analytical grade pure chemicals were bought from ROTH, Meck, Sigma, Invitrogen. VentR® high fidelity polymerase was from NEB (Schwalbach, Germany). Sequencing grade trypsin and Trition X-100 were from Roche Diagnostics, Mannheim, Germany. Lipofectamine™ 2000 was from Invitrogen.

3.1.4 Instruments

LiposoFast™-Basic and Stabilizer extruder	AVESTIN, Inc.
Zeiss Axiovert 200M Digital Research Microscopy System	Carl Zeiss AG, Göttingen
Fluorometer GENios	TECAN, Männedorf
Phosphoimager Cyclone Autoradiography System	PerkinElmer
UltiMate HPLC system	Dionex, Idstein
UV/UIS Spectro-photometer	Eppendorf, Hamburg
Probot Micro Fraction Collector	Dionex
Microtip System Sonoplus and Ultrasonic bath Sonorex	Brandelin, Berlin
UV photographic documentation system	Herolab, Wiesloch
4700 Proteomics analyzer	Applied Biosystems
T3 Thermocycler	Whatman Biometra

3.1.5 Kits

E.Z.N.A.® Cycle-Pure Kit	peQLab
E.Z.N.A.® Gel Extraction Kit	peQLab
Wizard ® Plus SV Minipreps	Promega.
Amplex Red Cholesterol Assay Kit	Molecular Probes
Midprep kit	Quiagen (Hilden)
QuickChange® Site-Directed Mutagenesis Kit	Stratagene

3.1.6 Buffer, solution and media

LB medium,1lt	10 g typtone, 5 g yeast extract, 5 g NaCl, 1 ml 1 N NaOH
2 YT medium,1lt	16 g tryptone, 10 g yeast extract, 5 g NaCl,1 ml 1 N NaOH
LB agar plate, 1lt	10 g typtone, 5 g yeast extract, 5 g NaCl, 20 g agar (autoclave, cool down, add antibiotics, aliquot into petri dishes, store at 4°C until use)
Fixative, 100 ml	Dissolve 4 g paraformaldehyde in PBS by stirring at 60°C. Cool down and filter. Store aliquots at -20°C until use.
10×TBE electrophoresis buffer, 1lt	108 g Tris base, 55 g boric acid, 20 mM EDTA pH 8.0
10×TBS	200 mM Tris-Cl pH 7.4, 1.4 M NaCl
10×PBS	1.37 M NaCl, 27 mM KCl, 43 mM Na_2HPO_4, 14 mM NaH_2PO_4
High salt PBS	20 mM $NaPO_4$ pH 7.4, 500 mM NaCl
GSDB (Goat serum dilution buffer)	25% Goat serum, 0.3% (w/v) Triton X-100 in high salt PBS
SDS-PAGE running buffer	246 mM Tris, 1.92 M Glycin, 10%SDS
SDS-PAGE 4×stacking gel buffer	0.4% SDS, 0.5 M Tris-Cl pH 6.8
SDS-PAGE 4×seperating gel buffer	0.4%SDS, 1.5 M Tris-Cl pH 8.8
Milk block solution	3% non-fat milk powder in TBS
Antibody solution	2% (w/v) analytical grade BSA and 0.02%NaN_3 in TBS
Ponceau staining solution	0.3 (w/v) Ponceau S in 1% acetic acid
Blot buffer	80% SDS-PAGE running buffer, 20% methanol
6×DNA loading buffer	0.05% bromophenol blue, 0.05% xylene cyanol, 30% glycerol
Coomassie staining solution	0.1% (w/v) Coomassie 250G, 10% acetic acid, 25% methanol in dH_2O
Destaining solution	10% acetic acid, 25% methanol in dH_2O
Ampicillin stock solution (500×)	50 mg/ml filtered and stored at -20°C
Kanamycin stock solution (200×)	10 mg/ml filtered and stored at -20°C
0.5 l Bradford reagent (2×)	Coomassie G250 70 mg, phosphatic acid (85%) 100 ml, EtOH 50 ml. Filtered, shed from light.
1 M imidazole	6.81 g/100 ml dH_2O adjust pH
120 mM Na_2HPO_4	10.67 g/500 ml of dH_2O
120 mM NaH_2PO_4	9.36 g/500 ml of dH_2O
120 mM Na-Phosphate pH 7.4	77.4 ml Na_2HPO_4 + 22.6 ml NaH_2PO_4
High salt PBS (HSPBS)	41.6 ml120 mM Na-Phosphate (20 mM)+25 ml of 5 M NaCl (500 mM)+7.5 ml 10% Triton X-100 (0.3%)+175.9 ml dH_2O to final volume of 250 ml
2×Glycerol stock solution	0.1 ml 1 M Tris/HCl pH 8.0 (10 mM)+0.5 ml 1 M $MgSO_4$(50 mM) +5 ml 100% Glycerol+4.6 ml dH_2O
100 mM EGTA pH 8-9	3.8 g/100 ml; titrate with 10 N NaOH
50 mM DTT	Freshly made; 0.3855 g/50 ml dH_2O
50 mM PMSF	Dissolved in EtOH and freshly made.

3.2 Methods

3.2.1 Molecular Cloning

3.2.1.1 Oligonucleotide primers

Chemically synthesized oligonucleotide primers were ordered from MWG Biotech (Martinsried, Germany). All primers used for the thesis are listed in Appendix table 2.

3.2.1.2 Plasmids

pET21b	Ampicillin	Novagen
pET28a	Kanamycin	Novagen
pGEX-4T-1	Ampicillin	GE Health Life Sciences
pSL301	Ampicillin	Invitrogen
pcDNA3	Ampicillin/neomycin	Invitrogen
pEGFP-N3	Kanamycin	BD Biosciences Clontech
pcHA2	Ampicillin/neomycin	By Yasuo Nemoto (Yale University)

HA (hemagglutinin)-tag sequence (ATG GCG TAC GAC GTC CCA GAC TAC GCG for MAYPYDVPDYA) was cloned into the Kpn1 and BamH1 restriction sites of pcDNA3 vector. The multiple cloning site from BamH1 to XhoI of pBluescript IISK+ vector was inserted.

pcFLAG Ampicillin/neomycin
Same procedure for pcHA2 generation but instead, a FLAG-tag (ATG GAC TAC AAG GAC GAC GAT GAC AAG for MDYKDDDDK) was inserted.

3.2.1.3 PCR (Polymerase Chain Reaction)

Standard PCR recipe and program for amplification

dH_2O	30.5 µl
10×Thermal buffer	4 µl
Template (100 ng/µl)	1 µl
dNTP mixture (5 mM/each)	1.5 µl
Forward primer (10 pmol/µl)	1.25 µl
Reverse primer (10 pmol/µl)	1.25 µl
Vent polymerase	0.5 µl
	40 µl

PCR program:

Step 1.	95°C	2 min
Step 2.	95°C	30 sec
Step 3.	57°C	30 sec
Step 4.	72°C	1 kb/min go back step 2 for 31 cycles
Step 5.	4°C	Pause

3.2.1.4 Agarose gel electrophoresis and gel extraction

PCR products or digested vectors were loaded on agarose gels and run at 100 V. Gels were stained with ethidium bromide (EB) and images were taken under UV light. Bands were cut and the DNA was extracted according to manufacture's kit instruction. PCR fragments and vectors were cut with restriction enzymes at 37°C for 4 h. Vectors were dephosphorylated with 1 µl of CIP (calf intestine alkaline phosphatase) at 37°C for another 15 min. Agarose electrophoresis was used to separate digested and not-digested vectors. Digested vectors were cut and gel-extracted.

3.2.1.5 Bacterial strains

E.coli stain TOP10 cells were bought from Invitrogen (Karlsruhe, Gemany). They are generally used for transformation of ligation and amplification of plasmid DNA. ER2566 was bought from NEB (Schwalbach, Germany). It contains an IPTG-activated gene for the inducible expression of genes driven by the T7 promoter.

3.2.1.6 Competent bacteria, ligation and transformation

To prepare competent *E.coli*, one fresh colony from an overnight agar plate was inoculated into 5 ml LB medium for overnight culture at 37°C. This overnight culture was transferred into 100 ml of LB medium for shaking at 37°C until the OD_{600} value reached 0.4. Bacteria were centrifuged at 5,000 rpm at 4°C for 10 min. The supernatant was discarded and pellets were re-suspended into 10 ml of ice-cold $CaCl_2$ for 10 min on ice. Bacteria were re-centrifuged at 5,000 rpm for 10 min, and re-suspended into 2 ml of cold 0.1 M $CaCl_2$. Glycerol was adjusted to a final concentration of 10%, 100 µl were aliquoted into 1.5 ml eppendorf tube. Bacteria were fast-frozen using liquid nitrogen and stored at -80°C until use.

For standard transformations, 50-100 ng of cut vector and 2-3 mole-folds of inserts were mixed in a final volume of 20 µl. Ligation was carried out in the presence of T4 ligase at 4°C overnight or 16°C for 4 h.

To transform competent *E.coli*, either ligation solutions or pure plasmids were incubated with 100 µl of bacteria on ice for 30 min, heat-shocked at 42°C for 90 sec, followed by addition of 900 µl of LB medium. Bacteria were shaken at 37°C for 1 h, spread onto agar-LB plates with supplemented antibiotics and incubated at 37°C for overnight (16-20 h).

3.2.1.7 Colony PCR screen

Six colonies were selected from the overnight grown transformed *E.coli* on agar-plate to inoculate 60 µl of LB medium in the presence of antibiotics in a 96-well plate. The plate was then shaken at 37°C for 1.5 h. The following protocol was used to prepare a single PCR screen reaction mixture:

dH_2O	0.5 µl
10×buffer	2.5 µl
E.coli culture	1 µl
dNTP mixture (5 mM/each)	0.5 µl
Forward primer (100 pmol/µl)	0.2 µl (vector derived primer)
Reverse primer (100 pmol/µl)	0.2 µl (vector derived primer)
Taq polymerase	0.06 µl
	25 µl

The master solution was prepared and then aliquoted. The PCR program used was the same as that mentioned above, except that the annealing temperature was set to 55°C. After agarose gel electrophoresis and EB staining, positive clones were chosen to inoculate small-scale LB medium for overnight culture at 37°C. Miniprep was performed according to the manufacture's instruction the next day. 2 µl out of 30 µl of eluted DNA were subjected to double restriction enzyme digestion for the confirmation of correct inserts. 1 µg plasmid DNA was dried and analyzed by DNA sequencing.

3.2.1.8 Miniprep for positive colony screen

Freshly grown *E.coli* colonies were picked and inoculated into 3 ml of LB medium supplemented with antibiotics for overnight shaking at 37°C. The next day, 1.5 ml was transferred to an Eppendorf tube and centrifuged at 13,000 rpm for 1 min. Supernatants were discarded and pellets were re-suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0, 10 µg/ml RNase A). Bacteria were lysed by adding 200 µl of 0.2 M NaOH-1%SDS solution at RT for 3 min. The lysate was neutralized by adding 150 µl of 3 M KAc (pH 4.8) and centrifuged at 13,000 rpm for 10 min at RT. Supernatants were transferred to a new 1.5 ml eppendorf tube and 800 µl of pre-cold isopropanol was added. After centrifugation at 13,000 rpm for 10 min, pellets were further washed with 500 µl of 70% ethanol and centrifuged. Supernatants were carefully discarded and pellets were dried. Finally, the pellets were re-suspended in 30 µl dH_2O. 2 µl was used for double digestion to confirm correct inserts.

3.2.2 Bioinformatics and software interfaces

PSIPRED Secondary structure prediction

http://bioinf.cs.ucl.ac.uk/psipred/

MultAlin Multi sequence alignment

http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html

Online Helical Wheel α-helical analysis

http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html

ExPASy Proteomics Server

http://www.expasy.org

MWG Biotech (Martinsried) Oligonucleotide primers and sequencing

http://www.mwg-biotech.com/html/all/index.php.

NCBI homepage Data base for DNAs, proteins and literature

http://www.ncbi.nlm.nih.gov/

Mascot Peptide Mass Fingerprint and MS/MS ion search engine

http://www.matrixscience.com/search_form_select.html

Slidebook 4.0.8 Digital Microscopy Software from Intelligent Imaging Innovations (Göttingen)

Applied Biosystems Data Explorer

Used to view and process data files from Applied Biosystems 4700 TOF/TOF instruments

DNA club For DNA sequence analysis and primer design

3.2.3 Biochemistry

3.2.3.1 Isolation of synaptic detergent-resistant membranes (DRMs) from highly purified synaptosomes (P2)

Two rat (8-12 week old) brains were freshly prepared in 10 ml homogenization buffer (320 mM sucrose, 4 mM HEPES-NaOH pH 7.4) supplemented with 100 µl of 100 mM PMSF and 20 µl protease inhibitor cocktail (Sigma, Deisenhofen, mammalian protease inhibitor). Brains were homogenized with glass Teflon homogenizer at 900 rpm for 10 strokes and centrifuged using a JA17 rotor at 1,000 g for 10 min. The supernatant was carefully transferred into a new tube to avoid the loose part just above pellets, and further centrifuged at 10,500 g for 10 min. Pellets were re-suspended into 12 ml homogenization buffer, overlaid onto 9 ml 1.2 M sucrose cushion in SW41 rotor tubes and centrifuged at 37,200 rpm for 20 min. Interfaces were collected, and the final volume of 12 ml was adjusted by adding homogenization buffer. 3 ml was overlaid onto a 9 ml 0.8 M sucrose cushion and centrifuged again at 37,200 rpm for 20 min. Pellets (P2, highly purifed fine synaptosomes) were re-suspended in TNE buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT).

For cholesterol depletion, P2 was extracted twice with 10 mM of methyl-β-cyclodextrin (MβCD) in TNE buffer sequentially at 37°C and on ice for 20 min. 450 µg of control and MβCD-extracted P2 were solubilized with cold 2% Triton X-100 (or 2% CHAPS) in a total volume of 1.5 ml on ice for 10 min, followed by mixing on a rotating wheel at 4°C for 20 min. Samples were adjusted to a final volume of 4 ml in 30% Optiprep (iodixanol) by adding 2.5 ml of 48% Optiprep in TNE buffer and loaded to the bottom of an SW41 ultracentrifuge tube. This bottom layer was overlaid with 7 ml of 25 % Optiprep and 1 ml of 5% Optiprep in TNE/ 2% Triton X-100 or 2% CHAPS. Gradients were centrifuged at 36,100 rpm using an SW41 rotor overnight. The next day, ten fractions of 1.2 ml were taken from top to bottom and subjected to trichloroacetic acid (TCA) precipitation. Re-suspended proteins were seperated by SDS-PAGE. Immunoblots were performed to verify the localization of DRM markers and other proteins.

3.2.3.2 TCA precipitation

Equal volumes of 20% TCA were mixed with fractions from the overnight Optiprep gradient, incubated on ice for 40 min and 60°C for 5 min. After centrifugation at 14,000 rpm at 4° C for 20 min, pellets were further washed on ice for 10 min first with 90% acetone/10% TCA

and then with 100% cold acetone. Final pellets were re-suspended into 50 l Laemmli sample buffers.

3.2.3.3 Immunoblot, Coomassie staining and autoradiography

Protein samples were separated by SDS-PAGE at 20 mA for 1.5 h. For Coomassie staining, gels were stained with Coomassie blue for overnight followed by destaining next day. For immunoblot, separating gels were blotted to nitrocellulose membrane (Whatman Biometra, Göttingen) using the Biometra blotting apparatus FastBlot B 44 in a semi-dry manner with the current of 1 mA/cm^2 for 2 h. The nitrocellulose membranes were stained with Ponceau S for 5 min on a shaker and destained with 1% acetic acid. Protein molecular weight markers were labeled and gels were documented by scanning before being blocked at RT for 1 h or at 4°C overnight. Membranes were washed with TBS three times for 10 min each on a shaker. Primary antibodies were applied to cover the membrane and incubated at RT for 2 h or at 4°C overnight. After being washed once with TBS and twice with blocking solution for 10 min each, membranes were incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies (1:5000) of either Goat×Rabbit (G×R) or Goat×Mouse (G×M) in blocking solution at RT for 1 h. Unbound secondary antibodies were washed away by washing the membrane three times with TBS. ECL Detection Reagents (GE Healthcare, Munich) were applied and the chemiluminescence was detected in dark rooms with Hyperfilm (GE Healthcare).

For alkaline phosphate (AP) detection, blots were washed 3×10 min with TBS after primary antibodies. AP-conjugated secondary antibodies, G×R or G×M, were diluted 1:1000 in blocking solution and applied to the membranes for incubation at RT for 1 h. Membranes were washed twice with TBS and once with TSM. Finally, blots were developed by adding 20 ml TSM+122 µl NBT+66 µl BCIP.

TSM: 100 mM Tris pH 9.5
100 mM NaCl
5 mM $MgCl_2$
NBT: 50 mg/ml in 70% DMF
BCIP: 20 mg/ml in dH_2O

Due to its non-linear characteristics, ECL is not suitable for quantification. Thus, autoradiography was used to quantify blots. We took advantage of the fact that binding ability of Protein A is more specific to rabbit IgG than to mouse IgG. The mouse monoclonal primary antibody signals were dectected and amplified by using unlabeled rabbit-anti-mouse antibody (1:2000). [^{125}-I]-Protein A (1:1000) was incubated in blocking solution at RT for 1 h on a shaker followed by washing three times with blocking solution and once with TBS.

Membranes were dried and exposed to a phosphoimager screen at RT for at least 4 h to overnight. Signals were detected and quantified using the Cyclone Autoradiography System (Packard Biosciences).

3.2.3.4 Crude synaptic vesicle (LP2) preparation and co-immunoprecipitation

To prepare crude synaptic vesicles (LP2), fine P2 synaptosomes (from 10 fresh rat brains) were first purified as described above. Final P2 pellets were re-suspended in 50 ml of homogenization buffer. Hypotonic force was generated by adding 450 ml of pre-cold dH_2O supplemented with 80 µl of cocktail mammalian protease inhibitors and 5 ml of PMSF to break synaptosomes. 5 ml of HEPES-NaOH pH 7.4 was added to reach the final concentration of 20 mM to prevent the acidification by broken endosomes. The solution was homogenized at 2,000 rpm for 5 strokes followed by 30 min of centrifugation at 16,500 rpm using a JA17 rotor. Supernatants were harvested and re-centrifuged at 50,400 rpm (180,000 g) using an SW60-Ti rotor for 1.5 h. Pellets were suspended into 4 ml of HKA buffer (10 mM HEPES-KOH pH 7.4, 140 mM potassium acetate, 1 mM $MgCl_2$, 0.1 mM EGTA). Protein concentrations were determined by the Bradford method.

For co-immunoprecipitation experiments, 250 µl of LP2 (2 mg/ ml) was solubilized by adding equal volumes of 4% CHAPS in HKA buffer on ice for 30 min (final 2 % CHAPS). Insoluble material was removed by centrifugation at 18,500 g for 15 min. The supernatant was applied to 30 µl of protein A/G sepharose beads (Santa Cruz Biotechnology, Inc) that were pre-coupled with antibodies against transferrin receptor, synaptotagmin 1, synaptophysin, synaptobrevin 2 or γ-glutamic acid decarboxylase (GAD), respectively and incubated by end-over-end rotation at 4°C for 4 h. Beads were washed three times with immuoprecipitation buffer and once with HKA buffer without CHAPS. Beads were extracted with 60 µl Laemmli sample buffer. 30 µl was used for immunoblot; the other 30 µl was used for autoradiography and signal quantification.

HKA buffer

2 M HEPES-NaOH pH7.4	0.5 ml
1 M KAc	14 ml
0.5 M EGTA	20 µl
1 M $MgCl_2$	100 µl
dH_2O	85.5 ml
	100 ml

3.2.3.5 Total lipid extraction and free cholesterol determination

100 µg of proteins from mock-treated or MβCD-extracted P2 or LP2 were transferred into a 15 ml Falcon tube. 0.5 ml of 2% KCl, 1.875 ml methanol and 1.875 ml of chloroform were added sequentially and mixed vigorously for 2 min. After incubation at RT for 10 min, 0.625 ml of chloroform was added and mixed for 30 sec. For the separation of the hydrophobic and hydrophilic phases, 0.625 ml of 2% KCl was added and mixed for 30 sec. After spinning at 2,500 rpm (1,200 g) for 5 min, the bottom layer was collected and transferred to a separate glass tube. 0.5 ml of chloroform was added to the supernatant and re-centrifuged. Two bottom fractions were combined. For free cholesterol determination, the extracted total lipid solution was evaporated and dried under nitrogen stream. The total lipids were then re-suspended in 30-50 µl of 1×working solution (Amplex® Red Cholesterol Assay Kit (Molecular Probes). Sequential dilutions were prepared. The principle of free cholesterol determination is summarized in **Figure 3-1.** Experiments were performed according to the manufacture's instruction. Fluorescence was measured with a fluorescence microplate reader (TECAN) using excitation and detection wavelengthes at 540 nm and 610 nm, respectively.

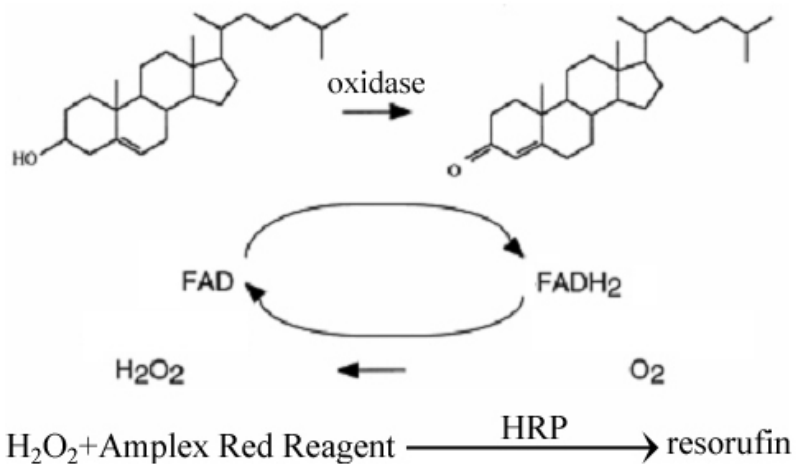

Figure 3-1 Principle of free cholesterol determination

Free cholesterol can be oxidized by cholesterol oxidase to yield H_2O_2 and the corresponding ketone products by using $FADH_2$ as reducing reagents. The H_2O_2 is then detected by 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent). In the presence of HRP, Amplex Red reagents react with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin.

3.2.3.6 Two-dimensional electrophoresis with immobilized pH gradients

Two dimensional polyacrylamide gel electrophoresis (2D PAGE), in which proteins are separated according to charge (pI) by isoelectric focusing (IEF) in the first dimension and

according to size (Mr) by SDS-PAGE in the second dimension, has a unique capacity for the resolution of complex mixtures of proteins. Mock and cholesterol-depleted synaptic DRMs were subjected to two-dimensional electrophoresis for the identification of DRM proteins in a cholesterol-dependent manner. Synaptic DRMs were prepared as described above. However, after TCA precipitation, the pellets were re-suspended in the first dimensional sample buffer, incubated at RT for 30 min and sonicated in a water bath for 1 min. Sample buffers with a pH gradient between 3-10 were applied to the first dimensional gels for equilibration for 30 min. After pre-electrophoresis at 200 V, 300 V and 400 V sequentially for 10 min each in the presence of running buffers, samples were loaded and overloaded with overlay buffers. Gels were run at 500 V at 4°C overnight. The first dimensional gel was pushed carefully out into a 10 cm petri dish filled with SDS sample equilibration buffers and washed three times for a total of 30 min. Equilibrated first dimension gel was then loaded and run in the second dimensional SDS-PAGE. Gels were stained with Coomassie blue.

Protein staining patterns were compared between mock and experimental groups. Only spots with decreased or disappearing signals in gels were cut out for MALDI-TOF peptide mass fingerprint analyses

First dimension acrylamide stock solution	48.56 ml of the Rotiphorese Gel 30 (37.5:1) 0.432 g of N,N'-Methylenbis-acrylamid Adjust volume to 50 ml with dH_2O
First dimensional sample buffer	
9.5 M urea	5.7 g
2.0% Triton X-100	2.0 ml 10% Triton X-100 stock
5% β-mercaptoethanol	0.5 ml
1.6% 5-7 ampholyte	400 µl
0.4% 3-7 ampholyte	100 µl
First dimensional sample overlay buffer	
9 M urea	5.41 g
0.8% ampholyte	200 µl
0.2% ampholyte	50 µl
Bromophenol blue	500 µl of a 0.05% (w/v) Bromophenol blue

Upper chamber buffer (100 mM NaOH)
Dissolve 0.2 g NaOH in 250 ml Milli Q water and degas thoroughly for 1 h.
Lower chamber buffer (10 mM H_3PO_4)
Dilute 1.36 ml concentrated H_3PO_4 in 2 l Milli Q water and degas thoroughly for 1 h.

First dimensional gel monomer solution	
9.2 M urea	5.5 g
4% acrylaminde (total monomer)	1.33 ml first dimension acrylaminde stock
2.0% Triton X-100	2.0 ml 10% Triton X-100
1.6% 5-7 ampholyte	400 µl 5-7 ampholyte
0.4% 3-10 ampholyte	100 µl 3-10 ampholyte
0.01% ammonium persulfate	10% APS (fresh)
0.1% TEMED	10 µl TEMED
	Adjust to final 10 ml

SDS sample equilibration buffer

0.0625 M Tris-HCl, pH 6.8	2.085 ml of 1.5 M Tris-HCl, pH 6.8
2.3% (w/v) SDS	11.5 ml 10% (w/v) SDS
5.0% (v/v) β-mercaptoethanol	2.5 ml
10% glycerol (w/v)	4 ml of glycerol
Milli Q water	29.915 ml
Total	50 ml

All solutions above containing urea were heated for dissolving (<45°C) in a water bath with swirling or directly degased thoroughly for 15 min.

3.2.3.7 16-BAC/SDS-PAGE two dimensional gel electrophoresis

Two dimensional gel electrophoresis 16-BAC (benzyldimethyl-*n*-hexadecy-lammonium chloride)/SDS-PAGE (Hartinger, Stenius et al. 1996) was used as a complementary method to IEF two dimensional electrophoresis for a better resolution of transmembrane and hydrophobic proteins potentially localized in synaptic DRMs. TCA precipitated pellets were dissolved in sample buffers and incubated at 60°C for 5 min for maximal solubilization. Samples were loaded to the first dimensional 7.5% 16-BAC gel and were run toward the cathode. Gels were fixed at RT for 1.5 h in the solution containing isopropanol:acetic acid:water in the ratio of 3.5:1:5.5 with several rounds of the fixative washing to remove the 16-BAC. After staining with Coomassie blue and destaining, the first dimensional gels were re-equilibrated three times with 100 mM Tris-Cl pH 6.8 for 10 min each. The lane of interest was cut out and loaded onto a preparative well in the second dimensional SDS-PAGE. Gels were finally stained with Coomassie blue and destained. The protocol of spot selection for mass spectrometry identification was the same as that for aforementioned 2D PAGE

10× running buffer for the first dimensional16-BAC gel

25 mM16-BAC	0.99 g
1.5 M glycine	11.26 g
500 mM phosphoric acid	3.4 ml (14.65 M)
	100 ml

1×Sample buffer

Urea	0.225 g
16-BAC	50 mg
Glycerol	50 µl
dH_2O	200 µl
DTT	25 µl (1.5 M DTT)
Pyronine Y	5 µl (5% w/v)
	1 ml

7.5% separating gel

Urea	1.8 g
Acrylamide (30%)	2.5 ml
Potassium Phosphate (pH2.1, 300 mM)	2.5 ml
H_2O	2.5 ml
Bisacrylamide (1.7% w/v)	0.35 ml
Ascorbic acid (80 mM fresh)	0.5 ml
Ferrous sulfate (5 mM fresh)	16 µl
16-BAC (250 mM)	100 µl
H_2O_2 (1:1200, diluted from 30% stock)	0.4 ml
	10 ml

4% stacking gel	
Urea	1 g
Acrylamide (30%)	1.33 ml
Potassium Phosphate (pH 4.1)(0.5 M)	2.5 ml
H_2O	3 ml
Bisacrylamide (1.7% w/v)	1.38 ml
Ascorbic acid (80 mM fresh)	0.5 ml
Ferrous sulfate (5 mM fresh)	8.5 µl
16-BAC (250 mM)	70 µl
H_2O_2 (1:750, diluted from 30% stock)	0.5 ml
	10 ml

3.2.3.8 Protein tryptic digestion and isotope labeling

DRM fractions obtained from flotation gradients derived from untreated or cholesterol-depleted synaptosomes were separated by SDS-PAGE side-by-side and stained with Coomassie. Gel lanes were cut into 16 slices of equal size. Excised gel slices were washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. The dried gel pieces were incubated with 120 ng trypsin (sequencing grade, Roche Diagnostics, Mannheim, Germany) in 50 µL of 5 mM ammonium bicarbonate. The enzymatic protein in-gel digestions were performed in the presence of $H_2{}^{18}O$ (Euriso-top GmbH, Saarbrücken, Germany, 95% ^{18}O) and $H_2{}^{16}O$ for mock-treated and MβCD-extracted DRM proteins, respectively. After 17 h incubation at 37°C, 50 µL of 0.3% trifluoroacetic acid (TFA) in acetonitrile was added, the samples were sonicated for 5 min, and the separated supernatant was dried under vacuum. Samples were reconstituted in 6 µL of 0.1% (v/v) TFA, 6% (v/v) acetonitrile in water. Samples from paired gel slices (^{16}O and ^{18}O samples of adjoining slices) were combined immediately before nanoLC-mass spectrometry.

3.2.3.9 NanoLC-MALDI-MS/MS and identification of proteins

An UltiMate HPLC system (Dionex, Idstein, Germany) was coupled off-line to MALDI-MS using a Probot Micro Fraction Collector (Dionex) deposition interface. For desalting and concentrating, the samples were loaded onto a precolumn (PepMap C18, 5 µm, 100 Å, 5 mm x 300 µm i.d., Dionex) using a Famos autosampler and a Switchos II system. Peptides were eluted onto an analytical column (PepMap C18, 3 µm, 100 Å, 150 mm x 75 µm i.d., Dionex) and separations were performed at an eluent flow rate of 200 nL/min. Mobile phase A was 0.1% TFA in acetonitrile-water (5:95, v/v) and B was 0.085% TFA in acetonitrile-water (8:2, v/v). Runs were performed using a gradient of 20-65% B in 40 min. The eluent was directly mixed in a micro-tee (Upchurch, Oak Harbor, WA) with matrix solution (2 mg of alpha-cyano-4-hydroxycinnamic acid in 1 ml of 0.1% TFA in acetonitrile/water, 7:3) at a 1:4 flow rate ratio and spotted onto blank target plates (Applied Biosystems). A total of 312 spots per

run were deposited in a 24 x 26 array in which each spot consisted of 10 s of chromatographic time.

MS/MS experiments was performed on a MALDI-TOF/TOF instrument (4700 Proteomics analyzer, Applied Biosystems, Framingham, MA, USA) equipped with an Nd:YAG laser (355 nm) operating at a frequency of 200 Hz. MS spectra were acquired in positive ion reflector mode by accumulation of 3000 consecutive laser shots. After completion of the acquisition of MS spectra the precursor ions for MS/MS analysis were selected automatically according to the selection criteria (a maximum of 5 peaks per spot, S/N > 35, 60 ppm fraction-to-fraction precursor mass tolerance). Both MS and MS/MS spectra were acquired using the instrument default calibration that was updated directly before the run. Fragmentation spectra were acquired with a minimum of 4000 and a maximum of 8000 laser shots (signal-to-noise of fragment ions-dependent stop condition was used). The precursor mass window was set to 80 (FWHM), the collision energy was 1 keV, and air was used as the collision gas. The GPS Explorer (version 3.5, Applied Biosystems) was used for processing and to submit the data to the MASCOT server (version 2.0, Matrix Science Ltd., London, UK) for in-house search against the NCBI non-redundant protein database (NCBINr 20050605). The maximum of two missed cleavages was allowed and the mass tolerance of precursor and sequence ions was set to 100 ppm and 0.15 Da, respectively. The search included variable modifications of cysteine with acrylamide, methionine oxidation, and the Carboxy-terminal $^{16}O/^{18}O$ exchange. A protein was accepted as identified if the total MASCOT score was greater than the significance threshold and at least 2 peptides appeared the first time in the report and were the first ranking peptides.

3.2.3.10 Quantification of proteins

Relative quantitation of proteins was performed using a previously described algorithm (Korbel, Schumann et al. 2005). Briefly, relative protein amounts were calculated from relative amounts of ^{18}O-labeled peptides which were identified by MS/MS with a score above MASCOT's homology threshold. Using signal intensities of tryptic peptides containing no, one, and two ^{18}O at m/z, (m+2)/z, and at (m+4)/z, respectively, the method considers that either one or both oxygen atoms of the Carboxyl group could be exchanged during in-gel digestion of the protein. The contribution of naturally occurring isotopes at (m+2)/z and at (m+4)/z as well as the isotopic purity of ^{18}O water (a = 0.95) were considered. Quantification of all proteins was based on calculations of isotope intensity ratios of at least two different

tryptic peptides and was performed twice; if the resulting ratio deviated by more than 30%, the experiment was repeated.

3.2.3.11 Recombinant protein expression and purification

6×His-tagged proteins were expressed and induced at 30°C for 4 h in the presence of a final concentration of 0.5 mM IPTG. Bacterial pellets were harvested by centrifugation at 5,000 rpm, pellets were suspended in 10 ml of Tris buffer (50 mM Tris-HCl, pH 8.0; 1 mM $MgCl_2$; 1 mM DTT; 0.25 mM PMSF, 6 µM GTP) and stored at -20°C. Protein expression and solubility were checked by SDS-PAGE and Coomassie blue staining. The protocol for protein purification was as follows: 100 µl of 100 mM PMSF, 10 µl of Benzonase, tips of lysozyme (Carl Roth GmbH, Karlsruhe) were added to the re-suspended bacteria on ice for 10 min. The bacteria were sonicated once (90 sec, 50% duty cycle, 60% power) on ice (to prevent heating) and 1% CHAPS was adjusted and added on ice for 10 min. After centrifugation at 17,000 rpm for 10 min, the supernatant was transferred to 400 µl of His-Select^{TM} Nickel Affinity gel (Sigma) in the presence of 10 mM imidazole pH 8.0 and incubated on a rotating wheel at 4°C for 1.5 h. The binding solution was centrifuged at 2,000 rpm for 1 min and beads were washed first with Tris buffer supplemented with 0.1% CHAPS and 10mM imidazole for 5 min. The centrifugation was repeated twice followed by washing with Tris buffer in the presence of 10 mM and 20 mM imidazole. Proteins were eluted into 1.5 ml of elution buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 6 µM GTP, 200 mM imidazole, 0.25 mM PMSF) at 4°C for 1.5 h. Eluted proteins were centrifuged at 4,000 rpm and supernatant was further centrifuged at 65,000 rpm (Beckman TLA110) for 15 min. The supernatant was dialyzed against dialysis buffer (20 mM HEPES-NaOH pH 7.4, 100 mM NaCl, and 1 mM $MgCl_2$ 10% glycerol) at 4°C overnight. Proteins were centrifuged at 65,000 rpm for 15 min and supernatant protein concentration was determined according to Bradford. Proteins were either stored at -80°C as aliquots or used freshly.

To produce recombinant myristoylated Arfs, competent *E.coli* strain ER2566 transformed with pBB131 plasmid containing yeast *N*-myristoyltransferase was prepared and used to express the desired proteins. 200 µM of myristic acid (90 mg was dissolved into 5 ml 100% ethanol and 2.5 ml was used for 1 l culture) was added after the OD_{600} reached 0.8. 10 min later, IPTG for protein induction was added for 4 h.

It has been suggested that small GTPase proteins are mostly in the GTP-bound form when purified from *E.coli*. In order to get a homogenous protein conformation with the same nucleotide bound, nucleotides were exchanged *in vitro*. Bacterial lysates were first incubated with Nickel agarose, and bound proteins were washed with 2 ml of Tris buffer supplemented with 2 mM EDTA, 100 µM GTP (or GDP), 10 mM imidazole and 0.1% CHAPS for 5 min. Immobilized proteins were then washed with Tris buffer supplemented with 10 mM imidazole, 5 mM $MgCl_2$ and 100 µM GTP (or GDP). The final wash also included 20 mM imidazole. The remainder of the purification procedure was the same as that for Arf protein purification without nucleotide exchange.

GST fusion proteins were either induced at 30°C for 4 h or at 16°C for overnight in the presence of 0.5 mM IPTG. Bacteria were harvested and suspended in PBS. Bacterial lysates were prepared as described above for 6×His tagged proteins. Supernatants were incubated with GST sepharose at 4°C for 1.5 h, washed three times with cold PBS for 5 min each. GST fusion proteins were eluted with PBS pH 7.4 supplemented with 20 mM of reduced glutathione (GSH) at 4°C for 1.5 h. Eluted proteins were centrifuged at 65,000 rpm for 15 min and then dialyzed against PBS with 10% glycerol at 4°C overnight. Proteins were stored at -80°C. For some proteins, the GST-tag has to be cleaved. To this purpose, on-bead cleavage was carried out. 3 U of thrombin per one mg of protein was used to elute GST-fusion proteins at 4°C for 3 h. PMSF was added to a final concentration of 1 mM at the end of elution to prevent further protein digestion. Supernatant was centrifuged at 65,000 rpm for 15 min and applied to a pre-equilibrated Supdex 75 (Amershan) column and run at 1 ml/min. Fractions were collected and checked by SDS-PAGE and Coomassie blue staining.

3.2.3.12 M9 medium preparation, protein expression labeled with ^{15}N and ^{13}C for NMR analysis

For NMR analysis of liposome-bound Arf proteins, recombinant ^{15}N- and ^{13}C-labeled proteins have to be expressed using M9 medium. The recipe to prepare M9 medium is following:

Salts (10×) pH 7.2-7.3 for 1 l of M9 medium (autoclaved)

$Na_2HPO_4 \times 2H_2O$	80 g
KH_2PO_4	20 g
NaCl	5 g

Trace elements for 500 ml stock solution (100×) pH 7.5-7.7 (autoclaved)

EDTA	(in 400 ml H_2O, NaOH is used to adjust to pH 7.0)	2.5 g
$FeSO_4$		250 mg

$ZnCl_2$	25 mg
$CoSO_4$	5 mg

1 l M9 medium

850 ml of autoclaved dH_2O was added in order with the following components:

Tracer element (A)	10 ml
1 M $MgSO_4$ (A)	1 ml
1 M $CaCl_2$ (A)	0,3 ml
M9 salts 10× (A)	100 ml
20% ^{13}C-Glucose (SF)	20 ml
Thiamin HCl (1 mg/ml) (SF)	1.5 ml
Biotin 0.1 mg/ml (SF)	15 ml
$^{15}NH_4Cl$ 5 g/20 ml (SF)	2 ml
	149.8 ml

(A) Autoclave (SF) sterile filter

3 g ^{13}C-Glucose and 0.5 g of $^{15}NH_4Cl$ were dissolved in 20 ml and 2 ml of autoclaved dH_2O, respectively, and passsed through 0.2 µm filters.

To express labeled proteins, 50 ml of LB media were inoculated with 1 ml of overnight culture. After 8 h shaking culture at 37°C, bacteria were spun down at 5,000 rpm for 10 min. The pellets were re-suspended in 50 ml of M9 medium supplemented with $^{15}NH_4Cl$ and ^{13}C-Glucose and grown at 37°C overnight. These 50 ml overnight M9 cultures were used to inoculate 1 l M9 medium containing $^{15}NH_4Cl$ and ^{13}C-Glucose and shaken at 37°C until OD_{600} reached 0.6. IPTG was added to the final concentration to 0.5 mM to induce protein expression at 30°C for 4 h.

3.2.3.13 Protonation of phosphoinositides using acid

The phosphoinositide powder was dissolved in chloroform, dried under nitrogen stream and desiccated at RT for 1 h. The powder was re-suspended in chloroform:methanol:1N HCl with a molar ratio of 2:1:0.01 and incubated at RT for 15 min. The solution was dried under nitrogen stream. The powder was desiccated again at RT for 1 h, dissolved in chloroform:methanol (3:1) and dried under nitrogen stream. The powder was dissolved in chloroform, dried and finally dissolved in chloroform. The solution was stored at -80°C until use.

3.2.3.14 Liposome preparation and protein binding assay

Large unilamellar vesicles (LUVs) were prepared as described (Ford, Mills et al. 2002). Briefly, for liposome preparation, 50% PC, 40% PE and 10% of tested lipids (w/v) were dissolved in chloroform, dried under nitrogen stream for the formation of a thin layer of lipid sheets on the wall of a glass tube. Residues of chloroform were prevented by applying vacuum at RT for at least 1 h (shed from light). Re-hydration was performed at 37°C using

300 µl of 0.3 M sucrose or buffer needed for 1 h. In the case of using sucrose, 1.7 ml of dH_2O was added after re-hydration and liposomes were harvested by centrifugation at 20,000 rpm using TLA110 rotors for 1 h. Pellets were re-suspended in desired buffer (20 mM HEPES-NaOH pH 7.4, 100 mM NaOH and 1 mM $MgCl_2$) and passed through 400 nm or 200 nm polycarbonate membranes eleven times using an extruder.

To check the protein binding ability, 4 µg of proteins in 50 µl of buffer were incubated with equal volumes of 2 mg/ml of liposomes in the presence of 100 µM GTP, GDP or GTPγS at RT for 10 min. Liposomes were recovered by centrifugation at 14,000 rpm for 15 min. Supernatants were removed to new 1.5 ml Eppendorf tubes and pellets were re-suspended in 120 µl Laemmli protein sample buffer. Proteins that bind to liposomes should co-pellet with liposomes. Protein binding profiles were evaluated by separating 30 µl of the pellet sample by 13% SDS-PAGE, followed by Coomassie blue staining. Negative controls were included by mixing 4 µg of corresponding proteins in 50 µl buffer with equal volume of buffers without liposomes. For $PI(4,5)P_2$ liposome binding, epsin ENTH domain was used as a positive control (Ford, Mills et al. 2002).

3.2.3.15 *In vitro* tubulation assay and EM negative staining

For tubulation assays, liposomes with two different compositions were used: Folch fraction I + 10% $PI(4,5)P_2$ or 45% PC + 20% PE + 20% PA + 5% cholesterol + 10% $PI(4,5)P_2$. Liposomes were passed through 400 nm polycarbonate membranes to induce a relatively uniform size distribution. 4 µM of proteins were mixed first with liposomes at RT for 1 min and then loaded on the carbon grid. Uranyl acetate was used as a contrasting reagent. An alternative way was to sediment liposomes on the carbon-coated grid at RT for 3 min, followed by addition of proteins for 1 min.

For live imaging of *in vitro* tubule formation from flat membrane sheets, an assay was established as illustrated in **Figure 3-2** (Itoh and De Camilli 2006). Glass coverslips (22 mm×40 mm) were cleaned by sonication in 1% 7× (MP Biomed., Germany). After rigorous washing with dH_2O, the coverslips were finally washed with 100% ethanol and stored until use. Coverslips were dried under nitrogen stream flux just before use. Two 1 µl droplets of 10 mg/ml lipid mixture were spotted onto each coverslip and put into vacuum (0.2 milli-torr) for at least 1 h. Lipids were pre-hydrated at 37°C in an incubator (10% CO_2, 100% humidity) for 30 min. A small chamber was built by inverting the coverslips on the glass slides with

double-sided strips as spacers. Lipid sheets were completely re-hydrated by injecting 20 µl of buffer. 5 µl of protein was injected from one side of the chamber and membrane deformation was recorded in differential interference contrast (DIC) mode.

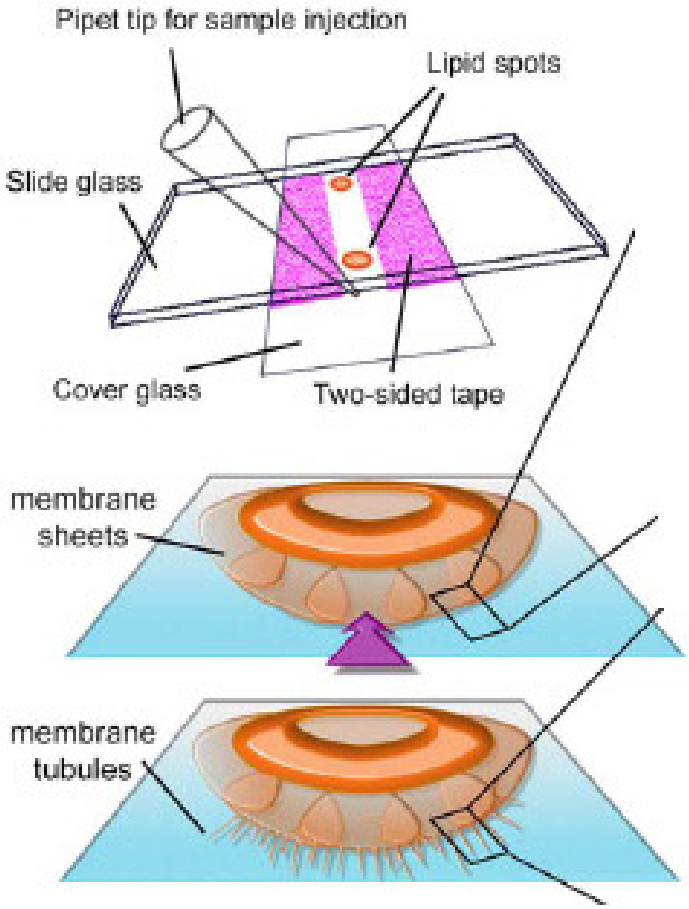

Figure 3-2 *In vitro* liposome deformation assay from lipid membrane sheets

A chamber was established between a coverslip with two spotted lipid and a glass coverslip with double-sided strips as spacers. After rehydration of the lipid sheets, proteins were injected into the chamber from one side of the chamber. If the protein could deform the membrane sheet, tubules should be seen at the proximal sides of the protein injection in the DIC mode. (Taken from Itoh *et al.* 2006).

3.2.3.16 Clathrin purification from pig brain

Pig brains were delivered and washed immediately with ice-cold tap water in a 4 l beaker several times until the water became clear. White matter and large vessels were removed. Brains were cleaned, rapidly frozen in liquid nitrogen and stored at -80°C until use.

One day before the experiment, brains were slowly thawed at 4°C overnight. 400 ml of buffer A (100 mM Mes pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3, 0.5 mM DTT) was filled in a blender and 5 ml of 100 mM PMSF was added. Thawed brains were mixed at low

and high speeds for 1 min sequentially with 2 min interval to prevent over-heating. The homogenate was poured into 500 ml tubes and centrifuged at 12,000 rpm (17,700 g) using the SLA-3000 rotor for 45 min. Supernatants were carefully transferred into new 500 ml tubes; pellets were re-suspended in 100 ml of buffer A and shaken vigorously. Both the supernatant and pellets were centrifuged at 12,000 rpm (SLA-3000) for another 30 min. The supernatants were transferred into a pre-cooled beaker on ice, divided into Ti45 tubes (fill completely to prevent collapse) and centrifuged at 150,000 g (44,000 rpm) for 1 h; this step was repeated until all supernatants were finished. Supernatants were discarded and several pellets were sequentially re-suspended in 10 ml of buffer A. The final volume for all re-suspended pellets was about 20 ml and then 200 µl of 100 mM PMSF was added. The re-suspensions were homogenized with 5-10 strokes (2,000 rpm) in a Teflon homogenizer. Final volume was adjusted to 25 ml using buffer A, and then equal volume of 12.5% Ficoll/12.5 sucrose was added and mixed. Samples were centrifuged using TLA60-Ti rotor tubes at 35,700 rpm (90,600 g) for 30 min. Supernatants were collected and 4 volumes of buffer A were added. Ti45 rotor tubes were used to centrifuge the diluted supernatants at 150,000 g (44,000 rpm) for 1 h. Supernatans were discarded and pellets were re-suspended in buffer A with total volume not more than 10 ml. After homogenization at 2,000 rpm for 5-10 strokes, the final volume was adjusted to 10 ml. 5 ml of buffer B and 150 µl of 100 mM PMSF (freshly prepared) were added and incubated at RT for overnight. The next day, 15 ml of dH_2O was added to reduce the sample Tris concentration to 0.4 M, diluted samples were centrifuged using TLA60-Ti rotor at 50,000 rpm for 1 h. Supernatants were carefully transferred to a new 50 ml Falcon tube except ~2 ml on top of the pellets which was collected in a separate tube and spin in a TLA110 tube at 100,000 rpm for 20 min. This supernatant was combined with the previous one (total volume would be ~30 ml). The combined supernatants were concentrated by centrifuging at 5,000 rpm using a 30 kD concentration tube (Amicon® Ultra) until the volume was around 5 ml. Concentrated proteins were further filtered by passing through a 0.2 µm filter. For gel filtration, HiPrep 26/60 Sephacryl S-400 HR column was operated by an ÄKTA™ prime liquid chromatography system. (Amersham Biosciences, GE healthcare). The column was pre-equilibrated with two bed volumes of running buffer. 5 ml of sample was injected and the chromatography was run at the speed of 1 ml/min with the pressure limit of 0.15 MPa.

Buffer A:	100 mM Mes pH 6.5	29.25 g	58.5 g	
	1 mM EGTA	30 ml	60 ml	100 mM stock
	0.5 mM $MgCl_2$	0.375 ml	0.75ml	2 M stock
	0.02% NaN_3	3 ml	6 ml	10% stock
	0.5 mM DTT	15 ml	30 ml	50 mM stock
		1.5 l	3 l	dH_2O

Buffer B:	2.4 M Tris-HCl pH 7.4	14.5 g	Tris base
	3 mM EGTA	1.5 ml	100 mM stock
	0.04% NaN_3	200 µl	10% stock
	1 mM DTT	1 ml	50 mM DTT
		50 ml	dH_2O
12.5% Ficoll/12.5% sucrose in buffer A:		7.5 g	Ficoll
		7.5 g	sucrose
		60 ml	Buffer A
Column running buffer:	0.5 M Tris-HCl, pH7.4	121.1 g	Tris base
	1.0 mM EGTA	20 ml	100 mM EGTA
	0.02% NaN_3	4 ml	10% stock
	0.5 mM DTT	20 ml	50 mM DTT
		2 l	dH_2O

* adjust pH prior to the addition of DTT and PMSF was freshly prepared!

3.2.4 Cell biology

3.2.4.1 Tissue culture cell lines and storage

COS7 African Green Monkey Kidney fibroblasts

DMEM (low glucose 1000 mg/L) (+GlutaMax+pyruvate) + 10% FBS + 1% P/S

HEK293 Human Embryonic Kidney fibroblast
DMEM (high glucose 4500 mg/L + L-Glutamine, -Pyruvate) + 10% FBS + 1% P/S

PC12
DMEM (high glucose 4500 mg/L + L-Glutamine, -Pyruvate) + 10% FBS + 5% HS + 1% P/S + 1% L-Glutamine

NIH 3T3 Mouse embryonic fibroblast
DMEM + 10% FBS +1% P/S + 1% L-glutamine

DMEM stands for Dulbecco's Modified Eagle's Medium (DMEM). FBS (Fetal Bovine Serum) is normally heat inactivated at 56°C for 30 min. HS stands for Horse Serum and is filtered before use. P/S stands for penicillin (50 units/ml) and Streptomycin (50 µg/ml)

Freezing medium: 20%DMSO, 50% FBS and 30% medium. Cells were kept in liquid nitrogen for long term storage.

3.2.4.2 Transfection, indirect immunofluorescence microscopy analysis

Cells were normally grown in a 10 cm dish and medium was regularly changed every 2-3 days. For transfection, cells were split and grown on 3.5 cm petri dishes until 70-80% confluent. Just before transfection, cells were changed to fresh medium. For transfection solution preparation, 1 µg of DNA and 10 µl of lipofectamine™ reagent (Invitrogen) were separately mixed with 150 µl of Opti-MEM® in poly-styrol tubes at RT for 5 min and then combined at RT for 30 min. 300 µl of transfection solutions were added to the fresh-medium fed cells at 37°C in a humidified incubator with 95% O_2 and 5% CO_2 for 4 h. Medium was changed once. After incubation of another 4 h, cells were washed briefly with pre-warmed PBS and treated with typsin/EDTA for detachment from the petri dish. Cells were split and grown on 18 mm coverslips in 37°C incubators overnight.

Immunofluorescence was performed the next day after transfection. Cells were briefly washed twice with PBS and fixed with 4% PFA at RT for 30 min or ice-cold methanol at -20°C for 5 min. Cells were washed twice with PBS and once with high-salt-PBS (HSPBS) for

5 min. Goat serum dilution buffer (GSDB) was used to permeabilize cells and prevent unspecific binding for 30 min. Primary antibodies were diluted into GSDB and applied to cells in a humidified chamber at RT for 2 h. Cells were washed three times with PBS. HRP-conjugated secondary antibodies were also diluted in GSDB and incubated in a dark humidified chamber at RT for 1 h (shed from light). Cells were further washed three times with PBS and mounted on to objective slides in the presence or absence of DAPI. After drying at RT, slides were kept at 4°C; otherwise slides were immediately analyzed by fluorescent microscopy.

A Zeiss Axiovert 200M Digital Research Microscope illuminated by a 150 W Xe lamp under the control of the 3i Inc. Ratio Imaging System (Stallion). With the aid of Slidebook 4.0.8 Digital Microscopy Software (Göttingen, Germany) three algorithms, no neighbor deconvolution, nearest neighbor deconvolution and constrained iterative deconvolution were used to obstain quasi-confocal images to improve z-resolution. DAPI, GFP and RFP channels were generally used to visualize fluorescence. 40× and 63× oil-immersion objectives were used.

3.2.4.3 Primary rat hippocampal neuron-glia co-cultures

Postnatal (DIV 0 or 1) rats were decapitated. Hippocampi with a banana shape were removed into a 3 cm petri dish with Hank's+20% FCS. Hippocampi were cut into small slices, transferred into a 15 ml Falcon tube and filled to 5 ml with Hank's+20% FCS. The small slices accumulated at the bottom automatically by gravity, the supernatant was discarded. This procedure was repeated once. Slices were washed twice with Hank's solution without FCS. 5-6 mg of trypsin was dissolved in 1 ml of digestion solution and 10 µl of DNase was added. Filtered trypsin solution (0.2 µm) was applied to hippocampal slices. Another 1 ml of digestion solution was filtered and combined with the previous one. Slices were digested at 37°C for 10 min. Supernatants were carefully discarded and pellets were washed twice with Hank's+20% FCS and twice with Hank's without FCS. Digested slices were collected at the tube bottom by gravity between washing steps. 1 ml of dissociation solution was supplemented with 10 µl of DNase, filtered and applied to the slices at 37°C for 10 min. Supernatants were removed and 2 ml of plating medium was added. The dissociated slices were passed through a Pasteur pipette several times until the solution became cloudy. The solution was further passed through a fire-polished Pasteur pipette several times. Dispersed hippocampal cells were counted using a hemacytometer and 30,000-50,000 cells in 250 µl plating medium were plated on to a Matrigel-coated 18mm coverslip for 1 h. 750 µl growth

medium was added afterwardss. Half of the volume was replaced with fresh medium twice a week.

Hank's solution
Hank's balanced salt solution w/o calcium or magnesium
1 mM HEPES-NaOH pH 7.4
50 mg/l $NaHCO_3$

Dissociation solution
Hank's solution
12 mM $MgSO_4$

Digestion solution
25 mM HEPES-NaOH pH 7.4
137 mM NaCl
5 mM KCl
7 mM Na_2HPO_4

Basic medium
Minimal essential medium
1.25 g/l glucose
50 mg/l $NaHCO_3$
25 mg/l transferrin

Plating medium
Basic medium
10% FCS
2 mM L-glutamine
25 mg/l insulin

Growth medium
Basic medium
10% FCS
0.5 mM L-glutamine
2% B27-supplement (Gibco)
2-4 μM cytosine arabinoside

3.2.4.4 *In vitro* targeted differentiation of neuron from ES cells

The protocol was modified from a previous study by Kawasaki *et al.* (Kawasaki, Mizuseki et al. 2000). ES14 cells were grown in 10 cm dishes coated with 0.1% gelatin (at least 1 h). 20 U/ml of LIF (leukemia inhibitory factor) was added to the culture medium and cells were split every second day 1:5 or 1:10 to prevent differentiation. For targeted neuron differentiation, ES cells were first trypsinized and 10 ml of differentiation medium was added to stop the reaction. Cell numbers were determined: 3,000 ES cells were diluted into 10 ml of differentiation medium and placed on a confluent monolayer of PA6 cells in a 10 cm dish. Medium was changed after 4 days and every day during the next following 4 days. On day eight, cell aggregates were dissociated by trypsinization and passed through a fire-polished paster pipette. Dissociated neurons were cultured on matrigel-coated coverslips for another 24 h until used for immunofluorescence.

ES14 cell medium
BHK 21 (Glasgow MEM)
1% FCS
10% knock out serum replacement (KSR, Gibco)
2 mM L-glutamine
1 mM sodium pyruvate
0.1 mM nonessential amino acids
0.1 mM β-mercaptoethanole
1% Pen/Strep

Differential medium
BHK 21
10% KSR
2 mM L-glutamine
1 mM sodium pyruvate
0.1 mM nonessential amino acids
0.1 mM β-mercaptoethanole

PA6 cell medium is: Alpha-MEM + 10% FCS + 2 mM L-glutamine + 1% Pen/strep

3.2.4.5 Triton X-100 extraction of primary hippocampal neurons

Mixed hippocampal neuron-glia co-cultures (14-20 DIV) grown on glass coverslips were washed with phosphate-buffered saline (PBS) and extracted with 500 µl of 120 mM Na-Phosphate pH 7.4 supplemented with 0.5% Triton X-100 either on ice or at 37°C for 10 min. Neurons were carefully washed twice with PBS, fixed and proceeded for immunostaining. Images were acquired on a Zeiss Axiovert 200M fluorescent microscope equipped with the Stallion system (3i Inc.) and analyzed by SlideBook™ software using nearest neighbor deconvolution.

3.2.4.6 Cholera toxin uptake

Hippocampal neurons prepared from E18 rat embryos (14 DIV) were briefly washed with Krebs-Ringer-HEPES (KRH) solution containing 128 NaCl, 25 HEPES, 4.8 KCl, 1.3 CaCl, 1.2 $MgSO_4$, 1.2 KH_2/K_2HPO_4 (all in mM) and 5.6% glucose, and then incubated with 10 µg/ml of FITC-CTB in KRH/high K^+ (110 mM K^+ and a corresponding reduction in Na^+) at 37°C for uptake for 5 min. Neurons were thoroughly washed with KRH buffer, fixed, and immunofluorescence was performed using monoclonal antibodies against flotillin 1, transferrin receptor or synaptotagmin 1.