Preparation of Brain Membrane Fractions

This protocol has been modified from one provided by Michel Ehlers (Duke University).

1- Crude Membrane/Cytosol Prep

Be sure that all procedures are done with precooled reagents at 4°C.
Dissect out brain regions of interest into ice-cold into 10 volumes of cold homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA, protease inhibitors, phosphatase inhibitors as below).
Homogenize using 10-15 strokes of a motor-driven glass-teflon homogenizer. Never use polytron.
Spin at 1000 x g for 15 min to remove pelleted nuclear fraction (P1).
Take supernatant (S1) and spin at ~200,000 x g (50,000 rpm for 30 min in 70.1 Ti rotor; 62,000 rpm for 15 min in TLA100.3 rotor) to yield crude cytosol (S2) and crude membrane pellet (P2).
Resuspend pellet in homogenization buffer.
Spin again at ~200,000 x g to yield washed crude membrane pellet (P2').
Resuspend pellet in HEPES-Lysis buffer (50 mM HEPES pH 7.4, 2 mM EDTA, protease/phosphatase inhibitors).
Measure protein concentration by BCA or Coomassie.
Can solubilize with detergents or store at –80°C.

2- Microsome Prep

Microsomes = vesicles derived from rough ER and various smooth membrane bound organelles (including Golgi stack components).

Homogenize brain in homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA, protease inhibitors, phosphatase inhibitors as below).
Spin at 1000 x g for 15 min to remove pelleted nuclear fraction (P1).
Take supernatant (S1) and spin at 10,000 x g for 20 min to remove pelleted mitochondria (P2).
Spin supernatant again at 12,000 x g for 30 min to doubly remove pelleted mitochondria (P2).
Take resulting S2 supernatant and centrifuge at 140,000 x g for 120 min to yield microsomal pellet (P3).
The resulting microsome pellet can be resuspended in homogenization buffer and further fractionated by layering over a discontinuous sucrose gradient (0.8, 1.0, 1.3, and 2.0 M sucrose) and centrifuging at 97,000 x g for 120 min.
I can't quite remember what organelles are present at each interface, but Wenthold typically uses the interfaces between 1.0 and 0.8 M and between 0.8 M and 0.32 M.

For reference, see Gurd et al., (1974). *J. Neurochem.* 22:281-290.

3- **Synaptic Plasma Membrane Prep**

This protocol is adapted from Blackstone et al. (1992) and Lau et al. (1996) and should be followed when an enriched membrane prep or PSD fraction is desired

All procedures should be done at 4°C using precooled reagents. For rat and mouse samples, immediately remove brain from the cranium into ice cold HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES pH 7.4) containing a freshly added protease inhibitor cocktail (required, see below) and phosphatase inhibitor cocktail (optional, see below). Different parts of the brain can be subdissected and enriched plasma membranes/PSD can be prepared as described below.

Protease inhibitors
PMSF (0.1 mM)
Aprotinin (1.5 μg/ml)
Antipain/leupeptin (10 μg/ml ea.)
Chymostatin/pepstatin (10 μg/ml ea.)
Benzamidine (0.1 mg/ml)

Phosphatase Inhibitors
EGTA (2 mM)*can interfere with protein determination
NaF (50 mM, VWR 1 mM)* can osmotically shock proteins
sodium pyrophosphate (10 mM)
β-glycerophosphate (20 mM) *100x, -20ºC
para-nitrophenylphosphate (PNPP) (1 mM)
* add fresh, hydrolyzes fastosmotically shock proteins
microcystin LR (optional) (1 μM)
Sodium orthovanadate (1 mM) *100x, 4ºC
Ammonium molybdate (0.1 mM) *100x, 4ºC

1. Add 10 volumes of HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES pH7.4) to the tissue and homogenize in a motor driven glass-teflon homogenizer at ~900 rpm (10-15 strokes). Never use polytron.
2. Centrifuge the homogenate (Hom.) at 800-1000 x g at 4°C to remove the pelleted nuclear fraction (P1). Note: Save 150 ul aliquot.
3. Spin resulting supernatant (S1) at 10,000 x g for 15 min (9200 rpm in SL50T rotor) to yield the crude synaptosomal pellet (P2).
4. Save supernatant (S2), which contains the cytosol and light membranes- Spin at 100,000xg for 15 min to produce the cytosolic fraction (S2') and the LM fraction.
5. Resuspend pellet from step 3 (P2) in 10 volumes of HEPES-buffered sucrose and then respin at 10,000 x g for another 15 min to yield the washed crude synaptosomal fraction (P2').
6. Lyse resulting pellet by hypoosmotic shock in 9 volumes ice cold H_20 plus protease/phosphatase inhibitors and three strokes of a glass-teflon homogenizer
7. Rapidly adjust to 4 mM HEPES using 1 M HEPES, pH 7.4 stock solution.
8. Mix constantly in cold room for 30 min to ensure complete lysis.
9. Centrifuge lysate at 25,000 x g for 20 min (14,500 rpm in SL50T rotor) to yield a supernatant (S3, crude synaptic vesicle fraction) and a pellet (P3, lysed synaptosomal membrane fraction). Save S3 for synaptic vesicle prep.
10. Resuspend P3 pellet in HEPES-buffered sucrose (~1.5ml for 2-4 P18 brains).
11. Using a Pasteur pipet, layer the resuspended membranes on top of a discontinuous gradient containing 0.8 to 1.0 to 1.2 M sucrose (top to bottom; equals 27%/34%/41%) in an ultraclear tube. [I use a 13 ml tube layered with 4 ml 1.2 M sucrose, 3 ml 1M, 3 ml 0.8 M and then approx 1.5 ml sample on top. Don't forget to add protease/phosphatase

inhibitors to the sucrose solutions! Always use a Pasteur pipet to pour layers and add sample].

12. Centrifuge the gradient at ~150,000 x g for 2 hr in a swinging bucket rotor (30,000 rpm in SW41 Ti; 36,000 rpm in SW50.1 Ti; 42,000 rpm in TLS-55; 28,0000 rpm in SW28).
13. Recover synaptic plasma membranes in the layer between 1.0 and 1.2 M sucrose. [Carefully puncture tube with needle and withdraw band with a syringe]
14. Dilute to 0.32 M sucrose by adding 2.5 volumes of 4 mM HEPES pH 7.4.
15. Pellet by centrifugation at 150,000 x g for 30 min. 42,000 rpm in 70.1 Ti; 55,000 rpm in TLA 100.3
16. Resuspend resulting pellet (SPM) in PBS (pH 7.4) or (50 mM HEPES pH 7.4, 2 mM EDTA) with protease and phosphatase inhibitors. Save 150 ul aliquot. Save the rest at -80°C.

Postsynaptic Densities

For references, see Carlin et al. (1980) and Cho et al. (1992).

1. Resuspend synaptic plasma membranes prepared as above in 3-5ml of ice cold 50 mM HEPES pH7.4, 2 mM EDTA, plus protease/phosphatase inhibitors. Add Triton X-100 to 0.5%
2. Rotate in cold room for 15 min.
3. Centrifuge at at 32,000 x g for 20 min (22,000 rpm in 70.1 Ti; 28,000 rpm in TLA 100.3) to obtain the PSD-1T pellet.
4. Resuspend PSD-1T in 2 mL ice-cold 50 mM HEPES pH7.4, 2 mM EDTA plus protease/phosphatase inhibitors). Save 200 ul aliquot.
5. To half of the remaining resuspended pellet (~1 mL), add Triton X-100 to 0.5% and rotate in cold room again for 15 min.
6. Centrifuge at 200,000x g for 20 min to obtain the PSD-2T pellet. 50,000 rpm in 70.1 Ti; 65,000 rpm in TLA 100.3. Resuspend PSD-2T pellet in 100 ul (depends on size of pellet) of 50 mMHEPES pH 7.4, 2 mM EDTA plus PIs. Note: PSD-2T pellets may need some SDS (0.2%) to dissolve completely. I generally add the SDS, then boil at 65 degrees for 5 min.
7. In a separate experiment resuspend the second half of the PSD-1T pellet and incubate for 10 min in ice-cold 3% sarcosyl (same as N-lauroyl sarcosine) in 50 mM HEPES pH7.4, 2 mM EDTA, plus protease/phosphatase inhibitors.
8. Centrifuge at 200,000 x g for 1 hr to obtain the PSD-1T+S pellet.
9. All pellets can be resuspended in PBS or 50 mM HEPES pH7.4, 2 mM EDTA plus protease/phosphatase inhibitors or SDS-PAGE sample buffer.

OTHER SYNAPTIC FRACTIONS

Synaptic Vesicle Prep

1. To prepare synaptic vesicles, prepare S3 fraction as in *Synaptic Plasma Membrane Prep* above.
2. Centrifuge S3 fraction at 165,000 x g for 2 hr. 55,000 rpm in TLA 100.3, 45,000 rpm in 70.1 Ti.
3. Resuspend in PBS or 50 mM HEPES pH7.4, 2 mM EDTA plus protease/phosphatase inhibitors.

Pure Synaptosome Prep

For crude synaptosomes, can simply use P2' fraction from the *Synaptic Plasma Membrane Prep*. For added purity:

1. layer P2' fraction onto 4 ml of 1.2 M sucrose.
2. Centrifuge at 230,000 x g for 15 min in swinging bucket rotor. 38,000 rpm in SW 41 Ti, 44,000 rpm in SW50.1, 52,000 rpm in TLS-55.
3. Collect gradient interphase.
4. Dilute to ~7-8 ml with ice-cold HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES pH 7.4).
5. Layer onto 4 ml of 0.8 M sucrose.
6. Centrifuge at 230,000 x g for 15 min.
7. Pellet contains pure synaptosomes.
8. For bioassays, wash and resuspend in appropriate incubation buffer:
   For electroporation or functional release assays: (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$, 20 mM HEPES pH 7.4, 10 mM dextrose (added fresh), ±1.8 mM $CaCl_2$, and optional ATP regenerating system.
   For digitonin permeabilization: 115 mM KOAc, 25 mM HEPES, pH 7.4, 5 mM NaOAc, 0.05 mM EGTA containing 0.025% digitonin, an ATP regenerating system, and 10 mM DTT. (see Meacham, Patterson, et al., Nature Cell Biology, 2001)

   ATP regenerating system – 5 mM Mg-ATP, 80 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase

9. Otherwise, can solubilize as desired, or subfractionate further into synaptosomal membranes and synaptic vescles by hypotonic lysis as described under *Synaptic Plasma Membrane Prep*.

Synaptoneurosomes

Advance Prep

a. Get rat
b. Prepare Dissection Buffer (~100 mL, see below) and oxygenate on ice.
c. Prepare Incubation Buffer (see below) and place on ice and 37°C if needed. Need ~30 mL/brain.
d. Put glass-glass Dounce homogenizer and 2-3 conical tubes on ice. (1 brain requires 15 mL Dounce homogenizer).
e. Prepare nylon syringe filters (100 μ and 50 μ). To do this, cut ends off of 30 mL syringes, warp double layer of nylon mesh over end and fasten tightly with rubber and (thick rubber bands for biohazard waste work well for this).
f. Prepare Millipore Mitex 10 μ filter (either the #LCWP 047 or LCWP 025) by fitting to Swinnex filter holders (Millipore). Be sure to tighten these HARD.
g. Get dissection tools/guillotine
h. Set up protein assay

1. Rapidly dissect out brain regions of interest in ice-cold oxygenated dissection buffer (see below) (212.7 mM sucrose, 2.6 mM KCl, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM dextrose, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, saturated with 95% O_2 and 5% CO_2). One can optionally include 20 μM CNQX plus 100 μM AP5 if excitotoxicity is a problem. Can also substitute kynurenic acid (2 mM) for CNQX and AP5.
2. Homogenize in 10 volumes ice-cold incubation buffer: 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$, 20 mM HEPES pH 7.4, 10 mM dextrose (added fresh), ±1.8 mM $CaCl_2$, and optional ATP regenerating system by 7 strokes with a loose pestle and 4 strokes with a tight pestle in a glass-glass tissue homogenizer (Kontes). Add protease inhibitors and phosphatase inhibitors if only using synaptoneurosomes for western. If using SN's for bioassay, leave out protease inhibitors and phosphatase inhibitors unless testing pharmacologically. Don't forget to add ubiquitin aldehyde for ubiquitination experiments!!!
3. SAVE ALIQUOT OF UNFILTERED BRAIN HOMOGENATE.
4. Pass homogenate over two layers of 100 μm pore nylon mesh filters (Small Parts, Inc.) pre-wetted with cold incubation buffer followed by two layers of pre-wetted 50 μm pore nylon mesh filters (optional) (Small Parts, Inc). This step is accomplished by cutting the ends of 20 mL syringes and fastening the nylon mesh tightly over using rubber bands. Homogenate is then placed in the syringe and gentle pressure applied.
5. Force homogenate through a final pass pre-wetted 10 μm pore LCWP 047 or LCWP 025 Millipore Mitex membrane filter (Millipore catalog # LCWP 047 00 and LCWP 025 00) fitted to Swinnex filter holders (Millipore). Make sure filter holder is VERY tight.
6. Collect the filtered particulate and spin at 1000 x g for 20 min at 4°C.
7. Discard supernatant.
8. Resuspend pellet in 5 volumes incubation buffer.
9. Respin at 1000 x g for 10 min at 4°C to obtain washed synaptoneurosomes.
10. Resuspend pellet in incubation buffer (~ 5 volumes) to a final protein concentration of ~ 0.5 – 1.5 mg/ml. Yield is approximately 20 mg SN/brain
11. Use synaptoneurosomes fresh for experiments (within 90 min) or freeze at –80°C if only to be used for western blot.
12. For bioassay experiments, may also want to add an ATP regenerating system, as well as some DTT (10 mM) if the synaptoneurosomes will be permeabilized or electroporated. See above section of synaptosomes for electroporation and digitonin solutions.

    ATP regenerating system – 5 mM Mg-ATP, 80 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase

Solutions

Stock Dissection Buffer 1L
KCl (2.6 mM)
NaH_2PO_4 (1.23 mM)
$NaHCO_3$ (26 mM)
Kynurenic Acid (2 mM)

Dissection Buffer	100 mL	made fresh
Stock Dissection Buffer	100 mL	
Sucrose (212.7 mM)	7.28 g	
Dextrose (10 mM)	0.18 g	
1M $MgCl_2$ (10 mM)	1 mL	
1M $CaCl_2$ (0.5 mM)	0.05 mL	

Stock Incubation Buffer	1L
NaCl (140 mM)	
KCl (5 mM)	
$NaHCO_3$ (5 mM)	
Na_2HPO_4 (1.2 mM)	
HEPES pH 7.4 (20 mM)	

Incubation Buffer	50 mL	100 mL	made fresh
Stock Incubation Buffer	50 mL	100 mL	
1M $MgCl_2$ (1 mM)	0.05 mL	0.1 mL	
1M $CaCl_2$ (1.8 mM)	0.09 mL	0.18 mL	
Dextrose (10 mM)	0.09 g	0.18 g	

Rotor Notes

Available rotors at Duke:

For our SuperT21 table top superspeed:
ST-H750 (swinging bucket, ~700 ml, max 3800 x g), SLC-250T (fixed angle, ~200 ml, max 20,000 x g), SL-50T (fixed angle, ~25 ml, max 41,000 x g).

For the floor model Beckman XL-90 ultracentrifuge
Dept. of Neurobiology
Type 70.1 Ti (fixed angle, max 450,000 x g), SW28 (swinging bucket, max 140,000 x g, 6 x 39 mL), NVT90 (near vertical, max 645,000 x g)
Dept. of Neurology
SW41 (swinging bucket, max 288,000 x g, 6 x 13.2 mL), SW 50.1 (swinging bucket, max 300,000 x g, 6 x 5 mL), Type 60 Ti (fixed angle, max 360,000 x g)

For the Beckman tabletop ultracentrifuge in Pate's lab
TLA 100.3 (fixed angle, max 540,000 x g), TLS-55 (swinging bucket, max 255,000 x g)