Published in final edited form as:

Curr Protoc Neurosci. 2012 April ; 0 2: Unit–2.722. doi:10.1002/0471142301.ns0207s59.

Characterizing Synaptic Vesicle Proteins Using Synaptosomal Fractions and Cultured Hippocampal Neurons

Jerome DiGiovanni^{1,2}, Tao Sun^{1,2}, and Zu-Hang Sheng¹

¹National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland

Abstract

Cloning and characterization of synaptic vesicle proteins and their binding counterparts on the presynaptic plasma membrane have greatly advanced our understanding of the molecular mechanisms involved in the synaptic vesicle cycle and neurotransmitter release. This unit discusses multidisciplinary approaches to characterize proteins from synaptosome-enriched subcellular fractions and localize them within cultured neurons. The first approach regroups methods used to isolate synaptic vesicles from rat brain synaptosomal preparations, allowing for specific biochemical investigation of synaptic vesicle proteins. The second is a detailed procedure for pre-embedding immunogold staining and electron microscopic observation, which permits the morphological identification of proteins in individual vesicles at intact synapses. Additionally, this chapter proposes methods for light microscopic examination of hippocampal neurons. It includes procedures for embryonic and postnatal hippocampal neuron culture and describes an immunocytochemical staining protocol used to investigate synaptic vesicle protein localization with respect to other proteins or subcellular structures.

Keywords

synaptosomes; hippocampal neurons; synapses; light microscopy; electron microscopy; immunocytochemistry; immunoblotting

INTRODUCTION

Chemical synapses are specialized intercellular junctions that allow neurons to communicate with each other and with other excitable cells. They utilize neurotransmitters as chemical signals that are released from presynaptic terminals and activate postsynaptic receptors with high temporal and spatial precision. A basic feature of presynaptic terminals is a cluster of neurotransmitter-loaded synaptic vesicles, some of which will respond to the action potential-driven Ca^{2+} influx into the nerve terminal by fusing with the plasma membrane and releasing their contents into the synaptic cleft. Synaptic vesicles that store non-peptide neurotransmitters (including glutamate, GABA, glycine, and acetylcholine) are small, membrane-bound, electron-lucent secretory vesicles (De Camilli et al., 2000). Within a

²These authors contributed equally to this work

given nerve terminal, these vesicles are of uniform shape and size (35 to 50 nm in diameter). The genes encoding many classes of synaptic vesicle membrane proteins have been cloned, and the functional roles of their protein products have been well characterized (Fernandez-Chacon and Südhof, 1999). Synaptic vesicle membrane proteins can be divided into two main groups based on their functions (De Camilli et al., 2000): those that transport ions and neurotransmitters across the synaptic vesicle membrane (e.g., the proton pump, neurotransmitter transporters, and SV2) and those that play a role in synaptic vesicle docking, priming, fusion, and recycling (e.g., synaptobrevin/VAMP2, synaptotagmin, Rab3-rabphilin, and synaptophysin). Molecular identification, biochemical and functional characterization, and specific distribution of synaptic vesicle proteins using multidisciplinary approaches have proven invaluable to understanding the molecular mechanisms mediating and regulating synaptic vesicle exocytosis and neurotransmission.

Three approaches are presented in this unit to identify and characterize synaptic vesicle proteins. In Basic Protocol 1, a subcellular fractionation method is used to isolate synaptic vesicles from a rat brain synaptosomal preparation, allowing a variety of biochemical studies on synaptic vesicle proteins. In Basic Protocol 2, a detailed procedure is given for pre-embedding immunogold staining and electron microscopy, affording the morphological identification of synaptic vesicle proteins in individual vesicles at the intact synapse. Finally, in Basic Protocol 3, immunocytochemical staining of cultured hippocampal neurons for light microscopy examination is described, allowing one to co-immunostain multiple synaptic vesicle proteins and determine their relative localization with pre- and post-synaptic plasma membrane proteins or other subcellular structures in neurons.

NOTE: All protocols using animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

BASIC PROTOCOL 1: ISOLATION AND SUBCELLULAR FRACTIONATION OF RAT BRAIN SYNAPTOSOMES

Four decades ago, Gray and Whittaker (1962) developed a biochemical procedure to pinch off nerve terminals from mammalian brain tissue by gentle homogenization and purify them from other subcellular structures by density gradient centrifugation. Although the resulting synaptosomes are separated from their neuronal cellular structure, they retain their functional integrity and are widely used to study stimulus-secretion coupling in vitro (Whittaker, 1993; Breukel et al., 1997; Cousin and Robinson, 1999). Synaptosomes are also the ideal starting material for isolation of synaptic vesicles, because they are purified from other small vesicular organelles, such as endoplasmic reticulum components, endosomes, or other small membrane fragments. Because of the abundance, size uniformity, and buoyant density of synaptic vesicles, it is possible to obtain a relatively pure, high-yield fraction of synaptic vesicles from cortical synaptosomes by using differential and sucrose gradient centrifugation (Whittaker et al., 1964; Leenders et al., 2008).

Synaptic vesicles can be further purified by methods such as permeation chromatography on controlled-pore glass (Huttner et al., 1983). In addition, membrane organelles such as

synaptic vesicles and endosomes can be further purified by immunoisolation using antibody-coated magnetic beads (Zhai et al., 2001; Cai et al., 2010). In particular, the latter technique is used to isolate particular vesicles based on their transmitter content using antibodies against the appropriate vesicular transporters. The protein content in the synaptic vesicle fractions can be investigated using SDS-PAGE and immunoblotting. Synaptic vesicle fractions also provide ideal starting material for proteomics to unveil the lipid and protein composition of synaptic vesicles by mass spectrometry and electron microscopy (Takamori et al., 2006).

The following protocol describes the isolation and purification of synaptosomes from rat brain cortex. Although other protocols have been described for isolation of synaptosomes (Gray and Whittaker, 1962; Cohen et al., 1977; Booth and Clark, 1978; Nagy and Delgado-Escueta, 1984; Dagani et al., 1985), this protocol, which replaces sucrose density gradient centrifugation with Percoll gradient centrifugation (Dunkley et al., 1988), yields the purest synaptosomal preparations. These preparations are suitable for subsequent fractionation (described below) and a variety of biochemical assays such as those for transmitter release, immunoprecipitation, and protein phosphorylation. They are also suitable for immunogold-labeled electron microscopy analysis (Basic Protocol 2).

Although this protocol yields relatively pure synaptic vesicles, it might not be suitable for all applications, notably because of its duration. Synaptic vesicles can also be obtained through a much quicker procedure involving floatation on Optiprep gradients as described by Hu et al. (2002). Such purified synaptic vesicles have been used successfully to reconstitute membrane fusion with proteoliposomes bearing plasma membrane SNARE proteins (Hu et al., 2002; Holt et al., 2008).

Materials

3- to 4-week old male rat (Wistar or Sprague-Dawley)

1× sucrose buffer (see recipe), ice cold

Percoll gradients (see recipe), ice cold

Wash buffer (see recipe), ice cold

Medium L (see recipe)

1 M KOH

1.0 M sucrose in Medium L (store up to 5 days at 4°C)

Sucrose gradients (see recipe) in thin-walled ultracentrifuge tubes (ultraclear tubes, 38.5-ml capacity, 25 × 89 mm)

Protease inhibitors (see recipe)

20 mM Tris-Cl, pH 7.4 (APPENDIX 2A)

Antibodies specific for subcellular fractions: e.g., anti-synaptophysin (Chemicon) or anti-VAMP (StressGen) for synaptic vesicles, anti-Na⁺/K⁺-ATPase (Transduction

Laboratories) for plasma membranes, and anti-lactate dehydrogenase (anti-LDH, Abcam) for cytosol

Rat guillotine

Surgical tools for isolation of cortex

25- and 55-ml Potter-Elvehjem homogenizers (Teflon-glass)

High-speed centrifuge (e.g., Sorvall RC-5C with SS-34 rotor and adaptors for eight 16 × 100-mm tubes)

50-ml (29 × 102-mm) polycarbonate high-speed centrifuge tubes

Ultracentrifuge capable of 100,000 × *g*, with:

Swinging bucket rotor (e.g., Beckman SW28) and 31-ml, 25 × 89-mm, thick-walled polycarbonate ultracentrifuge tubes

Fixed-angle rotor (e.g., Beckman 70.1 Ti or 50 Ti) with 10-ml, 16 × 76-mm thick-walled polycarbonate ultracentrifuge tubes

10,000 MWCO dialysis membranes

Centriprep-10 centrifugal concentrators (Millipore)

Additional reagents and equipment for rat anesthesia (*APPENDIX 4B*), dialysis, determining protein concentration (e.g., Simonian and Smith, 2006), SDS-PAGE (e.g., Gallagher, 2006), and immunoblotting (e.g., Gallagher et al., 2008)

NOTE: All isolation steps must be performed at 0° to 4°C, and all solutions, centrifuge tubes, and centrifuge rotors should be precooled below 4°C and kept on ice.

Isolate synaptosomes

- 1 Anesthetize rat (*APPENDIX 4B*) and decapitate with a guillotine. Remove brain and place in ice-cold 1× sucrose buffer.

A total of four to eight rats are recommended to prepare a concentrated synaptosome fraction for biochemical studies.

EDTA may be omitted when attempting to characterize Ca²⁺-dependent proteins that co-fractionate with synaptic vesicles.
- 2 Remove cerebellum, brain stem, midbrain, and white matter (Fath et al., 2009; Fig. 2.7.1). Pool four cortices together and place in 9 ml ice-cold 1× sucrose buffer.
- 3 Transfer to a 25-ml Potter-Elvehjem homogenizer and homogenize the cortex using ten slow up-and-down strokes of the homogenizer.
- 4 Centrifuge the homogenate 10 min at 1000 × *g* (3000 rpm in an SS-34 rotor), 4°C.
- 5 Collect supernatant and carefully load 2 ml supernatant onto each of four Percoll gradients. Centrifuge gradients for exactly 5 min at 32,500 × *g* (17,250 rpm in

an SS-34 rotor), 4°C, using a timer to start counting the time when 17,250 rpm is reached. Collect synaptosomes from the interface at 15%/23% using a Pasteur pipet.

Alternatively, the supernatant may be loaded onto a discontinuous sucrose gradient (see recipe) consisting of 1.2/1.0/0.8 M sucrose layers and centrifuged for 20 min at $48,200 \times g$ (Cohen et al., 1977). In that case, the synaptosome fraction will be collected from the interface between the 1.2 and 1.0 M sucrose layers.

Synaptosomes are visible as a cloud of material concentrated at the bottom interface (15%/23% for Percoll or 1.2/1.0 M for sucrose). Use a separate Pasteur pipet to remove upper layers before collecting the synaptosome layer.

- 6 Transfer the synaptosomes to a 50-ml polycarbonate centrifuge tube and add 30 ml chilled wash buffer. Centrifuge 15 min at $15,000 \times g$ (11,000 rpm in an SS-34 rotor), 4°C.
- 7 Carefully remove the supernatant and proceed immediately with fractionation (below) or immunogold labeling (see Basic Protocol 2).

Synaptosomes form a loose pellet. If a more firm pellet is desired, resuspend the pellet in 20 ml cold wash buffer and repeat centrifugation before proceeding with the analysis.

Both fractionation and immunogold labeling require freshly isolated synaptosomes.

Fractionate synaptosomes

The fractionation procedure is illustrated in Figure 2.7.2.

- 8 Lyse synaptosomes by resuspending the pellet in ice-cold hypoosmotic Medium L. For biochemical studies, combine synaptosomal preparations from six to eight rats in a single fraction and resuspend in a volume of 4 ml Medium L per rat.
- 9 Save a 0.5-ml aliquot of this synaptosomal suspension for validation of the fractionation procedure after purification (steps 18 to 21).
- 10 Manually homogenize four times slowly up and down in a 55-ml Potter-Elvehjem homogenizer, then incubate suspension on ice for 15 min.
- 11 Repeat homogenization once more, then adjust pH to 8.0 with 1 M KOH, and incubate homogenized synaptosomal suspension on ice for another 45 min.
- 12 Layer 5 ml synaptosomal suspension onto 5 ml of 1.0 M sucrose in Medium L in a 31-ml thick-walled polycarbonate ultracentrifuge tube (using a total of six to eight tubes). Centrifuge 30 min at $96,300 \times g$ (27,000 rpm in an SW28 rotor), 0° to 4°C. Collect the supernatant in fresh 31-ml ultracentrifuge tubes.

The pellet is a crude synaptosomal mitochondrial fraction. For further biochemical analysis, it can be resuspended in 20 mM Tris-Cl (pH 7.4) containing protease inhibitors and stored up to 1 year at -20°C .

- 13** Cover the supernatant tubes with Parafilm and gently mix by inverting (do not vortex). Centrifuge 14 hr at $25,000 \times g$ (14,000 rpm in an SW28 rotor), 4°C . Collect the supernatant (the cytosolic protein fraction) and place both it and the pellet on ice.

Dialyze and concentrate cytosolic proteins

- 14a** Dialyze the supernatant against 10 volumes of Medium L overnight at 4°C using a 10,000 MWCO dialysis membrane.

After setting up the dialysis, proceed to step 14b to process the pellet.

- 15a** The next day, centrifuge the dialyzed supernatant for 1 hr at $140,000 \times g$ (46,000 rpm in a 50 Ti rotor), 4°C .

- 16a** Collect the supernatant and concentrate with a Centriprep-10 centrifugal concentrator by microcentrifuging 30 min at maximum speed, 4°C . Collect concentrated product, load onto a fresh Centriprep-10, and repeat microcentrifugation.

- 17a** Collect the final concentrated cytosolic protein fraction from the Centriprep-10 and add protease inhibitors to final concentrations of 2 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM PMSF. Store in 50- to 100- μl aliquots for up to several years at -20°C . Avoid repeated freeze/thaw cycles.

If desired, a protease inhibitor cocktail (e.g., Sigma protease inhibitor cocktail, cat. no. P8340) can be used in place of individual aprotinin and leupeptin stock solutions.

Separate synaptic vesicles from plasma membranes

- 14b** After setting up the overnight dialysis, resuspend the pellet from step 13 in Medium L (1.0 to 2.0 ml for each cortex). Layer 5 ml of suspension onto separate sucrose gradients prepared in 38.5-ml thin-walled ultracentrifuge tubes. Mark phase interfaces on the tubes to facilitate recognition of the desired fractions after centrifugation.

- 15b** Centrifuge sucrose gradients 90 min at $68,000 \times g$ (23,000 rpm in an SW 28rotor), 0° to 4°C . Collect each fraction carefully with a Pasteur pipet.

The synaptic vesicle fraction is located above the 0.4 M sucrose layer. The fraction enriched with the presynaptic plasma membrane proteins is located at the phase interfaces between 0.6/0.8, 0.8/1.0, and 1.0/1.2 M sucrose. The 0.8/1.0 M interface usually contains the purest plasma membrane fraction.

- 16b** Transfer each fraction to a thick-walled 10-ml ultracentrifuge tube. Add Medium L to fill 3/4 of each tube. Centrifuge 45 min at $106,500 \times g$ (40,000 rpm in 50 Ti rotor), 0° to 4°C.
- 17b** Collect each pellet and resuspend in 0.5 to 1 ml of 20 mM Tris-Cl, pH 7.4, containing protease inhibitors as in step 17a. Store in 50- to 100- μ l aliquots for up to several years at -20°C. Avoid repeated freeze/thaw cycles.

Analyze protein distribution in fractions

- 18** Determine protein concentration of each fraction: synaptosomes (step 7), cytosol (step 17a), plasma membranes (step 17b), and synaptic vesicles (step 17b).
- 19** Analyze aliquots containing equal amounts of protein from each fraction by SDS-PAGE.
- Aliquots containing 5–10 μ g total protein should be sufficient to detect most synaptic vesicle proteins.
- 20** Detect protein(s) of interest by immunoblotting with the appropriate specific antibodies.
- 21** Determine relative purity of each fraction by sequential immunoblotting with antibodies against well-characterized markers (see Fig. 2.7.3).

BASIC PROTOCOL 2: VISUALIZATION OF SYNAPTIC VESICLE PROTEINS BY PRE-EMBEDDING IMMUNOGOLD ELECTRON MICROSCOPY

Because synaptic vesicles are only 30 to 50 nm in diameter, visualization of synaptic vesicles in synaptosomes or at nerve terminals requires the resolution of an electron microscope. Ultrastructural visualization can be achieved with sensitive and specific labeling of synaptic vesicle proteins by pre-embedding immunogold labeling. This protocol, adapted from Tanner et al. (1996), is for synaptosomal preparations, but can also be applied to cultured neuronal cells or freshly dissected brain tissue (Leenders et al., 2008).

Materials

Unfractionated rat brain synaptosomes (see Basic Protocol 1, step 7)

Wash buffer (see recipe)

0.1 M sodium phosphate buffer, pH 7.4 (see recipe)

4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer

PBS (*APPENDIX 2A*)

5% (v/v) normal goat serum/0.1% (w/v) saponin in PBS

5% (w/v) nonfat dry milk in PBS

Primary antibody against synaptic vesicle protein of interest

Nanogold-conjugated secondary antibody (Nanoprobes)

2% (w/v) glutaraldehyde in PBS

HQ silver enhancement kit (Nanoprobes)

Filter paper

Razor blade

12-well tissue culture plates

Additional reagents and equipment for preparing samples for electron microscopy(*UNIT 1.2*)

NOTE: All steps are performed at room temperature.

Fix synaptosomes

- 1** Resuspend synaptosome pellet purified from one rat cortex in 1 ml wash buffer, divide into four equal volumes, and microcentrifuge 5 min at maximum speed.

Use freshly prepared synaptosomes only.

Synaptosomes from one rat cortex should be divided into a minimum of four samples, as fixation has limited penetration into the pellet.

When using cultured neuronal cells, omit this step. Instead, remove culture medium from cells, wash cells with 0.1 M sodium phosphate buffer, then proceed to step 2.
- 2** Discard supernatant and fix pellet by adding 0.5 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer and incubating for 30 min.
- 3** Wash fixed pellet four times, each time for 5 min with 1 ml of 0.1 M sodium phosphate buffer.
- 4** Place each pellet on filter paper soaked with 0.1 M sodium phosphate buffer and divide into four to six pieces using a razor blade (total 16–24 pieces). Transfer the pieces to separate wells of 12-well tissue culture plates.

Label synaptic vesicle proteins

- 5** Block nonspecific binding sites and permeabilize synaptosomes by adding 1 ml of 5% normal goat serum/0.1% saponin in PBS to each well and incubating for 1 hr.

Normal serum should come from the same species as the gold-labeled secondary antibody. The Nanogold antibody used in this protocol is from goat.
- 6** Dilute the desired primary antibody as appropriate in 5% normal goat serum/0.1% saponin/PBS. Add 0.5 ml diluted primary antibody to each well and incubate for 1 hr.

Antibody may be incubated for a longer time period if done at a lower temperature (e.g., 4°C). The dilution and incubation time will need to be determined empirically for each antibody used.

- 7 Wash four times, each time for 5 min with 1 ml of 5% nonfat dry milk in PBS.
- 8 Dilute Nanogold-labeled secondary antibody 1:400 in 5% nonfat dry milk in PBS. Add 400 µl diluted secondary antibody to each well and incubate for 1 hr.
- 9 Wash as in step 7.
- 10 Post-fix by adding 1 ml of 2% glutaraldehyde in PBS to each well and incubating for 30 min.
- 11 Wash four times, each time for 5 min with 1 ml PBS.
- 12 Wash four times, each time for 5 min with distilled water.
- 13 Add 1 ml of silver enhancement solution to each well and incubate for 4 min.
- 14 Immediately wash four to six times for a total of 10 min, each time with 1 ml water.
- 15 Wash three times, each time for 5 min with 1 ml of 0.1 M sodium phosphate buffer.

Process samples for EM

- 16 Process for electron microscopy as described in *UNIT 1.2*, starting with osmium tetroxide treatment, but use 1 ml of 0.2% (instead of 1%) osmium tetroxide in 0.1 M sodium phosphate buffer.

Figure 2.7.4 shows immunogold electron micrographs of synaptosomes isolated from adult rat brain.

BASIC PROTOCOL 3: IMMUNOFLUORESCENCE STAINING OF SYNAPTIC VESICLE PROTEINS IN CULTURED HIPPOCAMPAL NEURONS

Immunocytochemical staining of cultured hippocampal neurons for light microscopy analysis allows one to co-immunostain multiple synaptic vesicle proteins and determine their relative localization with respect to other proteins or subcellular structures in neurons. The steps below describe labeling with a single primary antibody, but multiple antibodies can be used either simultaneously or sequentially. Whenever using multiple antibodies, extreme care must obviously be used to ensure that the species in which the antibodies were raised are different, so as to avoid cross-staining by secondary antibodies.

Materials

Hippocampal neurons cultured on coverslips (see Support Protocols 1 and 2)

Phosphate-buffered saline (PBS; *APPENDIX 2a*), pH 7.4

Fixative solution: 4% (w/v) paraformaldehyde/4% (w/v) sucrose in PBS

Blocking/permeabilization buffer: 10% (v/v) normal goat serum/0.2% (v/v) Triton X-100 *or* saponin in PBS (prepare fresh)

Primary antibody against protein of interest

Antibody dilution buffer: 10% (v/v) normal goat serum/0.05% (v/v) Triton X-100 *or* saponin in PBS (prepare fresh)

Fluorophore-conjugated secondary antibody (e.g., TRITC, FITC, Cy2, Cy3, or Cy5; store protected from light)

Anti-fading mounting medium (e.g., Fluoroshield, Accurate Chemical & Scientific Corp.)

12-well tissue culture plates

Microscope slides

Forceps

Fluorescence microscope with 40× and/or 63× oil-immersion objectives

NOTE: Fluorophores are light-sensitive reagents. All solutions and stained samples should be protected from light, e.g., using aluminum foil.

NOTE: All steps are performed at room temperature.

Fix cells

- 1 Transfer each coverslip containing cultured hippocampal neurons to 1 ml PBS in an individual well of a 12-well tissue culture dish.
- 2 Aspirate PBS, add 1 ml fixative solution to each well, and incubate 10–15 min.
Alternatively, cells can be fixed with ice-cold methanol for 10 min at -20°C .
- 3 Remove fixative and wash coverslips three times, each time for 5 min with 1 ml PBS, pH 7.4.
- 4 Remove PBS, add 1 ml blocking/permeabilization buffer to each well, and incubate for 1 hr to block nonspecific sites and permeabilize the cells.
Triton X-100 is commonly used as detergent in this step; however, saponin is the preferred alternative for some proteins, especially plasma membrane proteins.

Immunolabel synaptic vesicle proteins

- 5 Dilute primary antibody as appropriate in antibody dilution buffer. Add 400 μl diluted primary antibody to each well and incubate 2–3 hr.
Incubation may be extended to overnight at 4°C . Use the same detergent in the dilution buffer as used in the blocking/permeabilization

buffer. The optimal antibody dilution and incubation time will need to be determined empirically for each antibody used.

- 6 Remove antibody and wash coverslips as in step 3.
- 7 In a microcentrifuge tube, dilute fluorophore-conjugated secondary antibody as appropriate in a volume of 400 μ l antibody dilution buffer per coverslip, and then microcentrifuge 5 min at maximum speed to remove fluorophore aggregates.
Commercial preparations are typically diluted at 1:100 to 1:500.
- 8 Add 400 μ l diluted secondary antibody to each coverslip and incubate for 1–2 hr.
- 9 Remove antibody and wash coverslips as in step 3. After last wash, add 1 ml PBS to each coverslip.

Mount samples

- 10 Remove PBS from the coverslips, add 1 ml water, and leave for 10 min before mounting.
- 11 Label microscope slides and place 1 drop of mounting medium on each slide.
- 12 Using forceps, pick up one coverslip and gently blot off excess water by touching a corner to the edge of a Kimwipe. Carefully place the coverslip cell-side-down onto the drop of mounting medium on a slide.
- 13 Gently blot mounted coverslip with paper towel. Allow to air dry.

Fixed, immunostained, and mounted samples can be stored in the dark for 6 months to 1 year at 4°C.

Samples are ready to be viewed under a fluorescence microscope with a 40 $\times$ or 63 $\times$ oil-immersion objective. Figure 2.7.5 shows an immunofluorescence photomicrograph of cultured hippocampal neurons.

SUPPORT PROTOCOL 1: CULTURE OF HIPPOCAMPAL NEURONS FROM POSTNATAL RATS OR MICE

Generally, to culture embryonic or postnatal rat or mouse brain cells, the brains are removed, the particular tissue of interest is dissected and minced into small pieces, and the tissue is treated with enzymes that dissociate individual neurons and glial cells. After further mechanical dissociation, the resulting cell suspension is divided into individual wells of a cell culture dish containing nutritive media and a substrate (usually precoated glass coverslips) to which the cells can adhere and begin to grow. The following procedure is used to culture hippocampal neurons from postnatal rat or mouse brains. Typically, P0 mouse pups and 2- to 3-day-old rat pups are used. The younger the animals are, the fewer glia will be present in the preparation.

NOTE: A laboratory coat and gloves should be worn at all times during dissection. Surgical instruments should be soaked in 70% (v/v) ethanol prior to dissection and cleaned carefully after each use. The dissection should be performed on a laboratory bench covered with clean paper.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% CO₂ incubator unless otherwise specified. All culture media should be pre-equilibrated at 37°C, 5% CO₂.

NOTE: All solutions except culture media should be kept at 4°C on ice during the procedure.

Materials

Xylenes
100% (v/v) ethanol
Polyornithine (Sigma P4957)
Ultrapure water
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fibronectin (Sigma F1141-5MG)
P0 mouse pups or P2-3 rat pups
Hank's balanced salt solution (HBSS; see recipe)
HBSS containing 20% fetal bovine serum (FBS), ice cold, prepare fresh
50 mg/ml trypsin (type XI, Sigma, T1005) in H₂O (store 10-mg aliquots at –20°C)
20 mg/ml DNase (type IV, Sigma, D5025) in H₂O (store 1-mg aliquots at –20°C)
Digestion buffer (see recipe), ice cold
Dissociation buffer: 150 mg MgSO₄·7H₂O in 50 ml HBSS (final 12 mM), ice cold, prepare fresh
Plating medium (see recipe), 37°C
Feeding medium 1 (see recipe) with 3 μM and 6 μM Ara-C, 37°C
12- or 25-mm-diameter round coverslips
Sonicator
Gauze pads
Glass Petri dishes
250°F oven

Dissecting tools:

Spring scissors, straight and angled

Forceps, straight and curved

Angled dissecting bone chisel

Scalpel

Straight surgical scissors

35- and 60-mm tissue culture dishes

Dissecting microscope with fiber-optic lights

9-in. borosilicate glass pipets (Pasteur pipets)

Plastic transfer pipets

15-ml polypropylene (not polycarbonate) conical centrifuge tubes

0.22- μ m filter

Fire-polished, sterile, siliconized/plugged Pasteur pipets, 0.5- and 0.75-mm bore size

Clean and coat coverslips

- 1 Wash 12- or 25-mm round coverslips by sonicating in xylenes for ~30 min.
CAUTION: Perform this step in a chemical fume hood, and dispose of xylenes (including ethanol washes below) in appropriate waste containers.
- 2 Remove coverslips from xylenes and rinse two to three times with 100% ethanol to remove residual xylenes.
- 3 Sonicate coverslips for 1 hr in 100% ethanol, rinse in ethanol, and repeat for a total of 3 times.
- 4 Rinse at least 5 times with distilled, deionized water.
- 5 Separate coverslips manually and allow them to air-dry on clean gauze pads.
- 6 Transfer coverslips to a glass Petri dish and bake at 250°F for at least 2 hr, preferably overnight.
- 7 Dilute polyornithine 1:6 in sterile ultrapure water. Incubate coverslips in polyornithine in individual 35-mm dishes for 2–6 days in a sterile container shielded from light (an incubator works well).
- 8 One day before dissections, remove the polyornithine solution and rinse coverslips three times with PBS.
- 9 Dilute fibronectin 1:1000 in ultrapure water. Incubate coverslips in fibronectin solution in 35-mm dishes for 6–24 hr.
- 10 Just before plating cells, rinse coverslips twice with ultrapure water.

Dissect tissue

- 11 Decapitate a rat or mouse pup using sharp scissors. Holding the head tightly with one hand, carefully cut through the scalp with spring scissors from the point of decapitation towards the orbits. Use angled spring scissors to cut through the skull and expose the brain.
- 12 Using curved forceps, carefully remove the brain and place in a 60-mm tissue culture dish containing 8 ml ice-cold HBSS/20% FBS.
- 13 Using a dissecting chisel, hemi-dissect the brain and remove the brainstem and thalamus to expose the hippocampus. Under a dissecting microscope, remove the hippocampus, leaving some of the subiculum attached.

Tug and pull as little as possible. Always cut, don't crush the tissue, and be careful not to stab the tissue at any point.
- 14 Using the back end of a Pasteur pipet or a cut plastic transfer pipet for the gentlest handling, transfer hippocampi to a second 60-mm dish containing 8 ml ice-cold HBSS/20% FBS.
- 15 Using straight forceps, peel off all adhering membranes and blood vessels. Cut away the fimbriae with a scalpel or scissors, and transfer the cleaned hippocampi to a third dish containing 8 ml ice-cold HBSS/20% FBS.
- 16 Unroll the dentate gyrus. Use the subiculum to pin down the tissue while unrolling and cutting off the dentate gyrus with scissors. Then, cut away the hippocampi from the remaining subiculum and transfer the hippocampi to a fourth 60-mm dish containing 8 ml ice-cold HBSS/20% FBS.
- 17 Cut hippocampi into approximately ten thin slices and place on ice. Repeat steps 11–17 until all dissections are complete.

Each dissociated hippocampus yields $\sim 5 \times 10^5$ cells. It is important to complete the dissection for one animal before proceeding to the next, and to keep the tissue pieces on ice.
- 18 Under sterile conditions, use a plastic pipet to transfer the sliced hippocampal tissue to a 15-ml polypropylene conical tube. Let the tissue settle and discard the supernatant.
- 19 Wash once with 10 ml HBSS/20% FBS, allow tissue to settle to the bottom of the tube, and remove the supernatant. Repeat wash three more times with 10 ml HBSS alone.

Digest tissue

- 20 Add 200 μ l of 50 mg/ml trypsin (10 mg) and 50 μ l of 20 mg/ml DNase (1 mg) to 2 ml digestion buffer and filter-sterilize the solution directly onto the last pellet (step 19) using a 0.22- μ m filter. Incubate for 5 min at 37°C, occasionally shaking gently.
- 21 Discard solution and stop digestion by adding ~ 10 ml HBSS/20% FBS.

- 22 Wash once with 10 ml HBSS/20% FBS and once with 10 ml HBSS alone, as above.

Dissociate cells

- 23 Add 50 μ l of 20 mg/ml DNase (1 mg) to 2 ml dissociation buffer and filter-sterilize onto the last pellet as above.
- 24 Mechanically dissociate cells by gently triturating with two fire-polished, sterile, siliconized/plugged Pasteur pipets with successively smaller bore sizes (0.75 and 0.5 mm). Avoid bubbles and always pipet onto the wall of the tube. Allow clumps of undissociated tissue to settle for 2 min, then transfer the supernatant to another tube.

This step separates suspended cells from tissue particles.

CAUTION: Tips that are too small will lyse the cells; tips that are too large will not dissociate cell aggregates.

- 25 Add 3 ml HBSS/20% FBS to the cell suspension (5 ml total), and centrifuge for 10 min at $200 \times g$, 4°C.
- 26 Discard supernatant and resuspend pellet in prewarmed (37°C) plating medium. Determine cell concentration using a hemacytometer.

Plate and maintain cells

- 27 Place drops of suspension containing $\sim 1 \times 10^5$ cells individually onto 12-mm polyornithine/fibronectin-coated coverslips in separate 35-mm dishes. Incubate 1–2 hr to allow the cells to adhere.

The volume of suspension should be between 50 μ l (to avoid evaporation) and 200 μ l.

The first short cell processes should be visible within this time.

- 28 Add 2 ml prewarmed plating medium to each dish and incubate for 48 hr.
- 29 Change plating medium to selection medium by replacing 1 ml old medium with 1 ml fresh pre-equilibrated feeding medium 1 containing 6 μ M Ara-C.

For the first change of medium, the concentration of Ara-C is doubled, because it will be diluted by half in the plating medium. All subsequent feedings are done with medium containing 3 μ M Ara-C.

- 30 Feed cells twice a week by replacing one third or one half of old medium with fresh pre-equilibrated feeding medium 1.

Cells can be maintained in culture for up to 3 weeks. Always keep neurons covered with medium, as they are extremely sensitive and will die if exposed even briefly.

SUPPORT PROTOCOL 2: CULTURE OF HIPPOCAMPAL NEURONS FROM EMBRYONIC RATS OR MICE

Typically, E18 or E19 embryos are used to culture embryonic mouse or rat hippocampal neurons. It is best to order the pregnant animal so that it arrives the day before the dissections and is not subject to transport-induced stress immediately prior to being sacrificed.

NOTE: See Support Protocol 1 for important notes concerning culture conditions, sterility, and temperature of media and solutions.

Materials

18- or 19-day pregnant rat or mouse

70% (v/v) ethanol

Dissection buffer (see recipe), ice-cold

Papain solution (see recipe)

1/1 solution (see recipe)

10/10 solution (see recipe)

Plating medium 2 (see recipe), 37°C

Feeding medium 2 (see recipe), 37°C

Dissecting tools:

Straight wide-tipped forceps

Straight surgical scissors

Straight fine scissors

Forceps, curved and straight

Spring scissors, straight and angled

Dumont forceps (#5/45, 45° angled tip)

Dissecting microscope and fiber-optic lights

35- and 100-mm tissue culture dishes

15-ml polypropylene (not polycarbonate) conical centrifuge tubes

5-ml pipet

Polyornithine/fibronectin-coated 12- or 25-mm-diameter round coverslips (see Support Protocol 1, steps 1–10)

Additional reagents and equipment for rodent anesthesia and euthanasia (*APPENDIX 4B & 4H*) and counting cells with a hemacytometer (*APPENDIX 3B*)

Isolate embryos

- 1 Anesthetize pregnant rat or mouse with CO₂ or by another approved method, then euthanize by decapitation.
- 2 Sterilize abdomen with 70% ethanol.
- 3 Gripping the skin with straight wide-tipped forceps, cut the skin and muscle along the midline with surgical scissors.
- 4 Make two cuts to the sides and pull out both horns of the uterus. Cut the uterus and transfer to a 100-mm tissue culture dish.
- 5 Open the uterus with straight fine scissors. Remove the embryos and decapitate immediately with sharp scissors. Place heads in a 100-mm dish containing 15 ml cold dissection buffer.

Isolate hippocampi

- 6 Using curved forceps, hold one head firmly by inserting the tips of the forceps into the orbits. Using curved spring scissors, carefully cut through the skin and skull from the point of decapitation towards the orbits.
- 7 Holding the head with one pair of Dumont forceps, use another pair of Dumont forceps to peel back the skin and skull, exposing the brain. Work caudally from beneath the olfactory bulbs.
- 8 Using curved forceps, carefully remove the intact brain and place in a clean 35-mm dish containing dissection buffer.
- 9 Under a dissecting microscope with fiber-optic lights, separate the hemispheres of the brain by moving Dumont forceps through the midline.
- 10 One hemisphere at a time, remove the septum, thalamus, and hypothalamus from the cortex to expose the hippocampus, which will appear curved along the medial edge of the cortex.
- 11 Remove the olfactory bulb and striatum to further expose the hippocampus for easy removal.
- 12 Carefully remove the meninges and choroid plexus.

The meninges form a thin layer covering the surface of the brain. The choroid plexus attaches to the meninges at the edge of the hippocampus. Both of these layers are highly vascularized, which facilitates locating and removing them.
- 13 Using sharp Dumont forceps, cut or separate the hippocampus from the rest of the brain and place it in a 35-mm dish containing dissection buffer.
- 14 Repeat steps 10–13 with the other hemisphere, then repeat the entire procedure with the remaining heads. Place all hippocampi in the same 35-mm dish containing 3 ml dissection buffer.

It should take less than 1 hr from the time the animal is sacrificed to the time all hippocampi are completely dissected. After ~1 hr, cell viability decreases dramatically, and the brain tissue becomes more difficult to work with.

Dissociate cells

- 15 In a laminar-flow hood, remove the dissection buffer from the dish and mince tissue into fine pieces using spring scissors.
- 16 Using a 1000- μ l pipet tip, aspirate the minced tissue and place into a 15-ml tube containing 5 ml papain solution. Incubate in a 37°C incubator for 45 min with gentle agitation every 10 min.
- 17 Triturate tissue gently with a 5-ml pipet until pieces are broken up and solution appears cloudy.
- 18 Centrifuge at $200 \times g$ for 5 min. Gently remove supernatant by aspiration.
- 19 Resuspend pellet in 5 ml of 1/1 solution. Using a 5-ml pipet, gently pipet up and down until the tissue is totally dissociated (15–20 times). Do this slowly to avoid bubble formation and facilitate maximum trituration.
- 20 Allow suspension to settle for 10 min.

After 10 min, some tissue chunks will have fallen to the bottom of the tube.
- 21 Using a 1000- μ l pipet tip, gently remove the supernatant and layer it on 10 ml of 10/10 solution, rotating the 10/10 solution tube as the supernatant is layered.

The 10/10 solution column traps dead cells and debris. This ‘cushion’ should be ~8 to 10 ml in volume.

Plate and maintain cells

- 22 Centrifuge suspension at $200 \times g$ for 10 min. Discard the supernatant and resuspend the pellet in 2–6 ml plating medium 2. Determine cell density using a hemacytometer.
- 23 Just before plating cells, remove water from polyornithine/fibronectin-coated cover-slips. Add 2 ml plating medium 2 and plate $4\text{--}8 \times 10^5$ cells to each coverslip in a 35-mm dish.
- 24 On the second day (after 24–36 hr), remove half of the plating medium and replace with fresh pre-equilibrated feeding medium 2.
- 25 Twice a week, replace half of the medium with fresh pre-equilibrated feeding medium 2.

Cells can be maintained in culture for up to 3 weeks. Always keep the neurons covered with medium, as they are extremely sensitive to air and will die if exposed even briefly.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. Use sterile, tissue-culture-grade water and sterile PBS in recipes for all neuronal culturing experiments. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

1/1 solution

500 μ l ovomucoid inhibitor (Worthington, 3182)
0.5 vial (0.25 mg) DNase (Worthington, LK003172)
Add HBSS (no phenol red, Invitrogen, 14025-092) up to 5 ml
Filter-sterilize using a 0.22- μ m filter
Prepare fresh
Before use, dissolve 1 vial ovomucoid inhibitor in 32 ml HBSS.

10/10 solution

100 mg trypsin inhibitor (Sigma, T9253)
100 mg bovine serum albumin (Sigma, A9647)
Add HBSS (no phenol red, Invitrogen, 14025-092) up to 10 ml
Mix well and incubate at 37°C for 10 min to dissolve
Filter-sterilize using a 0.22- μ m filter
Prepare fresh

Basic medium

500 ml modified Eagle medium (MEM, Invitrogen, 51200-038)
2.5 g glucose
100 mg NaHCO₃
50 mg transferrin (Calbiochem, 616420)
Adjust pH to 7.4
Filter-sterilize using a 0.22- μ m filter
Store at 4°C

Digestion buffer

137 mM NaCl (400 mg/50 ml)
5 mM KCl (18 mg/50 ml)
7 mM Na₂HPO₄ (50 mg/50 ml)
25 mM HEPES (150 mg/50 ml)

Adjust pH to 7.2

Filter-sterilize using a 0.22- μ m filter

Store at 4°C

Dissection buffer

500 ml HBSS (Invitrogen, 14170-112)

5 ml 1 M HEPES (Invitrogen, 15360-080)

5 ml 100 $\times$ penicillin/streptomycin (Invitrogen, 15140-122)

Store at 4°C

Feeding medium 1

97 ml basic medium (see recipe)

1 ml 200 mM glutamine (Sigma, G7513, stored at -20°C)

2 ml 50 $\times$ B-27 supplement (Invitrogen, 17504-044)

3 μ M cytosine arabinoside (Ara-C, Sigma, C6645)

Filter-sterilize using a 0.22- μ m filter

Store up to 1 week at 4°C

Feeding medium 2

9.75 ml neurobasal medium (Invitrogen, 21103-049)

200 μ l 50 $\times$ B-27 supplement (Invitrogen, 17504-044)

50 μ l GlutaMAX-1 (Invitrogen, 25030-061)

Filter-sterilize using a 0.22- μ m filter

Prepare fresh

Hank's balanced salt solution (HBSS)

HBSS without calcium, magnesium, or sodium bicarbonate (Powder, Sigma, H2387)

350 mg/liter NaHCO₃ (4 mM)

1.3 g/liter HEPES (5 mM)

Adjust pH to 7.3–7.4

Filter-sterilize using a 0.22- μ m filter

Seal with Parafilm to help maintain consistent pH

Store up to 2 months at 4°C

Medium L, pH 8.0

0.174 g K₂HPO₄ (1 mM final)

2 ml 0.5 M EDTA (0.1 mM final)

1 liter H₂O

Store up to several months at 4°C

Papain solution

1 vial (100 units) papain (Worthington, 3176-PAP2)

0.5 vial (0.25 mg) DNase (Worthington, LK003172)

Add HBSS (no phenol red, Invitrogen, 14025-092) up to 5 ml

Filter-sterilize using a 0.22-µm filter

Prepare fresh

Percoll gradients

23% Percoll:

12 ml 100% Percoll and 14 ml H₂O in 26 ml 2× sucrose buffer (see recipe)

15% Percoll:

30 ml 23% Percoll in 16 ml 1× sucrose buffer

10% Percoll:

20 ml 15% Percoll in 10 ml 1× sucrose buffer

3% Percoll:

6 ml 10% Percoll in 14 ml 1× sucrose buffer

Adjust solutions to pH 7.4 and store up to 5 days at 4°C.

To create a discontinuous gradient, carefully layer 2 ml of each Percoll solution in an 11-ml (16 × 100-mm) polycarbonate high-speed centrifuge tube, starting with 23% at the bottom. Prepare gradients on the day of or the day before the experiment, and keep at 4°C until use.

Plating medium 1

89 ml basic medium (see recipe)

10 ml fetal bovine serum (FBS, heat-inactivated at 57°C for 30 min, stored at 4°C)

1 ml 200 mM glutamine (Sigma, G7513, stored at -20°C)

200 µl 12.5 mg/ml insulin (stock prepared from powdered insulin [Sigma, I5500] in 10 mM HCl and stored at -20°C)

Prepare fresh on day of use

Filter-sterilize using a 0.22-µm filter

Plating medium 2

9.23 ml neurobasal medium (Invitrogen, 21103-049)

0.5 ml FBS (heat-inactivated 30 min at 55°C)

200 µl 50× B-27 supplement (Invitrogen, 17504-044)

25 µl 12.5 mg/ml insulin (stock prepared from powdered insulin [Sigma, I5500] in 10 mM HCl and stored at –20°C)

25 µl GlutaMAX-1 (Invitrogen, 25030-061)

20 µl G5 supplement (Invitrogen, 17503-012)

Prepare fresh on day of use

Filter-sterilize using a 0.22-µm filter, then pre-equilibrate in a 37°C, 5% CO₂ incubator

Protease inhibitors

Aprotinin: Prepare 10 ml of an aqueous solution containing 20 mg aprotinin (2 mg/ml). Store in aliquots up to 1 year at 4°C. Where indicated, add immediately before use at a 1:1000 dilution (2 µg/ml final).

Leupeptin: Prepare 10 ml of an aqueous solution containing 10 mg leupeptin (1 mg/ml). Store in aliquots up to 1 year at –20°C. Where indicated, add immediately before use at a 1:1000 dilution (1 µg/ml final).

Phenylmethylsulfonyl fluoride (PMSF): Prepare 10 ml of 100% isopropanol containing 0.348 g PMSF (200 mM). Store in aliquots at –20°C (stable at least 9 months). Where indicated, add immediately before use at a 1:200 dilution (1 mM final).

Sodium phosphate buffer, pH 7.4, 0.1 M

11.4 g NaH₂PO₄·H₂O

85.79 g Na₂HPO₄·7H₂O

2 liters H₂O (final 0.2 M sodium phosphate)

Store up to several months at 4°C

Dilute 1:1 (final 0.1 M) immediately before use

Sucrose buffer, pH 7.4, 1×

2 × sucrose buffer:

21.9 g sucrose (0.64 M)

2 ml 0.1 M EDTA (2 mM)

240 mg HEPES (10 mM)

100 ml H₂O

Filter-sterilize using a 0.22-µm filter

Store up to 2 weeks at 4°C

1 × sucrose buffer:

Dilute 2× solution 1:1 with H₂O

Adjust pH to 7.4 with NaOH

Store up to 5 days at 4°C

Sucrose gradients

7 ml 1.2 M sucrose

6 ml 1.0 M sucrose

6 ml 0.8 M sucrose

6 ml 0.6 M sucrose

6 ml 0.4 M sucrose

Prepare sucrose solutions individually in Medium L (see recipe) and store up to 5 days at 4°C. On day of experiment, layer solutions on top of each other in a thin-walled, 38.5-ml, 25 × 89-mm ultracentrifuge tube, starting with 1.2 M at the bottom. Keep at 4°C until use.

Wash buffer

6.142 ml 2 M NaCl (122 mM final)

500 µl 1 M KCl (5 mM final)

460 µl 0.25 M NaH₂PO₄ (1.15 mM final)

4 ml 0.5 M PIPES (20 mM final)

0.1 g D(+)-glucose (1 mg/ml final)

100 ml H₂O

Adjust to pH 6.8

Prepare fresh on day of use and keep at 4°C

COMMENTARY

Background Information

The development of FM styryl dyes and the establishment of techniques permitting the staining and destaining of recycling synaptic vesicles at nerve terminals has advanced our knowledge of the synaptic vesicle cycle (Betz et al., 1992, 1996; Cousin and Robinson, 1999; also see *UNIT 2.6*). However, a number of challenges still remain in our elucidation of the molecular mechanisms underlying the synaptic vesicle cycle, particularly the modulation of these mechanisms by synaptic vesicle proteins. Thus, application of a multidisciplinary approach to visualizing and identifying the proteins on synaptic vesicles is critical to the field. Classical methods such as differential and density centrifugation and electron

microscopy are still very useful. When used in combination with immunostaining techniques as described herein, they provide biochemical and morphological tools to determine the relative intracellular localization of particular proteins in intact synapses (Ilardi et al., 1999; Su et al., 2001; Tian et al., 2003; Leenders et al., 2008).

Electron microscopy of pre-embedding immunogold-labeled material allows direct, high-resolution localization of specific proteins in subcellular compartments, such as synaptic vesicle proteins in synapses. Although extra care should be taken with the silver enhancement to preserve this high resolution, this method is preferred over the immunoperoxidase method, because it is easier to perform and more sensitive. However, it is not appropriate if ultrastructural studies require higher precision, as the diaminobenzidine (DAB) reaction product can spread beyond the antigen-localization site (Cuello, 1993).

Critical Parameters and Troubleshooting

Subcellular fractionation of synaptosomal preparations—When fractionating synaptosomal preparations, it is imperative to retain synaptic structural integrity and prevent proteins from degrading. One must be particularly cautious at several steps of the protocol. First, rapid dissection and homogenization are crucial for the recovery of functionally intact synaptosomes. When performing multiple dissections, protease inhibitors and/or 0.25 mM dithiothreitol must be added to the 1× sucrose buffer. Second, homogenizers, centrifuge tubes, buffers, and gradient reagents must be chilled prior to and during use. Finally, to minimize damage to the synaptic structure, excessive force should never be used on cortical tissue. The tissue should be homogenized at a maximum of 900 rpm, and a pipet should be used to re-suspend pellets instead of a vortex. Synaptosomes must be freshly isolated when used for fractionation or pre-embedding EM staining.

Immunostaining—Success in immunostaining depends upon the sensitivity and specificity of the antibodies used. The quality (specificity) and concentration of primary antibodies are key to good detection of the protein of interest at intact synapses and within cultured hippocampal neurons. Careful evaluation or preliminary testing of an antibody's specificity by immunoblotting of brain homogenates is a necessary step before immunostaining. Any potential cross-reaction with other related or nonspecific proteins must be excluded. If required, further purification of antibodies using a specific-antigen-coupled affinity column is highly recommended. A proper negative control with preimmune antiserum or normal IgG should be included with the specific antibodies during immunostaining. Primary antibodies can also be omitted for a negative control to exclude nonspecific staining by secondary antibodies. It is also advisable to use an antibody known to label the area of interest (e.g., β -tubulin for hippocampal neurons) as a positive control to ensure that the staining procedure works under the same conditions. Preliminary testing of the optimal antibody dilution is required for best immunocytochemical staining. In addition, the optimal dilution of the secondary antibody needs to be determined to maximize the signal-to-noise ratio. Other factors, including fixation method (e.g., paraformaldehyde vs. methanol), detergents, blocking reagents, duration of antibody incubation, and blocking buffer may need to be evaluated to optimize immunocytochemical studies. For further information on optimization in immunocytochemistry, see *UNIT 2.12*.

Hippocampal cultures—There are many critical parameters in the culturing of hippocampal neurons. Coverslips must be cleaned properly, rinsed well, and sterilized prior to use. The proper number of substrates on which cells will be grown (e.g., coverslips) must be coated with polyornithine and fibronectin in advance. When dissecting, it is imperative to remove the brain intact, then rapidly and accurately dissect out the hippocampi and place them in the appropriate buffer. To help prevent clumping of tissue (which occurs due to release of DNA from damaged cells), DNase is added to the chopping, trypsinization, and trituration solutions. Over-digestion and over-trituration of cells both lead to cell damage, so these procedures must be done carefully. When feeding cells, it is important to remove and replace medium slowly, so as not to disturb the neurons and their processes. Additionally, only medium warmed in a 37°C incubator should be used to make the one-third to one-half volume changes of feeding medium.

Anticipated Results

Typical results for each Basic Protocol are shown in Figures 2.7.3 to 2.7.5. In Figure 2.7.3, an immunoblot of protein markers for each subcellular fraction indicates the purity of the synaptic vesicle fraction, with both synaptophysin and VAMP2 as markers for the synaptic vesicle proteins. Both immunogold EM staining of intact synapses from synaptosomal preparations (Fig. 2.7.4) and immunofluorescent staining of cultured hippocampal neurons (Fig. 2.7.5) show specific staining of the synaptic vesicle membrane protein synaptophysin. The same monoclonal antibody against synaptophysin was used in all three experiments.

Time Considerations

Fractionation—Preparation of the solutions and Percoll gradients takes at least 2 hr. These solutions can be prepared the day before and stored at 4°C until use. If gradients are prepared at room temperature on the same day as the isolation, they must be given sufficient time to cool to 4°C. The isolation of synaptosomes takes 2 hr. For subsequent fractionation of synaptosomes, at least four animals are required. The fractionation protocol takes 3 days to complete. On the first day, isolate synaptosomes and perform the first steps of the fractionation procedure ending with the overnight (14 hr) centrifugation at step 13. It will then take several hours to complete the procedure. Immunoblotting for detection of proteins of interest and all fraction markers will take several days.

Pre-embedding immunogold labeling—Fixation and immunogold staining takes ~5 to 6 hr, and further processing for EM (e.g., osmium tetroxide stain, dehydration, embedding, and cutting of ultrathin sections; see *UNIT 1.2*) will take at least 5 days.

Immunofluorescence staining—Immunofluorescence staining of cultured hippocampal neurons requires a minimum of 5 hr. Actual time will depend on the length and the temperature of primary antibody incubations. Regardless, experiments should be planned in advance when using hippocampal neurons, since they require weeks to grow.

Hippocampal neuron culture—There are many time considerations in hippocampal neuron culture. It is necessary to plan at least 1 week in advance for the dissection day. It is necessary to prepare stripped, sterile coverslips in advance so that they can be coated with

polyornithine and fibronectin. On the dissection day, media should be freshly prepared and warmed, and the chopping buffer should also be freshly prepared. One should allow no more than 1 hr to dissect out the hippocampi, as dissecting beyond this time may reduce cell viability. Mincing, enzymatic digestion, washing, triturating, and counting the cells should take less than 1.5 hr. Again, it is very important to work as rapidly as possible to ensure cell viability. For maintaining neurons, time is needed to prepare fresh feeding medium, equilibrate it in a 37°C incubator, and feed the cultures. It is recommended that cultures be observed on feeding days and at other times to assess culture conditions (e.g., viability, health, and growth rate of the cells). It is typically sufficient to evaluate cultures every other day.

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Figure 2.7.1.
Illustration of rat brain anatomical features.

Figure 2.7.2.
Flow chart for fractionation of synaptosomes.

Figure 2.7.3.

SDS-PAGE of subcellular fractions from rat brain synaptosomes. Crude synaptosomes were sedimented from rat brain homogenate by differential centrifugation and separated into fractions enriched in presynaptic cytosol (PC), synaptic vesicles (SV), and synaptosome plasma membrane (PM) (see Basic Protocol 1). Equal amounts (8 μ g) of synaptosome fractions and 50 μ g of crude synaptosomes were analyzed by SDS-PAGE and then sequentially immunoblotted with the indicated antibodies. The relative purity of subcellular fractions was determined by staining for markers of synaptic vesicles (VAMP2 and synaptophysin), plasma membrane (Na/K-ATPase), and cytosol (lactate dehydrogenase, LDH).

Figure 2.7.4.

Immunogold electron micrographs of synaptosomes isolated from adult rat brain. Synaptophysin staining was visualized by immunogold labeling followed by silver enhancement (see Basic Protocol 2). Gold particles (dark) label synaptophysin on synaptic vesicles. Scale bars: 100 nm.

Figure 2.7.5.

Immunofluorescent staining of cultured hippocampal neurons. Cultured hippocampal neurons from E18 rat embryos were fixed at day in vitro (DIV) 35, stained with anti-synaptophysin antibody, and visualized by rhodamine-conjugated secondary antibody (see Basic Protocol 3). Scale bar: 10 μ m.