Video Article

Brain Membrane Fractionation: An *Ex Vivo* Approach to Assess Subsynaptic Protein Localization

Xavier Morató*[1,2], Marc López-Cano*[1,2], Paula M. Canas[3], Rodrigo A. Cunha[3], Francisco Ciruela[1,2]

[1]Unitat de Farmacologia, Departament Patologia i Terapèutica Experimental, Facultat de Medicina, IDIBELL, Universitat de Barcelona, L'Hospitalet de Llobregat

[2]Institut de Neurociències, Universitat de Barcelona

[3]Center for Neurosciences of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra

*These authors contributed equally

Correspondence to: Francisco Ciruela at fciruela@ub.edu

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Abstract

Assessing the synaptic protein composition and function constitutes an important challenge in neuroscience. However, it is not easy to evaluate neurotransmission that occurs within synapses because it is highly regulated by dynamic protein-protein interactions and phosphorylation events. Accordingly, when any method is used to study synaptic transmission, a major goal is to preserve these transient physiological modifications. Here, we present a brain membrane fractionation protocol that represents a robust procedure to isolate proteins belonging to different synaptic compartments. In other words, the protocol describes a biochemical methodology to carry out protein enrichment from presynaptic, postsynaptic, and extrasynaptic compartments. First, synaptosomes, or synaptic terminals, are obtained from neurons that contain all synaptic compartments by means of a discontinuous sucrose gradient. Of note, the quality of this initial synaptic membrane preparation is critical. Subsequently, the isolation of the different subsynaptic compartments is achieved with light solubilization using mild detergents at differential pH conditions. This allows for separation by gradient and isopycnic centrifugations. Finally, protein enrichment at the different subsynaptic compartments (*i.e.,* pre-, post- and extrasynaptic membrane fractions) is validated by means of immunoblot analysis using well-characterized synaptic protein markers (*i.e.,* SNAP-25, PSD-95, and synaptophysin, respectively), thus enabling a direct assessment of the synaptic distribution of any particular neuronal protein.

Video Link

The video component of this article can be found at https://www.jove.com/video/55661/

Introduction

Synaptic transmission relies on the physical integrity of the synapse, a concept that was envisaged as early as 1897 by Foster and Sherrington[1]. Thus, understanding the distribution of key neurotransmission components (*e.g.,* ion channels, receptors, *etc.*) is essential to elucidate synaptic function, both in normal and pathological conditions. Electron microscopy (EM) has contributed enormously to the current ultrastructural notion of prototypical central nervous system (CNS) synapses. In such a way, EM has finely established the differences between pre- and postsynaptic densities, which are separated by a cleft of a rather uniform distance (~25 nm)[2]. Interestingly, the postsynaptic apparatus exhibits a relatively continuous, electron-dense thickening below its plasma membrane, the so-called postsynaptic density, or PSD[2]. Conversely, at the presynaptic apparatus, a noticeable discontinuous cytomatrix network is arranged just beneath the plasma membrane, which is essential to the alignment and docking of synaptic vesicles to the plasma membrane active zone[3]. Hence, EM constitutes the golden experimental approach to survey the distribution of proteins within structurally preserved CNS synapses. However, the information provided by electron micrographs is static. Indeed, accumulating evidences show that *in vivo* synapses are extremely dynamic, thus experiencing dramatic structural changes upon sustained synaptic transmission. In addition, the morphology and composition of synapses can change throughout different CNS regions and upon development, maturation, aging, and the development of neuropathological conditions. Overall, a protocol focused on isolating proteins belonging to different synaptic compartments in physiological conditions represents a valuable tool for a more comprehensive study of synaptic functioning.

Here, we describe this kind of complementary experimental approach, which allows for the preparative biochemical enrichment of the different synaptic membrane compartments-namely, extra-, pre- and postsynaptic membrane domains. This membrane fractionation method, first described by Philips *et al*. (2001)[4], is based on a pH shift that weakens the adhesive interactions occurring within the pre- and postsynaptic apparatus. First, by using mild detergents at pH 6.0, it is possible to discern the adherens junction that holds the pre- and postsynaptic apparatus and that is maintained from the extrasynaptic membrane domain, which is solubilized and thus can be extracted from the synaptic contacts. Subsequently, raising the pH from 6.0 to 8.0 in the presence of mild detergents weakens the strength of the adherens junction that keeps the presynaptic active zone tightly bound to the postsynaptic density. Hence, the presynaptic compartment is solubilized and can be separated from the postsynaptic density, which is mostly preserved because the concentration of detergent used does not promote its solubilization[4].

Interestingly, the fractionation efficiency, eventually higher than 90%, can be confirmed by different subsynaptic markers: *i*) synaptosomal-associated protein 25 (SNAP-25), from the presynaptic active zone; *ii*) synaptophysin, from the extrasynaptic fraction (*i.e.,* outside the active zone and including microsomes); and *iii*) postsynaptic density protein 95 (PSD-95), from the postsynaptic density. Notably, this brain membrane fractionation method has been used successfully. Accordingly, it has been possible to precisely determine the subsynaptic localization of different receptors, such as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors[5], adenosine A_1 receptor (A_1R)[6], adenosine A_{2A} receptor ($A_{2A}R$)[7], adenosine triphosphate (ATP) P2 receptors[8], nicotinic acetylcholine receptor subunits[9], and Parkinson's disease-associated receptor GPR37[10]. However, a number of limitations may impede the proper assessment of the synaptic distribution of a particular neuronal protein. Thus, in this procedure, we not only fully describe the entire protocol, but we also highlight some critical points to be considered, such as the rather large amount of tissue needed, the low protein yield, and the mandatory requirement to validate the efficiency of each separation before performing the definite experiment.

Protocol

All animal experimental procedures were approved by the University of Barcelona Committee on Animal Use and Care (CEEA), in compliance with the guidelines described in the Guide for the Care and Use of Laboratory Animals[11] and following the European Community, law 86/609/CCE, FELASA, and ARRIVE guidelines. Thus, mice are housed in standard cages, with *ad libitum* access to food and water, and are maintained under controlled standard conditions (12 h dark/light cycle starting at 7:30 AM, 22 °C temperature, and 66% humidity).

1. Obtaining Mouse Brain Synaptosomes Using a Discontinuous Sucrose Gradient

Note: This method was reported previously[10].

1. Prepare fresh isolation buffer (IB), pH 7.4; 2 M sucrose; 1 M sucrose/0.1 mM $CaCl_2$; and 0.1 mM $CaCl_2$ (see **Table 1**). Chill the solutions on ice.
2. Sacrifice five mice by cervical dislocation. Decapitate and rapidly remove the brain of each animal. Dissect the brain region of interest *(i.e.,* the hippocampus, 0.25-0.35 g of fresh tissue) and place it in a 5 mL Potter-Elvehjem glass tube on ice with 1 mL of IB.
3. Homogenize the tissue using a homogenizing stirrer (10 strokes at 700-900 rotations per min), preferentially in an ice bath to preclude sample warming.
4. Place the homogenized tissue (1 mL) into a 15 mL tube containing 6 mL of 2 M sucrose and 2.5 mL of 0.1 mM $CaCl_2$ at 4 °C. Mix slowly by inverting the tube.
   Note: The addition of calcium is essential to maintaining the adhesive interactions between organelles and thus to preserving the structure of the different "densities."
5. Place the solution in a 12 mL centrifuge tube and slowly add 2.5 mL of 1 M sucrose/0.1 mM $CaCl_2$ on top of each tube to form the sucrose gradient.
   Note: Label the position of the gradient interface on the centrifuge tube using a permanent marker.
6. Weigh and equilibrate the centrifuge tubes with IB solution within their corresponding steel supports and with their closing lids.
7. Centrifuge the tubes for 3 h at 100,000 x *g* and 4 °C using a swinging bucket rotor centrifuge. Completely fill the rotor with all the steel supports (even empty, if required).
8. Discard the top layer containing myelin. With a Pasteur pipette, collect the white ring between the 1.25-M and 1-M sucrose interphase corresponding to the synaptosome fraction.
9. Dilute the synaptosomes with 9X their volume using IB in a centrifuge tube.
10. Weigh and equilibrate the centrifuge tubes with IB solution within their corresponding steel supports and with their closing lids.
11. Centrifuge the tubes for 30 min at 15,000 x *g* and 4 °C using a swinging bucket rotor centrifuge.
12. Discard the supernatant and resuspend the pellet with 1.1 mL of IB solution. Collect 100 µL of this synaptosomal solution and centrifuge for 5 min at 11,000 x *g* and 4 ºC.
13. Resuspend the synaptosomal pellet in 5% sodium dodecyl sulfate (SDS) and store this sample at -20 ºC.
    Note: This sample will correspond to the total synaptosome fraction for further Western-blot analysis. The remaining synaptosomal fraction (~1 mL) can either be frozen down at -20 ºC until use or processed for subsequent subsynaptic fractionation. The synaptosomes or purified synapses represent between 1 and 2% of the total hippocampal volume[12].

2. Pre-, Post- and Extrasynaptic-isolation

1. Prepare 2x fresh solubilization buffer, pH 6.0; 1x solubilization buffer, pH 6.0; solubilization buffer, pH 8.0; and 0.1 mM $CaCl_2$ (see **Table 1**). Chill the solutions on ice.
   Note: The pH of these solutions should be accurately adjusted to achieve a good subsynaptic fractionation.
2. Slowly dilute the 1-mL resuspended synaptosomal fraction (see step 1.12) with 5 mL of 0.1 mM $CaCl_2$.
3. Add the same volume (5 mL) of ice-cold 2x solubilization buffer, pH 6.0, and incubate for 50 min on ice under high agitation in a beaker on ice.
   Note: While 1% of Triton X-100 at pH 6.0 solubilizes all plasma membrane proteins, it preserves proteins within the synaptic contacts or junctions (*i.e.,* pre- and postsynaptic structures)[4].
4. Place the solution in a centrifuge tube. Weigh and equilibrate the tubes with equivalent volumes of 0.1 mM $CaCl_2$ and 2x solubilization buffer solution, pH 6.0, within their corresponding steel supports and with their closing lids.
5. Centrifuge the tubes for 30 min at 40,000 x *g* and 4 °C using a swinging bucket rotor centrifuge. The resulting supernatant represents the extrasynaptic fraction, and the pellet corresponds to the synaptic junctions (*i.e.,* pre- and postsynaptic fractions).
6. Concentrate the supernatant containing the extrasynaptic fraction to a final 200 µL volume using a 15 mL 10K filter tube centrifuged at 4,000 x g and 4 ºC.
7. Precipitate the resulting concentrated extrasynaptic fraction (200 µL) with 5 volumes (1 mL) of pre-chilled (-20 °C) acetone overnight at -20 °C.

8. The next day, centrifuge the extrasynaptic fraction for 30 min at 18,000 x *g* and -15 °C. After centrifugation, discard the acetone supernatant and dry the pellet to remove any trace of acetone.
9. Finally, resuspend the pellet containing the extrasynaptic proteins with 200 µL of 5% SDS. Sonicate the pellet, if required.
10. Without disrupting it, carefully wash the pellet containing the pre- and postsynaptic fractions with 2 mL of 1x solubilization buffer, pH 6.0. Discard the buffer.
11. Resuspend the pellet with 10 mL of 1x solubilization buffer, pH 8.0, using a glass Pasteur pipette.
Note: While 1% of Triton X-100 at pH 8.0 solubilizes the presynaptic specialization, it preserves the insoluble postsynaptic density[4].
12. Incubate the suspension for 50 min on ice under high agitation in a beaker on ice.
13. Place the solution in a centrifuge tube. Weigh and equilibrate the tubes with 1x solubilization buffer solution, pH 8.0, within their corresponding steel supports and with their closing lids.
14. Centrifuge the tubes for 30 min at 40,000 x *g* and 4 °C using a swinging bucket rotor centrifuge; the resulting supernatant corresponds to the presynaptic fraction and the pellet to the postsynaptic fraction.
15. Process the supernatant containing the presynaptic fraction, as described in steps 2.6-2.9.
16. Resuspend the pellet containing postsynaptic fraction with 200 µL of 5% SDS. Sonicate the pellet, if required.

3. Analyze the samples by immunoblot to validate the membrane fractionation

1. Determine the amount of protein in each fraction (*i.e.,* the total, extra-, pre- and post-synaptic fractions) using the bicinchoninic acid protein assay.
2. Take 20 µg of protein from each fraction and dilute it to a final volume of 50 µL in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Boil for 5 min at 100 ºC.
3. Separate the proteins by SDS-PAGE electrophoresis 10%, with a 4% concentrating gel under reducing conditions. Electrophorese at constant voltage of 80 V until the dye enters the lower gel and then increase the voltage to 120 V.
4. Transfer the proteins to a PVDF membrane and block with IB blocking solution for 45 min at room temperature under continuous shaking.
5. Incubate the membrane overnight at 4 ºC with the indicated primary antibody (*i.e.,* anti-SNAP-25, anti-PSD-95, anti-synaptophysin, or anti-GPR37) diluted in IB blocking solution under continuous shaking.
6. Wash the membrane three times (10 min each) with IB washing solution to eliminate unbound primary antibody.
7. Incubate with the indicated secondary antibody for 90 min in dark conditions at room temperature under continuous shaking.
8. Wash the membrane three times (10 min each) with IB washing solution to eliminate unbound secondary antibody.
9. Incubate the membrane with chemiluminescent substrate (prepare the mix under dark conditions and following the 1:1 proportion of solution A and B provided by the manufacturer).
10. Analyze the membrane in a chemiluminescent detection device.

Representative Results

The described methodology has been largely used for the subsynaptic analysis of neuronal proteins in general and for the isolation and biochemical characterization of synaptic receptors[5,6,7,8,9] in particular. Interestingly, the representative result displayed here show the usefulness of this experimental procedure for the analysis of the subsynaptic hippocampal distribution of an orphan G protein-coupled receptor- namely, the Parkinson's disease-associated receptor GPR37[10]. GPR37 was originally identified through searches for homologues for endothelin and bombesin receptors[13], although it was found not to bind to endothelins or related peptides. In its place, GPR37 was proposed to be activated by the head activator peptide[14,15,16] and, more recently, by the neuropeptides prosaposin and prosaptide[17], although these associations are yet to be universally accepted. GPR37 has received the most attention for its linkage to Parkinson's disease[18]. Thus, there is significant interest in knowing the neurobiology of this intriguing receptor, both under normal and pathological conditions. Therefore, uncovering the GPR37 subsynaptic localization might help to clarify its function within the brain. To this end, the hippocampus was first isolated from C57BL/6J (WT) and GPR37-KO mice at eight weeks of age. Then, the extra-, pre- and post-subsynaptic fractions were purified using the membrane fractionation protocol (see **Figure 1** for a schematic overview of the procedure). Subsequently, the purities of these subsynaptic compartments were verified by the segregation of the respective synaptic markers: *i*) the extrasynaptic vesicular marker (synaptophysin); *ii*) the presynaptic active zone marker (SNAP-25); and *iii*) the postsynaptic density marker (PSD95). Accordingly, the enrichments in synaptophysin, SNAP-25, and PSD95 within the extra-, pre- and post-subsynaptic fractions, respectively, were analyzed by immunoblot using specific antibodies against these proteins (**Figure 2**). Hence, a fractionation efficiency of at least 90% was found for each synaptic marker tested (**Figure 3**), similar to those described previously[6]. Interestingly, GPR37 immunoreactivity was more abundant (n = 3; P <0.001) in the extrasynaptic fraction when compared to the presynaptic (20 ± 4%) and the postsynaptic (36 ± 2%) fractions (**Figure 3**). In addition, our data indicated that, while present at the presynaptic active zone, GPR37 was mainly localized in the postsynaptic density (n = 3; P <0.05) (**Figure 3**). Overall, the brain membrane fractionation protocol allowed for the assessment of the subsynaptic distribution of GPR37 in the mouse hippocampus, thus providing valuable information for future manipulations of this orphan receptor.

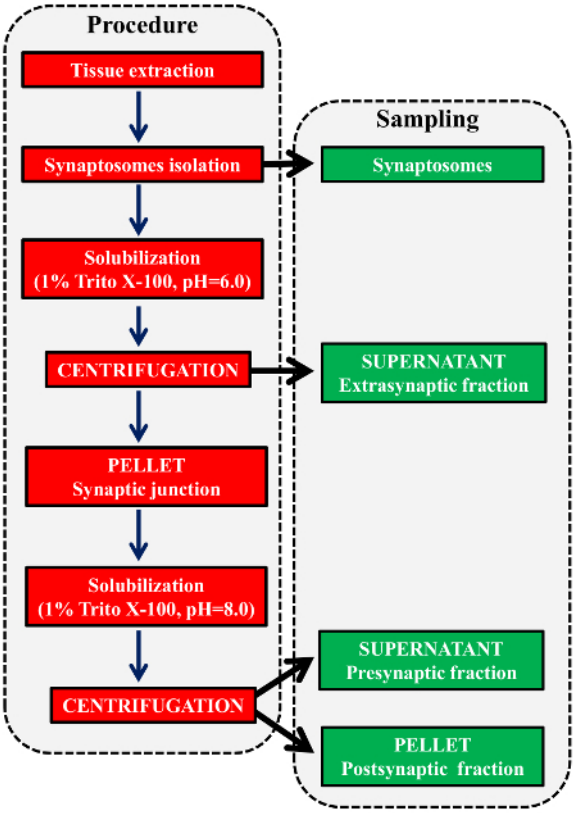

Figure 1: Schematic flow-chart representation of the membrane fractionation protocol. All experimental procedures are described in the left-hand column, while the sample collection is depicted in the right-hand column. Please click here to view a larger version of this figure.

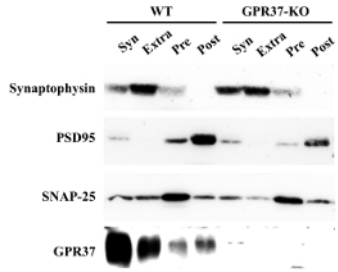

Figure 2: Subsynaptic distribution of GPR37 in the mouse hippocampus. Representative immunoblot showing Synaptophysin, SNAP-25 and PSD-95 as extra-, pre- and postsynaptic specific synaptic markers, as well as GPR37 immunoreactivity in hippocampal synaptic fractions of WT and GPR37-KO mice. Hippocampal synaptosomes (Syn) were fractionated into extrasynaptic (Extra) and presynaptic (Pre) active zones and postsynaptic density (Post) fractions. These were analyzed by immunoblot (20 µg of protein/lane) using the rabbit anti-synaptophysin (1:3,000), mouse anti-SNAP-25 (1:3,000), rabbit anti-PSD95 (1:3,000), and rabbit anti-GPR37 (1 µg/mL) antibodies. The primary bound antibody was detected using either an HRP-conjugated goat anti-rabbit (1/30,000) or a rabbit anti-mouse (1:30,000) antibody. These data are extracted from reference[10], with permission. Please click here to view a larger version of this figure.

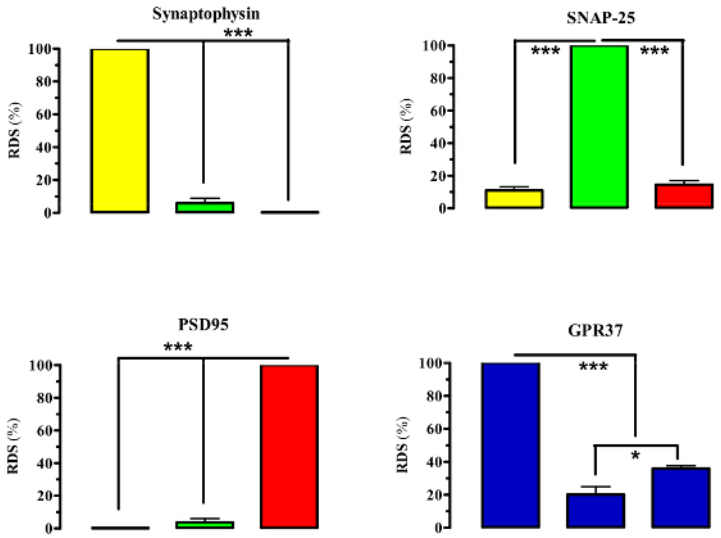

Figure 3: Relative quantification of GPR37 enrichment in hippocampal extra-, pre-, and postsynaptic fractions. The intensities of the immunoreactive bands on the immunoblotted membranes corresponding to extrasynaptic (Extra; yellow column), presynaptic (Pre, green column), and postsynaptic (Post, red column) fractions, shown in Figure 2, were measured by densitometric scanning. The densities were quantified from non-saturated bands. Values were normalized (in % of the relative densitometric scanning, RDS) using the amount of Synaptophysin, SNAP-25, PSD95, and GPR37 in the most enriched fraction and were presented as the means ± SEM of three independent experiments[10]. The asterisks denote significantly different data: *p <0.05, ***p <0.001 (1-way ANOVA with a Bonferroni's *post hoc* test). Please click here to view a larger version of this figure.

Discussion

The protocol presented here constitutes a powerful biochemical tool for the study of the subsynaptic distribution of specific proteins within any brain region. However, there are some drawbacks inherent to the technique that deserve to be highlighted here. For instance, one of the main limitations is the relatively large amount of tissue needed to purify a reasonable amount of protein in order to perform the immunoblot analysis of all subsynaptic fractions. This issue might be related to the fact that synapses (*i.e.,* synaptosomes) represent only 1-2% of the total hippocampal volume[12]. Indeed, between 1 and 1.5 g of fresh tissue (*i.e.,* the hippocampus) is needed to perform a successful fractionation; otherwise, the yield is too low to assess the identity, as well as the localization, of the protein being studied.

Conversely, if an excess of brain tissue is used, then the separation procedure will not be optimal. It is also critical to carefully adjust the pH of the solutions to ensure an optimal subsynaptic fractionation. Consequently, every time a brain membrane fractionation is performed, it is mandatory to validate the efficiency of each fraction before any further study. Importantly, the following protocol it is unlikely to be suitable for the subsynaptic fractionation of inhibitory synapses because of its differential structure and distribution. However, all these drawbacks do not overshadow the enormous usefulness of this experimental procedure, which, without any doubt, will become a universal methodology for the study of the synapse.

Disclosures

The authors have nothing to disclose.

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