Video Article

Electroconvulsive Seizures in Rats and Fractionation of Their Hippocampi to Examine Seizure-induced Changes in Postsynaptic Density Proteins

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Abstract

Electroconvulsive seizure (ECS) is an experimental animal model of electroconvulsive therapy, the most effective treatment for severe depression. ECS induces generalized tonic-clonic seizures with low mortality and neuronal death and is a widely-used model to screen anti-epileptic drugs. Here, we describe an ECS induction method in which a brief 55-mA current is delivered for 0.5 s to male rats 200 - 250 g in weight via ear-clip electrodes. Such bilateral stimulation produced stage 4 - 5 clonic seizures that lasted about 10 s. After the cessation of acute or chronic ECS, most rats recovered to be behaviorally indistinguishable from sham "no seizure" rats. Because ECS globally elevates brain activity, it has also been used to examine activity-dependent alterations of synaptic proteins and their effects on synaptic strength using multiple methods. In particular, subcellular fractionation of the postsynaptic density (PSD) in combination with Western blotting allows for the quantitative determination of the abundance of synaptic proteins at this specialized synaptic structure. In contrast to a previous fractionation method that requires large amount of rodent brains, we describe here a small-scale fractionation method to isolate the PSD from the hippocampi of a single rat, without sucrose gradient centrifugation. Using this method, we show that the isolated PSD fraction contains postsynaptic membrane proteins, including PSD95, GluN2B, and GluA2. Presynaptic marker synaptophysin and soluble cytoplasmic protein α -tubulin were excluded from the PSD fraction, demonstrating successful PSD isolation. Furthermore, chronic ECS decreased GluN2B expression in the PSD, indicating that our small-scale PSD fractionation method can be applied to detect the changes in hippocampal PSD proteins from a single rat after genetic, pharmacological, or mechanical treatments.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56016/>

Introduction

Electroconvulsive therapy has been used to treat patients with major depressive disorders, including severe drug-resistant depression, bipolar depression, Parkinson's diseases, and schizophrenia^{1,2}. In this therapy, seizure is generated by electrical stimulus delivered to the head of anesthetized patients via epicranial electrodes^{1,2,3}. Repetitive administration of ECS has been clinically beneficial to drug-resistant depressive disorders^{1,2,3}. However, the exact mechanism underlying the long-term efficacy of the antidepressant effect in humans has remained elusive. ECS is an animal model of electroconvulsive therapy and is widely used to investigate its therapeutic mechanism. In rodents, both acute ECS and chronic ECS treatment promote adult neurogenesis in the hippocampi and reorganize the neural network^{4,5}, which is likely to contribute to improvements in cognitive flexibility. Furthermore, global elevation of brain activity by ECS alters the abundance of transcripts, such as a brain derived neurotrophic factor⁶, and multiple proteins, including metabotropic glutamate receptor 1⁷ and the N-methyl-D-aspartate (NMDA) type glutamate receptor subunits⁷. These changes are involved in mediating long-term modification of synapse number, structure, and strength in the hippocampus^{7,8,9}.

In ECS models, electrical stimulation is delivered to rodents via stereotactically implanted electrodes, corneal electrodes, or ear electrodes to evoke generalized tonic-clonic seizures^{10,11}. Stereotaxic implantation of electrodes involves brain surgery and requires significant time to improve the experimenter's surgical skills to minimize injury. Less invasive corneal electrodes could cause corneal abrasion and dryness and require anesthesia. The use of ear-clip electrodes bypasses these limitations because they can be used on rodents without surgery or anesthesia and cause minimal injury. Indeed, we found that current delivered to awake rats *via* ear-clip electrodes reliably induces stage 4-5 tonic-clonic seizures and alters synaptic proteins in their hippocampi¹⁰.

To examine the ECS-induced abundance of synaptic proteins in the specific brain regions of the rodents, it is important to choose the experimental methods that are most suitable for their detection and quantification. Subcellular fractionation of the brain allows for the crude isolation of soluble cytosolic proteins; membrane proteins; organelle-bounds proteins; and even proteins in special subcellular structures, such as the PSD^{12,13,14}. The PSD is a dense and well-organized subcellular domain in neurons in which synaptic proteins are highly concentrated at and near the postsynaptic membrane^{12,13,15}. The isolation of the PSD is useful for the study of synaptic proteins enriched at the PSD, since

dynamic changes in the abundance and function of postsynaptic glutamate receptors, scaffolding proteins, and signal transduction proteins in the PSD^{12,15,16,17} are correlated with synaptic plasticity and the synaptopathy observed in several neurological disorders^{17,18}. A previous subcellular fractionation method used to purify the PSD involved the isolation of the detergent-insoluble fraction from the crude membrane fraction of the brain by the differential centrifugation of sucrose gradients^{14,19}. The major challenge with this traditional method is that it requires large amounts of rodent brains^{14,19}. Preparation of 10 - 20 rodents to isolate the PSD fraction per treatment requires extensive cost and time investment and is not practically feasible if there are many treatments.

To overcome this challenge, we have adapted a simpler method that directly isolates the PSD fraction, without sucrose gradient centrifugation^{20,21}, and revised it to be applicable to PSD isolation from the hippocampi of a single rat brain. Our small-scale PSD fractionation method yields about 30 - 50 µg of the PSD proteins from 2 hippocampi, sufficient for use in several biochemical assays, including immunoprecipitation and Western blotting. Western blotting demonstrates the success of our method for isolating the PSD by revealing the enrichment of postsynaptic density protein 95 (PSD-95) and the exclusion of presynaptic marker synaptophysin and soluble cytoplasmic protein α -tubulin. Our ECS induction and small-scale PSD fractionation methods are easily adaptable to other rodent brain regions and provide a relatively simple and reliable way to evaluate the effects of ECS on the expression of PSD proteins.

Protocol

All experimental procedures including animal subjects have been approved by the Institutional Animals Care and Use Committee at the University of Illinois at Urbana-Champaign.

1. Maintaining a Rat Colony

1. Breed Sprague-Dawley rats (see the **Table of Materials**) and maintain them in standard conditions with a 12-h light-dark cycle and ad libitum access to food and water.
2. Wean the rat pups at postnatal day (P) 28 and house them in groups of 2 - 4 male or female littermates.
3. Mark the tails of male rats with a non-toxic permanent black marker for identification.
4. Weigh the male rats 3 times per week and record their bodyweights.

2. Preparation of an ECS Machine

1. At 7:30 am, disinfect the bench in the animal preparation room and place an ECS machine (pulse generator) on the bench.
2. Place an individual male rat weighing 200 - 250 g in a clean, empty cage with a lid. Repeat this for all male rats to be treated with ECS induction. Let the rats habituate for 30 min.
3. While the rats are habituating in their cages, set a pulse generator for ECS induction to the frequency of 100 pulses/s, a pulse width of 0.5 ms, a shock duration of 0.5 s, and a current of 55 mA (**Figure 1A**).
4. Prepare the pulse generator by pushing the "RESET" button and ensuring that the "READY" button is lit. Make sure that the ear clips are not attached to a pulse generator and then press the "SHOCK" button for a few seconds.
NOTE: At this point, the pulse generator is ready for ECS induction.
5. Plug the ear clips into the pulse generator.

3. Induction of Acute ECS

NOTE: See **Figure 1B**, top panel.

1. Wet the ear clips with sterile saline and ensure that they are saturated.
2. Wet a rat's ears with sterile saline by wrapping them in saline-soaked gauze. Once they are wet, remove the gauze.
3. Attach one clip per ear, position beyond the main cartilage band.
4. Confirm on the ECS machine that a true loop is established; if not, an error message or a reading of "1" will appear on the machine.
5. Wear a thick, non-metal glove. While holding the rat gently in a gloved hand, press the "SHOCK" button for a few seconds and slowly release the grip on the rat to observe the seizure. For the sham "no seizure" (NS) control, handle the rat identically but do not deliver the current.
6. Disconnect the ear clips as clonus begins and record the seizure behavior according to a revised Racine's scale²² that includes "mouth and facial movements" (stage 1), "head nodding" (stage 2), "forelimb clonus" (stage 3), "rearing with forelimb clonus" (stage 4), and "rearing and falling with forelimb clonus" (stage 5). The seizure should last approximately 10 s; record the seizure duration using a timer.
7. Following seizure termination, return the rat to its home cage. Monitor the rat for another 5 min to make sure of the recovery of the rat from the seizures. Keep it singly housed in the cage and return the cage to the recovery room.
8. Repeat the ECS induction on the next rat.
9. Monitor the rats throughout the remainder of the day and at least once in the morning and once in the afternoon the following day until they are euthanized for experiments.

NOTE: The ECS induction method could lead to clinical signs as an incidental side effect, which requires attention. For example, the ECS induction protocol could induce seizures that last longer than 15 s and cause unnecessary distress to the rats. In this case, terminate the seizure using diazepam (10 mg/kg, i.p.) or pentobarbital (25 - 30 mg/kg, i.p.). If the rats develop respiratory distress or severe behavioral abnormalities following seizure cessation, euthanize them by carbon dioxide inhalation followed by decapitation.

4. Induction of Chronic ECS

NOTE: See **Figure 1B**, bottom panel.

1. Induce one ECS per day at the same time in the morning as described in steps 1 - 3, above, for seven consecutive days.
2. Monitor the rats twice a day after they are returned to their home cages.

5. Homogenization and Fractionation of Rat Hippocampi

NOTE: See **Figure 2**.

1. Prepare a fresh homogenization buffer (Solution A) that contains 320 mM sucrose, 5 mM sodium pyrophosphate, 1 mM EDTA, 10 mM HEPES pH 7.4, 200 nM okadaic acid, and protease inhibitors. Filter-sterilize the buffer using filters with a 0.22 μ m pore size for vacuum filtration and place it on ice.
2. At a given time point following acute ECS or chronic ECS (**Figure 1**), euthanize the rat by CO₂ inhalation for 5 - 10 min, followed by decapitation using a guillotine.
3. Remove the brain and dissect the hippocampi on the metal plate placed on ice, as described previously^{23,24}.
4. Place two hippocampi from one rat onto a 30-mm tissue culture dish and mince them into small pieces using scissors.
5. Transfer the minced hippocampi to a manual glass homogenizer using a 1 mL pipette and add 1 mL of ice-cold homogenization buffer (Solution A, step 5.1). Insert a round pestle into a glass homogenizer. While the glass homogenizer is on ice, gently and steadily stroke up and down on the pestle 10 - 15 times for 1 min, until small pieces of hippocampal tissue disappear.
6. Transfer the homogenate to a 1.7 mL microcentrifuge tube using a 1-mL pipette and centrifuge the homogenate at 800 x g for 10 min at 4 °C to separate the postnuclear supernatant (S1 fraction) from the pellet containing insoluble tissue and nuclei (P1 fraction). Transfer 50 μ L and 950 μ L of the S1 fraction to two separate, new 1.7 mL microcentrifuge tubes using a 1 mL pipette and store these tubes on ice. Save the P1 fraction pellet on ice.
7. Centrifuge the S1 fraction (950 μ L) for 10 min at 13,800 x g and 4 °C to separate the supernatant (S2 fraction), enriched with cytosolic-soluble proteins, and the pellet (P2 fraction), enriched with membrane-bound proteins, including synaptosomal proteins. Transfer the S2 fraction to a new 1.7 mL microcentrifuge using a 1-mL pipette and store it on ice.
8. Resuspend the pellet (P2 fraction) in 498 μ L of ice-cold purified water using a 1-mL pipette. Add 2 μ L of 1 M HEPES (pH 7.4) using a 20 μ L pipette to achieve a final concentration of 4 mM HEPES (pH 7.4). Incubate at 4 °C with agitation for 30 min. Store the resuspended P2 fraction on ice.
9. Determine the protein concentration of the S1, S2, and P2 fractions using a BCA assay. Add 50 mM HEPES (pH 7.4) to each fraction to achieve a 1 mg/mL concentration and store at -80 °C until use, or process the P2 fraction to isolate the PSD.

6. Isolation of the PSD from the Crude Membrane Protein (P2) Fraction

1. Centrifuge the P2 fraction (500 μ L) for 20 min at 25,000 x g and 4 °C to separate the lysed supernatant (LS2 fraction) and the lysed pellet (LP1 fraction). Transfer the LS2 fraction to a new 1.7 mL microcentrifuge tube using a 1 mL pipette and store it on ice.
 2. Resuspend the LP1 pellet in 250 μ L of 50 mM HEPES (pH 7.4) mixed with 250 μ L of 1% detergent in 1x PBS buffer using a 1 mL pipette. Incubate at 4 °C with gentle agitation for 15 min.
 3. Centrifuge the resuspended LP1 pellet for 3 h at 25,000 x g and 4 °C to separate the supernatant (non-PSD fraction) from the pellet (PSD fraction). Remove the supernatant to a 1.7-mL microcentrifuge tube and resuspend the PSD pellet in 100 μ L of 50 mM HEPES (pH 7.4) using a 200 μ L pipette.
 4. Determine the protein concentration of the LS2, non-PSD, and PSD fractions using a BCA assay. Add 50 mM HEPES (pH 7.4) to each fraction to achieve a 1 mg/mL concentration and store at -80 °C until use.
- NOTE: All solutions used in steps 5 - 6 (i.e., Solution A, HEPES buffer, and PBS buffer) should be made with purified water free of ionic and organic contaminants and particles.

7. Western Blotting

1. Thaw each protein fraction on ice. Transfer 12 μ L of each fraction (S2, P2, and PSD in 1 mg/mL) to a new 1.7 mL microcentrifuge tube using a 20- μ L pipette.
 2. Add 3 μ L of 5x SDS sample buffer and incubate at 75 °C for 30 min in a water bath. Cool the sample down to room temperature (RT).
 3. Load 10 μ L of the protein sample into each well of 4-20% gradient 15-well comb SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel using a 20 μ L pipette. Run the gel in the SDS-PAGE apparatus and at 80-100 V in running buffer (25 mM Tris, 190 mM glycine, and 0.1% SDS; pH 8.3).
- NOTE: Each gel should contain protein samples from NS rats and ECS rats at different time points following acute or chronic ECS.
4. Transfer the proteins from the SDS-PAGE gel to a polyvinylidene difluoride (PVDF) membrane in the transfer apparatus at 25 - 30 V (60 mA) for 9 - 12 h in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol; pH 8.3).
 5. Remove the PVDF membrane from the transfer apparatus and wash it in Tris-buffered saline (TBS) for 5 min on a multi-purpose rotator at RT.
 6. Block the membrane in 5% milk and 0.1% Tween-20 in TBS for 1 h. Incubate it in primary antibodies (**Table 1**) in washing buffer (1% milk and 0.1% Tween-20 in TBS) overnight on a multi-purpose rotator at 4 °C (see the **Table of Materials** for dilutions).
 7. Wash the membrane 4 times for 10 min each in wash buffer and then incubate it with horseradish peroxidase (HRP)-conjugated secondary antibodies in washing buffer for 1 h on a multi-purpose rotator at RT.
 8. Wash the membrane 4 times for 10 min each in wash buffer and then with TBS for 5 min.
 9. Incubate the membrane with enhanced chemifluorescence substrate for 1 min and expose it to X-ray film. Develop the exposed film with a film processor.

8. Quantification of Western Blots

1. Scan the Western blot as a TIFF file and save this file to the computer.
2. Open the Western blot file in the ImageJ program as a grayscale image, under "File," "Open image," right arrow "gray-scale."
3. Choose the rectangular selection tool from the ImageJ toolbar and draw a rectangle that covers a single Western blot band of a protein of interest.
4. Under "Analyze," hit "Measure" to obtain the area and the mean density of a selected band.
5. Move the rectangle to a background area without changing its size and shape. Repeat step 8.4 to the area and the mean density of a background.
6. Subtract the mean density value of a background band from that of a Western blot band, giving the background-subtracted band density of the protein of interest.
7. Repeat steps 8.2 - 8.6 for all Western blot bands of interest.
8. Divide the background-subtracted band density of the protein of interest by the background-subtracted band density of a housekeeping gene product, such as α -Tubulin; this step yields the normalized value of the protein of interest.

Representative Results

Using the detailed procedure presented here, one electrical shock (55 mA, 100 pulses/s for 0.5 s) delivered via ear-clip electrodes induced nonrecurring stage 4-5 tonic-clonic seizures in rats (**Figure 1A-B**). A total 8 of rats received acute ECS induction and displayed stage 4-5 tonic-clonic seizures. The seizures lasted about 10 s, and all rats recovered within 1 - 2 min of seizure cessation. Sham "no seizure" rats did not receive an electric shock and therefore did not display seizures. A total of 4 sham rats were used. For chronic ECS induction, the rats received one electric shock per day for 7 consecutive days and displayed stage 4-5 tonic-clonic seizures upon each electric shock (**Figure 1A-B**). A total 8 of rats received chronic ECS induction and a total of 4 sham rats were used as "no seizure" rats. Although most rats that received chronic ECS were behaviorally indistinguishable from sham "no seizure" rats, a few developed body tremors and reduced exploratory activity by the 7th electric shock.

To examine if ECS-induced global elevation of activity alters protein expression in the hippocampi, rats were sacrificed at 3 h and 24 h after acute ECS and at 24 h and 96 h after the final ECS in chronic ECS treatment (**Figure 1B**). Two hippocampi from each rat were rapidly dissected and subjected to our optimized small-scale fractionation protocol (**Figure 2**). The initial homogenate of two hippocampi free of insoluble tissue and nuclei (S1 fraction) was re-centrifuged at 13,800 x g for 10 min to separate the supernatant containing soluble cytoplasmic proteins (S2 fraction) from the crude membrane pellet containing synaptosomes (P2 fraction) (**Figure 2**). Our Western blot detected cytoplasmic protein α -tubulin in the S2 fractions, but not the P2 fractions, of the hippocampi from rats treated with acute and chronic ECS (**Figure 3** and **Figure 4**), demonstrating the successful fractionation of cytosolic soluble proteins from crude membrane pellets.

PSD95 is a member of the membrane-associated guanylate kinase (MAGUK) family and a core component of PSD^{12,18,25}. PSD95 and NMDA receptor subunit GluN2B were detected exclusively in the P2 fractions, but not the S2 fractions (**Figure 3** and **Figure 4**). Stronger expression of another glutamatergic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA2 was detected in the P2 fractions compared to the S2 fractions in the hippocampi (**Figure 3** and **Figure 4**), indicating that postsynaptic membrane proteins are enriched in the crude membrane P2 fraction pellets. Interestingly, synaptic vesicle protein synaptophysin and integral membrane protein Striatal Enriched Tyrosine Phosphatase 61 (STEP₆₁)²⁶ were detected equally in the S2 and P2 fractions (**Figure 3** and **Figure 4**). Considering the small size of synaptic vesicles-with an average diameter of 39 nm²⁷ and endosomes, the centrifugation to isolate the crude membrane pellet (P2 fraction) might not have been sufficient to pellet all synaptic vesicles and endosomes. These results together indicate that our crude fractionation protocol can enrich membrane-bound proteins and transmembrane proteins that are not associated with synaptic vesicles and possibly with endosomes in the crude membrane P2 fraction.

To examine if ECS-induced global elevation of activity alters the expression of postsynaptic proteins in the hippocampi, the crude membrane P2 fraction was lysed in ice-cold water, resuspended in HEPES buffer, and centrifuged at 25,000 x g for 20 min to isolate the lysed pellet (LP1) (**Figure 2**). The LP1 pellet was resuspended in HEPES buffer containing 1% Triton X-100 detergent and centrifuged at 25,000 x g over 3 h to obtain the PSD pellet (**Figure 2**). The subsequent Western blot of the PSD fractions detected PSD95, GluN2B, and GluA2 (**Figure 3** and **Figure 4**), which are known postsynaptic proteins^{17,25,28}. However, the PSD fractions lacked α -tubulin and, importantly, presynaptic marker synaptophysin (**Figure 3** and **Figure 4**). STEP₆₁, which dephosphorylates GluN2B and GluA2 and acts as their negative regulator^{29,30,31}, was not found in the PSD fractions (**Figure 3** and **Figure 4**), consistent with the recent report demonstrating the synaptic exclusion of STEP₆₁, mediated by PSD95 enriched in the PSD²⁶. Altogether, these results indicate that our small-scale fractionation method successfully isolates PSD proteins from the crude membrane P2 fraction.

The quantification of Western blotting revealed that GluN2B expression was unaltered in the P2 fraction but displayed an increasing trend in the PSD fraction at 3 h and 24 h after acute ECS (n = 4) (**Figure 3B**). GluA2 expression was not changed in both the P2 and PSD fractions at 3 h and 24 h following acute ECS (**Figure 3C**). At 24 h and 96 h after chronic ECS, GluN2B expression displayed a decreasing trend in the P2 fraction and was significantly reduced in the PSD fraction (24 h: $p < 0.05$, n = 4; 48 h: $p < 0.01$, n = 4) (**Figure 4B**). In contrast, chronic ECS did not affect GluA2 expression in the P2 and PSD fractions (n = 4) (**Figure 4C**).

Figure 1: Schema for the Induction of Acute ECS and Chronic ECS. (A) A rat was connected to a pulse generator via ear-clip electrodes and an electrical shock (55 mA, 100 pulses/s for 0.5 s) was applied to elicit stage 4-5 tonic-clonic seizures. (B) Acute ECS was induced by one electric shock. Chronic ECS was elicited by one electric shock per day for 7 consecutive days. The time points shown represent the duration following the induction of acute ECS or the last ECS for chronic ECS induction prior to dissection of the hippocampi. Sham "no seizure" (NS) control rats were handled identically but did not receive an electrical shock. [Please click here to view a larger version of this figure.](#)

Figure 2: Workflow of Subcellular Fractionation to Isolate the S2, P2, and PSD Fractions from the Hippocampi of a Single Rat. [Please click here to view a larger version of this figure.](#)

Figure 3: Examination of Synaptic Proteins in Hippocampal S2, P2, and PSD Fractions from the Rats that Received NS or Acute ECS. (A) Representative Western blots show the protein expression of NMDA receptor subunit GluN2B, AMPA receptor GluA2, and STEP₆₁ in the S2, P2, and PSD fractions from the hippocampi of sham "no seizure" (NS) rats and rats that received acute ECS. The cytoplasmic-soluble protein α-tubulin is enriched in the S2 fraction. Synaptophysin is a presynaptic vesicle protein and is enriched in the crude membrane P2 fraction, but not in the PSD fraction. PSD-95 is enriched in both the P2 and PSD fractions. (B-C) Quantification of GluN2B (B) and GluA2 (C) in the P2 and PSD fractions at 3 and 24 h after acute ECS (n = 4 rats per time point). GluN2B and GluA2 expression in the P2 fraction was normalized to α-Tubulin in the S2 fraction. GluN2B and GluA2 expression in the PSD fraction was normalized to PSD-95 in the PSD fraction. The data are presented as the percent ± SEM. [Please click here to view a larger version of this figure.](#)

Figure 4: Examination of Synaptic Proteins in Hippocampal S2, P2, and PSD Fractions from the Rats that Received NS or Chronic ECS. Representative Western blots of α-tubulin; synaptophysin; STEP₆₁; and postsynaptic proteins, including PSD95, GluN2B, and GluA2, in the S2, P2, and PSD fractions from the hippocampi of sham "no seizure" (NS) rats and rats that received chronic ECS. The cytoplasmic-soluble protein α-tubulin is enriched in the S2 fraction. Synaptophysin is a presynaptic vesicle protein and is enriched in the crude membrane P2 fraction, but not in the PSD fraction. PSD-95 is enriched in both the P2 and PSD fractions. (B-C) Quantification of GluN2B (B) and GluA2 (C) in the P2 and PSD fractions at 24 and 96 h after chronic ECS (n = 4 rats per time point). GluN2B and GluA2 expression in the P2 fraction was normalized to the α-Tubulin in the S2 fraction. GluN2B and GluA2 expression in the PSD fraction was normalized to PSD-95 in the PSD fraction. The data are presented as percent ± SEM; *p < 0.05, **p < 0.01 compared with control (ANOVA and Tukey's *post-hoc* test). [Please click here to view a larger version of this figure.](#)

Fraction	Protein fraction	Protein marker
P1	Nuclear	Histon H1
S1	Cytosol/membranes	a-tubulin (cytoskeleton) and GAPDH (cytosol)
P2	Crude synaptosomes	AMPA and NMDA receptor subunits
S2	Cytosol/light membranes	GAPDH (cytosol) and LAMP1 (lysosome)
Synaptosomal membrane	Synaptosome/mitochondria	AMPA and NMDAR receptor subunits, Synaptophysin (presynaptic marker)
PSD	PSD	AMPA and NMDA receptor subunits, PSD95, PICK1, CaMKII

Table 1: List of Protein Markers and Antibodies to Distinguish Subcellular Fractions.

Discussion

Here, we describe an ECS induction method in rats that elicits the global stimulation of neuronal activity in their hippocampi. ECS is an animal model of electroconvulsive therapy, which is clinically used to treat drug refractory depressive disorders in humans^{1,2,3}. Despite use of electroconvulsive therapy to treat severe depression, the precise underlying mechanism remains unclear. Because ECS induces anti-depressant-like behaviors in rodents and stimulates hippocampal neurogenesis^{4,32}, ECS has been extensively used to investigate if new adult-born neurons in the dentate gyrus of the hippocampus contribute to anti-depressant behavior^{5,32}.

ECS induces mild to severe generalized tonic-clonic seizures in rodents upon current delivery through stereotactically implanted electrodes, corneal electrodes, or ear-clip electrodes^{33,34}. EEG recordings in patients have revealed that bilateral electrical stimulation causes more prominent and longer generalized seizures than unilateral stimulation does^{35,36}. In our ECS induction method, tonic-clonic seizures are induced by current delivery through noninvasive ear-clip electrodes attached to both ears, mimicking seizures in humans evoked by bilateral stimulation³⁶. Although we haven't checked the efficacy of ECS in the rats to which sedation or anesthesia is applied, it is feasible that giving anesthetic drugs to the rats could reduce the degree of seizures induced by electrical stimulation, which might be due to the fact that anesthesia state is physiologically associated with enhanced inhibitory and dampened excitatory tone in the brain network. In addition, the critical step in the ECS induction method is to experimentally dial the current intensity to elicit stage 4 - 5 generalized tonic-clonic seizures based on the age, weight, and the species of rodent. Our ECS induction method has been optimized for inducing stage 4 - 5 seizures within a few seconds in awake male Sprague-Dawley rats weighing 200 - 250 g. If the rats under anesthesia state are used for ECS induction, the intensity, duration, and frequency of delivering current should be modified for successful induction of seizure ranging from stage 4 to 5.

ECS also offers the advantage of stimulating hyperactivity in neurons and causing acute transient seizures with very low mortality³³, in contrast to chemoconvulsants, including pilocarpine and kainite, which induce status epilepticus and cause chronic manifestation of spontaneous recurrent seizures and severe histological alterations^{37,38}. Although ECS has been routinely utilized to screen anti-epileptic drugs^{33,34}, acute ECS and chronic ECS do not result in the generation of chronic epilepsy and thus cannot be used in an animal model to study epileptogenesis. Instead, ECS has been widely employed to examine the extent to which the broad elevation of brain activity alters expression and/or posttranslational modifications of synaptic proteins *in vivo*, which contribute to persistent changes in synaptic strength and structures (**Figure 3** and **Figure 4**)^{10,40}. In this study, we only used male rats to exclude the unexpected effects of the estrus cycle phase of female rats on the amount of PSD protein after ECS. However, it was observed that there is no sex difference in the scaffolding proteins, including PSD-95 and SAP102 in the PSD region of the frontal cortex and hippocampus³⁹, implying that hormonal changes governing estrus cycle might not affect the basal amount of PSD proteins.

Multiple methods have been employed to examine the extent to which ECS induces changes in neurogenesis, synaptogenesis, and synaptic plasticity^{5,6,7,8,9,11,40}. Electrophysiological recordings of excitatory postsynaptic current (EPSC) are widely used to detect the changes in the synaptic strength of glutamatergic excitatory synapses²¹. For example, miniature EPSC recordings have revealed the homeostatic downscaling of excitatory synaptic strength in the cortical pyramidal neurons of layers II-III of mice following acute ECS⁴¹. However, the identification of synaptic proteins that contribute to ECS-induced synaptic plasticity is challenging because electrophysiological recordings must be paired with the genetic knockout or knock-down of specific candidate synaptic proteins. Changes in the level of glutamate receptors at the postsynaptic membrane and synaptic proteins enriched in the PSD regulate the strength and efficacy of excitatory synaptic transmission^{17,18,25}. Although immunohistochemistry can be used to examine ECS-induced changes in the expression of synaptic proteins, this technique can examine only 1 - 2 candidate synaptic proteins at a time and requires well-validated antibodies that specifically recognize them without causing non-specific staining.

The subcellular fractionation of brain tissue in combination with Western blotting offers advantages over electrophysiology and immunohistochemistry in identifying synaptic proteins that are altered by ECS. The subcellular fractionation of brain tissue is a rapid and crude biochemical method to separate soluble cytosolic proteins (S2 fraction) from crude membranes (P2 fraction), including ER and Golgi membranes, membrane-bound organelles, plasma membranes, and synaptic terminal membranes that reseal to form synaptosomes^{42,43,44}. The PSD fraction can be further isolated from the P2 fraction to enrich the synaptic proteins^{42,43,44}. The unbiased proteomic analysis of the PSD fraction could identify all PSD proteins whose levels are altered by ECS. Western blotting can be performed rapidly to examine the expression changes of the PSD proteins, which can be easily identified from non-specific bands.

The previous method of brain fractionation requires a large amount of rodent brain tissue^{42,43,44}, making this technique challenging for use when examining soluble or membrane proteins from an individual rodent brain or a specific brain region from a single brain. There is an increasing demand to quantitatively compare the proteome from one brain region to another and from a control animal to a transgenic animal or an animal that has undergone a specific treatment. Hence, we have revised and optimized the traditional brain fractionation method to isolate crude

soluble and membrane fractions from two hippocampi of a single rat. Our small-scale crude fractionation protocol successfully isolated crude P2 membrane fractions from cytosolic S2 soluble fractions, as indicated by the lack of cytoplasmic protein α -tubulin in the P2 fractions, whereas membrane-bound PSD95 was detected in the P2 but not S2 fractions (**Figure 3A** and **Figure 4A**). Using the method described here, we have quantitatively shown that acute ECS significantly increases STEP₆₁ expression and decreases tyrosine phosphorylation of its substrates, GluN2B and the extracellular signal-regulated kinase 1/2 in the crude membrane P2 fraction of rat hippocampi at 48 h following acute ECS¹⁰.

Unexpectedly, the S2 fractions contained synaptophysin; STEP₆₁; and, to a much lesser extent, GluA2 (**Figure 3A** and **Figure 4A**). STEP₆₁ is a glycosylated integral membrane protein²⁶ associated with the endoplasmic reticulum (ER) and PSD⁴⁵. It is possible that the centrifugation to isolate the crude membrane pellet (P2 fraction) might not have been sufficient to pellet all synaptic vesicles containing synaptosomes, as well as endosomes and lysosomes containing GluA2 and STEP₆₁. Nonetheless, clear enrichment of GluN2B, GluA2, and PSD95 and the lack of α -tubulin in the P2 fractions indicate that our fractionation protocol can enrich membrane-bound proteins and transmembrane proteins that are not associated with synaptic vesicles and endosomes in the crude membrane P2 fraction.

Although the crude membrane P2 fraction contains synaptosomes, one limitation of examining activity-dependent alterations of synaptic proteins in the P2 fraction is that the exact location of their changes cannot be determined. If synaptic proteins enriched in the PSD were to be determined, then further biochemical fractionation can be used to isolate the PSD. The previous method of PSD fractionation requires a large amount of rodent brain tissue (*i.e.*, 10 - 20 rodent brains) and sucrose gradients^{42,43,44}. Because this traditional method is inadequate to isolate a sufficient amount of the PSD fraction from two hippocampi of a single rat, we have adapted a simpler method that directly isolates the PSD fraction, without a sucrose gradient^{20,21}. This method yields about 30 - 50 μ g of the PSD protein, sufficient for several biochemical assays, including Western blotting. Our method enriches PSD95, GluN2B, and GluA2, which are known to be concentrated in the PSD fraction, and detects changes in GluN2B expression in PSD fraction following chronic ECS induction (**Figure 3** and **Figure 4**).

Here, our quantitative Western blot analysis revealed no significant change in GluA2 expression in the P2 and PSD fractions at 3 h and 24 h following acute ECS (**Figure 3C**). The GluN2B expression was unaltered in the P2 fraction but displayed an increasing trend in the PSD fraction at 3 h and 24 h after acute ECS (**Figure 3B**), suggesting the possible differential regulation of synaptic versus extrasynaptic GluN2B-containing NMDA receptors. We also observed that GluN2B expression displayed a decreasing trend in the P2 fraction after chronic ECS and was significantly reduced in the PSD fraction at 24 and 96 h following chronic ECS (**Figure 4B**). Though highly speculative, such removal of GluN2B from the PSD following repetitive induction of ECS may be mediated by multiple mechanisms, including direct internalization from the PSD membrane or lateral diffusion to extrasynaptic sites. Considering our previous report that prolonged enhancement of neuronal activity markedly increases STEP₆₁ expression and reduces Tyr-phosphorylation of GluN2B in the P2 fractions of cultured hippocampal neurons⁴⁶, our findings suggest that a decrease in GluN2B expression in the P2 and the PSD fractions after chronic ECS may be likely due to enhanced STEP₆₁ expression and the subsequent removal of GluN2B from the extrasynaptic membrane.

In summary, we have demonstrated that our small-scale PSD fractionation method, in combination with an ECS induction protocol, allows us to differentiate *in vivo* the activity-dependent regulation of glutamate receptors at the postsynaptic membrane versus total plasma membranes, including the extrasynaptic membranes. This protocol can easily be applied to any synaptic proteins at excitatory synapses and can be modified for other rodent hippocampi or other brain regions by adjusting the volume of each solution based on the tissue weight. Thus, our small-scale PSD fractionation method is versatile and can be adopted for future applications to examine the *in vivo* changes in the postsynaptic proteins in each animal upon genetic, pharmacological, or mechanical treatment.

Disclosures

The authors declare that they have no competing financial interests.

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