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IN-DEPTH ANALYSIS OF THE SYNAPTIC PLASMA MEMBRANE PROTEOME OF SMALL HIPPOCAMPAL SLICES USING AN INTEGRATED APPROACH

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Abstract—Comprehensive knowledge of the synaptic plasma membrane (SPM) proteome of a distinct brain region in a defined pathological state would greatly advance the understanding of the underlying biology of synaptic plasticity. The development of innovative approaches for studying the SPM proteome of small brain tissues is highly desired. This study presents a suitable protocol that integrates biotinylation-based affinity capture of cell surface-exposed proteins, isolation of synaptosomes, and biochemical extraction of SPM proteins from biotinylated hippocampal slices. The effectiveness of this integrated method was initially confirmed using immunoblot analysis of synaptic markers. Subsequently, we used highly sensitive mass spectrometry and streamlined bioinformatics to analyze the obtained SPM protein-enriched fraction. Our workflow positively identified 241 SPM proteins comprising 85 previously reported classical proteins from the pre- and/or post-synaptic membrane and 156 nonclassical proteins that localized to both the plasma membrane and synapse, and have not been previously reported as SPM proteins. Further analyses revealed considerable similarities in the physicochemical and functional properties of these proteins. Analysis of the interaction network using STRING indicated that the two groups showed a relatively strong functional correlation. Using MCODE analysis, we observed that 65 nonclassical SPM proteins formed 12 highly interconnected clusters with 47 classical SPM proteins, suggesting that they were the more likely SPM candidates. Taken together, the results of this study provide an integrated tool for analyzing the SPM proteome of small brain tissues, as well as a dataset of putative novel SPM proteins to improve the understanding of hippocampal synaptic plasticity. **© 2017 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: biotinylation, bioinformatics, hippocampal slice, proteome, synaptic plasma membrane.

INTRODUCTION

Synapses are highly specialized intercellular junctions for chemical transmission in the central nervous system (Phillips et al., 2001; Schrimpf et al., 2005; Li et al., 2009). The synaptic framework and its adaptations involve numerous highly ordered and complicated molecular events (MacDonald et al., 2012). Presynaptically, proteins are co-assembled, which mediates synaptic vesicle clustering, exocytotic fusion, and membrane retrieval, whereas the postsynaptic compartment of the junction harbors neurotransmitter receptors and their associated signaling components (Pischedda et al., 2014). Generally, the synaptic architecture and plasticity are involved in multiple signaling mechanisms with robust crosstalk and protein interactions between pre- and postsynaptic membranes (Tanaka et al., 2012). Although synaptic plasma membrane (SPM) proteins have always attracted extensive attention in the field of neuroscience, they are still underrepresented (Li et al., 2009).

Traditionally, for investigation of SPM proteins, researchers primarily enrich the SPM fraction from biological tissues with ultracentrifugation-based subcellular fractionation because of the low cellular abundance of SPM proteins (Phillips et al., 2001; Schrimpf et al., 2005; Dahlhaus et al., 2011). In our sample preparation, synaptosomes of postmortem animals and human brain tissues were isolated using the sucrose (Stevens et al., 2003; Prokai et al., 2005; Dahlhaus et al., 2011; Vegh et al., 2012) or Ficoll (Sidhu et al., 2011, 2016) density gradient centrifugation method, followed by osmotic shock washing to obtain the SPM-enriched fraction. However, this common approach generally leads to weak enrichment and contamination from other synaptic compartments such as the synaptic mitochondria and

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Abbreviations: ACSF, artificial cerebrospinal fluid; GOBP, gene ontology biological process; GOCC, gene ontology cellular component; GOMF, gene ontology molecular function; IDA, information dependent acquisition; KEGG, Kyoto Encyclopedia of Genes and Genomes; MCODE, Molecular Complex Detection; NeutrA, NeutrAvidin; NeutrA-PD, NeutrAvidin pull down; PM, plasma membrane; PSD, postsynaptic density; SPM, synaptic plasma membrane; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TEM, transmission electron microscopy; TMDs, transmembrane domains.

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vesicles (Burre and Volknandt, 2007; Li et al., 2009). The low abundance of SPM proteins makes their identification extremely difficult in the presence of contaminating compartments. In addition, this approach requires relatively high sample loads, which is probably a major disadvantage in this area of neuroscience research (Schindler et al., 2008; Schindler and Nothwang, 2009). Because the brain is highly complex, it is usually not investigated in its entirety, and individual brain areas such as the hippocampus are the focus of neuroscientists (Schindler et al., 2008; Schindler and Nothwang, 2009; Distler et al., 2014). For these specific regions, the obtainable tissue amount is usually limited, especially in brain disease research. Proteomic analyses of SPM proteins from these regions are hindered by the load disadvantage mentioned above (Smolders et al., 2015). Therefore, there is a substantial need to effectively analyze the SPM proteome of small and distinct brain areas.

Affinity purification methods based on cell surface-exposed protein biotinylation have already been widely used to specifically extract and enrich plasma membrane (PM) proteins from nerve cell cultures (Chen et al., 2006; Pischedda et al., 2014). Furthermore, for fresh brain tissues, an ex vivo method for the cell surface biotinylation of acute brain slices has been developed, which created a strategy for investigating the trafficking of PM proteins under more physiological and natural conditions (Smolders et al., 2015). Researchers can also simultaneously perform ex vivo slice examinations by combining this strategy with methods such as electrophysiological recording (Thomas-Crusells et al., 2003). The ex vivo slice biotinylation method has the potential to address the two major issues of poor preparation efficiency and high sample consumption associated with the conventional SPM proteome analysis method. Accordingly, in this study, we present an integrated strategy aimed at conducting an effective proteomic analysis of the SPM from small brain tissue samples. The combined protocol involves a set of procedures including biotinylation-based affinity capture of cell surface-exposed proteins, isolation of synaptosomes, and biochemical extraction of SPM proteins from biotinylated hippocampal slices.

EXPERIMENTAL PROCEDURES

Animals and acute hippocampal slice preparation

Neonatal (2-week-old) Sprague–Dawley rats (n = 5) of either sex weighing 15–20 g were obtained from the Animal Facility of Chongqing Medical University, and the animal protocols were approved by the local research ethics committee. All of the animals received care in accordance with the approved guidelines and regulations. The rats were maintained under constant laboratory conditions with a 13-h light/11-h dark daily photoperiod, whereas water and food were provided *ad libitum* during the experiment.

The acute hippocampal slices were prepared according to our previously described method with slight modifications (Chen et al., 2015). Briefly, after cervical dislocation, neonatal rats were decapitated; the brains were rapidly dissected and then placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF, 1.25 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 11 mM D-glucose, pH 7.4). The hippocampal tissues were quickly removed and cut into 300-μm-thick sections, which is the thickness required for effective biotinylation of an adequate number of intact and normal cells in the slices. The acute hippocampal slices were then transferred to an incubation chamber filled with ACSF, and a continuous flow of 95% O_2 and 5% CO_2 was provided for 90 min to ensure recovery of the slices.

Surface biotinylation of slices

The slices were biotinylated using a Pierce cell surface protein isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The slices were placed on ice and supplemented with CO_2 and O_2 throughout the entire biotinylation procedure. Before beginning surface biotinylation, the hippocampal slices were washed twice (5 min each) with chilled ACSF to gradually cool the cells. Then, the slices were suspended with 0.5 mg/mL EZ-link sulfo-NHS-SS-biotin in ACSF for 45 min and washed twice with a quenching solution to block the excess non-reactive NHS-SS-biotin.

Isolation of synaptosomes

Synaptosomes were isolated using Syn-PER synaptic protein extraction reagent (Thermo Fisher Scientific) as previously described (Wang et al., 2014). Immediately before use, the Syn-PER reagent was supplemented with a protease inhibitor cocktail (BBI, Canada). The slices were weighed and homogenized in Syn-PER reagent using a Dounce tissue grinder on ice, and the homogenate was centrifuged for 10 min at 1200g to remove the cell debris. The resulting supernatant was collected and saved as the homogenate sample (H). After 20 min of centrifugation at 15000g, the supernatant was divided into cytosolic (C) and synaptosomal (S) fractions and using our previous method, the S pellet was fixed for 4 h in 4% glutaraldehyde and analyzed using transmission electron microscopy (TEM) (Hu et al., 2013).

NeutrAvidin pull-down (NeutrA-PD)

The S pellet was suspended in a Triton lysis buffer (1% Triton X-100, 20 mM Tris–HCl, and 150 mM NaCl, pH 7.5, including a protease inhibitor cocktail). After 10 min of centrifugation at 40,000g, the proteins were quantified using a Pierce bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific) and the extracted protein sample was diluted with the lysis buffer to a final total protein concentration of 1 μg/μL. Then, NeutrAvidin (NeutrA) agarose resin was loaded onto a spin column and washed twice with the wash buffer. A brief centrifugation at 1000g followed each wash. The diluted lysate was added to the NeutrA agarose resin at a protein-to-agarose ratio of 2 μg/μL and mixed using a head-over-head shaker at 4 °C overnight. After

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