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ARTICLE *in* JOURNAL OF PROTEOME RESEARCH · JUNE 2009

Impact Factor: 4.25 · DOI: 10.1021/pr900101d · Source: PubMed

research articles Journal of proteome research

Proteomic Screen for Multiprotein Complexes in Synaptic Plasma Membrane from Rat Hippocampus by Blue Native Gel Electrophoresis and Tandem Mass Spectrometry

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Received February 8, 2009

Neuronal synapses are specialized sites for information exchange between neurons. Many diseases, such as addiction and mood disorders, likely result from altered expression of synaptic proteins, or altered formation of synaptic complexes involved in neurotransmission or neuroplasticity. A detailed description of native multiprotein complexes in synaptic plasma membranes (PM) is therefore essential for understanding biological mechanisms and disease processes. For the first time in this study, two-dimensional Blue Native/SDS-PAGE electrophoresis, combined with tandem mass spectrometry, was used to screen multiprotein complexes in synaptic plasma membranes from rat hippocampus. As a result, 514 unique proteins were identified, of which 36% were integral membrane proteins. In addition, 19 potentially novel and known heterooligomeric multiprotein complexes were found, such as the SNARE and ATPase complexes. A potentially novel protein complex, involving syntaxin, synapsin I and Na^+/K^+ ATPase alpha-1, was further confirmed by co-immunoprecipitation and immunofluorescence staining. As demonstrated here, Blue Native-PAGE is a powerful tool for the separation of hydrophobic membrane proteins. The combination of Blue Native-PAGE and mass spectrometry could systematically identify multiprotein complexes.

Keywords: hippocampus • synaptic plasma membrane • multiprotein complexes • proteomics • Blue Native PAGE • tandem mass spectrometry

Introduction

The hippocampus is one of the most important brain structures of the limbic system. It is critical for learning and memory processes. In the central nervous system, neuronal synapses are specialized sites of information exchange between neurons. Many diseases, such as addiction and mood disorders, likely result from altered expression of synaptic proteins that are involved in neurotransmission or neuroplasticity.[1] In principle, the function of the hippocampus could be defined in terms of the number, type, and location of its proteins or multiprotein complexes. Therefore, a comprehensive catalogue of protein and protein complexes in its synapses would provide a novel platform for the study of hippocampus functions.

Proteomic analyses of brain tissues are becoming an integral component of neuroscience.[2] Because of its accessibility and biological interest, the synapse has been a focus of neuroproteomics studies. A great deal of effort has been made to identify the protein components of synapses, including synaptosomes, synaptic membranes, postsynaptic densities, synaptic vesicles, and the presynapse.[1,3,4] Many known and new components of synaptic proteins have been identified. As we know, proteins usually exist in multiprotein complexes, ranging from a few subunits to more than 100 components. Therefore, the list of available proteins is not sufficient to describe biological processes. The characterization of protein complexes should provide further information about molecular organization as well as cellular pathways, making it one of the hot topics in proteomics research.[5−7]

Biochemical, genetic, and bioinformatics strategies have been used to characterize multiprotein complexes.[8] More recently, with the development of biological mass spectrometry, a combination of affinity purification and mass spectrometry has been used to advance our understanding of the compositions of multiprotein complexes.[7,9] In neuroscience, the more common strategy for isolating protein complexes relies on affinity methods. Antibodies were used to capture protein complexes, such as the NMDA receptor-adhesion protein signaling complexes,[10] metabotropic glutamate receptor 5 complexes,[11] NMDAR/MAGUK-associated signaling complexes,[12] and ion channel complexes.[13] However, this approach is limited by a requirement for prior detailed knowledge of candidate constituents, and by the availability of antibodies. Blue native PAGE (BN-PAGE) is an alternative biochemical method for the isolation of physiological protein−protein interactions. When combined with MS, BN-PAGE can identify

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10.1021/pr900101d CCC: $40.75

Published on Web 05/11/2009

and determine the compositions of potential protein complexes. It has been proven generally successful for the identification of integral membrane proteins and protein complexes, such as those from mitochondria,[14] plasma membranes[15] and whole cellular lysates.[16] However, no such systematic strategies for elucidating the synapse proteome have yet been reported.

Most of the known protein complexes in the nervous system are related to the postsynaptic proteome.[17] However, the multiprotein complexes in the presynaptic plasma membrane (PM) remain poorly understood. It was reported that synaptic signal conversion occurs at active zones, highly specialized sites of the presynaptic nerve terminal, which can be biochemically purified and reassembled *in vitro* in a pH-dependent manner.[18] The individual proteins expressed abundantly in these regions have been profiled.[19] Further defining the multiprotein complexes involved in these important processes will help to elucidate the mechanisms of synaptic vesicle exocytosis.

In this study, we applied two-dimensional Blue Native-SDS gel electrophoresis, combined with mass spectrometry, to screen for the multiprotein complexes in the synaptic plasma membrane (SPM) from rat hippocampus. To reduce contaminants from mitochondria, we optimized aqueous two-phase partition methods and successfully integrated them in the sample preparation. Moreover, a prefractionation strategy of adjusting the pH during the Triton X-100 extraction of isolated SPM was used. Several known and potentially novel synaptic membrane proteins and their multiprotein complexes were found and discussed.

Materials and Methods

Materials. Bio-Rad DC protein assay kit and electrophoresis reagents were bought from Bio-Rad Laboratories (Hercules, CA). Anti-synaptophysin, anti-synapsin and anti-syntaxin antibodies were obtained from Stressgen (Victoria, Canada). Anti-Na^+/K^+ ATPase monoclonal antibody and anti-Prohibitin monoclonal antibody were purchased from Abcam (Cambridge, U.K.). Protease inhibitor cocktail, PEG-3350, Dextran T-500, trypsin, Triton X-100, Hoechst 33258, penicillin, streptomycin and sucrose were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were bought from Gibco-BRL (Grand Island, NY). High molecular protein marker was from Amersham (Piscataway, NJ). Milli-Q Plus water purification system and Immobilon transfer membranes (PVDF) were from Millipore (Bedford, MA). All other reagents were analytical grade. Male rats (about 250 g weight) were purchased from Centre South University (Changsha, China).

Isolation of Synaptic Plasma Membrane from Rat Hippocampus. The rats were killed by decapitation, and the hippocampus were dissected quickly and stored on ice. The synaptic plasma membrane (SPM) fraction was isolated essentially as described.[20] First, the hippocampi from 10 rats were combined and homogenized in solution A (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$ containing protease inhibitor cocktail) at 4 °C using a hand-held disperser (IKA products, T8 ULTRA-TURRAX, Germany). A low speed (1400*g* for 10 min) pellet was obtained from the resultant homogenates and washed by resuspending the pellet in the solution A and then homogenized again. All the centrifugation in this experiment was carried out at 4 °C. The second centrifugation was performed at 710*g* for 15 min. The supernatants were pooled and centrifuged at 13 800*g* for 20 min. The resulting pellet was suspended in solution B (0.32 M sucrose and 1 mM $NaHCO_3$ containing protease inhibitor cocktail). The sucrose gradients, composed of suspended material, 0.85, 1.0, and 1.2 M sucrose were then centrifuged for 2 h at 82 500*g*. The bands between 1.0 and 1.2 M sucrose were collected and then diluted with solution B. The synaptosome was spun down at 32 800*g* for 20 min. The synaptosomal pellet was washed and resuspended in 12 mL of hypotonic solution containing 12 mM Tris-HCl (pH 8.1) and osmotic shock was carried out on ice for 45 min under gentle stirring. The resulting SPM was obtained after centrifugation at 20 000*g* for 30 min.

To reduce the possible contaminations from other organelles especially from mitochondria, an established aqueous two-phase partition method was used in the SPM preparation.[21] The two-phase systems consisted of 6.4% polyethylene glycol and 6.4% dextran. The upper phase, enriched in SPM, was rewashed by addition to the fresh lower phase taken from the second tube, and a fresh upper phase was repartitioned against the original lower phase. Finally, the two upper phases and two lower phases were combined and diluted 5-fold and 8-fold, respectively, with 1 mM $NaHCO_3$. Two resulting pellet from upper (UPM) and lower phase (LPM) were finally obtained by centrifugation at 37 500*g* for 20 min at 4 °C.

Prefractionation with Different pH Treatment. The obtained UPM sample from two-aqueous partition purification was extracted sequentially by 2% Triton X-100 solutions with different pH value according to the previous work.[18] First, the fractions were treated with 2% Triton X-100 in solution C (50 mM NaCl, 50 mM Imidazole, 2 mM 6-Aminohexanoic acid, and 1 mM EDTA, pH 6) on ice for 20 min. After centrifugation at 18 000*g* for 20 min at 4 °C, the supernatant (pH 6 fraction) was collected, and the pellet was resuspended in the same solution and centrifuged again. The supernatant were then combined and the pellet was solubilized again with 2% Triton X-100 in solution C at pH 8 for 20 min. The supernatant (pH 8 fraction) was recovered by centrifugation as before, and the last pellet (LP) was collected. Aliquots of the prepared subfractions were fixed with 2.5% glutaraldehyde for the transmission electron microscope (TEM) analysis.[22,23]

Blue Native-PAGE and SDS-PAGE. BN-PAGE was performed according to the published protocols with minor modifications.[24] Briefly, a 4−10% gradient separation gel with a 3.5% stacking gel was poured and stored at 4 °C until further use. The cathode buffer (7.5 mM Imidazole, 50 mM Tricine) containing 0.02% (w/v) Coomassie Brilliant Blue G250 and the anode buffer (25 mM Imidazole, pH 7.0) were chilled to 4 °C before use. For BN-PAGE, 3 μL of 50% Glycerol and 3 μL of 5% G-250 were added to 30 μL of sample, which was then loaded onto the sample well. Electrophoresis was begun at 100 V at 4 °C. After about 1 h, the cathode buffer was replaced by the same buffer containing 0.002% of G250, and the electrophoresis was continued with voltage at 200 V at 4 °C. The BN gel was fixed and stained with Coomassie Brilliant Blue G250.

For further separation in a second-dimensional SDS-PAGE, the excised lanes from BN-PAGE were denatured in SDS loading buffer for 2 h. The lanes were then rinsed briefly with 20 mM Tris buffer. SDS-PAGE was performed using 7.5−12.5% acrylamide gradient gels. The excised lanes were then placed into the SDS-PAGE and sealed with hot agarose solution. Two gel strips from the pH 6 and pH 8 fractions were loaded side by side on the same gel to facilitate further comparison of protein profiles. Electrophoresis was performed at 20 mA until

the front passed into the separation gel and then continued at 45 mA. Proteins were visualized using standard silver staining protocol.[25]

1-D SDS PAGE and Immunoblot Analysis. The same amounts of protein from different fractions were added with a trace of bromophenol blue, incubated at 100 °C for 10 min and then subjected to SDS-PAGE using 11.5% separation gel and 4.8% stacking gel. The SDS-PAGE and further Western blotting methods were performed as before.[22,26,27]

In-Gel Digestion and Mass Spectrometry Analysis. For identification, both the gel slices from BN-PAGE and the spots from the SDS-PAGE were subjected to trypsin digestion. The in-gel digestion was performed according to our previous work.[22,23,27] The final solution was pooled and lyophilized in a SpeedVac to about 10 μL.

For ion trap tandem mass spectrometry analysis, the digested peptides were injected into a capillary LC system (Agilent 1200) and first desalted and preconcentrated on a precolumn (C18 PepMap, 0.3 mm i.d., 5 mm length, LC Packings). The outlet of the LC system was directly connected to the nanoelectrospray source of an HCT ultra IT mass spectrometer (Bruker Daltonics, Germany). The gradient profile consisted of a linear gradient from 5% B (0.1% formic acid/4.9% H_2O/90% acetonitrile, v/v) to 50% B in 65 min followed by 85% B for 10 min, then the column was reconditioned with 95% A (0.1% formic acid/4.9% acetonitrile/95% H_2O, v/v) solution for 10 min. Instruments were controlled using Chemstation B01 (Agilent) and Esquirecontrol 6.0 (Bruker Daltonics) software. The flow rate was 5 μL/min. The peptides were detected in the positive ion MS mode or the data-dependent MS/MS mode. Data-depend mode was conducted using survey scans (m/z 400−3000) to choose the three most intense precursor ions (with charge states ≥ 2) for a collision-induced dissociation (CID).

Data Processing and Bioinformatics Analysis. For data processing, raw spectra were formatted in DataAnalysis 3.4 software (Bruker Daltonics). The produced.mgf files were analyzed using Mascot 2.1 software (www.matrixscience.com). Search parameters were set as follow: the International Protein Index (IPI) rat combined forward/reverse database (version 3.26, www.ebi.ac.uk/IPI, 82 988 entries); trypsin enzyme, allowing up to 1 missed cleavage; peptide mass tolerance was 2 Da and MS/MS mass tolerance was 0.8 Da; fixed modification parameter was carbamidomethylation, and the variable modification parameter was oxidation (Met). Peptide matches to a query appearing in the report for the first time are shown as bold red. In our output result, only the red bold peptides with their individual ion score above 15 in the mascot report contributed to protein identification. Proteins were considered to be identified until the first hit of the reverted database appears at the 95% confidence level. Therefore, it meant that false positive rate was kept to the lowest using the reverse database strategy. We also kept the principle of using the minimum set of protein sequences to account for all observed peptides. When a group of proteins were identified from the same peptides, only the protein with the most detailed GO annotations was retained. For proteins identified by only one peptide exhibiting the above criteria, the peptide sequence was systematically checked manually to confirm or cancel the MASCOT suggestion.

The molecular mass values and the number of peptides used to identify a protein as well as the sequence and charge state of each peptide were retrieved from Mascot output files. The grand average of hydropathicity (GRAVY) for identified proteins was calculated using the ProtParam software available at http://us.expasy.org by submitting each FASTA file in batch.[28] The mapping of putative transmembrane domains was carried out using the transmembrane hidden Markov model (TMHMM v 2.0) algorithm. Protein abundance estimates were determined using exponentially modified protein abundance index (emPAI) values,[29] which was estimated by normalizing the number of peptides per protein by the theoretical number of peptides per protein and had been successfully used in several similar studies.[27,30,31] In semiquantitative analysis, when a protein was identified for several times, only the protein with the highest Mascot score was taken into consideration. The subcellular location and function of the identified proteins were elucidated by the combination of literatures and the cellular component and function annotations of gene ontology (GO) (http://www.geneontology.org/) respectively.

Co-Immunoprecipitation. The synaptic plasma membrane fraction were solubilized with 2% Triton X-100 in solution D (50 mM NaCl, 50 mM Imidazole, 2 mM 6-Aminohexanoic acid, and 1 mM EDTA, pH 7) on ice for 20 min and cleared by ultracentrifugation. The supernatant protein (1 mg) was prewashed with protein A-Sepharose for 10 min on ice. The supernatant was then incubated with appropriate immobilized affinity-purified mouse anti-syntaxin or negative control nonspecific normal serum overnight at 4 °C, followed by the addition of 20 μL of protein A for 1h at room temperature. After brief washing, the bound proteins were eluted with SDS loading buffer.

Cell Culture and Immunofluorescence Staining. The SH-SY5Y (human neuroblastoma) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 50 units/mL of penicillin and 50 μg/mL of streptomycin, and kept in humidified atmosphere of 5%CO_2/95% O_2 at 37 °C.

For immunofluorescence staining analysis, SH-SY5Y cells were cultured on glass coverslips and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, and then permeabilized with 0.2% Triton X-100 for 5 min. Fixed cells were blocked in blocking solution (2% BSA, 0.1% Tween-20, PBS) for 30 min at 37 °C. Cells were then incubated with appropriate primary antibodies for 1.5 h at room temperature. Excess antibody was removed by incubation with PBS and 0.1% Tween-20 for 15 min. Cells were then incubated with Alexa 594 goat anti-mouse and Alexa 488 goat anti-rabbit secondary antibodies. Hoechst 33258 was used to stain the nuclei. After 3 washes with PBS, the cells were viewed under a Zeiss Axioskop2 plus fluorescence microscope (Carl Zeiss, Jena, Germany), and the digitized images were processed using AxioVision 3.1 software (Carl Zeiss, Jena, Germany).

Results

Synaptic Plasma Membrane (SPM) Fractionation and Purification. The experiment workflow of this study was shown in Figure 1. The SPM was first isolated using differential centrifugation and density gradient centrifugation. The SPM preparation method was carried out to isolate postsynaptic densities from brain regions as previously described.[20] In our pilot study, a high percentage of possibly contaminating proteins from other organelles, especially from mitochondria, was identified (data not shown). The aqueous two-phase partition method was a valuable alternative to density centrifugation for selective PM extraction, which had originally been integrated into proteomics research on PM proteins from mammalian tissue by our group.[21] We followed the established

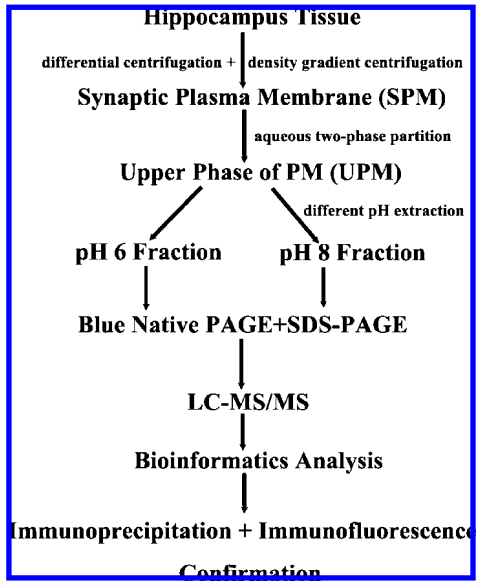

Figure 1. Schematic representation of the strategy used to identify the multiprotein complexes for synaptic plasma membrane.

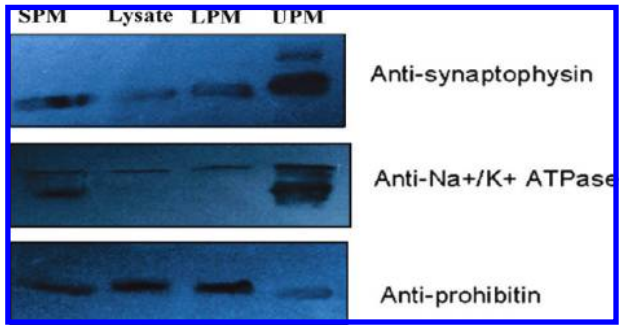

Figure 2. The Western blotting analysis of the synaptic plasma membrane after two aqueous phase partition method. SPM, the synaptic plasma membrane from sucrose density centrifugation; Lysate, the homogenate; LPM, the synaptic plasma membrane fraction from the lower phase of two aqueous phase partition method; UPM, the synaptic plasma membrane fraction from the upper phase of the two aqueous phase partition method. The same amount of proteins (25 μg) was loaded on each lane.

protocol to further improve the purity of our SPM preparations. About 70% of the initial membrane was recovered from the upper phase of the aqueous two-phase partition (UPM). The results of Western blotting indicated that the UPM was highly enriched in the two synaptic PM markers, synaptophysin and Na^+/K^+-ATPase, compared to the SPM fraction in Figure 2. The UPM was especially enriched compared to the lysate control. The mitochondrial marker prohibitin was greatly reduced in the UPM fraction (Figure 2). Therefore, only the UPM was used for the detection of multiprotein complexes.

It has been reported that there are synaptic active zones in the SPM, which have critical roles in the release of neurotransmitter from nerves terminals. This region has a dense matrix of proteins which are not soluble at pH 6, but are completely soluble above pH 8.[18] To learn the architectures of the protein complexes located in the synaptic PM, and to identify low-abundance proteins, two detergent solutions with different pH values were used in the sample extractions. Characterization of each fraction was determined by TEM and Western blotting. As shown in Figure 3A,B, the membrane structure of the SPM

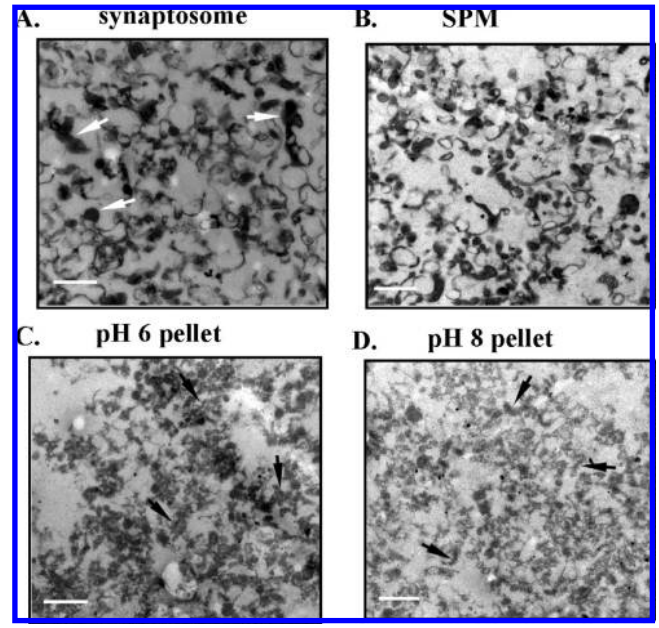

Figure 3. (A) The synaptosome fraction; (B) the synaptic plasma membrane fraction from the lower phase of two aqueous phase partition method; (C) the pellet fraction of the LPM after the treatment of 2% Triton X-100 solution at pH 6; (D) the pellet fraction of the pH 6 pellet after the treatment of 2% Triton X-100 solution at pH 8. The representative structures were labeled with arrows. The bar means 0.5 μm.

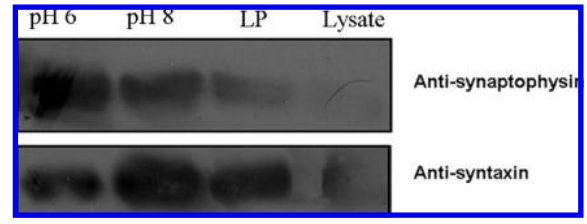

Figure 4. The Western blotting analysis of the synaptic plasma membrane fractions after the treatment of Triton X-100 solution. pH 6, the supernatant fraction of the LPM after the treatment of 2% Triton X-100 solution at pH 6; pH 8, the supernatant fraction of the pH 6 pellet after the treatment of 2% Triton X-100 solution at pH 8; LP, the pellet fraction of the pH 6 pellet after the treatment of 2% Triton X-100 solution at pH 8; Lysate, the homogenate.

fraction was more visible after osmotic shock than the membrane of the synaptosome fraction, which may release materials from the synaptic membrane vesicles. Moreover, mitochondrial fragments were relatively reduced in the SPM fraction. After treatment with a detergent solution at pH 6, the pellet exhibited few or no membrane attachments, and filamentous material attached to a probable postsynaptic density (PSD) structure were observed (Figure 3C). PSD structures in Figure 3D became clear after detergent treatment at pH 8, as previous reported.[18,20] The various pH treatments were further examined biochemically. Each fraction was separated on SDS-PAGE and incubated with the representative markers of synaptic vesicles (synaptophysin) and synaptic active zone (syntaxin). As shown in Figure 4, the pH 6 fraction was relatively enriched with synaptophysin compared to pH 8 fraction, and syntaxin proteins were mainly located in the pH 8 fraction, showing that our fractionation

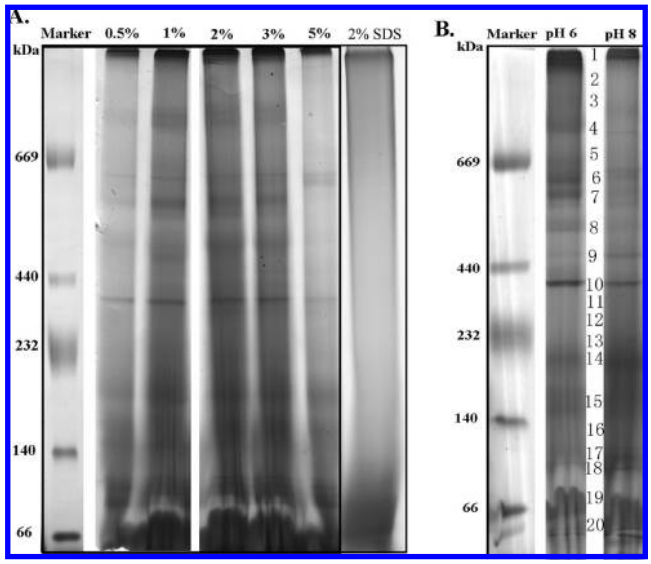

Figure 5. The Blue Native PAGE of the synaptic plasma membrane fractions. (A) The Blue Native PAGE of the LPM fraction solubilized with Triton X-100 solutions at different concentrations or with 2% SDS solution; (B) the Blue Native PAGE of the pH 6 and pH 8 fractions, the number of the bands for mass spectrometry analysis was shown in the center.

was effective, to some extent, in preparing the different membrane regions.

Separation of Multiprotein Complexes with BN-PAGE and SDS-PAGE. Triton X-100 is one of the most widely used nonionic detergents for solubilizing biological membranes for BN-PAGE analysis.[18,19] To determine the optimal detergent concentrations for subsequent research, a series of Triton X-100 concentrations at pH 7 were evaluated. Considering the yield and stability of the multiprotein complexes, 2% Triton X-100 was found to be the best choice, and was used for the subsequent UPM solubilization (Figure 5A). Most of the high MW bands (above 140 KDa) from the BN-PAGE seemed to be formed due to specific interactions as indicated from the control sample treated with 2% SDS (Figure 5A).

To give partial architectural information for the multiprotein complexes, two detergent solutions (pH 6 and pH 8) were used serially to recover the protein complexes from UPM. As indicated in Figure 5B, the two fractions had different patterns, especially for the intensity of some protein bands, which suggested that various expression or oligomerization states of the protein complexes were present. To get quantitative information on the components of the protein complexes, the BN-PAGE gel proteins were denatured and run on a second-dimensional SDS-PAGE. Meanwhile, to compare the architectures of the protein complexes in the special functional regions, the protein complexes from the pH 6 fraction and the pH 8 fraction were examined by the same SDS-PAGE. A representative 2-D BN/SDS-PAGE map of the pH 6 and pH 8 fractions is shown in Figure 6. With the use of standard silver staining, more than 100 spots were detected in the pH 6 fraction, and more than 60 spots were in the pH 8 fraction.

Protein Identification and Physicochemical Analysis. With the use of a strict database-searching algorithm, 514 nonredundant proteins were identified in gel bands and spots from two duplicated experiments, and only the proteins identified in both of the gels for the corresponding bands or spots were reported here, of which 427 (83%) proteins had two or more unique peptides (Supplementary Table S1). In our identification list, 387 unique proteins were from the BN-PAGE, and 339 were from the SDS-PAGE. The overlap of the proteins from the two strategies was shown in Figure 7A. As two pH fractions from SPM were considered, 439 proteins were identified from the pH 6 fraction, and 306 proteins were from the pH 8 fraction, with the overlap shown in Figure 7B.

To evaluate our separation methods and examine the characteristics of the different SPM fractions, all of the identified proteins were analyzed with respect to physical parameters such as molecular weight, isoelectric point, number of transmembrane segments and global hydrophobicity (Supplementary Table S1). Two hundred and fifty-seven (50%) of the proteins we identified had sizes and p*I*'s beyond the typical resolution limits of traditional 2-D electrophoresis (10 kDa < MW < 100 kDa and 4.5 < p*I* < 8.5). With regard to transmembrane (TM) helices, 183 (36%) proteins comprised at least one putative TM helix, of which 119 (65%) proteins had two or more TM helical domains. These numbers were higher than most of the proteome data sets from synaptosomes.[1,3] Another parameter commonly used to describe the hydrophobicity of proteins is the GRAVY index. One hundred and fifteen (22%) proteins in our results showed positive GRAVY, of which 30 (26%) exhibited a GRAVY above 0.4. When the separation methods and different SPM fractions were compared, the BN-PAGE demonstrated its relative superiority in separating proteins with MW < 10 kDa or TM ≥ 1 compared to the second dimension SDS-PAGE. The pH 6 and pH 8 fractions showed unbiased distributions versus molecular weight, isoelectric point, number of transmembrane segments, and global hydrophobicity (Supplementary Table S1).

Subcellular and Functional Classification of the Proteins. The identified proteins were annotated using gene ontology (GO) and the literature to gain insight into the potential subcellular localizations and biological functions (Supplementary Table S1). As expected, among the 423 (82%) proteins having known localization, 315 (74%) were PM or membrane-associated proteins, implying that our sample preparation was effective (Figure 7C). As shown in Figure 7C, most of the possible contamination came from mitochondria (10%). A few proteins annotated with location in proteasomes and the endoplasmic reticulum, ribosome and nucleus were also identified.

To our surprise, proteins involved in transport represented the largest functional category (20%) among the 445 (87%) annotated proteins with different molecular functions (Figure 7D). In the remaining proteins, apart from the ambiguous annotation of the binding (16%) and catalytic (17%) proteins, signal transduction proteins (18%) accounted for the largest percentage, followed by proteins involved in traffic (9%), and metabolism (8%). Other important proteins were related to structure (6%), adhesion (4%) and scaffolding (3%).

Confirmation of the Potential Novel Multiprotein Complexes among Syntaxin, Synapsin I, and Na^+/K^+ ATPase Alpha-1. Several known and possible novel protein complexes were found in our BN/SDS PAGE and identified by LC-MS/MS (Table 1 and Supplementary Table S1). Because of the multiple associations in the BN/SDS-PAGE and the important functions of synapses in neurobiology, the multiprotein complexes involved in syntaxin were further analyzed using co-immunoprecipitation (Co-IP). As shown in Figure 8, several protein bands were shown to specifically bind to syntaxin. All these bands were identified by LC-MS/MS. As we anticipated from the BN/SDS-PAGE results, many proteins found in the BN-

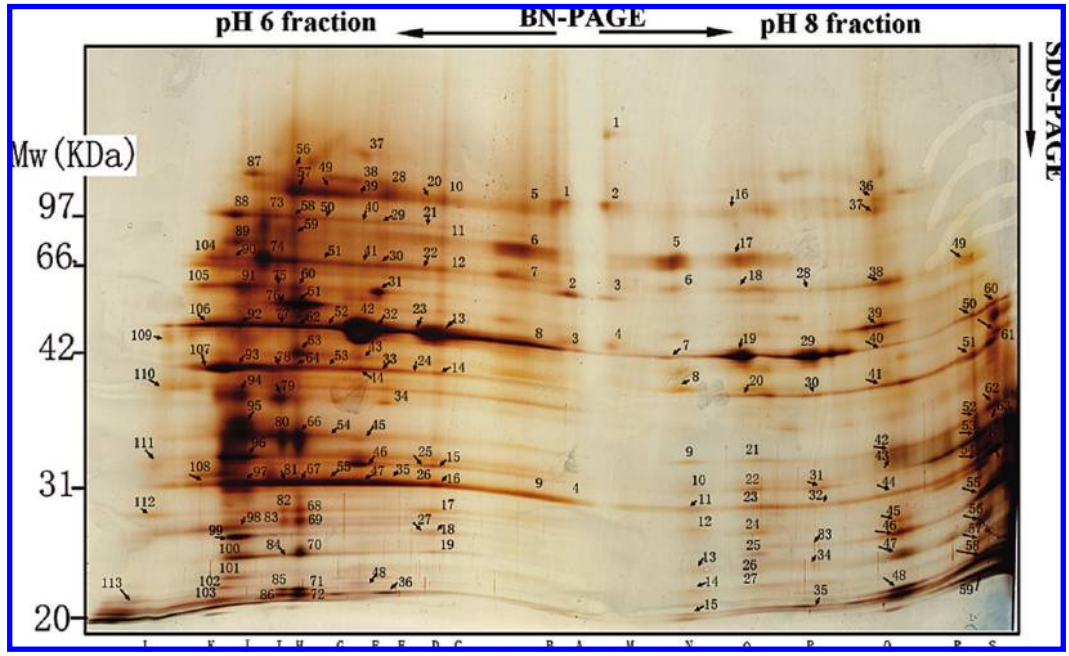

Figure 6. The SDS-PAGE of the multiprotein complexes bands from Blue Native PAGE. The pH 6 fraction was separated on the left; the pH 8 fraction was separated on the right. The gel spots for the mass spectrometry identification were labeled with numbers. The potential multiprotein complexes were labeled in the bottom.

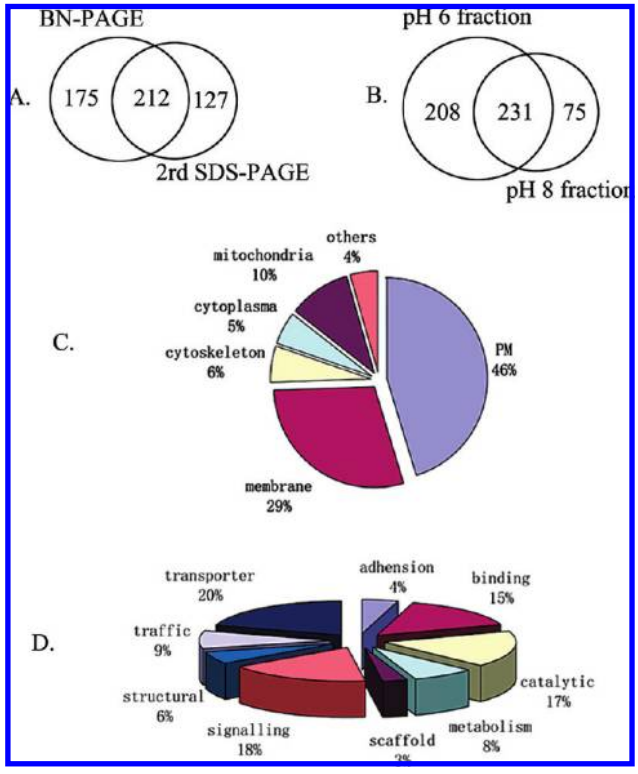

Figure 7. The overlap and catalogue of the identified proteins. (A) The overlap of the identified proteins from Blue Native PAGE and the SDS-PAGE; (B) the overlap of the identified proteins from pH 6 fraction and pH 8 fraction; (C) the subcellular locations of the identified proteins according to the GO annotations and literature; (D) the functions of the identified proteins according to the GO annotations and literature.

PGAE were also identified in the immunoprecipitation experiment, such as synapsin I, Na^+/K^+ ATPase alpha-1, and synaptotagmin (Table 1). At the same time, some novel possible interacted proteins were found, such as Isoform 1 of syntaxin-binding protein 1, NSF Vesicle-fusing ATPase, and Cadherin EGF LAG seven-pass G-type receptor 3 precursor (data not shown).

To further confirm the possible multiprotein complexes among syntaxin, synapsin I, and Na^+/K^+ ATPase alpha-1, these proteins were analyzed by co-localization in SH-SY5Y cells. Figure 9 showed that synapsin I and Na^+/K^+ ATPase alpha-1, synapsin I and syntaxin were partially overlapping, indicating their possible interaction during the biological processes of the synapse.

Discussion

Blue Native PAGE of Synaptic PM from Rat Hippocampus. It is known that the composition of multiprotein complexes varies over cell types, tissues, regions of tissues and between species. Therefore, proteins enriched in a particular tissue or cell type can be identified as potentially more relevant to the physiology of that particular tissue or cell type. However, synaptic protein complexes previously identified using mass spectrometry were usually affinity-purified from the whole brain or the forebrain.[10,11,32] In this study, we analyzed protein complexes only from the rat hippocampus, the key structure in the process of learning and memory. We are also aware that 90−95% of the cells in hippocampal tissue are not neurons but glia, which provide support or insulation for neurons, and that a majority of these glia are astrocytes.[2] It is likely, therefore, that many of the proteins previously identified in whole brain tissue preparations are of glial and not neuronal origin. To circumvent this problem, we previously profiled the PM proteins from primary hippocampus neurons.[23] In this study, we improved our ability to resolve the proteome of neurons by using a well-established procedure for isolating the subcellular fraction containing the intercellular communication junction between nerves, the synapse. Preparations of SPM fractions should be greatly enriched in proteins involved in synaptic transmission and

Table 1. All the Proteins Identified from Putative Complex H in Figure 5

ID[a]	description	score[b]	peptides[c]	emPAI[d]	TM[e]	subcellular[f]	function[f]
IPI00189655	Oxytocin receptor	41	1	0.009	7	PM	signaling
IPI00190364	Similar to calsequestrin-1 precursor	61	1	0.011	0		
IPI00191391	Solute carrier family 2, facilitated glucose transporter member 1	55	5	0.055	12	PM	transporter
IPI00191730*	Syntaxin-1b2	198	12	0.086	1	PM	traffic
IPI00192034	Similar to dihydropyrimidinase- related protein 2	274	22	0.098	0	membrane	catalytic
IPI00194222	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial precursor	209	18	0.215	1	membrane	metabolism
IPI00194324	Pyruvate dehydrogenase e1 component subunit beta	203	12	0.132	0	mitochondria	catalytic
IPI00196107*	Atp synthase b chain, mitochondrial precursor	222	18	0.160	0	membrane	catalytic
IPI00197696*	Malate dehydrogenase, mitochondrial precursor	1030	31	0.308	0	mitochondria	catalytic
IPI00197711*	L-lactate dehydrogenase a chain	451	18	0.137	0	cytoplasma	catalytic
IPI00198327*	Voltage-dependent anion-selective channel protein 2	99	7	0.087	0	membrane	transporter
IPI00200069	Sideroflexin-3	197	9	0.092	2	PM	transporter
IPI00200466*	Adp/atp translocase 2	385	22	0.161	2	membrane	binding
IPI00202184	Na^+-dependent amino acid transporter asct2	60	3	0.027	9	PM	transporter
IPI00205693*	Sodium/potassium-transporting atpase alpha-2 chain precursor	1184	69	0.223	8	PM	transporter
IPI00206170*	Synaptotagmin-1	149	19	0.110	1	PM	traffic
IPI00208117	Isoform 1 of choline transporter-like protein 1	49	3	0.019	9	PM	transporter
IPI00208205*	Heat shock cognate 71 kda protein	530	29	0.113	0	cytoplasma	binding
IPI00209115*	Slc25a3 protein	272	19	0.158	2	membrane	transporter
IPI00209908	Cytochrome c oxidase subunit 2	267	9	0.866	2	membrane	metabolism
IPI00210156	Cd44i (fragment)	59	2	0.019	1	PM	adhesion
IPI00210920*	Aspartate aminotransferase, mitochondrial precursor	529	30	0.200	0	PM	catalytic
IPI00213546	Heat shock 70 kda protein 1l	67	5	0.019	0	cytoplasma	binding
IPI00214030	Monocarboxylate transporter 4	43	1	0.012	12	PM	transporter
IPI00214462	Sodium- and chloride-dependent GABA transporter 3	102	4	0.036	10	PM	transporter
IPI00231023*	Isoform ib of synapsin-1	188	20	0.122	0	PM	traffic
IPI00231451*	Sodium/potassium-transporting ATPase alpha-3 chain	1140	72	0.270	8	PM	transporter
IPI00231611	Isoform mitochondrial of fumarate hydratase	185	13	0.085	0	mitochondria	catalytic
IPI00231651	Brain acid soluble protein 1	79	8		0	membrane	
IPI00231734	Fructose-bisphosphate aldolase a	355	34	0.303	0		catalytic
IPI00231783*	L-lactate dehydrogenase b chain	383	19	0.174	0	cytoplasma	catalytic
IPI00231927	ADP/ATP translocase 1	377	24	0.179	4	PM	transporter
IPI00326305*	Sodium/potassium-transporting ATPase alpha-1 chain precursor	755	44	0.153	10	PM	transporter
IPI00339124*	Sodium/potassium-transporting ATPase subunit beta-1	323	16	0.121	1	PM	transporter
IPI00339148	60 kDa heat shock protein, mitochondrial precursor	973	47	0.210	0	mitochondria	binding
IPI00358163	Similar to calcium-binding mitochondrial carrier protein aralar2	162	9	0.040	0	membrane	binding
IPI00363182	Similar to solute carrier family 25, member 5	105	9	0.061	2	membrane	transporter
IPI00363727	ER lumen protein retaining receptor	136	1	0.025	4	membrane	traffic
IPI00365423	Protein phosphatase 2 (formerly 2a), regulatory subunit a (pr 65), alpha isoform	45	1	0.005	0		
IPI00365545	Dihydrolipoyl dehydrogenase, mitochondrial precursor	425	25	0.157	0	cytoplasma	catalytic
IPI00365986	Similar to cg1814-pa, isoform a	226	21	0.098	0		
IPI00454475*	Keratin, type ii cytoskeletal 1b	118	5	0.025	0	cytoskeleton	structural
IPI00464440	Sideroflexin 1	148	12	0.132	2	PM	transporter
IPI00566672	Similar to heat shock protein 8	153	16	0.061	0		
IPI00568168	112 kDa protein	1414	66	0.240	8	PM	transporter
IPI00734667	35 kDa protein	142	9	0.083	1	membrane	binding
IPI00768849*	Similar to voltage-dependent anion channel 1	98	4	0.078	0	PM	transporter

[a] The IPI number of the identified protein (*, indicated the protein was also identified in the further co-immunoprecipiation experiment). [b] The highest Mascot score for the identified protein. [c] The unique peptide number for the identified protein. [d] The emPAI value for the identified protein. [e] The predicted transmembrane domain of the identified protein. [f] The subcellular location or function of the identified protein according to GO annotations and literature.

reception. Through this strategy, some low-abundance protein complexes in the synapse could be detected and identified.

To date, most comprehensive proteome analyses of the neuronal system either considered proteins located within different parts of the cell, or considered differential expression in response to development or disease.[1,3,4] Those studies lack information about the interaction pattern of these proteins, a very important aspect of the functions that the proteins perform. Capturing multiprotein complexes in sufficient quantity and purity, while maintaining their integrity, is a challenge. In neuroproteomics, the affinity purification strategy was frequently used to isolate protein complexes in the postsynaptic fractions, including ion channels and GPCR complexes.[13,17] Affinity purification is heavily dependent on the availability of ligands or antibodies. It is not a generic approach, and must be tailored to a particular complex of interest. Therefore, biochemical methods are needed to complement the affinity purification strategy.

BN-PAGE offers great promise for the purification of intact multiprotein complexes. It can separate multiple protein complexes at one time, and provide for the analysis of homooligomer complexes. Furthermore, the results can be visualized to analyze the size, the abundance, and the stoichiometry of subunit compositions. Combined with mass spectrometry, BN-PAGE has been widely used to discover the molecular composition of protein complexes. However, this strategy has been limited to complexes that were relatively abundant in the starting material. In our analysis, we first used subcellular

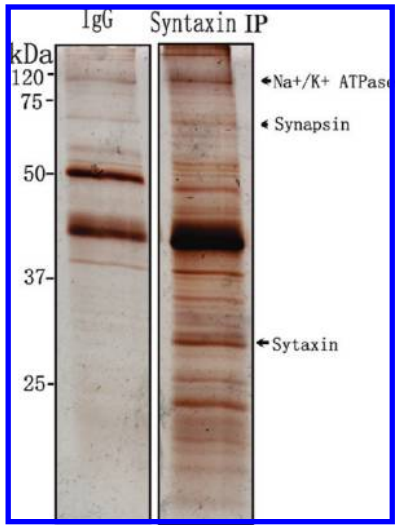

Figure 8. The SDS-PAGE of the potential multiprotein complexes involved with syntaxin using co-immunoprecipiation. The bands for the identification of synapsin I, syntaxin and Na^+/K^+ ATPase alpha-1 in the immunoprecipitation result were labeled with arrows and shown in the right.

fractionation to isolate the SPM structure of hippocampus, enriched in multiprotein complexes, and further purified it through the aqueous two-phase partition method. Because we used the nonionic detergent Triton X-100 and a native buffer system, several potential protein complexes were detected using BN PAGE according to their MW. All the bands or spots from the BN/SDS-PAGE were digested and subjected to LC-MS/MS. Five hundred and fourteen nonredundant proteins were identified from two duplicated experiments in this study, which provided the most comprehensive data set of membrane multiprotein complexes using BN PAGE.[33]

Considering the resolution of the BN-PAGE and the complexity of the SPM fractions, two detergent solutions with different pH values were used to fractionate the SPM fraction, and to give the architecture of the SPM proteins or protein complexes. The fraction extracted from the pH 6 solution was composed mainly of molecules involved in the synaptic vesicles, and molecules loosely connected to the synaptic junctional scaffold. The fraction extracted with the pH 8 buffer contained the pre- to postsynaptic scaffold and presynaptic web. As shown in our transmission electron microscopy and Western blots, each sample preparation was relatively enriched in different proteins. In the identification results, each fraction contained significant numbers of proteins thought to be restricted to the structures of the SPM. Marker proteins were uniquely identified or had greater abundance in the corresponding fractions. However, some proteins from the presynaptic cytomatrix, such as bassoon and piccolo, were not found in our data set. Their absence might result from the low abundance or low solubility of these proteins in our detergent solution.

BN-PAGE can not only provide data on multiprotein complex composition, but it can also increase our knowledge of the integral membrane protein content of a proteome of choice. Coomassie G250 binds on the surface of all membrane proteins as a result of hydrophobic interactions, thereby inducing a negative charge shift. Membrane proteins are soluble in aqueous media despite the lack of detergents, and migrate toward the anode in an electric field. Under these conditions, charge repulsion effects minimize agglomeration and precipitation of the very hydrophobic proteins. Considering the MW and the p*I* distribution of the proteins in our analysis, near 50% of them could not have been detected by conventional 2-DE, which uses isoelectric focusing as the first separation dimension. Moreover, 36% of the identified proteins, more than most of the synaptosome proteome,[3] were integral membrane proteins, having at least one trans-membrane domain.

Deciphering the Multiprotein Complexes in the BN/SDS-PAGE. Because of the nonionic detergents used in solubilization of the SPM and the mild electrophoresis conditions, multiprotein complexes assemblies were retained during Blue Native PAGE. Combined with the apparent masses of the complexes separated in the BN gel, the size, abundance and stoichiometry of protein subunits can be assessed from the second-dimensional gels. The high sensitivity of the MS used here identified several unique proteins in single 1-D gel bands or even single spots in the SDS-PAGE. Therefore, we analyzed the potential protein complexes first according to the protein profile of the SDS-PAGE, and then the information from the corresponding BN-PAGE bands was taken into consideration. The stoichiometry of the protein subunits was principally estimated from the emPAI value. According to these principles, at least 19 potential heterooligomeric protein complexes were found in the SDS-PAGE (Figure 6). Several known and putatively novel multiprotein complexes were found in our analysis.

Multiprotein Complexes Involved in Membrane Trafficking and Exocytosis. SNAREs play a role in the docking and fusion of synaptic vesicles to the active zone. The cores of SNARE complexes are composed of syntaxin, synaptobrevin, and SNAP-25. SNAREs exhibit multiple configurational, conformational, and oligomeric states. These different states allow SNAREs to interact with their matching SNARE partners, auxiliary proteins, or with other SNARE domains, often in a mutually exclusive fashion.[34] In the SDS-PAGE of our analysis, syntaxin was identified in the Q and H complexes (Figure 6, Table 1, and Supplementary Table S1). In the Q complexes, we also found several known SNARE-associated proteins, such as the calcium channel alpha-2 delta-3 subunit, isoform 1 of the syntaxin-binding protein 1, and synaptotagmin-1.[35]

Because of their low molecular weights, SNAP-25 and VAMP (synaptobrevin) were not identified in the Q complex. However, VAMP3 and two SNAP-25 proteins were identified in the corresponding BN band 17 (Figure 5). Synaptotagmins may be thought of as auxiliary calcium-dependent v-SNAREs because they bind both bSNAP and NSF, and because they interact with the t-SNAREs, syntaxin and SNAP-25.[36] The synaptotagmin−syntaxin 1−SNAP-25−VAMP complex constitutes the molecular correlation of a synaptic vesicle docked at the plasma membrane and awaiting the calcium influx. All four proteins may be associated with N-type calcium channels. This hypothesis was extended to include the possible interaction of synaptic complexes with P/Q-calcium channels.[36] We confirmed the existence of the complexes using a biochemical strategy here. Several potentially novel SNARE-associated proteins were also found, such as synapsin, Na^+/K^+ ATPase, and protein phosphatase 2.

The synapsins are a family of four closely related neuronal phosphoproteins, which are implicated in regulating the efficiency of neurotransmitter release, and in synapse formation.[37] In addition to their interactions with vesicles, the synapsins have multiple binding activities for cytoskeletal proteins, including actin, tubulin, spectrin and neurofilaments.

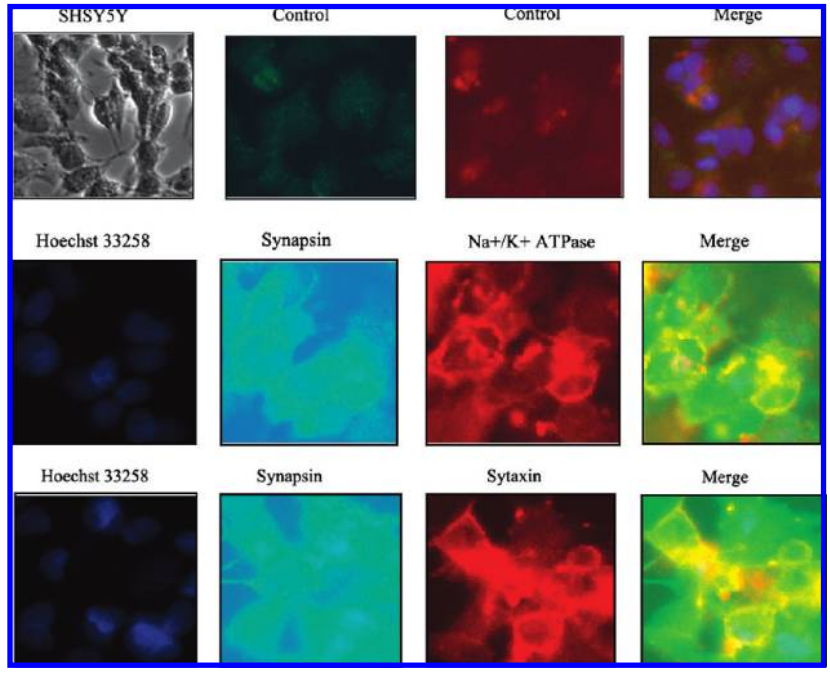

Figure 9. The immunofluorescence staining of SH-SY5Y cells under fluorescence microscope (× 200). The corresponding staining regents or antibodies were shown above the respective pictures. Control means no primary antibodies were added. Merge represents the overlap of the two fronting fluorescence.

In the SDS-PAGE, the potential J and R complexes contained synapsin (Supplementary Table S1). Several actin and tubulin proteins are also involved in these complexes. Reclustering of synapsins may be guided by interactions with endocytic proteins.[37] In the J complex, Munc-18, synaptotagmin, synaptophysin and VAMP were associated with synapsin, and a list of novel proteins was found, such as syntaxin, Na^+/K^+ ATPase, and rab GDP dissociation inhibitor alpha.

14-3-3 proteins appear to play important roles in various cellular functions, including protein kinase C regulation and exocytosis. There are seven identified 14-3-3 isoforms in mammals. They can form homodimers with other 14-3-3 isoforms,[38] which can also interact with over 200 target proteins in phosphoserine-dependent and phosphoserine-independent manners. All seven isoforms were identified in the R complex of the pH 8 fraction, along with their own unique peptides (Figure 6 and Supplementary Table S1). Other proteins in this lane included proteins that are known to interact, such as uncoupling protein UCP-4, H^+-transporting ATPase, and actin. The lane contained other proteins with potentially novel interactions, such as S100 calcium binding protein, creatine kinase, and prohibitin.

There are two types of G proteins: heterotrimeric G proteins, which bind G-protein-coupled receptors, and monomeric G proteins (or small G proteins). Heterotrimeric G protein consists of three subunits: α, β and γ.[39] We found 11 G-proteins, comprised of 6 α-subunits, 4 β-subunits and 1 γ-subunit. The heterotrimeric G proteins were simultaneously identified in bands 16 and 17 of the BN PAGE. Fourteen small G-proteins, such as Rab3A and Rap1A, are involved in the transport of synaptic vesicles to the PM.[39] Rab3A was identified in almost all the BN bands of the pH 6 fraction, suggesting that Rab3A might interact with proteins in multiple functions beyond its interactions with its known partners, such as synapsin and syntaxin.

Multiprotein Complexes Participating in Receptors and Ion Channels. Most excitatory synapses in the brain use glutamate as a neurotransmitter. Fast signaling at these synapses occurs primarily by activation of amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type and *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. Because Triton X-100 much more effectively solubilizes synaptic AMPA receptors (AMPARs) than NMDA receptors as reported previously,[40] only AMPARs were identified here, in our pH 8 fraction. AMPAR channels comprise heterotetramers of subunits GluR1−4, and were reported to interact with cytoskeletal protein, BiP, AP-2, and NSF.[41] Three membranes containing AMPA-type glutamate receptor were identified from our analysis. AP-2 was the only protein identified in the corresponding gels. After synaptic release, clearance of glutamate from the extracellular space is accomplished by high-affinity transporters. GLT is the most abundant glutamate transporter in the brain, operating as a monomer, dimer, or trimer.[42] We found four GLT proteins in almost all the BN PAGE bands, implicating it in interactions with many multiprotein complexes.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain. GABA is cleared from the synaptic cleft by specific and high-affinity Na^+- and Cl^--dependent transporters, which are thought to be located in the presynaptic terminals.[43] Two Na^+- and Cl^--dependent transporters was identified in this study. Only Na^+- and Cl^--dependent GABA transporter 3 were identified in the H Complex of the 2-D SDS-PAGE (Table 1), along with its known partner syntaxin.[44]

ATPase Complexes. There are three types of ATPases: (1) P-type, which transports Na^+, K^+ and Ca^{2+} through the eu-

karyotic plasma membrane; (2) V-type, which uses ATP to form H^+ gradients across vesicles; and (3) F-type, (ATP synthase) which runs in reverse to convert H^+ gradients into ATP in mitochondria and bacteria. Four Na^+/K^+-ATPase α and two Na^+/K^+-ATPase β subunits were found in our SPM. Some subunits existed in most of the potential complexes in the BN-PAGE and in the following 2-D SDS-PAGE, implying important roles for them in SPM functions. Two families of Ca^{2+} transport ATPases are involved in the maintenance of Ca^{2+} homeostasis in the nervous system: the plasma membrane Ca^{2+}-ATPase (PMCA) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase.[45] These proteins were identified from the BN-PAGE of only the pH 6 fraction. All four isoforms of PMCA (1, 2, 3 and 4) were identified in our extracts. PMCAs 1, 2, and 3 were found in several bands of the BN-PAGE, while PMCA 4, the least abundant, was identified in only one band.

V-ATPase is a large macromolecule, which uses ATP hydrolysis to pump protons into the synaptic vesicles, acidifying this compartment for neurotransmitter transport. Eleven H^+ transport ATPase subunits, 4 from the V0 subunit and 7 from V1 subunit, were identified.[46] In the D complex of our 2-D SDS-PAGE profile, 6 V-ATPases, including three V0- and three V1-ATPases, were found. The relative quantities of the A, B, C, E and H subunits of the V1-ATPase subunits were 0.19, 0.18, 0.06, 0.06 and 0.05, consistent with the stoichiometry of the complex. This consistency supports the feasibility of using the emPAI value for quantitating the proportion of subunits in the BN/SDS-PAGE strategy.

An F-type ATP synthase, namely, Complex V, resides mostly within the inner membrane of mitochondria. Like the V-type ATP synthase, the F-type ATP synthase is composed of two linked multisubunit complexes. Synapses are packed with mitochondria, both in pre- and postsynaptic terminals. Both terminals have roles in energy metabolism, cell signaling, and calcium homeostasis.[47] Although the aqueous two-phase partition method was used in our sample preparation to reduce the possible mitochondria complexes, as shown in Figure 2, a high percentage of putative mitochondrial proteins were still present in our extract (Figure 7C). Four F1 and four F0 subunits of ATP synthase were identified in our analysis. The stoichiometry of the F1 subunits is $\alpha_3\beta_3\gamma$. The emPAI values of these subunits were 0.3, 0.36 and 0.1, respectively.

Putative Homooligomeric Complexes. In addition to the heterooligomeric complexes, many proteins were found in bands with MW several times the MW of the monomer, suggesting that these proteins may exist in homooligomeric states in the synaptic plasma membrane. Indeed, this result reflects not only the current situation in the neurons, but also the tendency of evolution to produce homooligomeric protein complexes.[48] Blue Native PAGE is superior to most other methods of detecting homooligomeric proteins, and many proteins have been detected as homooligomers by this strategy.[48,49] From the MW information in the BN-PAGE and the second-dimensional SDS-PAGE in Figures 5 and 6, our comprehensive native data here provided a resource and a starting point for searching for homooligomeric states.

For example, recent evidence suggests that GPCRs may form dimers as part of their normal trafficking and function.[50] The possible homodimer formation of olfactory receptor olr215 was also suggested in our BN/SDS-PAGE (spots 107 in Figure 6 and corresponding band 16 in Figure 5). Enolase is present in homodimers, and in heterodimers formed from three distinct subunits. In addition to the possible heteroligomeric complexes with α-enolase at about 600−700 kDa, β-enolase and γ-enolase were found to form homodimers and heterodimers at about 90 kDa (spot 89 in Figure 6 and corresponding band 15 in Figure 5). The stoichiometry of γ-enolase was about 3 times that of β-enolase, according to the emPAI value.

In this study, the multiprotein complexes in the synaptic PM from rat hippocampus were screened by BN-PAGE and identified by LC-MS/MS. Nineteen potential multiprotein complexes and their components were found and identified. Several known multiprotein complexes were detected, such as the SNARE protein, the AMPA-type receptor, and different kinds of ATPase complexes. Some potentially novel components of known multiprotein complexes and of less well-known complexes were also implied from the data. However, co-migration of proteins on BN PAGE is not final proof of native association because physically distinct complexes may have similar molecular masses, and therefore may appear in the same protein bands. Moreover, spots which are located side by side in a horizontal row might represent the same proteins that are localized in complexes of different molecular mass. Such proteins were generally of high abundance in our sample, including calcium-binding mitochondrial carrier protein, ATP synthase, ubiquinol-cytochrome-c reductase complex core protein and ADP/ATP translocase, all of which might interact with protein complexes nonspecifically. However, the resolution of this technique is much higher than conventional gel filtration chromatography. If the sample has been well fractionated to limit complexity, we consider this 2D gel technique is an excellent line of evidence for association. Most importantly, the real interaction among these proteins should be experimentally validated using other orthogonal approaches eventually. Confirmation can be obtained using immunoprecipitation or co-migration under different conditions (e.g., forms of chromatography that are not based on size). In this study, the potential multiple proteins involved in the syntaxin multiprotein complexes were confirmed by co-immunoprecipitation and tandem mass spectrometry. Several proteins associated with syntaxin in the Blue Native PAGE were also identified in the co-immunoprecipitation experiments. Synapsin I, syntaxin, and Na^+/K^+ ATPase alpha-1 were all found in the co-immunoprecipitation, implying a potential interaction among the three proteins. Further evidence came from immunofluorescence staining, which indicated at least a partial co-localization of synapsin I and Na^+/K^+ ATPase alpha-1, or synapsin I and syntaxin. It should be kept in mind that these complexes are likely to be highly dynamic and temporary. Proteins may exist in many different complexes at a single time point, which could be the reason we detected one protein in many different bands or lines.

When the true composition of a complex is determined, the next key issue is its biological function, and how the complex changes under physiological and pathological conditions. Locations and functions of synapsin I, syntaxin, and Na^+/K^+ ATPase alpha-1 have been described in detail.[34,35,37,51] The potential novel multiprotein complexes involving these three proteins might participate in the fusion or recycling of synaptic vesicles during exocytosis. The Na^+/K^+ ATPase alpha-1 protein might be engaged in providing ATP for assembly of protein complexes that facilitate signaling pathways. Further experiments are needed to determine the functions of such complexes.

In summary, multiprotein complexes in the synaptic plasma membrane were screened by BN PAGE, and identified by tandem mass spectrometry from a highly enriched and pre-

fractionated synaptic sample. Several known and potential novel proteins and their multiprotein complexes were identified. To the best of our knowledge, this was the first synaptic plasma membrane proteome, and the largest data set determined by the BN PAGE strategy. It contributes promising starting points for further studies.

Acknowledgment. We thank all the members of our group for their kind help and suggestions. This work was supported by National Basic Research Program of China (2007CB516809, 2007CB914203) and National Natural Science Foundation of China (30770437).

Supporting Information Available: Table S1 contains all the identified proteins and potential protein complexes with their identification information and some detailed annotations. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR900101D