In-depth protein profiling of the postsynaptic density from mouse hippocampus using data-independent acquisition proteomics

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Located at neuronal terminals, the postsynaptic density (PSD) is a highly complex network of cytoskeletal scaffolding and signaling proteins responsible for the transduction and modulation of glutamatergic signaling between neurons. Using ion-mobility enhanced data-independent label-free LC-MS/MS, we established a reference proteome of crude synaptosomes, synaptic junctions, and PSD derived from mouse hippocampus including TOP3-based absolute quantification values for identified proteins. The final dataset across all fractions comprised 49,491 peptides corresponding to 4558 protein groups. Of these, 2102 protein groups were identified in highly purified PSD in at least two biological replicates. Identified proteins play pivotal roles in neurological and synaptic processes providing a rich resource for studies on hippocampal PSD function as well as on the pathogenesis of neuropsychiatric disorders. All MS data have been deposited in the ProteomeXchange with identifier PXD000590 (http://proteomecentral.proteomexchange.org/dataset/PXD000590).

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The hippocampal formation has gained special attention due to its importance for learning and memory [1]. With its well-described trisynaptic circuit and a uniform neuronal population, the hippocampus continuously serves as a model brain region for the analysis of synaptic transmission. The majority of synaptic connections within the hippocampus are excitatory and glutamatergic and each of these contacts is mainly characterized by an electron-dense structure at the postsynaptic membrane called postsynaptic density (PSD) [2]. This spatially organized and dynamically regulated macromolecular complex is essential for the stability of postsynaptic architecture [3]. Importantly, molecular alterations of the PSD are key features of neuropsychiatric disorders such as autism.
intellectual disability, or schizophrenia [4]. Therefore, the comprehensive analysis of molecular PSD composition is essential to obtain a deeper insight into disease-relevant mechanisms. We focused on hippocampal PSD at approximately 3 weeks of age, a developmental stage corresponding to early adolescence in humans, which is critical for postnatal development of synaptic networks in rodents and humans alike and which is within the age range typically used for electrophysiological analyses. Altered synaptic homeostasis at this age window has been discussed to have detrimental consequences on further brain development, for example leading to epileptic seizures [5] and cognitive disability [6].

Hence, we isolated the PSD from juvenile mouse hippocampus and performed detailed proteomic characterization applying our novel data-independent acquisition (DIA) LC-MS workflow called UDMS* and label-free quantification using the software package ISOQuant [7].

PSD was isolated as recently described [8] based on protocols by Siekevitz [9, 10] (Fig. 1A). After intraperitoneal injection of ketamine at an overdose, mice (C57BL/6, postnatal day 19) were decapitated; the hippocampus was dissected, immediately frozen on dry ice, and stored at −80°C.

Animal experiments were conducted in accordance with national laws and approved by the local authorities. Subcellular fractionation was performed from three samples, each containing hippocampi pooled from 10 to 11 mice. All buffers were supplemented with protease and phosphatase inhibitor mix (Roche). All steps were either performed at 4°C or on ice. Briefly, tissue was homogenized in homogenization buffer (320 mM sucrose, 5 mM HEPES, pH 7.4) using a Teflon douncer. The homogenate was centrifuged for 10 min at 1000 × g to remove cell debris and nuclei (pellet P1). The supernatant was further centrifuged for 20 min at 12 000 × g to obtain the soluble fraction and the crude synaptosomes (P2). P2 was resuspended in a Tris buffer (320 mM sucrose, 5 mM Tris/HCl, pH 8.1) and centrifuged in a sucrose density gradient (0.8/1.0/1.2 M) for 2 h at 85 000 × g. Purified synaptosomes were collected from the 1.0/1.2 border; diluted in five volumes of 1 mM Tris/HCl, pH 8.1; lysed for 30 min by stirring; and centrifuged for another 30 min at 33 000 × g. The resulting pellet (P3, synaptosomal membranes) was resuspended in Tris buffer containing 0.5% Triton X-100, stirred for 15 min, and centrifuged for 30 min at 33 000 × g to obtain a last pellet containing a one-Triton extracted “crude” PSD...
fraction (PSD). Prior to tryptic digestion and LC-MS analysis, the purity of one-Triton extracted “crude” PSD preparation was verified by Western blot demonstrating highly enriched levels of the major PSD scaffold protein ProSAP2/Shank3 (Fig. 1B).

For LC-MS analysis, the same subcellular fractionation protocol was used as described above to obtain synaptosomal membranes (P3). P3 was resuspended in 5 mM Tris/HCl, pH 8.1, and centrifuged in another sucrose density gradient (0.8/1.0/1.2 M) for 2 h at 85 000 × g for additional purification. Synaptic junctions (SJ) were collected from the 1.0/1.2 border, resuspended in Tris buffer containing 0.5% Triton X-100, stirred for 15 min, and centrifuged for 30 min at 33 000 × g to obtain a pellet containing a one-Triton extracted “pure” PSD fraction (PSDII). The Triton X-100 extraction step was repeated with resuspended PSDII to obtain a two-Triton extracted “pure” PSD fraction (PSDII, Fig. 1A).

For quantitative analysis, we included the LC-MS characterization of the crude synaptosomes (P2) and the synaptic junctions (SJ) in the present dataset to assess enrichment efficiency and purity of isolated PSDI and PSDII fractions. Prior to tryptic digestion, PSD fractions were solubilized in lysis buffer (7 M urea, 2 M thiourea, 5 mM DTT, 2% CHAPS) by sonication for 10 min at 4°C. Protein amounts were determined using the Pierce 660nm Protein Assay (Thermo). Proteins (20 μg for PSDI, SJ, P2 and, due to limiting source material, approximately 5 μg for PSDII) were digested applying a modified FASP (filter-aided sample preparation) protocol [7]. Prior to LC-MS analysis, peptides were dissolved in 0.1% formic acid and spiked with 20 fmol/μL of enolase 1 MassPREP protein digestion standard (Waters).

Tryptic peptides were analyzed in triplicates using a nanscale UPLC system (nanoAcquityUPLC (Waters)) coupled to a Synapt G2-S HDMS mass spectrometer (Waters). Peptides were separated on a CSH C18 1.8 μm, 100 μm × 250 mm reversed-phase column (Waters) using direct injection mode as described before [7]. Water containing 0.1% formic acid was used as mobile phase A and ACN containing 0.1% formic acid as mobile phase B. Peptides were eluted with a gradient of 5–40% mobile phase B over 90 min at a flow rate of 300 nL/min and a temperature of 45°C. Afterward, the column was rinsed with 90% mobile phase B and re-equilibrated resulting in a total analysis time of 120 min. Eluted peptides were analyzed in positive mode ESI-MS using MS3 in combination with online ion-mobility separation (UDMS) as described in detail by Distler et al. [7]. The data were postacquisition lock mass corrected using [Glu1]-Fibrinopeptide B.

LC-MS data were processed using ProteinLynxGlobalServer version 3.0.2 (PLGS, Waters Corporation) searching against the Uniprot KB/Swissprot mouse database (UniProtKB release 2014.02, 16 648 entries), which was concatenated to a reversed decoy database. For database search, precursor- and fragment-ion mass tolerances were automatically determined by PLGS3.0.2. Ion mass tolerance was typically below 5 ppm (3.3 ppm RMS) for precursor and below 10 ppm for fragment ions. The following search criteria were set for peptide identification: (i) trypsin as digestion enzyme, (ii) up to two missed cleavages allowed, (iii) fixed carbamidomethylcysteine and variable methionine oxidation as modifications, and (iv) minimum three identified fragment ions. The initial false discovery rate (FDR) for protein identification was set to 1% in PLGS.

Data were postprocessed using the software package ISOQuant [7] performing retention-time alignment, exact-mass retention-time and ion-mobility clustering, signal annotation, normalization, and protein isofrom/homology filtering. Within ISOQuant, the peptide-level FDR for cluster annotation was set to 1%. For further analysis, only proteins identified by at least two peptides (minimum length: six amino acids) were considered resulting in an FDR of 0.27% on protein level for the complete dataset. Further on, to be included in the final dataset a protein had to be identified in all biological replicates of at least one fraction (either P2, SJ, PSDI, or PSDII). Absolute in-sample amounts were calculated for each protein based on the TOP3 approach [11]. For display, we defined the relative amount of each protein with respect to the sum overall detected proteins (ppm: parts per million (w/w) of total protein). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [12] with the dataset identifier PXD000590.

Data were further processed in the R scripting and statistical environment and GraphPadPrism (version 5.0a for Mac OS X). Venn diagrams were generated with the Venny web application (http://bioinfogp.cnb.csic.es/tools/venny/index.html). For functional annotation, protein lists were analyzed using DAVID Bioinformatics Resources (version 6.7; http://david.abcc.ncifcrf.gov/home.jsp) [13]. Pathway analysis was conducted using the KEGG and PANTHER pathway databases [13,14].

Using a novel DIA LC-MS approach [7], we identified in total 49 491 peptides and 4558 proteins across hippocampal P2, SJ, PSDI, and PSDII fractions derived from three independent, pooled biological samples (Supporting Information Tables 1 and 2). Of these, 2102 proteins could be found in PSDII and 3560 proteins in PSDI in at least two biological replicates (Fig. 1C). Over 4000 proteins were identified in both, SJ and P2 fraction, in at least two biological replicates (Supporting Information Fig. 1). TOP3-based label-free quantification indicated high reproducibility of sample preparation and LC-MS workflow (Supporting Information Fig. 2). For identification, only proteins found in all biological replicates of at least one fraction (either P2, SJ, PSDI, or PSDII) with a minimum of two peptides were considered. The majority of proteins (60%) were identified by at least six peptides, 36% even by 20 or more peptides (Fig. 1D). The dynamic range of identified proteins across all fractions spanned over four orders of magnitude (Fig. 2A).

Although 1990 proteins were identified in all four fractions in at least two replicates for each fraction
Figure 2. Comparison of PSDII, PSDI, SJ, and P2 fractions derived from mouse hippocampus. (A) Dynamic range of detected mouse hippocampal PSDII, PSDI, SJ, and P2 proteins. (B) Overlap of proteins identified in PSDII, PSDI, SJ, and P2. Only proteins identified in at least two biological replicates per fraction are considered. (C) Correlation of proteins abundances for all identified proteins in PSDII, PSDI, SJ, and P2. (D and E) Proteins enriched (D) and depleted (E) in PSDI and PSDII.

(Fig. 2B), they highly differed in their relative abundances (Supporting Information Table 1). Comparing the fractions, we observed distinct differences between purified PSDI/PSDII and the SJ/P2 fractions (Fig. 2C, Supporting Information Fig. 3). Isolated PSDI and PSDII displayed an enrichment of most of the typical PSD proteins such as glutamate receptors of the NMDA- and AMPA-types, PDZ-domain containing scaffolding proteins (e.g. DLG4/PSD-95, ProSAP2/Shank3), non-PDZ-domain containing scaffolding proteins (e.g. Homer1), and major kinases...
Figure 3. (A) Functional annotation of identified hippocampal P2, SJ, PSDI, and PSDII proteins displayed in a heat map according to their relative abundance in each fraction (normalized to the average abundance over all fractions). Selected GO terms for cellular components (CC), biological processes (BP), and molecular functions (MF) are displayed. (B and C) KEGG and PANTHER pathways enriched (B) and depleted (C) in PSDI and PSDII based on the number of identified proteins.

(e.g. CaMKII (calcium/calmodulin-dependent protein kinase type II subunit alpha), SynGAP1 (synaptic Ras GTPase-activating protein 1); Fig. 2D, Supporting Information Fig. 4 and Table 1) [15–17]. Concomitantly, major mitochondrial and presynaptic proteins (e.g. Synaptogamin-1, Synaptophysin) were less abundant or completely absent in PSDI and PSDII (Fig. 2E, Supporting Information Table 1).

Comparing our PSDII dataset to two recently described consensus PSD datasets, one for rodents in general [16] and one for mouse [17], we found an overlap of 90.0% and 71.6%, respectively (Supporting Information Fig. 5). Among the 1311 proteins detected only in the present study, we found proteins, which play a pivotal role in the PSD, such as AMPA receptor associated proteins (e.g. Stargazin and NEDD-4), scaffolding proteins (e.g. SAPAP-4, Homer-3) as well as proteins associated with the PSD cytoskeleton (e.g. Actin-binding protein 1, heterogeneous nuclear ribonucleoprotein K) and PSD signaling like the Trk-B receptor, which is involved in synapse maturation [18].

Compared to the P2 and SJ factions, proteins associated with cell organelles (i.e. mitochondrion, ER, etc.) were highly decreased in PSDI and PSDII (Fig. 3A). In contrast, the percentage of PSD membrane, cytoskeletal as well as synapse proteins increased in PSDI and PSDII. GO analysis of
biological processes revealed that the relative abundance of proteins associated with cellular and metabolic processes was markedly higher in P2 and SJ (Fig. 3A). In contrast, proteins involved in cell communication, developmental processes, and intracellular signaling cascades were enriched in PSDI and PSDII (Fig. 3A). Molecular functions increased in PSDI and PSDII as compared to the other fractions were enzyme regulator, transmembrane receptor, ion channel, as well as kinase activities (Fig. 3A). Pathway analysis of the hippocampal P2, SJ, PSDI, and PSDII proteome revealed a higher enrichment of pathways associated with cell communication (e.g. tight junction, gap junction) and synaptic signaling (e.g. glutamate receptor pathways) in PSDI and PSDII as compared to SJ and P2 (Fig. 3B, Supporting Information Tables 3–10). Furthermore, in PSDII, we detected most of the proteins described to be involved in long-term potentiation and long-term depression (Supporting Information Fig. 6 and Table 3), two key synaptic processes [19]. Across all fractions, we found proteins involved in neurological disorders (e.g. Parkinson’s, Huntington’s, and Alzheimer’s diseases; Supporting Information Tables 3–10). Proteins involved in mitochondrial functions (i.e. oxidative phosphorylation) and metabolic processes were decreased in PSDI and PSDII (Fig. 3C).

To our knowledge, the present dataset provides the most comprehensive coverage of a mouse hippocampal PSD proteome acquired without any protein and/or peptide prefractionation steps prior to LC-MS analysis [20–23], indicating the high sensitivity of our approach. In previous studies, hippocampal PSD fractions were either isolated from hippocampal slices [20] resulting in fewer detected proteins, or applied extensive prefractionation of tryptic digests derived from hippocampal PSD proteins requiring significantly more source material compared to the present study (Supporting Information Table 11) [21–23]. Notably, the present dataset also includes quantitative information for all detected proteins across all investigated fractions including crude synaptosomes (P2) synaptic junctions (SJ) and PSDI/II, thereby allowing to distinguish between PSD components and potential contaminants based on their relative abundance in the individual fractions. This enables to define a high-confidence PSD dataset (Supporting Information Fig. 7 and Table 12) based on protein correlation profiling [24]. Notably, this dataset contains 879 proteins, is highly enriched in PSD-characteristic molecules, and includes less than 5% of mitochondrial proteins (Supporting Information Fig. 8 and Table 12).

In summary, the high sensitivity of our DIA workflow [7] allowed the detailed characterization of hippocampal PSD with less than 20 μg PSD material per sample, facilitating a comprehensive analysis of PSD composition from low amounts of source tissue. Thus, our approach enables (i) the analysis of PSD material of different brain structures such as the olfactory bulb, basal ganglia, or hypothalamic region and (2) the study of alterations in the PSD composition in these regions upon genetic manipulation, both cases, in which primary brain material is limited.

Taken together, our high-confidence PSD dataset including TOP3-based quantification of all PSD components provides a rich resource for systems biology approaches to model-specific PSD functions. However, future studies will be required to pinpoint the relative abundances of all PSD components identified in this study across a variety of synaptic subtypes within the hippocampus including the well-studied hippocampal subregions dentate gyrus and cornu ammonis.

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