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Quantitative Mass Spectrometry Measurements Reveal Stoichiometry of Principal Postsynaptic Density Proteins

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Abstract

Quantitative studies are presented of postsynaptic density (PSD) fractions from rat cerebral cortex with the ultimate goal of defining the average copy numbers of proteins in the PSD complex. Highly specific and selective isotope dilution mass spectrometry assays were developed using isotopically labeled polypeptide concatemer internal standards. Interpretation of PSD protein stoichiometry was achieved as a molar ratio with respect to PSD-95 (SAP-90, DLG4), and subsequently, copy numbers were estimated using a consensus literature value for PSD-95. Average copy numbers for several proteins at the PSD were estimated for the first time, including those for AIDA-1, BRAGs, and densin. Major findings include evidence for the high copy number of AIDA-1 in the PSD (144 ± 30)—equivalent to that of the total GKAP family of proteins (150 ± 27)—suggesting that AIDA-1 is an element of the PSD scaffold. The average copy numbers for NMDA receptor sub-units were estimated to be 66 ± 18 , 27 ± 9 , and 45 ± 15 , respectively, for GluN1, GluN2A, and GluN2B, yielding a total of 34 ± 10 NMDA channels. Estimated average copy numbers for AMPA channels and their auxiliary sub-units TARPs were 68 ± 36 and 144 ± 38 , respectively, with a stoichiometry of $\sim 1:2$, supporting the assertion that most AMPA receptors anchor to the PSD via TARP sub-units. This robust, quantitative analysis of PSD proteins improves upon and extends the list of major PSD components with assigned average copy numbers in the ongoing effort to unravel the complex molecular architecture of the PSD.

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Notes

Certain commercial equipment, instruments, and materials are identified in this paper to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

The authors declare no competing financial interest.

Supporting Information

Supplemental Table I: MRM fragmentation transitions and optimized fragmentation parameters of targeted peptides and proteins of interest. Supplemental Table II: Normalization factors to strep tag and DLG4 for polypeptides from each sample preparation. Supplemental Table III: The origin of quantitative variability is shown through measurement means, SDs, and %CVs. Supplemental Figure I: Primary amino acid sequences of polypeptides expressed from each of eight QconCAT clone constructs. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00109.

Graphical Abstract

Keywords

postsynaptic density; protein stoichiometry; multiple reaction monitoring; copy numbers

INTRODUCTION

The postsynaptic density (PSD), an electron-dense structure lining the postsynaptic membrane, is a large protein complex with an average total mass of about 1 million kDa.¹ The complex contains neurotransmitter receptors, signaling molecules, and cell-adhesion molecules, organized through a number of specialized scaffold proteins. The number and specific organization of these components within the PSD determine the type and strength of the postsynaptic response to neurotransmitter release. The identity and quantity of the molecular makeup for the PSD, however, are not completely established.

A first step in the elucidation of the molecular architecture of the PSD is identification of its components and determination of their stoichiometry. This task has been greatly aided by the existence of cell fractionation techniques that allow for the isolation of the PSD complex. Treatment of synaptosomal fractions with mild detergents solubilizes synaptic membranes, releasing mostly intact PSDs which can subsequently be purified through centrifugal² and affinity-based strategies.³ Biochemical identification of proteins in these fractions and verification of their localization in intact cells by electron microscopy lead to the enumeration of major PSD components.

The large number of qualitative studies on the composition of the PSD has stimulated the creation of SynProt.org, a Web database detailing observed protein constituents with associated links to documentation.⁴ Comparative studies using stable isotope labels such as ICAT and iTRAQ, as well as label-free strategies based on spectral counting and intensity-based quantification approaches, have been used to estimate relative quantities of proteins in PSD fractions from different brain regions,^{5,6} from different species,⁷ or from different activity states⁸ as well as to compare levels of proteins in sub-cellular fractions to assess enrichment in PSDs.^{9,10}

Estimation of the stoichiometry and actual numbers of proteins within the PSD complex has been relatively challenging (reviewed in ref 11). Through analysis of isolated PSDs by scanning transmission electron microscopy (STEM) and quantitative Western immunoblotting, Chen et al.¹ estimated the number of copies of PSD-95, a member of the membrane-associated guanylate kinase (MAGUK) family, to be ~300 per PSD. By a green fluorescent protein (GFP)-based calibration technique, Sugiyama and colleagues determined absolute numbers for a few scaffold proteins at single synapses in intact cells.¹² Their estimate of close to 300 MAGUK family proteins per synapse is in reasonably good agreement with the numbers obtained by Chen et al.¹ Peng's group applied a mass spectrometric strategy for quantification of selected proteins in PSD fractions using stable-isotope-labeled synthetic peptides.^{5,13} The molar ratios of targeted proteins to PSD-95 obtained in these studies were used to estimate their average copy numbers at the PSD.¹⁴

While the quantitative estimates for the PSD-95/MAGUK family of proteins obtained using orthogonal strategies are in agreement, estimates in the literature for certain other PSD components show discrepancies. For example, quantitative estimates for AMPA-type glutamate receptors, obtained by Cheng et al.⁵ using an isotope dilution-based mass spectrometric (ID-MS) strategy, are lower than those from other methods, including electrophysiology and electron microscopy.^{15,16} These differences may stem from differences in cell types under consideration but also may be due to methodological bias.

A previous non-targeted mass spectrometric analysis, tracking enrichment of proteins in a PSD-95 affinity-purified PSD fraction, from a parent, conventional PSD fraction, allowed differentiation of genuine PSD components from contaminants and added new candidates to the roster of core PSD components.¹⁷ Enumeration of new potential core proteins, as well as the availability of improved mass spectrometric techniques, prompted the present targeted quantitative study. Many of the co-purified proteins observed in our previous affinity-purification study,¹⁷ including specialized scaffolds, NMDA and AMPA receptors, small G-protein regulators, and various signaling molecules, were targeted in the present study for quantitative stoichiometric analysis using a more definitive strategy.

An improved ID-MS approach¹⁸ was used to limit methodological biases associated with sample preparations that often lead to quantitative errors. Two peptides per target protein were selected for expression in eight ¹³C,¹⁵N-labeled biosynthetic concatenated polypeptides. These standards were designed to mimic the tryptic digestion properties of the native full-length proteins by including native sequences adjacent to their tryptic sites. The labeled biosynthetic standards contained *equimolar* quantities of each target peptide as well as a 10-mer standard peptide (strep tag) common to all eight concatenated polypeptides, as required for normalization between polypeptide standards. With these labeled concatenated standards, multiple-reaction monitoring (MRM) using a liquid chromatography–electrospray ionization–triple quadrupole mass spectrometer (LC–ESI–QQQ–MS) now provides methodology for accurate relative quantification and stoichiometric analysis of multiple proteins in the rat PSD complex, concurrently. Forty-two proteins were quantified relative to the abundance of PSD-95, a marker protein for the PSD. Copy numbers for several proteins were estimated for the first time in this work, and stoichiometry of other PSD components was validated or re-defined.

EXPERIMENTAL SECTION

Materials

All chemicals and solvents were purchased through Sigma (St. Louis, MO) unless otherwise noted below.

Fused Labeled Polypeptide Standards

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) peptide assays require enzymatic cleavage of proteins, and the rate of enzyme proteolysis is influenced significantly by amino acids flanking the cleavage residues,^{18–21} here the tryptic lysine and arginine residues. Fused labeled polypeptide standards (Supplemental Figure I) were biosynthesized under contract by the Protein Expression Laboratory (NCI-Frederick) as previously described.²² The genetically encoded fusions contain equimolar quantities of each tryptic peptide flanked by six-amino-acid native upstream and downstream sequence regions, a C-terminal His6, and an N-terminal Strep tag, together with a 17-amino acid tryptophan-rich tag for UV quantitation. Cell-free protein expression^{22,23} incorporated labeled arginine and lysine amino acid precursors $^{13}\text{C}_6$, $^{15}\text{N}_2$ -L-lysine and $^{13}\text{C}_6$, $^{15}\text{N}_4$ -L-arginine (99% purity). The Protein Expression Laboratory recovered the labeled, fused polypeptide concatemers by centrifugation, buffer-washed them to remove *in vitro* expression system contaminants, and provided them as frozen pellets. During the course of these and prior studies, concatemers were observed to be invariably insoluble in the *in vitro* translation system buffer and were dissolved with heating in 8 mol/L urea or 6 mol/L guanidinium-HCl for dilution as internal standards.

Postsynaptic Density (PSD) Fractionation

The PSD fraction was prepared essentially by the method of Carlin et al.² that includes two Triton X-100 extraction steps, with modifications as described previously.²⁴ Brains from adult Sprague–Dawley rats anesthetized with CO_2 were custom collected by Pel-Freez Biologicals (Rogers, AR) or Rockland Immunochemicals (Gilbertsville, PA), frozen immediately in liquid nitrogen, and shipped on dry ice. Brains from 16 rats were pooled for each PSD preparation. Four different PSD preparations were analyzed (biological replicates). Brains were thawed by a 1 min immersion in isotonic sucrose at 37 °C and dissected on ice. Tissue consisting mainly of cerebral cortices, obtained by removing white matter from forebrains, was then rapidly homogenized in 0.32 mol/L sucrose. Dissection and homogenization were completed within 4 min. Nuclear fraction (P1) was removed by centrifugation, and a synaptosome/mitochondria-enriched pellet (P2) was obtained by further centrifugation (13800g for 10 min). P2 was re-suspended and fractionated on a sucrose density gradient, and a synaptosome fraction was collected from the 1 mol/L–1.2 mol/L sucrose interface. The synaptosome fraction was treated with Triton X-100 (0.5% v/v final concentration) for 15 min on ice with gentle mixing and was centrifuged to obtain a detergent-insoluble pellet. The pellets were resuspended in 0.32 mol/L sucrose and layered on a sucrose gradient. Following centrifugation, the PSD-enriched fraction was collected from the 1.5 mol/L–2.1 mol/L sucrose interface and subjected to a second extraction with Triton X-100 (0.5% v/v final concentration) and KCl (75 mmol/L final concentration). PSD-enriched fractions were collected by centrifugation on a 2.1 mol/L sucrose cushion. In

preparation for mass spectrometric analysis, PSD samples (0.5 mg protein) were re-suspended into 1 mL of 20 μ mol/L HEPES buffer and pelleted by centrifugation.

Sample Preparation

PSD pellets isolated from rat brains were thawed to room temperature from temporary storage at -20°C . Pellets (0.5 mg protein) were reconstituted in 50 μL of freshly prepared 8 mol/L urea in 50 mmol/L ammonium bicarbonate (NH_4HCO_3). To ensure complete solubilization of the PSD fraction, 20 μL of 0.1 g/L sequencing grade modified porcine trypsin (Promega, Madison, WI) in 50 mmol/L NH_4HCO_3 was added to each sample (250:1 protein:trypsin), and samples were shaken at 37°C for 30 min. Two 0.5 mg solubilized samples were pooled together in each biological replicate for further processing. A total of 200 pmol of an external standard (non-labeled strep-tag synthetic peptide) was added to each PSD sample, which was then thoroughly mixed prior to splitting into eight identical aliquots in 0.5 mL Eppendorf tubes. A 10 μL aliquot ($\sim 1.7 \mu\text{g}$) of ^{15}N stable-isotope-labeled polypeptide from each of eight different quantitative concatemer clones was added to one of each of the eight samples, respectively.

PSD pellets and labeled internal standard polypeptides were digested using a standard protocol. Briefly, dithiothreitol (DTT) was added to a final concentration of 5 mmol/L with shaking at 50°C for 30 min to reduce disulfide linkages. Samples were allowed to cool, iodoacetamide (IAM) was added to a final concentration of 15 mmol/L, and samples were stored in the dark for 30 min. Before the first addition of trypsin (3.2 μg /sample; 40:1 protein:trypsin), samples were diluted with NH_4HCO_3 to reduce the urea concentration to 1 mol/L. Samples were shaken at 37°C for 4 h. A second addition of trypsin (3.2 μg /sample) was allowed to react overnight at 37°C . The following morning, 20 μL of 1 mol/L HCl was added to quench the reaction, and samples were mixed thoroughly and dried. Dry samples were reconstituted in water and purified using C_{18} ZipTips (Millipore) based on the manufacturer's protocol.

Mass Spectrometry Analysis

Liquid chromatographic separation was achieved using a Zorbax (Agilent) stable bond (SB)- C_{18} reversed-phase analytical column (2.1 mm $\times$ 150 mm, 3.5 μm particles) at a flow rate of 200 $\mu\text{L}/\text{min}$. Mobile phases A and B consisted of 0.1% (v/v) formic acid in water or acetonitrile, respectively (Honeywell, Burdick and Jackson, MS grade). Peptide elution was accomplished using a linear gradient over 25 min from 3% to 50% B (v/v), followed by a column wash at 95% B (v/v) and re-equilibration. Column temperature was maintained at 30°C ; autosampler plate temperature control was set at 5°C . An Agilent 1200 HPLC system (Santa Clara, CA) was coupled inline with an Applied Biosystems API 5000 triple-quadrupole mass spectrometer (Foster City, CA) equipped with a standard microflow source. Ions were detected using a MRM method in positive polarity with a dwell time of 40 ms.

During data acquisition, all source and fragmentation parameters were set identically for non-labeled/labeled transition pairs. Source conditions were as follows: collision gas, 2.7×10^4 Pa (4 psi); unit resolution in Q1 and Q3; curtain gas (CUR), 7.5×10^4 Pa (11 psi); intensity threshold, 0; ion source gas 1 (GS1), 2.1×10^5 Pa (30 psi); settling time, 3 ms; ion

source gas 2 (GS2), 2.8×10^5 Pa (40 psi); pause between mass ranges, 3 ms; ion-spray voltage (IS), 4500 V; capillary temperature (TEM), 400 °C. Fragmentation conditions were as follows: for all peptides, the entrance potential was set to 10 V; exit cell potential was set to 15 V. Collision energies and declustering potentials were compound dependent and are detailed in Supplemental Table I. Data acquisition and peak integration were performed using Analyst v1.5 software (Applied Biosystems). Peaks were identified manually and were automatically selected by the Analyst Quantitation Wizard; peak areas were integrated by Analyst using a bunching factor = 1, number of smooths = 0, and all other parameters set to default values. All peak integrations were visually inspected and manually integrated when necessary. Blank injections were monitored for sample carry-over.

A total of 534 fragmentation transitions were monitored among 89 peptides from 42 proteins (plus the strep tag) in both the PSD samples and the fused labeled polypeptides, split among eight LC-MRM methods. Peptide fragmentation was predicted using Skyline (2.5), developed at the University of Washington (MacCoss Lab).²⁵ Initially, 6–8 MRM transitions were screened for each peptide by LC–MS/MS analysis of control samples, and the optimal transitions were selected for the final analytical method design. Three fragmentation transitions were monitored per peptide for both the native non-labeled PSD peptides and the labeled peptides—two transitions were used for quantification, and the third transition was used as validation only.

Experimental Design and Statistical Analysis

A summary of the experimental design for the quantitative approach is provided in Figure 1. Eight distinctive fused polypeptide clones propagated in *E. coli* were designed in-house and expressed in cell-free media, each coding for 11 or 12 unique tryptic peptide sequences with flanking amino acids (six N- and C-terminal flanking residues) from known PSD protein components. Fused peptide expression was performed in a cell-free expression system using minimal media enriched with ¹⁵N-labeled lysine and ¹⁵N-labeled arginine residues with the aim of using the “heavy” polypeptides as internal standards for MS quantification. Fused polypeptide design is provided in Supplemental Figure I, along with the peptide sequences targeted by MRM. Each fused polypeptide was designed with an identical C-terminal strep tag 10-mer tryptic peptide (SAWSHPQFEK) for normalization between expression products and an N-terminal hexa-His tag initially intended for purification, but which was found to be unnecessary with the *in vitro* expression system.

A synthetic, unlabeled (light) analogue of the strep-tag peptide (SAWSHPQFEK) was spiked into the entire PSD preparation prior to partitioning the solubilized pellet into eight fractions. Recombinantly expressed, labeled (heavy) fused polypeptide standards were spiked separately into each of the eight identical PSD fractions prior to tryptic digestion. Because the quantity of concatemer was unavoidably variable, the strep tag peptide on each concatemer was used to normalize differences in concatemer quantity. This necessitated that each concatemer was analyzed separately being spiked into eight equivalent PSD fractions each containing a different concatemer. After sample cleanup, the SAWSHPQFEK peptide was quantified by LC–MS/MS (MRM) analysis in each sample, and the average peak area ratio of light/heavy strep tag (Supplemental Table II) was used to normalize measurements

between samples with fused polypeptide standards from different clones. Overall, normalization factors were moderately variable between fused polypeptide standards from different clones as a result of differences in digestion efficiency²⁶ and/or polypeptide quantity. This variability was, however, demonstrated to be consistent for independent, quadruplicate sample preparations, thereby validating the need for this normalization approach.

Proteins were subsequently normalized within each sample preparation to the high-abundant protein, PSD-95 (SAP90, DLG4). For quantification of each protein from PSD fractions, average copy numbers and standard deviations (SD) of the mean were propagated through three levels of measurement precision: (1) two (or more) MRM transitions per peptide, (2) two (or more) peptides per protein, and (3) four different PSD preparations (biological replicates) (Supplemental Table III). SDs at each level of measurement precision were calculated using all data points, rather than using accrued mean values, to ensure propagation of error.

RESULTS

A quantitative, targeted LC–MS/MS analysis of the rat PSD complex was performed, with the ultimate goal of estimating the relative amounts of the major proteins of this neuronal protein complex and defining their copy numbers. The intensities of proteotypic peptides were determined from well-known PSD complex protein constituents (PSD-95 and other scaffolds, kinases, and receptors), likely contaminants (bassoon, synaptophysin), and one biological negative control (mouse IgG). Relative molar abundances for 42 proteins were determined relative to PSD-95 and are provided in Table 1, along with SDs and coefficients of variation (%CV) for each protein measurement. Supplemental Table III is provided to demonstrate the sources of the propagated measurement variability. The measurement mean, SD, and %CV are provided for each level of quantification at the “within transition”, “within peptide”, and “within protein” levels. The levels represent variability due to sample preparation, variability among ions from the same peptide, and variability among peptides from the same protein, respectively. Total variability, described as “within protein” (35.0%), necessarily encompasses “variability within peptide” (28.2%) and “variability within transition” (28.0%), as a result of the propagation of error. However, variability calculated at each level separately demonstrates that PSD sample preparation, peptide, and protein variability all contribute significantly to the total error. Differences in the composition of individual PSD preparations and due to downstream sample preparation (solubilization being one likely cause) may contribute to the variability between biological replicates. Digestion variability is the major factor contributing to variability within protein, and matrix interferences are the major factor contributing to variability within peptide.

A representative MRM mass chromatogram is provided in Figure 2 for the targeted analysis of PSD proteins in rat brain preparations. As expected, PSD-95 was observed to be the most abundant quantified protein with the exception of two members of the CaMKII family (KCC2A and KCC2B, Table 1). No other protein targeted in this study was determined to have an abundance greater than 50% relative to PSD-95. The median measurement precision (median %CV for all proteins) was determined to be 34%. To our knowledge, average copy

numbers per PSD for several proteins were estimated for the first time in this work including AIDA-1, densin, GluN2B, TARP sub-units, CaMKII- δ and - γ , PP1A catalytic sub-unit, CYLD, and members of the BRAG family.

Labeled, fused polypeptides are designed specifically for quantitative proteomics to mimic the tryptic digestion environment of native proteins in biological samples following sample denaturation.^{18,27} However, due to differences between protein expression in a cell-free system based on bacterial ribosomal translation machinery and mammalian tissue, such as post-translational modifications (PTMs) or folding, expressed recombinant polypeptides occasionally differ from their mammalian analogues structurally, or in their accessibility to enzymatic digestion. Two peptides from the same protein may therefore differ quantitatively if mammalian (light) proteins are normalized to recombinantly expressed (heavy) peptides. In this study, the largest variability found between peptides from the same protein was consistently observed among proteins in the AMPA-type glutamate receptor (GRIA1, GRIA2, and GRIA3) family (2.5-, 2.2-, and 3.1-fold, respectively). This family of proteins is reported to exhibit multiple post-translational modifications, including ubiquitination^{28,29} and phosphorylation on multiple residues (reviewed in ref 30), and to exist in mixtures of homo- and hetero-tetramers.^{31–34} Further investigations are necessary to identify the source of the observed inconsistencies. However, it should be noted that the average fold-change for related peptides from all proteins was determined to be 0.34 (calculated as average $[(1 - ([\text{peptide}_1]/[\text{peptide}_2]))]$).

Two of the 42 proteins targeted—bassoon (BSN) and synaptophysin (SYPH)—are presynaptic proteins that may contaminate the PSD fraction. The ratio of bassoon to PSD-95 is fairly high (0.21 ± 0.07), indicating contamination from the presynaptic active zone matrix. However, no synaptophysin is detected (0.00 ± 0.01), indicating absence of contamination from synaptic vesicles. The abundance ratio observed for IGHG1 (an Ig gamma chain protein) was 0.05 ± 0.03 , providing an estimate for analytical “noise” and the contribution of biological noise.

The estimate of relative protein stoichiometry in the PSD fraction was subsequently expressed as a copy number in the PSD (given the consensus copy number of PSD-95). The abundance of PSD-95 at the PSD as estimated previously by quantitative Western blot analysis of isolated PSD fractions¹ agrees well with the numbers obtained by Sugiyama et al. in 2005, using quantitative fluorescence imaging in intact neurons.¹² Based on these studies, it is generally accepted that an average PSD contains ~300 PSD-95 molecules.¹⁴ We used this copy number for PSD-95 to calculate average copy numbers for selected components at the PSD (Table 2). The proteins in Table 2 are grouped into functional categories, and, where appropriate, estimated copy numbers of polymeric units are included. For example, the relative copy number of total NMDA receptors, composed of two GluN1 and two GluN2 (either GluN2A or GluN2B) sub-units, is estimated as 35 ± 10 . The fact that the estimated number of GluN1 sub-units (66 ± 18) is statistically indistinguishable from the estimated total number of GluN2 sub-units (72 ± 24), yielding the expected ~1:1 stoichiometry, lends additional confidence to the estimates for individual proteins. The average relative copy number for AMPA receptors, which are tetrameric units composed of

any combination of GluA1–4, is calculated as total number of GluA's divided by four $[(45 + 144 + 81)/4 = 68 \pm 36]$.

DISCUSSION

PSD Scaffold

PSD complex contains high quantities of specialized proteins with multiple protein–protein interaction domains that bind to each other to form a so-called PSD scaffold. The PSD scaffold maintains the molecular architecture of the PSD by anchoring elements such as receptors and signaling molecules at specific positions. Scaffolding proteins at the PSD are organized in lateral layers at different distances from the postsynaptic membrane.³⁵ Closest to the membrane, and well within the electron-dense core of the PSD are the family of MAGUKs, comprised of PSD-95 (DLG4), PSD-93 (DLG2), SAP102 (DLG3), and SAP97 (DLG1). GKAPs (SAPAPs), another group of scaffolds that bind to both MAGUKs and Shanks, are thought to peg the core PSD scaffold containing MAGUKs to the deeper layer of the PSD, where Shanks and Homer are located.³⁶ The present quantitative study suggests that AIDA-1 (ANS1B, Q8BZM2 in ref 17) may represent another major class of scaffolding protein at the PSD. AIDA-1 is observed with a stoichiometry of about one to every two PSD-95 molecules, with an average copy number equal to that of all GKAP subtypes combined. AIDA-1, like GKAPs, binds to PSD-95³⁷ and is located within the same layer as PSD-95³⁸ at the dense core of the PSD. AIDA-1 contains two sterile alpha motifs (SAMs) known to serve as protein–protein interaction domains. The estimated high copy numbers, taken together with the specialized molecular structure and electron microscopic localization, suggest that AIDA-1, in addition to its previously described role in synapse to nucleus signaling,³⁷ may serve as a major scaffolding element at the PSD core.

The data presented here on the relative abundance of MAGUKs, with PSD-95 being the most abundant type, are in agreement with the findings of Cheng et al. in 2006.⁵ The relative abundances of PSD-93, SAP102, and SAP 97 are also consistent with the Cheng study.⁵ The finding of very low quantities of SAP97 in the two studies by quantitative mass spectrometry is in contrast with an earlier study by quantitative Western blot analysis.¹ However, the specificity of MRM-MS techniques relative to immunoassays, along with quantitative agreement from two distinct peptides of SAP97, support the current observation.

The stoichiometry of total GKAPs to PSD-95 is estimated as ~1:2, and is in close agreement with the study by Cheng et al., 2006⁵ although there is some difference between the two studies on the relative contributions of different GKAP subtypes. Another study by quantitative fluorescence microscopy on cultured hippocampal neurons estimated average copy numbers of GKAPs and MAGUKs to be 273 ± 38 and 171 ± 53 , respectively,¹² reasonably close to the estimates by quantitative mass spectrometry in adult cerebral cortex.

In contrast to MAGUKs and GKAPs, estimated copy numbers for Shanks (SHAN1, SHAN2, and SHAN3) and Homer (HOME1) at the deepest layer of the PSD differ substantially between strategies. Estimates from two studies^{5,14} including this work using quantitative mass spectrometric analysis of PSD fractions are 2–6 times lower compared to estimates by fluorescence techniques on intact cells.¹² One explanation for the discrepancy

could be loss of these proteins during isolation of PSD fractions, especially upon detergent extraction of synaptosomes.

Glutamate Receptors

PSDs at glutamatergic neurons contain NMDA and AMPA types of ionotropic glutamate receptors. NMDA receptor channels are tetramers composed of two GluN1 and two GluN2 sub-units. In the present study, the copy numbers of GluN1 and GluN2 were found to be about equal, thus providing additional validation to estimates for individual subunits, and account for an average of 34 ± 10 copies of NMDA receptor channels per PSD. The abundance of NMDA receptors at the PSD estimated here is somewhat higher than previous estimates: 20 per PSD by quantitative mass spectrometry,^{5,14} 5–30 per PSD by electrophysiology,¹⁵ and 16–25 per PSD by EM tomography.¹⁶ The number of tetrameric AMPA receptor channels (any tetrameric combination of GRIA1, GRIA2, and GRIA3) per PSD in the present study, 68 ± 36 , is considerably higher than a previous estimate (~15) by quantitative mass spectrometry,^{5,14} but is compatible with predictions by quantitative immuno-EM (<3 to 140),³⁹ EM tomography (30–100),¹⁶ and two reports by electrophysiology (58–70) and up to 150/spine.^{15,40}

Glutamate receptors anchor to the PSD through association with PSD-95 and other MAGUKs. While NMDA receptors attach directly to PSD-95, association of AMPA receptors are through auxiliary sub-units, the most important category of which are TARPs of type I ($\gamma 2$, $\gamma 3$, $\gamma 4$, $\gamma 8$; CCG2, CCG3, CCG4, CCG8).⁴¹ TARPs can associate with all four types of AMPA receptor sub-units and the stoichiometry of association varies with cell type,^{42–44} even though one TARP per tetramer would be enough for anchoring of the AMPA receptor channel to the PSD. The present study estimates for the first time abundances of all four types of type I TARPs in PSD fractions. We find a stoichiometry of roughly two TARPs per AMPA receptor in PSDs isolated from cerebral cortex, a number that suggests most AMPA receptors at the PSD must be anchored through TARP auxiliary sub-units. More than half of all TARPs are of $\gamma 3$ sub-type, in agreement with a previous finding of its preferential expression of $\gamma 3$ in the cerebral cortex.⁴⁵

Enzymes

In addition to scaffolds and glutamate receptors, PSD fractions contain several types of enzymes and signaling molecules, the most prominent of which is CaMKII. Two anchoring proteins for CaMKII—densin (LRRC7) and GluN2B (NR2B)—have been quantified for the first time in PSD fractions. Densin may anchor CaMKII to the PSD in a calcium-independent manner.⁴⁶ GluN2B, the other PSD anchor for CaMKII, binds the kinase in the presence of Ca^{2+} or upon its autophosphorylation on Thr-286.^{47,48} The molar ratio of densin to GluN2B is 1:2, and taken together these two proteins account for a maximum of ~70 CaMKII anchoring sites. The higher estimated copy numbers for CaMKII in the PSD fraction (275 holoenzymes) may be due to self-association of the holoenzymes at the PSD, as well as contamination by CaMKII clusters that form during tissue dissection.²⁴ Indeed, examination of the PSD fraction by electron microscopy reveals presence of particulate contaminants that appear to consist of self-associated CaMKII.^{3,24} Another protein kinase, PKC (KPCG), is also fairly abundant at the PSD, with a molar ratio exceeding 1:10 with

respect to PSD-95. Protein phosphatase 1 (PP1), which reverses the phosphorylation of several PSD components, is a crucial player in activity-dependent synaptic modification.⁴⁹ The present study reveals high average copy numbers for PP1A at the PSD, with a molar ratio of PP1A to PSD-95 greater than 1:6.

PSDs contain substantial quantities of two types of small G-protein regulators, SynGAP and BRAGs. Estimates for SynGAP copy numbers could not be obtained in the present study but an earlier study¹⁴ concluded that it is even more abundant than PSD-95 in the PSD fraction. BRAGs (IQEC1, IQEC2), guanine nucleotide exchange factors regulating Arf6 function, are within a group of proteins shown to copurify with PSDs.^{17,50} The stoichiometries for IQEC1 and IQEC2 with respect to PSD-95 in the PSD fraction were estimated for the first time in the present study. The total copy number for BRAGs was observed to be 69 ± 24 per average PSD, with about equal contributions of the two sub-types. The relatively high abundance suggests a prominent role of BRAGs in postsynaptic function, possibly in the regulation of the actin cytoskeleton that abuts the PSD.

The present study using state-of-the-art techniques for quantitative mass spectrometric analysis provides new information on the stoichiometry of PSD components that should help in the elucidation of the molecular architecture of the PSD. Copy numbers for several proteins have been estimated for the first time, and pre-existing numbers for others have been confirmed or, in a few cases, challenged. As suggested by the discussion above, contributions of orthogonal strategies for the determination of copy numbers of PSD components are essential, as each strategy provides distinct strengths and biases. While proteomic studies lay the background by providing average quantitative profiles, they should ultimately be complemented by microscopy studies that can pinpoint synapse-to-synapse variability as well as fluctuations during specific activity states for each component.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase
EM	electron microscopy
GFP	green fluorescent protein
GKAP	guanylate-kinase-associated protein

GRIA	glutamate receptor
ID-MS	isotope dilution mass spectrometry
MAGUK	membrane-associated guanylate kinase
MRM	multiple-reaction monitoring
NIST	National Institute of Standards and Technology
NMDA	<i>N</i> -methyl-D-aspartic acid
PSD	post-synaptic density
SAP-90	synapse-associated protein 90
TARP	TCR gamma alternate reading frame protein

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Figure 1. Schematic representation of the analytical workflow used to fractionate PSD proteins from rat brain and prepare samples for quantitative analysis.

Figure 2. Representative MRM mass chromatogram for the analysis of targeted peptide transitions of digested PSD proteins from rat brain preparations, and found on fused polypeptide (clone) 3.

Table 1

Relative Molar Abundances of Selected Proteins in PSD Fractions from Rat Brain

protein		relative molar abundance normalized to PSD-95	SD	%CV
UniProtKB	common name			
KCC2A_rat	CaMKII- α	8.19	3.01	36.8
KCC2B_rat	CaMKII- β	1.91	0.42	21.9
DLG4_rat	PSD-95	1.00	0.17	16.6
KCC2D_rat	CaMKII- δ	0.49	0.24	48.5
GRIA2_rat	GRIA2	0.48	0.24	49.4
ANS1B_rat	AIDA-1	0.48	0.10	21.8
KCC2G_rat	CaMKII- γ	0.39	0.13	33.5
HOME1_rat	Homer1	0.30	0.07	23.1
GRIA3_rat	GRIA3	0.27	0.18	64.3
CCG3_rat	TARP γ -3	0.26	0.05	19.7
DLG2_rat	PSD-93	0.24	0.05	21.2
BAIP2_rat	IRSp53	0.24	0.08	34.1
NMDZ1_rat	GluN1	0.22	0.06	28.5
BSN_rat^a	bassoon	0.21	0.07	34.3
PP1A_rat	PP1A	0.18	0.07	38.9
DLGP3_rat	SAPAP3	0.16	0.03	15.9
NMDE2_rat	GluN2B	0.15	0.05	30.8
GRIA1_rat	GRIA1	0.15	0.08	54.9
DLGP2_rat	SAPAP2	0.14	0.03	22.7
SHAN3_rat	Shank3	0.13	0.06	48.5
D4A6H8_rat (CTNA2)	α -Catenin	0.12	0.03	21.6
DLG3_rat	SAP-102	0.12	0.03	21.8
KPCG_rat	PKC- γ	0.12	0.10	85.3
M0RBD0_rat (IQEC1)	BRAG2	0.12	0.03	26.5
SHAN1_rat	Shank1	0.11	0.04	36.6
CCG8_rat	TARP γ -8	0.11	0.04	35.7
D4A4I4_rat (IQEC2)	BRAG1	0.11	0.05	47.9
DLGP4_rat	SAPAP4	0.10	0.03	25.0
DLGP1_rat	SAPAP1	0.10	0.02	20.7
NMDE1_rat	GluN2A	0.09	0.03	38.1
SHAN2_rat	Shank2	0.09	0.03	29.9
CCG2_rat	TARP γ -2	0.08	0.02	22.4
LRRC7_rat	densin	0.08	0.04	45.8
D4A7T8_rat (FA81A)	hypothetical protein	0.07	0.02	23.6
NLGN3_rat	neuroligin 3	0.07	0.04	57.3
Estimate for Analytical "Noise" and/or Contribution of Biological Noise				
IGHG1_mouse^b	immunoglobulin G1	0.05	0.03	57.0
CCG4_rat	TARP γ -4	0.03	0.02	47.2

protein		relative molar abundance normalized to PSD-95	SD	%CV
UniProtKB	common name			
DTBP1_rat	dysbindin	0.03	0.02	61.3
ERBB4/ERBB2_rat	erbB-4, erbB-2	0.02	0.01	59.6
DLG1_rat	SAP-97	0.01	0.04	163.1
CYLD_rat	CYLD	0.01	0.02	177.4
SYPH_rat^a	synaptophysin	0.00	0.01	313.8

^aKnown presynaptic contaminants.

^bNegative control.

Table 2

Estimated Average Number of Copies of Selected Proteins at the PSD, Assuming 300 Copies for PSD-95

*category	protein name (UniProtKB)	average no. of copies ($\pm$ SD of the mean)	immuno-EM validation ref ^b
Scaffolds			
MAGUKs	PSD-95 (DLG4)	300 (literature consensus)	35
	PSD-93 (DLG2)	72 $\pm$ 15	51
	SAP-102 (DLG3)	36 $\pm$ 7	51
	SAP-97 (DLG1)	3 $\pm$ 12	52
GKAPs (SAPAPs)	SAPAP1 (DLGP1)	30 $\pm$ 6	
	SAPAP2 (DLGP2)	42 $\pm$ 7	
	SAPAP3 (DLGP3)	48 $\pm$ 7	
	SAPAP4 (DLGP4)	30 $\pm$ 7	
	total SAPAP =	150 $\pm$ 27	53
AIDA	AIDA-1 (ANS1B) ^a	144 $\pm$ 30	38
shanks (ProSAPs)	Shank1 (SHAN1)	33 $\pm$ 12	54
	Shank2 (SHAN2)	27 $\pm$ 9	54
	Shank3 (SHAN3)	39 $\pm$ 18	55
	total shank =	99 $\pm$ 39	56
homer	Homer1 (HOME1)	90 $\pm$ 21	57
Receptors			
NMDA receptors	GluN1 (NMDZ1)	66 $\pm$ 18	58
	GluN2A (NMDE1)	27 $\pm$ 9	59
	GluN2B (NMDE2) ^a	45 $\pm$ 15	59
	total NMDAR tetramers =	35 $\pm$ 10	
AMPA receptors	GluA1 (GRIA1)	45 $\pm$ 22	60
	GluA2 (GRIA2)	144 $\pm$ 71	61
	GluA3 (GRIA3)	81 $\pm$ 54	62
	total AMPAR tetramers =	68 $\pm$ 36	
AMPA auxiliary sub-units (TARPs)	TARP γ -2 (CCG2) ^a	24 $\pm$ 6	63
	TARP γ -3 (CCG3) ^a	78 $\pm$ 15	
	TARP γ -4 (CCG4) ^a	9 $\pm$ 5	
	TARP γ -8 (CCG8) ^a	33 $\pm$ 12	64
	total TARP =	144 $\pm$ 38	45
Kinase/Phosphatase	CaMKII- α (KCC2A)	2457 $\pm$ 903	65
	CaMKII- β (KCC2B)	573 $\pm$ 126	66
	CaMKII- δ (KCC2D) ^a	147 $\pm$ 71	
	CaMKII- γ (KCC2G) ^a	117 $\pm$ 36	
	total CaMKII dodecamers =	275 $\pm$ 94	67
	PKC- γ (KPCG)	36 $\pm$ 29	68
	PP1 (cat. sub-unit) (PP1A) ^a	54 $\pm$ 21	69

*category	protein name (UniProtKB)	average no. of copies ($\pm$ SD of the mean)	immuno-EM validation ref ^b
Cell-Cell Interaction	Neurologin 3 (NLGN3) ^a	21 $\pm$ 11	
	α -Catenin, CTNA2 (D4A6H8)	36 $\pm$ 8	
Other PSD Proteins	BRAG2, IQSEC1 (M0RBD0) ^a	36 $\pm$ 9	70
	BRAG1, IQSEC2 (D4A4I4) ^a	33 $\pm$ 15	71
	Densin (LRRC7) ^a	24 $\pm$ 11	^c
	IRSp53 (BAIP2)	72 $\pm$ 23	72
	CYLD (CLYD) ^a	3 $\pm$ 6	73
	Fam81a, hypothetical (D4A7T8) ^a	21 $\pm$ 6	

^aTo our knowledge, average copy number at the PSD estimated for the first time.

^bImmuno-electron microscopy studies demonstrating specific labeling for the targeted proteins at the PSD. The earliest reference is cited when more than one study was found.

^cTao-Cheng, personal communication.