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A sandwich enzyme-linked immunosorbent assay for the quantification of insoluble membrane and scaffold proteins

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ABSTRACT

Enzyme-linked immunosorbent assays (ELISAs) are applied for the quantification of a vast diversity of small molecules. However, ELISAs require that the antigen is present in a soluble form in the sample. Accordingly, the few ELISAs described so far targeting insoluble proteins such as integral membrane and scaffold proteins have been restricted by limited extraction efficiencies and the need to establish an individual solubilization protocol for each protein. Here we describe a sandwich ELISA that allows the quantification of a diverse array of synaptic membrane and scaffold proteins such as munc13-1, gephyrin, NMDA R1 (*N*-methyl-D-aspartate receptor subunit 1), synaptic vesicle membrane proteins, and SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors). The assay is based on initial solubilization by the denaturing detergent sodium dodecyl sulfate (SDS), followed by partial SDS removal using the detergent Triton X-100, which restores antigenicity while keeping the proteins in solution. Using recombinant standard proteins, we determined assay sensitivities of 78 ng/ml to 77 pg/ml (or 74–0.1 fmol). Calibration of the assay using both immunoblotting and mass spectroscopy revealed that in some cases correction factors need to be included for absolute quantification. The assay is versatile, allows parallel processing and automation, and should be applicable to a wide range of hitherto inaccessible proteins.

Despite recent advances in quantitative mass spectrometry (MS)[1] [1,2], the quantification of individual proteins and peptides is widely founded on immunoassays that rely on the binding of specific antibodies. Since the introduction of radioimmunoassays [3] and, somewhat later, enzyme-linked immunosorbent assays (ELISAs), countless variations of quantitative immunoassays have been developed and standardized [4–6]. Together, these assays have revolutionized clinical diagnostics by allowing the detection and quantification of an increasingly large array of peptide and nonpeptide biomolecules at high sensitivity in complex biological samples. To date, however, such assays have been confined largely to biomolecules that are soluble and, thus, can be easily and selectively captured by immobilized antibodies [7]. In contrast, it is much more difficult to quantify proteins that are bound to cellular structures, such as membranes and multiprotein scaffolds, or to insoluble aggregates/polymers, such as the extracellular matrix [8]. To measure such proteins by immunoassays, they need to be made accessible to antibody binding and to be dissociated from the macromolecular structure with which they are associated; hence, they need to be solubilized.

Currently, there is no unifying protocol that allows the nondenaturing solubilization of insoluble proteins owing to the fact that the chemical nature of binding is highly variable. For instance, integral membrane proteins are usually solubilized by nondenaturing detergents, resulting in a colloidal solution of mixed micelles of detergents, including both membrane lipids and proteins. The most widely used detergent is Triton X-100, a mixture of nonionic surfactants composed of polyoxyethylene oxides of variable length that are linked to the 4-(1,1,3,3-tetramethylbutyl)phenyl group as hydrophobic moiety [9]. Although this strategy works well for membrane proteins that are freely mobile in the plane of the membrane, it is inefficient if the proteins are stably anchored to scaffold

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[1] *Abbreviations used:* BKG, background; MS, mass spectrometry; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; NMDA R1, *N*-methyl-d-aspartate receptor subunit 1; mAb, monoclonal antibody; pAb, polyclonal antibody; SNAP, synaptosome-associated protein; VGLUT, vesicular glutamate transporter; TMB, tetramethylbenzidine; NTA, nitrilotriacetic acid; cDNA, complementary DNA; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SJC, synaptic junctional complexes; iTRAQ, isobaric tag for relative and absolute quantitation; PSD, postsynaptic density; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G; TP, tryptone/peptone; TBS, Tris-buffered saline; TBST, TBS (pH 7.4) and 0.05% Tween 20; HRP, horseradish peroxidase; TEAB, triethylammonium bicarbonate; TFA, trifluoroacetic acid; SCX, strong cation exchanger.

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proteins, if the proteins are associated in detergent-resistant microdomains ("rafts"), and/or if the interactions are mediated primarily by interactions that are not exclusively hydrophobic. The only universally available reagent effective for solubilizing most of such complexes is sodium dodecyl sulfate (SDS), a surfactant with a single negative charge. Although SDS forms mixed micelles and thus acts as surfactant, its unique features are owed to the fact that it binds directly to the polypeptide backbone of any protein, thereby stabilizing the unfolded state on denaturation while preventing aggregation due to the introduction of multiple negative charges [10]. However, SDS is generally not compatible with antigen–antibody interactions because (i) epitopes are at least partially destroyed and (ii) not only the proteins to be analyzed but also the detecting antibodies are denatured.

Considering the versatility of SDS as a general solubilizing agent, an attractive possibility is to remove SDS after the initial solubilization so as to restore antigen–antibody interaction. However, because proteins at best undergo only partial refolding after the removal of SDS, removal usually results in aggregation. Consequently, the only widely used method for immunodetection after SDS solubilization is immunoblotting (or "Western" blotting). Here the proteins are first separated by denaturing SDS–PAGE (polyacrylamide gel electrophoresis) according to molecular mass and then transferred to adsorbing membranes while washing out bound SDS, followed by antibody detection of the immobilized (and aggregated) protein. Indeed, immunoblotting must currently be considered as the standard procedure for relative (and occasionally absolute) quantification of all proteins for which other immunoassays are not available. However, there are several limitations that so far have prevented its use for widespread and standardized routine analysis. First, transfer efficiency and detection sensitivity are variable between experiments. Consequently, both standards and analyzed samples need to be separated on the same gel and need to be transferred to the same membrane, severely limiting sample throughput. Second, due to the limitation in the resolving power of one-dimensional SDS–PAGE, antibody binding to rare proteins is quenched by more abundant proteins migrating at the same position, thereby limiting sensitivity and/or increasing the chance of nonspecific signals, with the latter being particularly relevant if high-sensitivity detection systems such as enhanced chemiluminescence are used.

In the current study, we explored the general usefulness of previously described methods for partial renaturing of SDS-solubilized proteins [11,12] that prevent aggregation and, thus, allow capturing such proteins with antibodies in solution. It has been known for many years that the addition of excess Triton X-100 to SDS-containing samples results in SDS capture within mixed micelles; this not only prevents denaturation of newly added proteins such as antibodies but also strips SDS from the proteins, at least partially, and restores secondary structure while preventing aggregation [13]. As examples, we used a set of synaptic proteins that are either "free" integral membrane proteins (vesicular glutamate transporters), membrane proteins known to associate with other membrane proteins (SNAREs [soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors] and synaptotagmin 1), membrane proteins that are tightly bound to protein scaffolds (NMDA R1 [*N*-methyl-D-aspartate receptor subunit 1] and glycine receptor), or "classical" insoluble scaffold proteins (munc13-1 and gephyrin) [8,14]. We systematically explored solubilization efficiency and preservation of antigen–antibody binding, and we describe a modified ELISA that is based on antigen capture with specific mouse monoclonal antibodies followed by detection with specific rabbit antibodies that are then read out by a standard enzyme-linked secondary antibody. Our results show that with the newly developed assay, quantification of insoluble proteins is possible at a medium-throughput scale.

Materials and methods

Materials

Mouse monoclonal antibody (mAb) and rabbit polyclonal antibody (pAb) specific for the following proteins were used for ELISA or Western blot in this study (all available from Synaptic Systems, Göttingen, Germany): gephyrin (mAb 3B11, pAb C-terminal epitope), glycine receptor (mAb GlyR4a, unmodified and biotinylated), munc13-1 (mAb 266, pAb N-terminal epitope), NMDA R1 (mAb M68, pAb 1), SNAP-25 (mAb 71.2, pAb C-terminal epitope), synaptotagmin 1 (mAb 41.1, pAb cytoplasmic epitope), VGLUT1 (mAb 317D5, pAb C-terminal epitope), and VGLUT2 (mAb 321A8, pAb C-terminal epitope). Whole mouse brains from adult (10-week-old) VGLUT1 knockout mice, embryonic (E18) munc13-1/2 double knockout mice, and controls were provided by S.M. Wojcik and J.S. Rhee [15,16], and whole mouse brains from newborn SNAP-25 knockout mice and controls were provided by J.B. Sørensen [17]. SDS–PAGE and Western blot were performed according to standard protocols (Laemmli SDS gels or NuPage 4–12% Bis–Tris gradient gels from Invitrogen [USA] and chemiluminescence detection by FujiFilm BAS-1000 camera). Maxi-Sorb microtiter plates were obtained from Nunc (Denmark), and secondary antibodies were obtained from Dianova (Hamburg, Germany) and Bio-Rad (USA). TMB (tetramethylbenzidine) reagent for the ELISA was prepared according to Ref. [18].

Standard proteins

Recombinant proteins from rat were expressed in *Escherichia coli* as His6-tagged proteins and purified over an Ni^{2+}–NTA (nitrilotriacetic acid) affinity resin (Qiagen, Hilden, Germany) or were expressed as Strep-tagged proteins and purified over a Strep-Tactin affinity resin (IBA, Göttingen, Germany) [19]. Both purifications were followed by ion exchange chromatography (Äkta System, GE Healthcare, USA). Only SNAP-25 and synaptotagmin 1 were purified as full-length proteins. For the purification of full-length synaptotagmin 1, 10% cholate in the homogenization buffer and 0.01% dodecyl maltoside in the subsequent buffers were used. The following fragments were used as standard for the other proteins: gephyrin, amino acids 294–736; munc13-1, amino acids 3–317; NMDA R1, amino acids 17–35 fused to amino acids 660–811 (complementary DNA [cDNA] was kindly provided by P. Seeburg, Heidelberg, Germany); VGLUT1, amino acids 456–560; and VGLUT2, amino acids 510–582. Inclusion bodies from *E. coli* were solubilized in the presence of 6 M urea. For ELISA, recombinant proteins were diluted in mouse liver homogenate (or in bovine serum albumin [BSA] for the ubiquitously expressed gephyrin) and treated with detergent in the same way as the endogenous proteins.

Homogenization and solubilization

To obtain a complete tissue homogenate, brain or liver was homogenized in phosphate-buffered saline (PBS) with protease inhibitors by 10 strokes in a Potter–Elvehjem homogenizer clamped to an overhead stirrer (Eurostar Digital, IKA Labortechnik, Staufen, Germany) at 2000 rpm. Subcellular fractionation of brain tissue was carried out according to Refs. [20] and [21]. Briefly, rat or mouse brain was homogenized in 0.32 M sucrose and 10 mM Hepes–NaOH (pH 7.4) with protease inhibitors (12 ml/rat brain) and was centrifuged for 10 min at 800*g*. The supernatant was centrifuged for 15 min at 9200*g*. The resulting pellet was washed once with sucrose buffer, yielding a crude synaptosomal pellet (P2) that was resuspended in 1.2 ml of sucrose buffer and lysed by the

addition of 9 volumes of distilled water followed by homogenization. Hepes–NaOH buffer was added to a final concentration of 10 mM at pH 7.4. After 30 min of incubation, the fraction was spun at 25,000*g* for 20 min. The pellet fraction of this centrifugation (LP1) was used for all initial experiments of the study and is referred to as synaptic junctional complexes (SJC) because it is enriched in presynaptic membranes and presynaptic active zones, including attached synaptic vesicles, and postsynaptic membranes, including postsynaptic density (PSD) proteins. Major additional contaminations include mitochondria and myelin [20]. For the comparison of ELISA, Western blotting, and iTRAQ (isobaric tag for relative and absolute quantitation) quantification, we used the supernatant of a 35,000*g* centrifugation of rat brain homogenate (S1). All brain tissue samples or membrane protein fractions were solubilized at 3 mg/ml protein in 10 mM sodium phosphate (pH 7.4), 130 mM NaCl, and 5 mM ethylenediaminetetraacetic acid (EDTA) containing protease inhibitors and 1.2% SDS (modified from Ref. [11]) for 15 min at room temperature and diluted with 5 volumes of ice-cold 1.2% Triton X-100 in PBS. Insoluble content was pelleted by centrifugation at 100,000*g* for 30 min and was resuspended in SDS sample buffer for further analysis. The supernatant was either used for immunoprecipitation/ELISA or precipitated by the addition of 4–6 volumes of acetone (−20 °C) for further analysis by SDS–PAGE or MS.

Immunoprecipitation

Protein G–Sepharose (GE Healthcare) was loaded with 2–4 μl of ascites or 6 μg of purified immunoglobulin G (IgG) and was incubated with 100 μg of solubilized SJC overnight at 4 °C. Beads were collected, washed three times with PBS and 0.2% Triton X-100, and resuspended in 50 μl of SDS sample buffer. Samples of starting material and supernatant were precipitated by the addition of 3 volumes of acetone (−20 °C) and were resuspended in an equal volume of SDS sample buffer before analysis by SDS–PAGE and Western blot. Nonreducing SDS sample buffer was used if the molecular weight of the antigen was close to 50 or 25 kDa to avoid interfering antibody signals.

Immunoassay

All incubations were performed at room temperature and 700 rpm on a microplate shaker (SSM5, Stuart, UK) unless indicated otherwise. Microtiter plates were coated with 100 ng of goat anti-mouse IgG in 0.1 M sodium carbonate (pH 9.6) for 3 h and were blocked with 1% tryptone/peptone (TP) in carbonate buffer for 1 h. After three washes with Tris-buffered saline (TBS, pH 7.4) containing 0.05% Tween 20 (TBST), the plates were transferred to 4 °C and incubated with mouse mAb diluted in TBST overnight (~75–100 ng/well). On the next day, plates were washed twice with TBST and twice with the antigen buffer (0.2% Triton X-100, 0.05% TP, and TBS) before the addition of the antigen for 2 h. This was followed by two washes with the antigen buffer, and one wash and an incubation with the blocking buffer 0.5% BSA, 0.5% gelatin, 0.5% TP in TBST for 30 min to minimize unspecific binding of the rabbit pAb. The pAb was then added for 1 h as a 1:1000 dilution in blocking buffer. After three washes, the horseradish peroxidase (HRP)-coupled goat anti-rabbit detector antibody was applied for 1 h as a 1:10,000 dilution in blocking buffer. Finally, the plates were washed three times with TBST and developed with TMB reagent for 30 min before the reaction was stopped by the addition of 1 M H_2SO_4. Absorbance at 450 nm was measured in a Tecan Genios Pro plate reader with a reference wavelength of 650 nm.

Tryptic digestion and iTRAQ labeling

Acetone pellets of solubilized samples were resuspended in 28 μl of RapiGest SF Buffer (Waters, USA) and digested in-solution by trypsin as described previously [22]. In short, the proteins were first reduced by adding 2 μl of reducing agent (iTRAQ Reagent Kit, Applied Biosystems, USA). After incubation for 1 h, 1 μl of 200 mM iodoacetamide was added and vortexed for 20 min. Subsequent digestion was done by adding trypsin (1:20 trypsin/protein ratio) dissolved in 100 mM triethylammonium bicarbonate (TEAB) buffer to each sample. The samples were digested overnight at 37 °C while shaking. The tryptic peptides were then tagged with iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems). The brain extract and the standard protein mix were tagged with iTRAQ 116 and 117, respectively. After iTRAQ labeling, the samples were mixed and acidified by 5% trifluoroacetic acid (TFA, pH 2.0) and incubated at 37 °C for 30 min. The samples were then centrifuged at 4 °C for 30 min at 21,000*g* to remove the RapiGest SF.

After digestion, the samples were fractionated on an ICAT SCX (strong cation exchanger) column according to the manufacturer's instructions (Applied Biosystems). Briefly, the iTRAQ-labeled samples were dissolved in 2 ml of loading buffer (10 mM KH_2PO_4 in 25% acetonitrile, pH 3.0) and subsequently washed with 1 ml of loading buffer. The peptides were then step eluted with 500 μl of a KCl solution (5, 100, 150, 200, 300, 400, 500, 600, 800, and 1000 mM) in 25% acetonitrile (pH 3.0). Each fraction was dried in

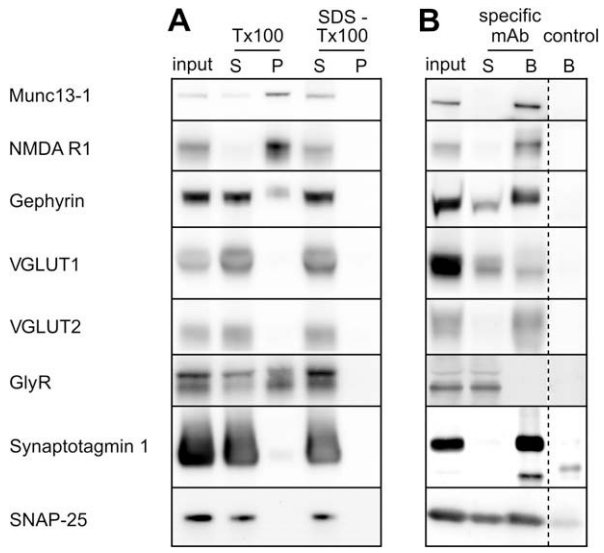

Fig. 1. (A) Detergent extraction of membrane and scaffold proteins from a fraction enriched in SJC. Here 600 μg of SJC was solubilized at 3 mg/ml in 1.2% SDS and diluted by the addition of 5 volumes of ice-cold 1.2% Triton X-100 (Tx100). A control sample treated in parallel but with SDS being omitted is shown for comparison. The extracts were centrifuged at 100,000*g* to pellet insoluble material (P). Input and supernatant (S) were concentrated by acetone precipitation, and all fractions were resuspended in an equal volume of SDS sample buffer. Samples corresponding to 15 μg of starting material from each fraction were separated by SDS–PAGE, followed by immunoblotting for the proteins indicated. The blots are representative of four independent experiments. GlyR, glycine receptor. (B) Immunoprecipitation after SDS extraction. Here 100 μg of SDS-solubilized SJC was incubated with 2–4 μl of mouse ascites or 6 μg of purified IgG and 30 μl of protein G–Sepharose overnight at 4 °C. Beads were washed three times and resuspended in 50 μl of SDS sample buffer (B). Input and supernatant (S) were concentrated by acetone precipitation. Samples corresponding to 10–20 μg of input were analyzed by SDS–PAGE/immunoblotting. Control incubations were carried out using an unspecific mouse IgG fraction (except for GlyR and NMDA R1 where the control contained no IgG). The blots are representative of at least two independent experiments. The dotted line indicates that samples were run on the same gel but not in adjacent lanes.

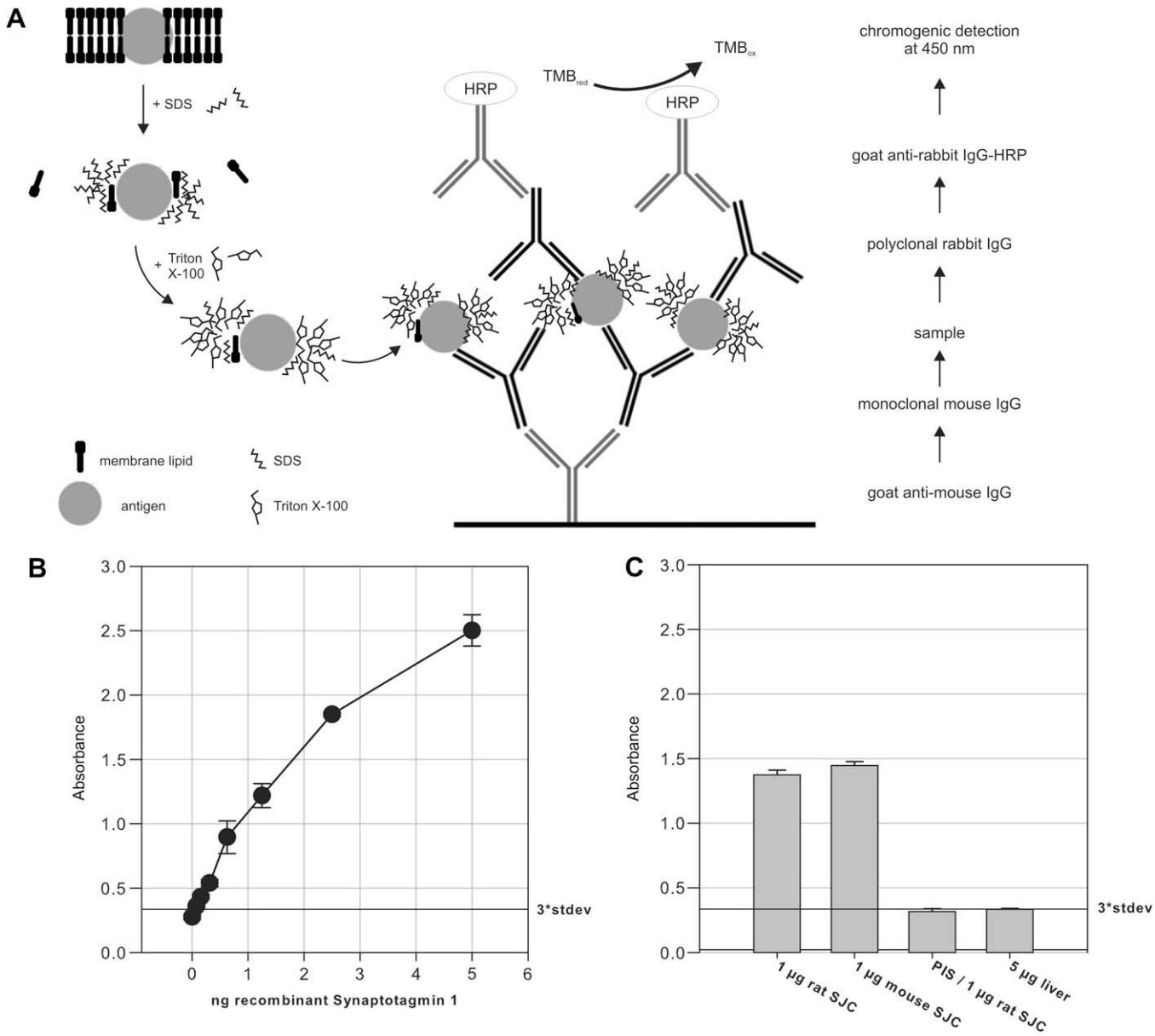

Fig. 2. ELISA for insoluble proteins: Assay principle and quantification of synaptotagmin 1, an integral membrane protein of synaptic vesicles. (A) Schematic overview of the procedure (see text for details). Following SDS extraction and the addition of Triton X-100, the samples were incubated on microtiter plates that were coated with goat anti-mouse IgG and decorated with the capture mAb. Sequential incubations were then carried out as indicated on the right. (B) Standard curve obtained with recombinant full-length synaptotagmin 1. (C) Analysis of extracts of rat and mouse SJC fractions for synaptotagmin 1. Controls included using an unspecific rabbit serum (PIS) instead of the detecting antibody and liver extracts where synaptotagmin 1 is not expressed. The graphs represent a typical experiment with average and standard deviations from triplicate values. The level of three standard deviations of the background absorbance (3 stdev) is shown to indicate the detection limit.

a vacuum centrifuge and redissolved in 0.3% TFA. To remove the salt from the samples prior to MS analysis, the samples were desalted on a handmade microcolumn with Poros Oligo R2 RP material as described previously [23]. The samples were washed with 70 µl of 0.1% TFA, eluted with 20 µl of 50% acetonitrile in 5% formic acid, and dried in a vacuum centrifuge.

MS and quantification

For liquid chromatography–tandem mass spectrometry (LC–MS/MS), the dried fractions were redissolved in 10% acetonitrile and 0.15% formic acid and were analyzed on a Thermo LTQ XL Orbitrap (Thermo Fisher Scientific, Bremen, Germany) coupled to an Agilent 1100 series LC system (Agilent Technologies, USA). Peptides were separated at a flow rate of 200–300 nl/min on a self-made reversed phase column (C18, Reprosil, Maisch, Germany). Elution of the peptides was done with a 118-min gradient from 7.5% to 37.5% mobile phase B (80% acetonitrile and 0.15% formic acid). Peak lists were searched against the NCBI RefSeq database using the Mascot search engine (version 2.2.04). Mass accuracy was 10 ppm for the parent ion and 30 ppm for fragment ions. The peptides were constrained to be tryptic with a maximum of two missed cleavages. Carbamidomethylation of cysteines was considered as a fixed modification, whereas oxidation of methionine residues was considered as a variable modification. Quantification was done using Mascot (version 2.2.04). The protein ratio was calculated as a weighted median ratio where peptides with scores above 15 were used for quantification. Only proteins quantified with unique peptides and a minimum of three peptides have been included.

Results

Solubilization and immunoprecipitation of synaptic proteins

To establish a solubilization protocol for insoluble proteins, which is compatible with subsequent immunoprecipitation, we took advantage of the fact that SDS can be "quenched" by the addition of cold Triton X-100, partially restoring antigenicity while preventing denaturation of added antibodies [11,12]. For testing, we analyzed a rat brain fraction enriched in SJC for a panel of synaptic proteins that are thought to be either free integral membrane proteins (vesicular glutamate transporters), integral membrane proteins participating in protein–protein interactions within the membrane (the SNARE SNAP-25 and the vesicular Ca^{2+} sensor synaptotagmin 1), integral proteins linked to protein scaffolds (NMDA R1 and glycine receptor), or scaffold proteins of the presynaptic active zone (munc13-1) or of the PSD (gephyrin) [8,14]. The membrane proteins were treated with SDS at different detergent/protein ratios, from which we chose 4:1 (w/w) as the minimal detergent concentration required for complete solubilization. Following Refs. [11] and [13], the extract was diluted with Triton X-100 at a Triton/SDS ratio of 5:1 (w/w) and was centrifuged to separate soluble and insoluble content, shown in Fig. 1A compared with solubilization by Triton X-100 alone. As expected, Triton alone was not capable of quantitatively extracting scaffold or scaffold-anchored proteins. Similar results were obtained with other nondenaturing detergents, including octylglucoside, sodium cholate, and the zwitterionic detergent SB3-10 (data not shown). In contrast, all proteins are efficiently solubilized by SDS and remain soluble after the addition of Triton (Fig. 1A).

Next, we screened mouse mAbs for their ability to precipitate synaptic proteins from the SDS/Triton extract. Using a panel of 37 different mAbs, the samples were incubated overnight with the solubilized proteins, followed by capture of the immunocomplexes by incubation with protein G–Sepharose. Of the 37 mAbs examined, 29 (78%) resulted in specific immunoprecipitation of the respective antigen. Representative experiments are shown in Fig. 1B. For several proteins, immunoprecipitation was nearly quantitative (e.g., munc13-1, NMDA R1, VGLUT2, synaptotagmin 1). For others, such as VGLUT1, gephyrin, and SNAP-25, precipitation was not quantitative, although an excess of both antibodies and protein G–Sepharose was used. Finally, some proteins, such as the glycine receptor, could not be precipitated by any of the available antibodies. We also carried out immunoprecipitations using lower (2.5:1) and higher (10:1) ratios of Triton X-100/SDS, yielding virtually identical results (not shown). Together, our results show that effective solubilization and immunoprecipitation can be achieved with the SDS/Triton extraction protocol, a prerequisite for the development of an ELISA.

Table 1
Characterization of ELISA for a subset of proteins.

	Detection limit	*S/N* =2	Concentration in mouse brain	Spiking recovery (%)
Gephyrin	8 ng/ml (0.09 nM)	3 ng	2.5 ± 0.1 ng/μg (liver 0.9 ± 0.1 ng/μg)	63–138
Munc13-1	28 ng/ml (0.14 nM)	13 ng	Quantifiable only in membrane fraction	82–130
NMDA R1	78 ng/ml (0.74 nM)	19 ng	4.8 ± 0.2 ng/μg	39–84
SNAP-25	6 ng/ml (0.25 nM)	2 ng	2.4 ± 1.6 ng/μg	61–86
Synaptotagmin 1	0.24 ng/ml (5.1 pM)	37 pg	102 ± 20 pg/μg	87–120
VGLUT1	77 pg/ml (1 pM)	42 pg	26 ± 2 pg/μg	104–143
VGLUT2	11 ng/ml (0.17 nM)	5 ng	Quantifiable only in membrane fraction	76–111

Note. The standard curve of each protein is described by the detection limit, which was determined as the protein amount corresponding to background absorbance plus 3-fold standard deviation. In addition, the amount of standard protein corresponding to an absorbance signal-to-noise ratio of 2 ($S/N = 2$) is given to delineate the optimal measurable range. Protein expression in adult mouse brain was averaged from at least three experiments. The percentage of spiking recovery indicates how much of the expected signal increase was obtained by the addition of different amounts of standard protein to brain extract.

Development of ELISA for synaptic proteins solubilized by the SDS/Triton procedure

To establish an assay for quantification, we turned to a classical sandwich immunoassay using two specific antibodies: a

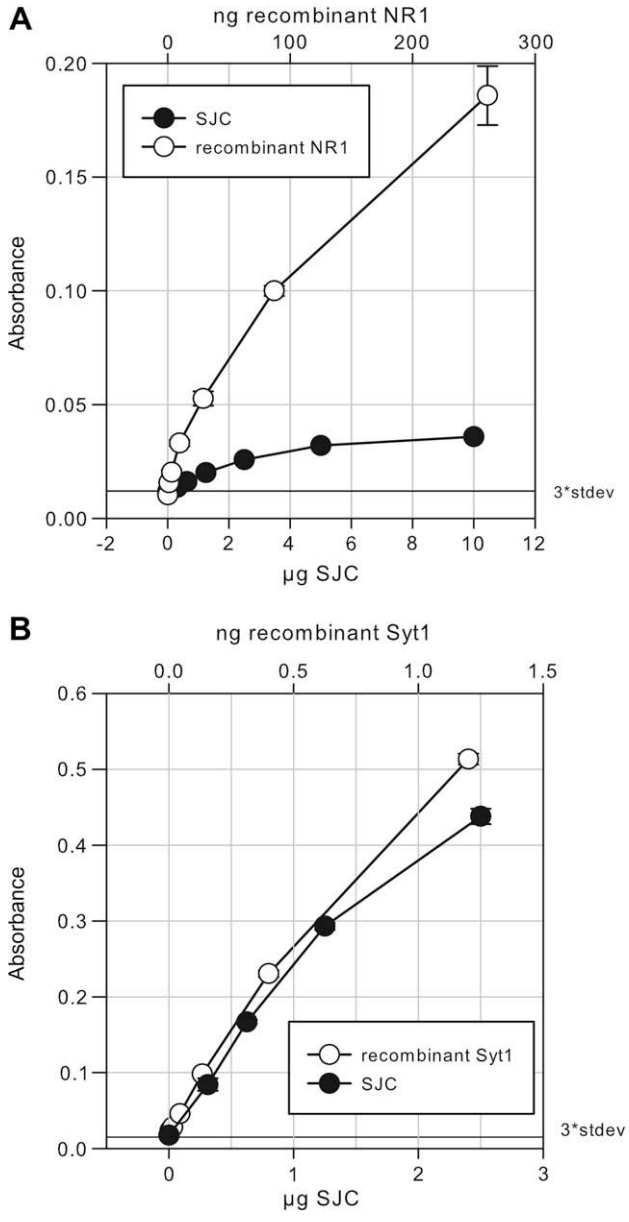

Fig. 3. Concentration dependence of the ELISA signal with two different antigens using purified standard proteins and membrane extracts. Increasing amounts of SJC extracts were assayed for NMDA R1 (NR1) (A) and synaptotagmin 1 (Syt1) (B) and were compared with the signals obtained with recombinant purified standard proteins. One of three independent experiments is plotted with the averages and standard deviations from triplicate values.

monoclonal capture antibody and a polyclonal detector antibody (Fig. 2). Because the commonly applied adsorptive binding of a protein to a surface is associated with partial denaturation and loss of function [24], we coated microtiter plates with goat anti-mouse IgG to allow binding of the capture antibody in an active form. For measuring the polyclonal detector antibody, goat anti-rabbit IgG coupled to HRP was added as a secondary detector, thereby avoiding the need for direct coupling of the specific antibody. Because proteins may lose antigenicity or aggregate when frozen and stored as Triton X-100 extracts, the SDS/Triton solubilization was performed directly before the ELISA. Samples were then diluted to an appropriate protein concentration and to 0.2% (v/v) Triton X-100. During development of the ELISA protocol, we found that most of the background was due to unspecific binding of the polyclonal rabbit serum. We tested the blocking efficiency of several compounds and finally decided to use a mixture of TP, BSA, and gelatin because each one performed best for a subset of antigens.

In total, 17 antibody pairs were found to be principally suitable for the quantification of synaptic proteins using our ELISA protocol. Of these 17 antibody pairs, 7 were selected for further standardization (Table 1), as exemplified for synaptotagmin 1 in Fig. 2. For calibration, recombinant synaptotagmin 1 (Fig. 2B) was diluted in liver extracts to approximate the sample complexity of the brain extracts. As can be seen from the figure, a nearly linear dependence of the signal on the amount of synaptotagmin was observed over a 100-fold measuring range, with the detection limit being 0.24 ng/ml. To control for the specificity of the detection, preimmune rabbit serum was used (Fig. 2C). As a negative control, we used extracts from rat liver (where synaptotagmin is not expressed) that were processed in parallel. In addition, extracts from knockout mice were tested if available (SNAP-25 and VGLUT1) and did not show a signal response above background (data not shown).

For each of the seven antibody pairs, we optimized antibody concentrations and determined the average detection limit for the purified recombinant standard proteins. Intraassay variation coefficients of the standard curve in the working range were generally below 10%, whereas interassay variation coefficients were between 2% and 20%. VGLUT2 and munc13-1 did not show a linear dose–response when brain homogenate was used as starting sample. Thus, these proteins could be accurately measured only in enriched membrane preparations (data not shown). To further test whether the tissue extracts contained factors interfering with the detection of the proteins, we performed internal standardization using the recombinant proteins. The amounts of the standard proteins were adjusted to be within the measuring range of the assay and were mixed with the tissue extracts. As shown in Table 1, signal recoveries for most proteins were high but variable, ranging from 60% to 140%. Exceptions include NMDA R1 and SNAP-25, where recoveries were lower, suggesting that antibody binding to the recombinant protein is partially shielded by interfering components present in the brain extract (Table 1). Moreover, we observed that in some cases the standard proteins had a higher linear range and yielded much higher signals at saturation than those obtainable by the extracts (Fig. 3). Although some improvement was observable when the concentration of detergent in the antigen buffer or the concentration of the specific pAb was increased, a 2-fold (or larger) difference remained (data not shown). Intriguingly, such deviation was seen only when the recombinant standard protein represented only a fragment of the native protein. In contrast, the ELISAs for synaptotagmin 1 and SNAP-25, for which recombinant full-length proteins were used as standard, did not show this behavior (Fig. 3) (see Discussion).

Comparison with other quantification techniques and possible applications

To further validate the ELISA quantification, we analyzed the same sample in parallel by ELISA, Western blotting, and quantitative MS following isobaric labeling of standard and test samples (Fig. 4and Table 2). Western blotting is commonly used for relative quantification and occasionally absolute quantification, depending on the (frequently not validated) assumption that there is no interference by sample factors after SDS–PAGE and transfer. Furthermore, sample concentrations need to be adjusted to ensure that the signals are in the linear range of the detection system. Isobaric labeling by iTRAQ reagents is based on chemical tagging of the (tryptic) peptides derived from different samples with specific isobaric tags (e.g., iTRAQ 116 and 117) that are subsequently mixed and analyzed by LC–MS/MS [2]. Along with the fragmentation, the tags are split and release characteristic reporter ions, whose ratios can be accurately measured for each peptide in the MS/MS spectrum.

Six standard proteins were combined in one mastermix standard and employed to determine the concentration of a rat brain preparation (S1). Both standard and sample were solubilized and either used directly for ELISA or precipitated for Western blotting and iTRAQ labeling. Despite the large complexity of the sample, three of the endogenous proteins (NMDA R1, SNAP-25, and synaptotagmin 1) could be quantified by iTRAQ. Again, the analysis revealed differences for different proteins. For NMDA R1, the ELISA

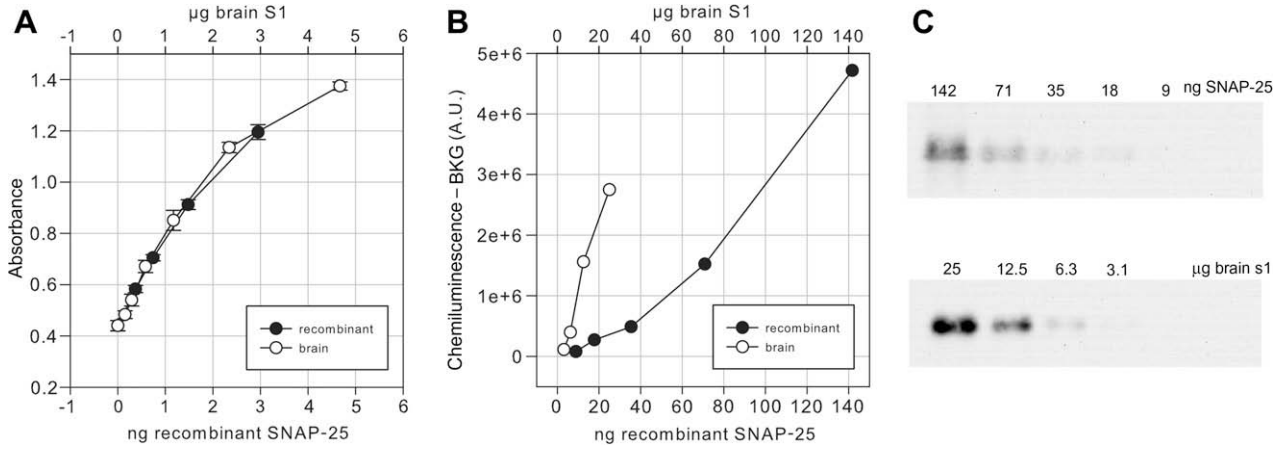

Fig. 4. Comparison of ELISA with Western blot (SNAP-25). Purified recombinant SNAP-25 and rat brain extract were measured by ELISA (A). The same samples were analyzed in parallel by SDS–PAGE and immunoblotting, followed by densitometry (B) as well as by iTRAQ labeling followed by two-dimensional LC–MS/MS (Table 2). A.U., arbitrary units. The blots used for panel B are shown in panel C.

Table 2
Comparison of quantification by ELISA, Western blot, and iTRAQ labeling

ng/μg Total protein	ELISA	iTRAQ	Western blot
NMDA R1	2.8 ± 0.5	3.4	7.9 ± 0.7
SNAP-25	0.69 ± 0.09	3.8 ± 0.6	3.9 ± 0.9
Synaptotagmin 1	0.38 ± 0.07	1.4 ± 0.2	1.8 ± 0.7

Note. The concentration of three proteins in rat brain S1 was determined by normalization to a standard protein mastermix. Aliquots of the same samples were processed for each technique. Values were obtained as averages and standard deviations of serial dilutions within the working range of the standard curve (n = 2–5 [see Fig. 4]). In the case of NMDA R1, the protein could only be quantified from one dilution by MS. The absolute values are given as ng of the full-length protein (NMDA R1, SNAP-25, or synaptotagmin 1) per μg of total protein (ng/μg total protein).

agreed with the iTRAQ/MS analysis, whereas the Western blot values were approximately 2-fold higher. In contrast, for SNAP-25 and synaptotagmin 1, the ELISA values were several times lower than those determined by the other methods (Fig. 4 and Table 2) and, therefore, would need to be multiplied by factors of 5.5 and 3.7, respectively, to obtain absolute quantification.

Because expression profiling is a routine element of knockout mouse analysis, we applied our protocol to measure relative protein expression and compare mouse brain samples (Fig. 5). When synaptotagmin 1 expression was assayed in embryonal and adult brain homogenate, average values obtained from two experiments were 6 ± 2 pg/μg in embryonal brain and 102 ± 20 pg/μg in adult brain, revealing a 17-fold increase during development. In contrast, gephyrin expression did not differ between embryonal and adult ages because the average expressions from two experiments were 2.1 ± 0.6 ng/μg for embryonal brain and 2.5 ± 0.1 ng/μg for adult brain. To investigate whether our sandwich antibody pairs would be suitable for a multiplexed assay, we used the same brain extract sample to detect six proteins successively in variable order (Fig. 6). Regardless of the position of the protein in the series, the absorbance level reached at least 80% of the initial signal, demonstrating that each antibody pair causes only minimal disturbance of the other measurements, thereby enabling efficient use of samples.

Discussion

Despite rapid advances in high-throughput proteomics, quantification of proteins in complex samples has remained a major bottleneck for systems biology. Although isotope labeling combined with MS/MS analysis holds the potential for filling this gap

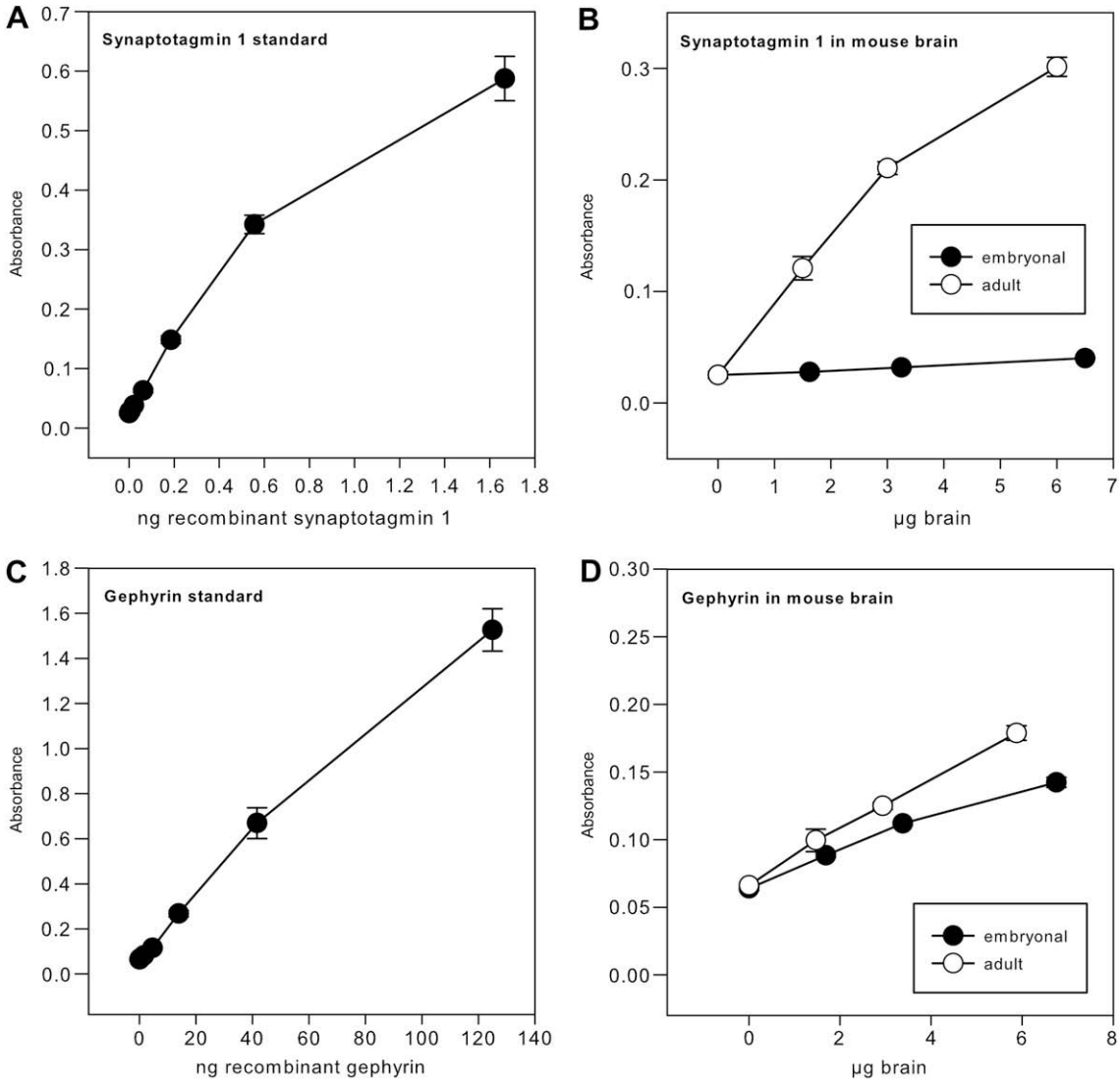

Fig. 5. Quantification by ELISA of synaptotagmin 1 and gephyrin in mouse brain. One of three independent experiments is plotted with the averages and standard deviations from triplicate values.

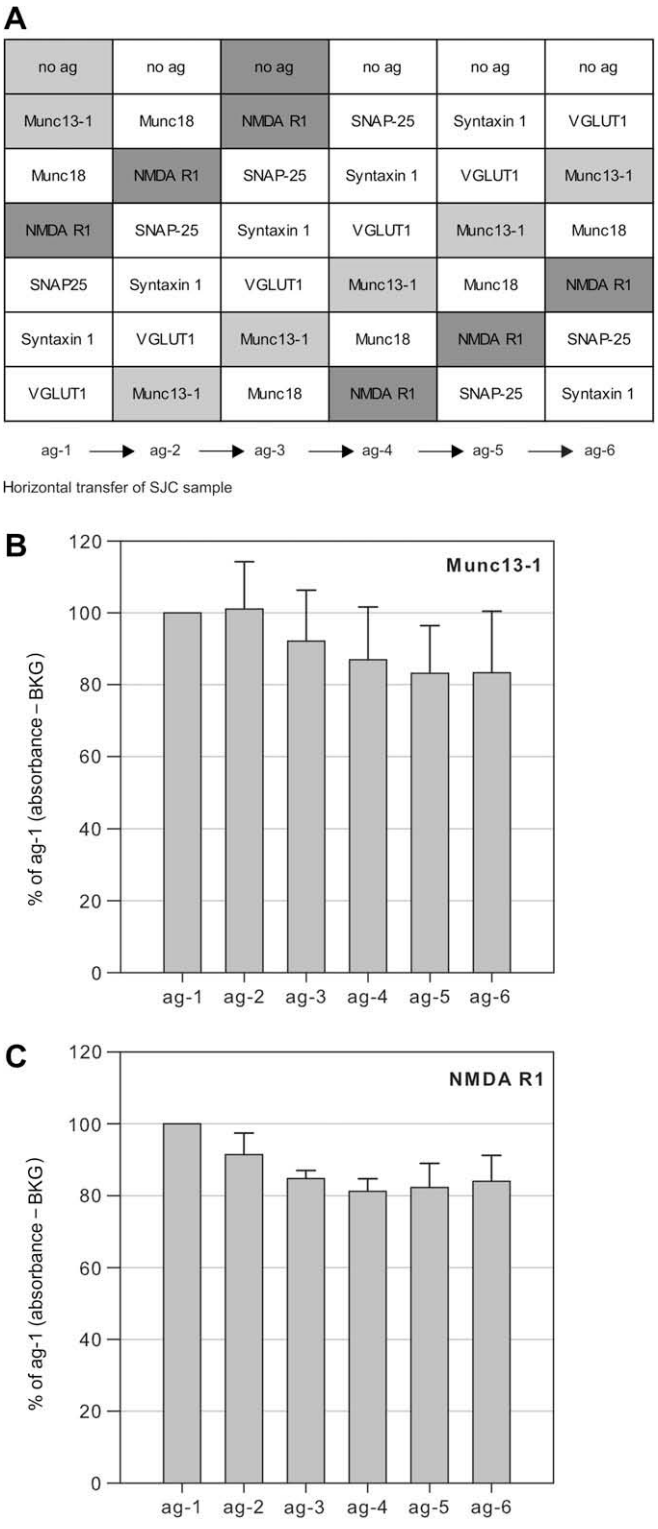

Fig. 6. Sequential measurement of six different antigens in the same sample. (A) Scheme of setup. Six different capture antibodies were coated on a microtiter plate as indicated. SJC extract was applied to columns 1 and 2 for 30 min and were successively transferred to columns 3 and 4, columns 5 and 6, and so forth. On a second microtiter plate, the order of antibodies was inverted. After development, values for each incubation were averaged from both plates and normalized to the signal obtained in the first incubation (ag-1). Plots for munc13-1 (B) and NMDA R1 (C) show the results from two independent experiments.

in the foreseeable future [25], it is currently limited to the comparison of few samples, requires access to modern MS facilities with cutting-edge instrumentation, and is likely to depend on the availability of mass-tagged peptide libraries for standardization [26,27]. In particular, the analysis of insoluble proteins, such as membrane and scaffold proteins, has been lagging behind. For instance, until recently, membrane proteins have been vastly underrepresented in proteomic analyses of complex biological samples (see, e.g., Ref. [14] for a discussion), thereby frequently leaving Western blotting as the only alternative for qualitative and (semi)quantitative detection of such proteins.

Thus, the ELISA procedure developed in this study fills a gap in the analytical repertoire for insoluble proteins, considerably extending the capacity and reducing sample amounts in comparison with previous methods such as Western blotting. Previous studies of ELISA in a similar direction [6,28–30] either did not examine solubilization efficiency or established a particular extraction protocol for each protein, whereas we have presented a universal procedure suitable for different types of proteins. We focused specifically on "difficult" proteins, that is, proteins that are membrane-anchored by single or multiple transmembrane domains (VGLUTs, NMDA R1, and synaptotagmin), part of stable and insoluble protein scaffolds (NMDA R1, gephyrin, and munc13-1), or associated with other proteins in complexes of very high stability (SNAP-25). The selection of these proteins was also motivated by our interest in synaptic function. Accordingly, the set includes representative proteins of synaptic vesicles, the presynaptic active zone, postsynaptic receptors, and postsynaptic scaffold proteins [8,14], thereby allowing the monitoring of multiple synaptic parameters in a single assay.

For assay development, we originally favored direct fluorescent labeling of tissue extracts followed by capture of the antigens using single specific antibodies, a strategy commonly used for chip-based assays with protein arrays (antibodies in this case). However, this approach turned out to be not feasible because of high unspecific background (unpublished observations). Therefore, we resorted to a classical sandwich ELISA (Fig. 2) and successfully adapted the method for 17 antibody pairs. To enable convenient handling, all incubations were adjusted so that the assay can be carried out in less than 2 days. The assay sensitivity varied between individual proteins, ranging from tens of ng/ml to tens of pg/ml (or 74–0.1 fmol per well), which generally agrees with ELISAs for soluble antigens [4]. To our knowledge, NMDA R1 is the only protein of the set studied here for which a comparable assay has been published, with longer incubation times and a higher sensitivity being reported [29].

The systematic investigation of variables allows a comprehensive assessment of both the advantages and limitations of the new procedure. Although the sandwich assay itself largely follows standard protocols (except for the need to use detergents) [4], sample preparation and sample complexity turned out to be critical factors. The following conclusions can be drawn. First, the combination of SDS extraction followed by quenching of SDS by excess Triton X-100 appears to be generally applicable for (i) solubilizing proteins that otherwise are nonextractable and (ii) recovering antigenicity after SDS quenching while keeping the proteins in solution. Recovery of antigenicity after SDS denaturation is not surprising given that Western blotting is based on the same principle. Our finding that most proteins can be immunoprecipitated from such extracts was a prerequisite for the development of a capture assay. However, our data also show that some proteins cannot be completely recovered by immunoprecipitation even if saturating amounts of antibody are used (e.g., SNAP-25, VGLUT [see Fig. 2]), and in rare cases no immunoprecipitation was observable. Thus, it is mandatory to carry out such an initial characterization for each new protein to be analyzed by this procedure.

Second, our internal standardization experiments revealed that protein recovery is generally high, showing that after solubilization

the samples are stable, with little interference by macromolecular complexation or aggregation that could mask antigen accessibility taking place. The lower recovery of SNAP-25 can be easily explained by the ability of this protein to form stable SNARE complexes that are resistant to SDS [31] and in which the SNAP-25 epitope is masked [32].

Third, caution needs to be exerted in extrapolating the measuring range from the standard to the sample. We found that in some cases the signals obtainable from complex extracts saturate at much lower levels than the recombinant standard proteins. It is possible that antigen accessibility is different if a recombinant fragment is used as standard. And although we applied the standard as a mixture with liver homogenate to imitate the matrix conditions of the sample, we cannot exclude brain-specific interactions shielding the endogenous protein, thereby lowering the detection range of the assay.

Finally, our data show that particular care needs to be applied if not only comparison of parallel samples will be carried out (relative quantification) but also the absolute amounts of the proteins need to be determined (absolute quantification). Our examples show that even internal standardization (generally considered to be sufficient for quantification) does not safeguard against a significant underestimation of the total amount. Again, it appears that the conditions are different for each protein, requiring at least initial calibration of the assay against other methods using a defined set of standards that then allow determination of a correction factor. In fact, this often overlooked problem is inherent to all antibody-based assays.

To our knowledge, this is the first description of an ELISA detecting multiple membrane and scaffold proteins in raw tissue extracts. The assay is highly reproducible, is easy to handle because it requires only standard equipment for ELISAs, allows medium-scale throughput, and is suitable for both relative and absolute quantification. As in all assays of this kind, each new antigen and each antibody pair need to be carefully evaluated and compared with standards, but we are confident that the assay can easily be expanded to a large array of insoluble proteins.

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