Journal of Visualized Experiments

High resolution quantitative synaptic proteome profiling of mouse brain regions after auditory discrimination learning

--Manuscript Draft--

Manuscript Number:	JoVE54992R2
Full Title:	High resolution quantitative synaptic proteome profiling of mouse brain regions after auditory discrimination learning
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	auditory learning; synaptosomes; quantitative mass spectrometry; label-free quantification; phospho-proteomics; Bioinformatics; meta-analysis
Manuscript Classifications:	2.1.50.150.900.649.865.635.505.500: Mice; 5.5.17: Animal Experimentation; 5.5.196.181: Chromatography; 5.5.196.181.400: Chromatography, Liquid; 5.5.196.566.600: Spectrometry, Mass, Electrospray Ionization; 5.5.196.566.880: Tandem Mass Spectrometry; 5.5.196.59: Analytic Sample Preparation Methods; 95.51.4: animal models; 95.51.6: biochemistry
Corresponding Author:	Thilo Kaehne, Ph.D University Hospital Magdeburg Magdeburg, Saxony-Anhalt GERMANY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	kaehne@med.ovgu.de
Corresponding Author's Institution:	University Hospital Magdeburg
Corresponding Author's Secondary Institution:	
First Author:	Angela Kolodziej, Ph.D
First Author Secondary Information:	
Other Authors:	Angela Kolodziej, Ph.D
	Karl-Heinz Smalla, Ph.D
	Sandra Richter
	Alexander Engler
	Rainer Pielot, Ph.D
	Daniela C Dieterich, Ph.D, Prof.
	Wolfgang Tischmeyer, Ph.D
	Michael Naumann, Ph.D, Prof.
Order of Authors Secondary Information:	
Abstract:	The molecular synaptic mechanisms underlying auditory learning and memory remain largely unknown. Here, the workflow of a proteomic study on auditory discrimination learning in mice is described. In this learning paradigm, mice are trained in a shuttle box Go/NoGo-task to discriminate between rising and falling frequency-modulated tones in order to avoid mild electric foot-shock. The protocol involves the enrichment of synaptosomes from four brain areas, namely the auditory cortex, frontal cortex, hippocampus, and striatum, at different stages of training. Synaptic protein expression patterns obtained from trained mice are compared to naïve controls using a proteomic approach. To achieve sufficient analytical depth, samples are fractionated in three different ways prior to mass spectrometry, namely 1D SDS-PAGE/in-gel digestion, in-

	solution digestion and phospho-peptide enrichment. High-resolution proteomic analysis on a mass spectrometer and label-free quantification are used to examine synaptic protein profiles in phospho-peptide-depleted and phospho-peptide-enriched fractions of synaptosomal protein samples. A commercial software package is utilized to reveal proteins and phospho-peptides with significantly regulated relative synaptic abundance levels (trained/naïve controls). Common and differential regulation modes for the synaptic proteome in the investigated brain regions of mice after training were observed. Subsequently, meta-analyses utilizing several databases are employed to identify underlying cellular functions and biological pathways.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

TITLE:
High resolution quantitative synaptic proteome profiling of mouse brain regions after auditory discrimination learning

AUTHORS:
Kolodziej, Angela
Leibniz Institute for Neurobiology (LIN)
Magdeburg, Germany
angela.kolodziej@lin-magdeburg.de

Smalla, Karl-Heinz
Leibniz Institute for Neurobiology (LIN)
Magdeburg, Germany
smalla@lin-magdeburg.de

Richter, Sandra
Institute of Experimental Internal Medicine
Medical School, Otto von Guericke University Magdeburg, Germany
sandra.richter@med.ovgu.de

Engler, Alexander
Leibniz Institute for Neurobiology (LIN)
Magdeburg, Germany
alexander.engler@lin-magdeburg.de

Pielot, Rainer
Institute of Pharmacology and Toxicology
Medical School, Otto von Guericke University
Magdeburg, Germany
Rainer.Pielot@med.ovgu.de
And
Leibniz Institute for Neurobiology (LIN)
Magdeburg, Germany
pielot@lin-magdeburg.de

Dieterich, Daniela C.
Institute of Pharmacology and Toxicology
Medical School, Otto von Guericke University
Magdeburg, Germany
daniela.dieterich@med.ovgu.de

Tischmeyer, Wolfgang
Leibniz Institute for Neurobiology (LIN)
Magdeburg, Germany

tischmeyer@lin-magdeburg.de

Naumann, Michael
Institute of Experimental Internal Medicine
Medical School, Otto von Guericke University Magdeburg, Germany
naumann@med.ovgu.de

Kähne, Thilo
Institute of Experimental Internal Medicine
Medical School, Otto von Guericke University Magdeburg, Germany
kaehne@med.ovgu.de

CORRESPONDING AUTHOR:
Kähne, Thilo
kaehne@med.ovgu.de

KEYWORDS:
auditory learning, synaptosomes, quantitative mass spectrometry, label-free quantification, phospho-proteomics, bioinformatics, meta-analysis

SHORT ABSTRACT:
The identification of molecules and pathways controlling synaptic plasticity and memory is still a major challenge in neuroscience. Here, a workflow is described addressing the relative quantification of synaptic proteins supposedly involved in the molecular reorganization of synapses during learning and memory consolidation in an auditory learning paradigm.

LONG ABSTRACT:
The molecular synaptic mechanisms underlying auditory learning and memory remain largely unknown. Here, the workflow of a proteomic study on auditory discrimination learning in mice is described. In this learning paradigm, mice are trained in a shuttle box Go/NoGo-task to discriminate between rising and falling frequency-modulated tones in order to avoid a mild electric foot-shock. The protocol involves the enrichment of synaptosomes from four brain areas, namely the auditory cortex, frontal cortex, hippocampus, and striatum, at different stages of training. Synaptic protein expression patterns obtained from trained mice are compared to naïve controls using a proteomic approach. To achieve sufficient analytical depth, samples are fractionated in three different ways prior to mass spectrometry, namely 1D SDS-PAGE/in-gel digestion, in-solution digestion and phospho-peptide enrichment.

High-resolution proteomic analysis on a mass spectrometer and label-free quantification are used to examine synaptic protein profiles in phospho-peptide-depleted and phospho-peptide-enriched fractions of synaptosomal protein samples. A commercial software package is utilized to reveal proteins and phospho-peptides with significantly regulated relative synaptic abundance levels (trained/naïve controls). Common and differential

regulation modes for the synaptic proteome in the investigated brain regions of mice after training were observed. Subsequently, meta-analyses utilizing several databases are employed to identify underlying cellular functions and biological pathways.

INTRODUCTION:

Learning is based on the formation of memory traces and their maintenance. It is widely accepted that one underlying mechanism may represent an activity-dependent formation of new and/or rearrangement of existing synaptic contacts between neurons. On the molecular level, various protein modifications, subcellular relocalizations and changes in the turnover of synaptic proteins have been described[1-4]. However, most studies so far focused on selected proteins rather than on the global but complex synaptic proteome composition. The present approach allows an unbiased screening for synaptic proteome changes in mouse brain regions after a learning experiment. It is suitable to render time-point dependent molecular snapshots of the learning-induced reorganization of the synaptic architecture. The described workflow requires a particular teamwork of different specialists in animal behavior, protein biochemistry, mass spectrometry and bioinformatics.

The chosen learning paradigm, i.e. frequency-modulated tone discrimination (FMTD), is a well-characterized auditory discrimination task in rodents[5]. Learning and long-term memory formation in this shuttle box Go/No-Go-task involves mechanisms depending on increased cortical dopamine signaling and protein synthesis. Accordingly, recent proteomic studies on gerbils and mice revealed dopamine- and learning-induced plastic rearrangements of synaptic components in cortical, but also in more basal brain regions that supposedly interact during FMTD learning and memory[6-8]. This illustrates that memory formation involves a complex interplay of various brain regions and thus, might be differentially regulated within these regions on the proteome level. Therefore, dissection of selected cortical and subcortical mouse brain regions is included in the workflow.

Furthermore, the reliable characterization even of weak changes in synaptic protein composition requires an enrichment of pre- and postsynaptic compartments rather than the analysis of homogenates or crude membrane fractions[9]. Therefore, the preparation of synaptosomes utilizing established protocols prior to proteomic analysis is described in order to increase the detection level and the dynamic range for synapse-specific proteins[10,11].

An essential prerequisite to use label-free high-resolution mass spectrometry for quantitative purposes is a high degree of similarity of protein samples. As rather minor changes in synaptic protein composition are expected to occur after learning, a label-free approach will be appropriate to compare corresponding protein samples obtained from trained and naïve mice. Alternatively, condition-specific label strategies of proteins/peptides using stable isotopes (e.g. TMT, iTRAQ , ICPL and SILAC) as well as MS2-based label free quantification (SWATH) are useful, but they are more expensive than the chosen label-free approach or need special mass spectrometric hardware.

Since proteomic screenings often yield complex data sets, bioinformatic processing is recommended for appropriate data interpretation. Additional meta-analyses may support a better understanding of potential molecular mechanisms underlying paradigm-related changes and the identification of involved key cellular processes and signaling pathways. Appropriate methodologies are also described below.

PROTOCOL:

All procedures including animal subjects were performed in accordance with the regulations of the German Federal Law, the respective EU regulations and NIH guidelines, and have been approved by the ethics committee of the Landesverwaltungsamt Sachsen/Anhalt (42502-2-1102 IfN).

1. Auditory learning

1.1. Auditory discrimination learning in the shuttle box (FMTD paradigm)

1.1.1. House C57Bl6/J mice in groups of three or four with free access to food pellets and tap water in clear polycarbonate cages. Maintain a 12 h light:dark cycle in the animal facility. If animals are received from another lab or from a company allow at least one week of acclimation and settling in.

1.1.2. Perform one shuttle box training session per day.

1.1.2.1. Take the mouse from its home cage in the animal facility and place it in a dimly lit shuttle box within a sound proof chamber.

1.1.2.2. Use a fully computer-controlled learning schedule for auditory discrimination learning. Begin with a habituation period of 3 min of silence, and then start the training session.

1.1.2.2.1. Use sequences of the rising tone (4-8 kHz, CS+) as the Go-stimulus during Go-trials: The animal has to cross the hurdle within 6 s of tone presentation (correct response, hit). Punish a miss by a mild foot-shock of 50-300 µA, delivered via the grid floor of the shuttle box.

1.1.2.2.2. Use sequences of the falling tone (8-4 kHz, CS-) as the No-Go-stimulus during No-Go-trials: The animal has to remain in the current compartment of the shuttle box during the 6 s of tone presentation. Punish a false alarm by a mild foot-shock of 50-300 µA, delivered via the grid floor of the shuttle box.

1.1.2.3. Use intertrial intervals of 20 $\pm$ 5 s.

1.1.2.4. Perform 30 Go-trials and 30 No-Go-trials per session in a pseudo-randomized order, so that one session consists of 60 trials and lasts about 25 min.

1.1.3. Put the trained animal back into its home cage in the animal facility.

1.2. Brain dissection

1.2.1. Euthanize the animal at the desired time point after a desired number of training sessions using cervical dislocation (*e.g.* 24 h after completion of the first session). Decapitate the animal.

1.2.2. Quickly dissect the brain via the following steps: Cut first the skin and then the skull with straight scissors along the *Sutura sagittalis.* Completely remove the parts of the bone which cover the brain tissue using strong forceps. Take out the brain with a spattle.

1.2.3. For dissection, place brain onto a Petri dish filled with ice. Dissect the auditory cortex, the frontal cortex, the striatum and the hippocampus under a stereomicroscope using a scalpel and a needle.

1.2.3.1. Localize the auditory cortex using visual landmarks on the brain surface such as blood vessels and the shape of the surface (Bregma -2.06 to -3.4, size rostrocaudal 2 mm, dorsoventral 1.3 mm) and bilaterally dissect as a rectangular tissue block with the thickness of the cortex.

1.2.3.2. Dissect the frontal cortex as a brain slice between Bregma 3.56 and 1.54 using the *chiasma opticum* as a landmark and excluding tissue from *bulbus olfactorius*.

1.2.3.3. Dissect the striatum as a brain slice between Bregma 1.54 and 0.5 and carefully remove cortical tissue.

1.2.3.4. Dissect the hippocampus by fixing the brain with the needle through the cerebellum and uncoiling the cortex starting at the occipital lobe.

1.2.4. Shock-freeze dissected brain samples in liquid nitrogen and store at -80 °C.

2. Preparation of synaptosomes or alternatively a post-synaptic-density (PSD)-enriched fraction

Note: During all procedures, keep samples and buffers at 0 - 4 °C. Buffers contain freshly diluted protease inhibitor cocktails in order to prevent proteolytic degradation of proteins. If protein phosphorylation is also studied, phosphatase inhibitor cocktails have to be added. All g-values indicated are given as g (average) throughout the whole protocol.

2.1. Preparation of a crude membrane fraction (Figure 3A)

2.1.1. Transfer dissected brain tissue into a homogenization vessel containing 1 mL ice cold buffer A (5 mM HEPES, 320 mM sucrose, pH 7.4) and homogenize tissue at 900 rpm with 12 strokes.

2.1.2. Centrifuge samples at 1000 x g for 10 min. Keep the supernatants.

2.1.3. Re-homogenize pellets at the same conditions in the same volume of homogenization

buffer as before and centrifuge samples again at 1000 x g for 10 min. Combine corresponding supernatants. Discard the pellets P1, which mainly contain nuclei and cell debris.

2.1.4. Spin the combined supernatants for 20 min at 12 000 x g. Discard supernatants or use for further fractionation[11].

2.1.5. Resuspend pellets in the same volume of homogenization buffer as before using the homogenizer with 6 strokes at 900 rpm and spin at 12 000 x g for 20 min. Discard supernatants. The pellets P2 represent the crude membrane fractions.

2.2. Purification of synaptosomes from crude brain membrane fractions (Figure 3A)

Note: Crude brain membrane fractions can be separated into myelin, light membranes, synaptosomes and mitochondria using sucrose density step gradient ultracentrifugation. For this 5 mM Tris/HCl pH 8.1 buffers containing sucrose at either 0.32 M, 1.0 M or 1.2 M concentration are required.

2.2.1. While performing the centrifugation to produce the P2 fractions, prepare sucrose step gradients in the ultracentrifuge tubes. Start with 2.5 mL 1.0 M sucrose buffer and sublayer with 1.5 mL 1.2 M sucrose buffer using a glass Pasteur pipet.

2.2.2. Re-homogenize P2 fractions in 0.5 mL of 0.32 M sucrose buffer manually with 6 strokes and load on top of the gradient.

2.2.3. Spin at 85 000 x g for 2 h in an ultracentrifuge using a swinging bucket rotor.

2.2.4. Discard the top 0.32 M sucrose layer including the material at the interface to the 1.0 M sucrose buffer (myelin, light membranes). Collect synaptosomes at the 1.0/1.2 M sucrose buffer interface. The pellet at the bottom of the tube contains mitochondria.

2.2.5. Add 0.32 M sucrose buffer to the synaptosomal fraction at 1:1 ration, mix carefully and spin at 150 000 x g for 1 h. Synaptosomes are in the pellet and can now be resuspended in a buffer required for further processing.

2.3. Preparation of a PSD-enriched fraction (Figure 3B)

2.3.1. Homogenize each specific brain area from a single animal in 100 µL extraction buffer (5 mM Tris/HCl pH 8.1, 0.5% Triton X-100) in a 200 µL ultracentrifuge tube with a PTFE (polytetrafluorethylene) pestle at 2000 rpm with 12 strokes.

2.3.2. Add 100 µL extraction buffer, mix and incubate for 1 h at 4 °C. Spin down at 100 000 x g for 1 h and collect the supernatant S1 carefully with a 200 µL pipet.

2.3.3. Re-homogenize pellet P1 in the same tube with 100 µL extraction buffer again with a PTFE pestle at 2000 rpm with 12 strokes.

2.3.4. Add 100 µL extraction buffer and mix well with a pipette and spin at 100 000 x g for 1 h.

2.3.5. Combine the supernatant S2 with S1 to the soluble protein fraction. This fraction contains cytosolic proteins, 0.5% Triton X-100 soluble membrane proteins and extracellular matrix molecules.

2.3.6. Resuspend the remaining pellet in 50 µL 5 mM Tris/HCl pH 8.1. This fraction contains PSDs, detergent-resistant membranes, insoluble cytoskeletal elements, mitochondria and cell debris including nuclei. It is enriched in PSDs which form the core of postsynaptic structures but also important parts of the presynaptic cytomatrix at the active zone. The factor for enrichment of PSDs is around 4 and the enrichment of PSD components has been demonstrated previously.[12]

3. Sample preparation for mass spectrometry

3.1. Lysis and sample normalization

Note: Sample normalization concerning the protein concentration is a very crucial step to finally achieve reliable quantitative data even for weak synaptic protein expression changes.

3.1.1. Dissolve synaptosomes or PSD-enriched preparations of each brain area of an animal in 20-50 µL (dependent on total amount of material: for auditory cortex with 5-15 mg tissue use 20 µL) of 8 M urea and incubate on ice for 1 h in an ultrasonic bath.

3.1.1.1. For in-gel digest, dissolve synaptosomes directly in the SDS-sample buffer. Carefully calculate the loaded amount to avoid overload of the gel. Consider that in this case, the high abundant scaffold proteins will be lost during the gel electrophoresis and in-gel digest.

3.1.2. Dilute with 1% of a removable detergent to ensure a final concentration of 2 M urea. Avoid any temperature higher than 30 °C to prevent protein carbamylation.

3.1.3. Perform SDS-PAGE with an aliquot (*e.g.* 10 µL) of the sample according to standard procedures[13,14].

3.1.4. Stain the gel with Coomassie Blue according to manufacturer's protocol. The procedure combines the fixing and staining step with methanol and acetic acid.

3.1.5. Determine the optical density of each sample for the whole lane with a calibrated gel scanner in transmission mode and calculate the relative protein amount.

3.1.6. Normalize the samples according to these calculations.

3.1.7. Split each sample into two different parts. Use one third for the in-gel digest and two thirds for the in-solution digest.

3.2. In-gel digest

3.2.1. **Gel separation**

3.2.1.1. Perform a second SDS-PAGE utilizing the concentration-adjusted samples. Stain and quantify the gels for a second time to check the normalization quality.

3.2.1.2. Cut out each lane of a sample within the gel in different areas (8/lane) but exclude the molecular weight range above 170 kDa. Transfer the gel pieces into separate tubes.

3.2.1.3. Cut the areas in smaller pieces (approx. 1 x 1 mm) with a sharp scalpel to facilitate in-gel digestion efficacy.

3.2.2. **Digest**[15]

3.2.2.1. Wash the gel pieces several times (depending on staining intensity) for 10 min with 50-150 µL of a buffer consisting of 50% acetonitrile (ACN) and 50 mM ammonium hydrogen carbonate (NH_4HCO_3).

3.2.2.2. Remove supernatants. Cover the gel pieces with ACN and incubate at 20 °C until gel pieces become white and shrink.

3.2.2.3. Remove the ACN and rehydrate the gel pieces for 5 min with 50 µL of 0.1 M NH_4HCO_3. Add the same volume of ACN and incubate for further 15 min at 37 °C.

3.2.2.4. Remove and discard liquid completely. Dry the gel pieces in a vacuum centrifuge.

3.2.2.5. Rehydrate gel pieces in 50 µL of NH_4HCO_3 containing 10 mM dithiothreitol (DTT) and heat samples for 45 min at 56 °C to reduce cysteine residues.

3.2.2.6. Remove supernatants and add 50 µL NH_4HCO_3 containing 55 mM iodoacetamide (IAA) for 30 min in the dark to carbamidomethylate reduced cysteines.

3.2.2.7. Remove and discard all liquid above the gel pieces and wash them twice with 50 µL NH_4HCO_3 and ACN (1:1) for 10 min to remove any residual IAA. Dry samples in a vacuum centrifuge.

3.2.2.8. For limited digestion of proteins add 25 mM NH_4HCO_3 containing 12.5 ng/µL of trypsin. The required volume depends on size and amount of the gel pieces. Incubate for a few minutes and check if the buffer is absorbed. Add more buffer if necessary, gel pieces should be completely covered. Incubate at 37 °C overnight (min. 12 h).

3.2.3. Peptide extraction

3.2.3.1. Overlay gel pieces with 10-20 µL of 25 mM NH_4HCO_3 and add the same volume of ACN. Incubate for 10 min on ice using ultrasonic bath. Afterwards remove and collect supernatants which contain most of the generated peptides.

3.2.3.2. Add 100 µL of extraction buffer containing 30% ACN/0.1% trifluoroacetic acid

(TFA) to the gel pieces. Repeat incubation in an ultrasonic bath and carefully collect this supernatant.

3.2.3.3. Repeat the last extraction steps by increasing the ACN concentration to 50%. After 10 min of ultrasonic bath spin down and collect supernatants.

3.2.3.4. Combine all three corresponding supernatants of the extraction steps and dry them in a vacuum centrifuge. Note that as a result of the gel separation the 8 areas per lane/sample are combined to one sample again in this step.

3.3. In-solution digest

3.3.1. Digest

3.3.1.1. Use the calculated amount (e.g. 100 µL of a 150 µL lysate, depends on the amount of material and the volume required for resuspension of a sample from a specific brain area) of normalized samples to obtain sufficient starting material for at least three technical replicates to perform label-free mass spectrometry.

3.3.1.2. Add 2 mM DTT in 25 mM NH_4HCO_3 and gently vortex the sample. Reduce the samples for 45 min at 20 °C.

3.3.1.3. Add 10 mM IAA to carbamidomethylate the cysteine residues. Mix and incubate for 30 min in the dark at 20 °C.

3.3.1.4. Finally, add 1 µL of a trypsin stock solution (1 µg/µL trypsin in 25 mM acetic acid) and incubate at 20 °C for 12 h.

3.3.2. **Solid-phase extraction (SPE)-Purification**

3.3.2.1. To remove the acid cleavable detergent, adjust samples to a final concentration of 1% TFA and incubate for 1 h at 20 °C.

3.3.2.2. Centrifuge samples at 16 000 x g for 10 min and carefully collect supernatants.

3.3.2.3. Place the SPE column in a rack and equilibrate the matrix with 2 mL methanol. Wash two times with 2 mL of 0.1% TFA in water (buffer B).

3.3.2.4. Add 2 mL of buffer B and load the sample. Wash another three times.

3.3.2.5. Elute the peptides by adding 200 µL 70% ACN/0.1% TFA. Repeat this step.

3.3.2.6. Pool both eluates and dry them down in a vacuum centrifuge.

3.4. Phospho-peptide-enrichment by TiO_2 chromatography[16]

3.4.1. Dissolve peptides produced by in-gel or in-solution digest in 150 µL of 80% ACN/2.5% TFA (buffer C) and equilibrate ~2 mg of the TiO_2 beads in 50 µL of buffer C.

3.4.2. Add beads to the sample and incubate in a rotating device for 1 h at 20 °C. Afterwards, spin beads down (16 000 x g, 1 min) and collect supernatants.

3.4.3. Wash the beads three times with 100 µL of buffer C by gently mixing and spinning down after 5 min. Collect supernatants. Repeat this step three times with 100 µL of 80% ACN/0.1% TFA followed by three washes with 100 µL of 0.1% TFA (without ACN), respectively.

3.4.4. Combine all ten supernatants, dry them in a vacuum centrifuge and handle them as the **phospho-peptide-depleted** fraction for further purification according step 3.5.

3.4.5. Elute the bound phospho-peptides with 20 µL of 400 mM NH_4OH/30% ACN from the beads. Repeat this step three times and collect all supernatants after spinning down the beads.

3.4.6. Combine the eluates of the in-gel digest and of the in-solution digest of a sample and handle them as the **phospho-peptide-enriched** fraction. Dry them in a vacuum centrifuge to a final volume of 4-8 µL.

3.5. Concentrating and desalting of phospho-peptide-depleted fractions by micro-SPE

3.5.1. Dissolve the dried peptides in 20 µL of 0.1% TFA.

3.5.2. Equilibrate the fixed C_{18}-matrix by drawing 20 µL ACN into the tip. Wash the matrix by drawing 0.1% TFA in water into the tip. Repeat the process three times.

3.5.3. Slowly load acidified sample into the tip (repeat this step three times).

3.5.4. Wash the C_{18}-matrix three times with 20 µL 0.1% TFA in water and discard the washing solution.

3.5.5. Elute peptides from the pipette tip by repeatedly (3 times) drawing 20 µL of 70% ACN/0.1% TFA and collect this elution solution in a separate tube.

3.5.6. Combine the eluates of a sample and dry them in a vacuum centrifuge.

4. Proteome analysis

Note: Proteome analysis is performed on a hybrid dual-pressure linear ion trap/orbitrap mass spectrometer equipped with an ultra HPLC. The HPLC is composed of a cooled autosampler with a 20 µL injection loop, a binary loading pump (µL flow range), a binary nano flow separation pump, a column heater with two micro switching valves and a degasser. Samples are firstly subjected to a trapping column (*e.g.* 100 µm x 2 cm) at a flow rate of 7 µL/min followed by separation on a column (*e.g.* 75 µm x 25 cm) at 250 nL/min. The separation column outlet is directly coupled to a coated Pico emitter tip positioned in a nano-spray interface at the mass spectrometer ionization source.

4.1. Nano-liquid chromatography and tandem mass spectrometry

4.1.1. Dissolve peptide samples in 12 µL 2% ACN/0.1% TFA for at least 30 min. Spin down for 15 s and transfer 11 µL supernatant to autosampler vials (conical, reduced diameter).

4.1.2. Set up an automated regime for sample application, chromatographic separation and tandem mass spectrometry at controlling software (*e.g.,* Xcalibur) as follows.

4.1.2.1. Use the following for Temperature: Autosampler: 5 °C; Column oven: 45 °C.

4.1.2.2. Use the following for Injection: Volume: 10 µL; Flow rate: 7 µL/min (2% ACN, 0.1% TFA); Time: 8 min; Valve setting: trap column – waste; mass spec acquisition: off.

4.1.2.3. Use the following for Separation: Flow rate: 250 nL/min Valve setting: trap column-separation column; mass spec acquisition: on.

0 min-100 min: 2% ACN, 0.1% formic acid – 40% ACN, 0.1% formic acid
100 min-105 min: 40% ACN, 0.1% formic acid – 95% ACN, 0.1% formic acid
105 min-109 min: 95% ACN, 0.1% formic acid
109 min-120 min: 2% ACN, 0.1% formic acid

4.1.2.4. Use the following for mass spectrometry settings: Full MS: FTMS; resolution 60 000; m/z range 400-2000; MS/MS: Linear Iontrap; minimum signal threshold 500; isolation width 2 Da; dynamic exclusion time setting 30 s; singly-charged ions are excluded from selection; normalized collision energy is set to 35%, and activation time to 10 ms.

Note: An full MS scan is followed by up to 15 LTQ MS/MS runs using collision-induced-dissociation (CID) of the most abundantly detected peptide ions.

4.1.3. Run three technical replicates for all samples.

4.2. Protein identification and label free quantification

4.2.1. Process mass spectrometric raw data towards protein identification and label-free quantification utilizing a commercial software suite (e.g., PEAKS Studio). In contrast to most other proteome software packages this particular software uses a *de novo*-sequencing algorithm prior to protein database alignments. However, this step can be easily substituted by other popular software packages.

4.2.2. Use essential settings listed in Table 2.

4.3. Phospho-proteomics

Note: Efficient and reliable phospho-peptide acquisition requires a few essential changes of the proteomic workflow setup.

4.3.1. After phospho-peptide enrichment, never dry samples completely. Always keep samples

dissolved.

Note: The phospho-ester bond of phosphorylated tyrosines or serines is very fragile. During collision-induced fragmentation within the ion trap this results in a neutral loss of phosphate. This prevents any further fragmentation of the peptide, which in turn is required for identification. Permitted *wideband-activation* in the mass spectrometry setup allows the fragmentation of phospho-peptides even after a neutral loss of the phosphate group. It performs a time saving "pseudo-MS^3". Phospho-site determination in MS/MS data requires a particular verification and evaluation and can be performed by phosphoRS 3.0.

5. Bioinformatics – Meta-Analysis

Note: Before performing functional annotation and network analysis, the protein lists have to be preprocessed. First merge the lists of regulated proteins and phospho-peptides for each brain region separately. Then remove all duplicate UniProt-IDs for each fraction to prevent misinterpretation.

5.1. Singular enrichment analysis with GeneCodis[17]

5.1.1. Open the web-based tool of GeneCodis (http://genecodis.cnb.csic.es).

5.1.2. Select "Mus musculus" as organism and "GO Biological Process" as annotation.

5.1.3. Paste a list of UniProt-IDs of a certain fraction. Submit and wait until the analysis is performed. Click on "Singular Enrichment Analysis of GO Biological Process" and view results.

5.1.4. Repeat step 5.1.3 for the other three fractions.

5.1.5. To see any duplications and intersections between the result lists use a scripting language like Perl or Python to filter the data needed. Similar tools for a singular enrichment analysis are DAVID (https://david.ncifcrf.gov/) and Cytoscape (http://www.cytoscape.org/) with the PlugIns BiNGO (http://apps.cytoscape.org/apps/bingo) and ClueGO (http://apps.cytoscape.org/apps/cluego).

5.2. Generating a force based graph out of GeneCodis data with Gephi (https://gephi.org/)

Note: The data for the graphs has to be provided by the user, either in a graph format (.gexf, .graphml, .dot, .gv, .gml) or entered by hand.

5.2.1. Generating the graph nodes

5.2.1.1. By hand: Open Gephi and click on "Data Laboratory". Create nodes. Click on "Nodes" on the left to switch to the "Nodes" table. Click on "Add node". Enter the name of the Term. Click "OK"/Press Enter.

5.2.1.2. Alternative: Save GeneCodis result to PC. Open the .txt with a spreadsheet program. Delete all rows except from "Item_Details" (term names). Change header

“Item_Details” to “Label”. Save Spreadsheet as “.csv”. Now in Gephi, click on “Import Spreadsheet”. Choose spreadsheet from the file browser of Gephi. Click “Next”. Click “Finish”.

5.2.2. Connecting nodes via edges.

5.2.2.1. Click on “Edges” on the left to switch to the “Edges” table. For every node (Term): look up gene names in other Terms. If one or more genes are shared -> create edge.

5.2.2.2. Click on “Add Edge”. Select “Undirected”. Select source and target node out of drop down lists. Click “OK”/Press Enter. If more than one gene is shared, enter abundance in “Weight” (table).

5.2.3. Force based graphical layout.

5.2.3.1. Open graph data file, set the graph type to “undirected” or use the data as entered by hand, click on “Overview” if not already selected.

5.2.3.2. Resize nodes depending on the abundance of interconnections. Click on Statistics, run either “Average Degree” (unweighted edges) or “Avg. Weighted Degree” (weighted edges) under “Network Overview”. In “Appearance”, click on “Nodes”, then on the Size Button, next choose “Attributes” and set the Attributes parameter to “Avg. Weighted Degree” or “Average Degree”. Click Apply.

5.2.3.3. Finally: Select “Force Atlas” in “Layout” and run; change “Repulsion strength” if nodes are colliding.

5.2.4. Export to picture.

5.2.4.1. Screenshot feature: Click on “Overview”, change graph layout, edge thickness, label size and scaling with the menu at the bottom of “Graph” window. Click the camera left button, and save picture.

5.2.4.2. Export feature of “Preview”: Click “Preview”. Change Presets to “Default straight”. Change Settings according the chosen preferences and click on “SVG/PDF/PNG” to export.

REPRESENTATIVE RESULTS:

Figure 1 summarizes the complete workflow of quantitative synaptic proteome profiling of mouse brain regions after auditory discrimination learning. It starts with the animal training in a shuttle box. In the example shown in **Figure 2**, mice started to show significant FM tone discrimination in the 4^{th} training session, indicating efficient learning. Animals are sacrificed at selected time points for brain area dissection. The required enrichment of synapses can either be achieved by the preparation of synaptosomes or alternatively by the preparation of a PSD-enriched fraction, both described in detail in **Figure 3.** The PSD-enrichment method has been

developed for low tissue amounts, *e.g.* 1-2 hippocampal slices from rat brain[12, 18]. It requires small tubes, PTFE pestles fitting to these tubes, and a laboratory drilling drive for powering the pestle.

Due to the particular protein composition of synaptosomes, it is strongly recommend to perform the sample preparation in two different but complementary ways. Scaffolds of the PSDs are often very high molecular weight proteins occurring in high stoichiometry. In-solution digest is the best way to extract them efficiently but may lead to an oversampling of the generated peptide mixture. The in-gel digest performed of the same sample in parallel can exclude those high molecular weight proteins and favor the analysis of proteins with medium and lower molecular weight. For a comprehensive analysis both types of proteolytic digests are recommended.

The different amounts of tissues of the brain areas investigated require an adjustment of the applied material for better comparison. Within the four investigated brain areas the auditory cortex is generally the limiting factor. The material of all other brain areas should carefully be adjusted to the amount of the auditory cortex after preparation of synaptosomes or PSD-enriched fractions (see 3.1.1.). Typical weights of freshly prepared brain areas from mice are as following: auditory cortex (AC): ~50 mg; hippocampus (HIP): ~90 mg; striatum (STR): ~120 mg and frontal cortex (FC): ~100 mg.

The PSD-enrichment method described in section 2.3 allowed the identification of approximately 1500 different proteins and approximately 250 different phospho-peptides per brain region on the level of a single animal (**Table 1**). Proteomic analysis 24 h after the first training session revealed that 7.3% of the identified proteins and 5.8% of the phospho-peptides showed significant ($p<0.05$) quantitative changes in their synaptic expression compared to naïve controls (**Table 1**). A conspicuous tendency for down regulation of synaptic scaffolds may point to a pronounced rearrangement of the synaptic architecture during early stages of FMTD learning. The vast majority of the regulated proteins were altered in a brain region-specific manner, whereas only 22% were found to be regulated in two or more brain areas. Six selected examples are shown in **Figure 4**.

Meta-analysis of the complex results by IPA provides evidence for the particular participation/manipulation of the following canonical pathways: “Clathrin-mediated Endocytosis Signaling”, “Axonal Guidance Signaling”, “Calcium Signaling”, “RhoA Signaling”, “Notch Signaling”, “Remodeling of Epithelial Adherens Junctions”, “Glutamate Receptor Signaling”, “GABA Receptor Signaling”, “Dopamine Receptor Signaling” and “Synaptic Long-Term Potentiation”.

Single enrichment analysis revealed significant overrepresented biological processes in the frontal cortex concerning protein transport, cell adhesion, phosphorylation, endocytosis, vesicle-mediated transport, forebrain development and axonogenesis (**Figure 5**). In the auditory cortex biological processes including ion transport, translation, mRNA transport, protein transport and learning were noticeable. The analysis of the protein fraction of the hippocampus

detects significantly enriched processes related to ion transport, cell cycle, translation, phosphorylation and nervous system development. In the striatum, overrepresented biological processes including mRNA transport, vesicle-mediated transport, axonogenesis, proteolysis, protein transport and endocytosis were found.

Figure 1: Systematic workflow of the methodological approach
This figure schematically summarizes the workflow of high resolution quantitative profiling of brain area specific synaptic protein composition.

Figure 2: Example of the performance of mice in the FM tone discrimination task
Animals show an increasing rate of hits (blue curve) and a decreasing rate of false alarms (black curve) in the course of training sessions. Significant discrimination occurs from the fourth session. Error bars are provided as SEM.

Figure 3: Preparation of the synaptosome and the PSD-enriched fraction
A: Synaptosome preparation
B: PSD-enriched fraction preparation
Both figures explain the detailed workflow of preparation of synaptosomes or alternatively PSD-enriched fractions from brain tissues.

Figure 4: Selected quantitative proteomic results
The relative synaptic abundances of selected proteins are compared between mice trained on the FMTD task (AV, n=6) and naïve control mice (NV, n=6) 24 h after the first training session. The abundance values were calculated as median of the peak areas of the three most intense peptides of a protein. Proteins with significant abundance changes (AV/NV; t-test) are marked within the plots: * $p<0.05$, ** $p<0.01$, *** $p<0.005$. Error bars are provided as SD.

Figure 5: Visualization of biological pathways for frontal cortex by GeneCodis/Gephi.
Only significant terms of the Gene Ontology (GO) database (http://geneontology.org) related to “Biological process” with a minimum protein number of three are shown here. Nodes represent GO terms, the size of the node, the line width and number of connections of a certain node depict the number of proteins, which share this GO term with other nodes. Due to the “Force Atlas” method of Gephi, related nodes are clustering closely together.

Table 1: Summary of a proteomic result
This table summarizes a representative proteomic experiment of trained mice (AV, n=6) 24 h after the first training session compared to their naïve controls (NV, n=6). The sum of 459 regulated proteins includes overlapping regulations. 283 different regulations were determined as brain specific. In detail, 57 proteins are regulated in two brain regions, 18 protein regulations were detected in three brain regions and only 2 proteins are regulated in all four investigated brain areas.

Table 2: Settings for protein identification (step 4.2.2)

DISCUSSION:

The study presents a methodological workflow optimized for an accurate quantitative profiling of synaptic protein expression changes during learning and memory consolidation in different brain areas of mice. The setup provides the opportunity to study the protein expression on the level of a single animal despite of the required application of at least three technical replicates per sample for mass spectrometric analysis.

The methodology takes into account the particular protein composition of the pre- and postsynapse consisting of high molecular weight scaffold proteins but also of important mediator proteins bearing medium or lower molecular weights. The in-solution digests of synaptosomal preparations result in an efficient generation and, hence, an over-representation of scaffold-derived peptides. This, in turn, may suppress the analysis of smaller or lower abundant proteins. The suggested preparation of SDS-PAGE fractions from an aliquot of each sample combined with an in-gel digestion procedure in parallel facilitates the analysis of medium and low abundance proteins and represents a highly recommended complementary method. After separate mass spectrometric application of all fractions derived from a sample (*e.g.* in-solution digest, in-gel digest, combined phospho-enriched fractions) the corresponding MS/MS data sets can be combined and further calculated for protein identification and quantification by PEAKS software or alternative popular software packages.

Alternatively, the individual application of in-gel-digestion-derived fractions of a sample (separately processed gel-areas of a sample lane) and fractions generated of the in-solution digested sample (e.g. by ion exchange chromatography) to mass spectrometry can increase the analytical depth. However, this extended workflow dramatically increases the required time for LS-MS/MS data acquisition. For generation of a detailed molecular sequence of synaptic protein rearrangements during learning and memory formation a specified time course of the proteomic profiling is required. This time course may start immediately after or even during the first training session and covers a close-meshed time frame until the animals' performance reached the asymptotic level of the learning curve after approx. 8-10 days of training (see **Figure 2** for details).

The analysis of phosphorylation changes of synaptic proteins requires a particular focus on the selected time frames during FMTD learning. On the one hand signaling cascades initiating synaptic protein rearrangements known to be triggered by protein phosphorylations and dephosphorylations are expected at very early stages of animal training. On the other hand, there are long lasting modifications of multiple phosphorylated synaptic proteins known which regulate the connectivity and assembly within the synaptic architecture[19, 20]. Those posttranslational modifications are expected even at later time points of memory consolidation.

The complex datasets generated by this proteomic workflow require bioinformatic processing to identify participating molecular pathways and key molecules. Meta-analysis shows significant overrepresented pathways, which play a role in learning and memory processes.

ACKNOWLEDGMENTS:
We wish to thank Yvonne Ducho and Kathrin Pohlmann for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 779) and by the State Saxony-Anhalt / European Regional Development Fund (ERDF) via the Center for Behavioral Brain Sciences (CBBS).

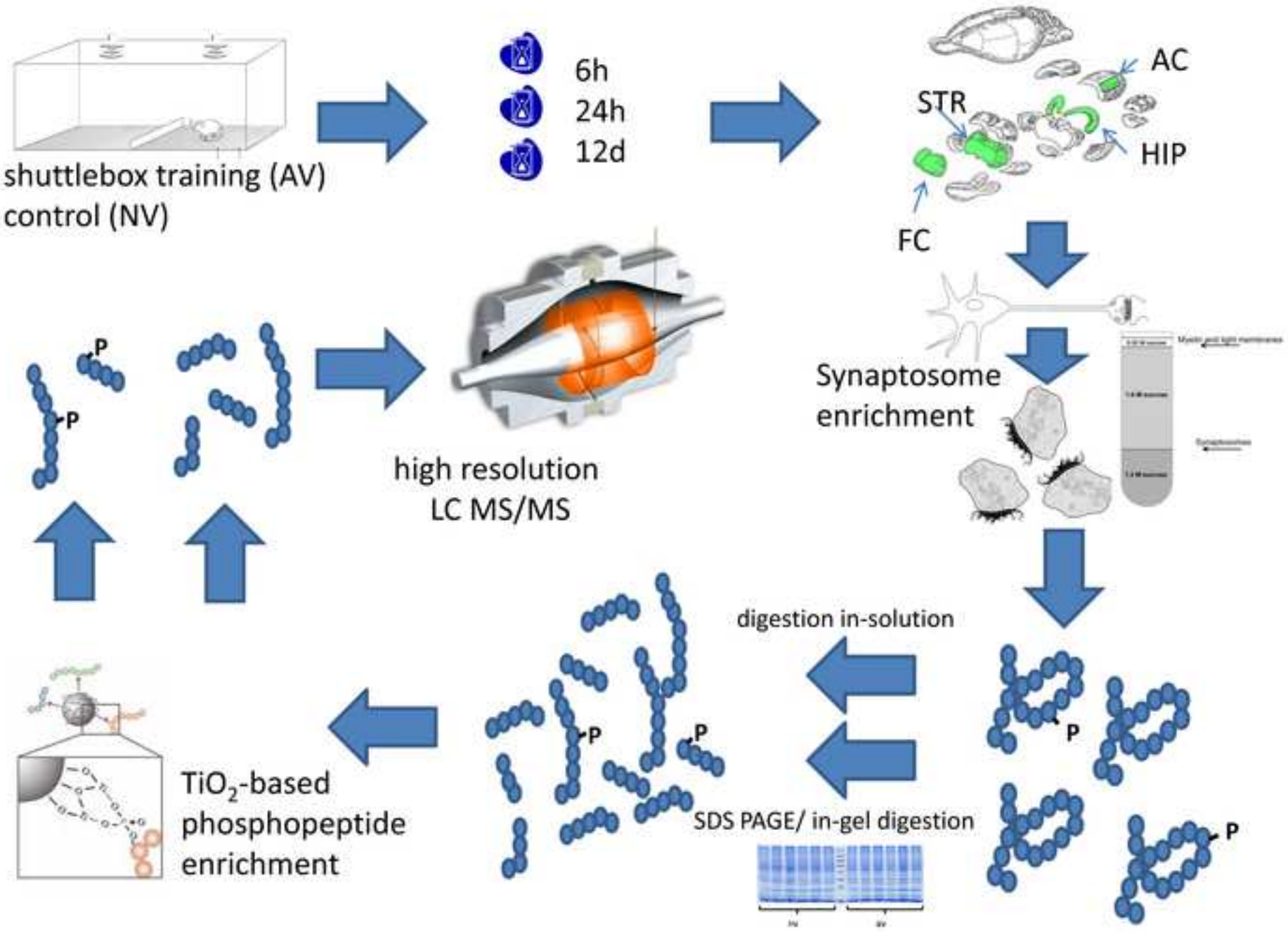
DISCLOSURES:
The authors have nothing to disclose.

REFERENCES
1. Lamprecht, R. & LeDoux, J. Structural plasticity and memory. *Nat Rev Neurosci*. **5** (1), 45-54, doi:10.1038/nrn1301(2004).
2. Bingol, B. & Schuman, E. M. Synaptic protein degradation by the ubiquitin proteasome system. *Curr Opin Neurobiol*. **15** (5), , 536-541, doi:10.1016/j.conb.2005.08.016 (2005).
3. Richter, J. D. & Klann, E. Making synaptic plasticity and memory last: mechanisms of translational regulation. *Genes Dev*. **23** (1), 1-11, doi:10.1101/gad.1735809 (2009).
4. Rosenberg, T. *et al.* The roles of protein expression in synaptic plasticity and memory consolidation. *Front Mol Neurosci*. **7**, 86, doi:10.3389/fnmol.2014.00086 (2014).
5. Scheich, H. *et al.* Behavioral semantics of learning and crossmodal processing in auditory cortex: the semantic processor concept. *Hear Res*. **271** (1-2), 3-15, doi:10.1016/j.heares.2010.10.006 (2011).
6. Kähne, T. *et al.* Synaptic proteome changes in mouse brain regions upon auditory discrimination learning. *Proteomics*. **12** (15-16), 2433-2444, doi:10.1002/pmic.201100669 (2012).
7. Reichenbach, N. *et al.* Differential effects of dopamine signalling on long-term memory formation and consolidation in rodent brain. *Proteome Sci*. **13**, 13, doi:10.1186/s12953-015-0069-2 (2015).
8. Kähne, T. *et al.* Proteome rearrangements after auditory learning: high-resolution profiling of synapse-enriched protein fractions from mouse brain. *J Neurochem*, doi:10.1111/jnc.13636 (2016).
9. Li, K. *et al.* Organelle proteomics of rat synaptic proteins: correlation-profiling by isotope-coded affinity tagging in conjunction with liquid chromatography-tandem mass spectrometry to reveal post-synaptic density specific proteins. *J Proteome Res*. **4** (3), 725-733, doi:10.1021/pr049802+ (2005).
10. Carlin, R. K., Grab, D. J., Cohen, R. S. & Siekevitz, P. Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities. *J Cell Biol*. **86** (3), 831-845 (1980).
11. Smalla, K. H., Klemmer, P. & Wyneken, U. in *The Cytoskeleton - Imaging, Isolation, and Interaction* (ed R. Dermietzel) 265-282 (Humana Press, 2012).
12. Smalla, K. H. *et al.* The synaptic glycoprotein neuroplastin is involved in long-term potentiation at hippocampal CA1 synapses. *Proc Natl Acad Sci U S A*. **97** (8), 4327-4332, doi:10.1073/pnas.080389297 (2000).
13. Shapiro, A. L., Vinuela, E. & Maizel, J. V., Jr. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem Biophys Res Commun*. **28** (5),

815-820 (1967).
14. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227** (5259), 680-685 (1970).
15. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem*. **68** (5), 850-858 (1996).
16. Thingholm, T. E., Larsen, M. R., Ingrell, C. R., Kassem, M. & Jensen, O. N. TiO(2)-based phosphoproteomic analysis of the plasma membrane and the effects of phosphatase inhibitor treatment. *J Proteome Res*. **7** (8), 3304-3313, doi:10.1021/pr800099y (2008).
17. Carmona-Saez, P., Chagoyen, M., Tirado, F., Carazo, J. M. & Pascual-Montano, A. GENECODIS: a web-based tool for finding significant concurrent annotations in gene lists. *Genome Biol*. **8** (1), R3, doi:10.1186/gb-2007-8-1-r3 (2007).
18. Bonn, S., Seeburg, P. H. & Schwarz, M. K. Combinatorial expression of alpha- and gamma-protocadherins alters their presenilin-dependent processing. *Mol Cell Biol*. **27** (11), 4121-4132, doi:10.1128/MCB.01708-06 (2007).
19. Tweedie-Cullen, R. Y., Reck, J. M. & Mansuy, I. M. Comprehensive mapping of post-translational modifications on synaptic, nuclear, and histone proteins in the adult mouse brain. *J Proteome Res*. **8** (11), 4966-4982, doi:10.1021/pr9003739 (2009).
20. Li, L. *et al.* The differential hippocampal phosphoproteome of Apodemus sylvaticus paralleling spatial memory retrieval in the Barnes maze. *Behav Brain Res*. **264**, 126-134, doi:10.1016/j.bbr.2014.01.047 (2014).

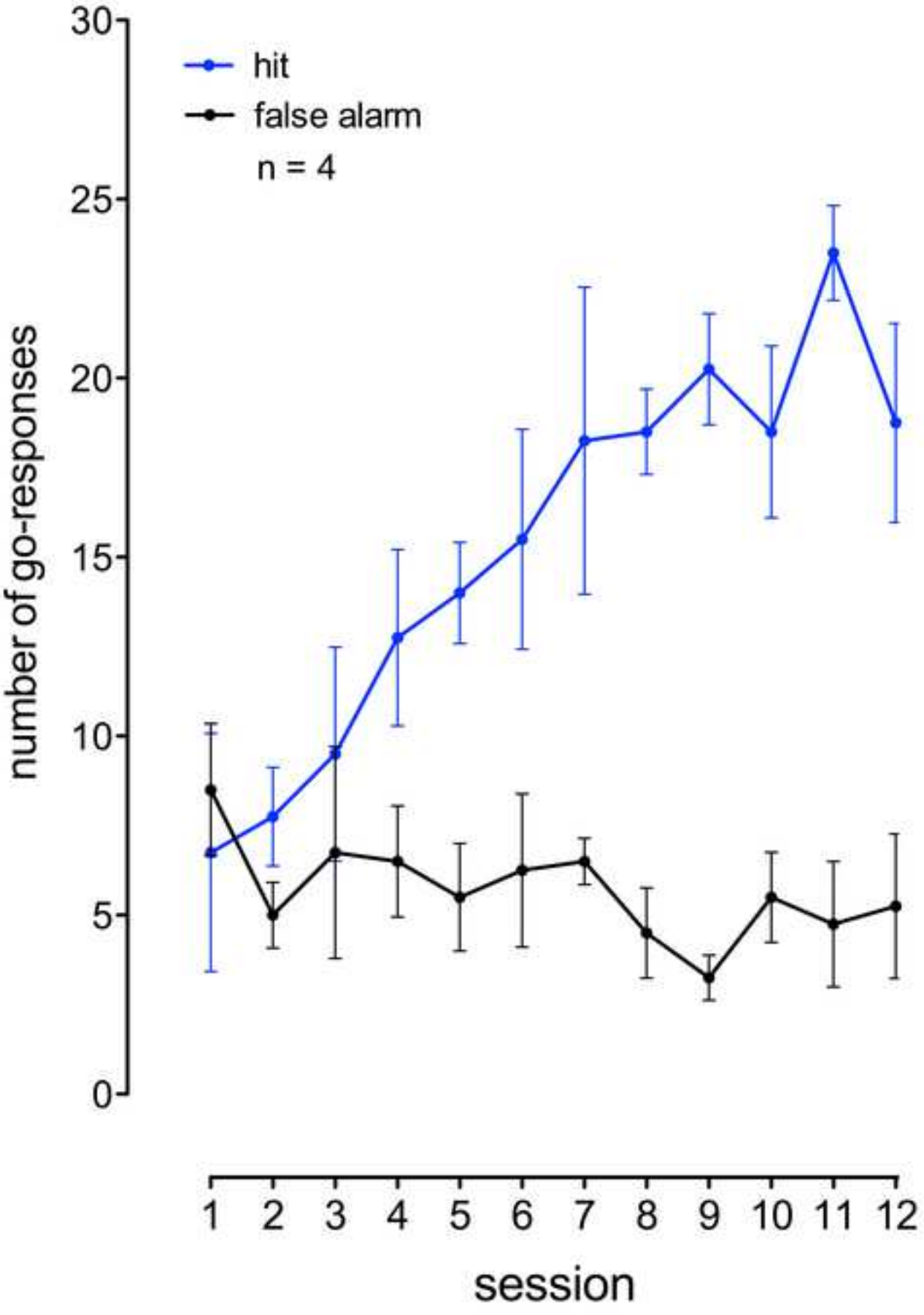
Figure

Click here to download Figure Figure1.tiff



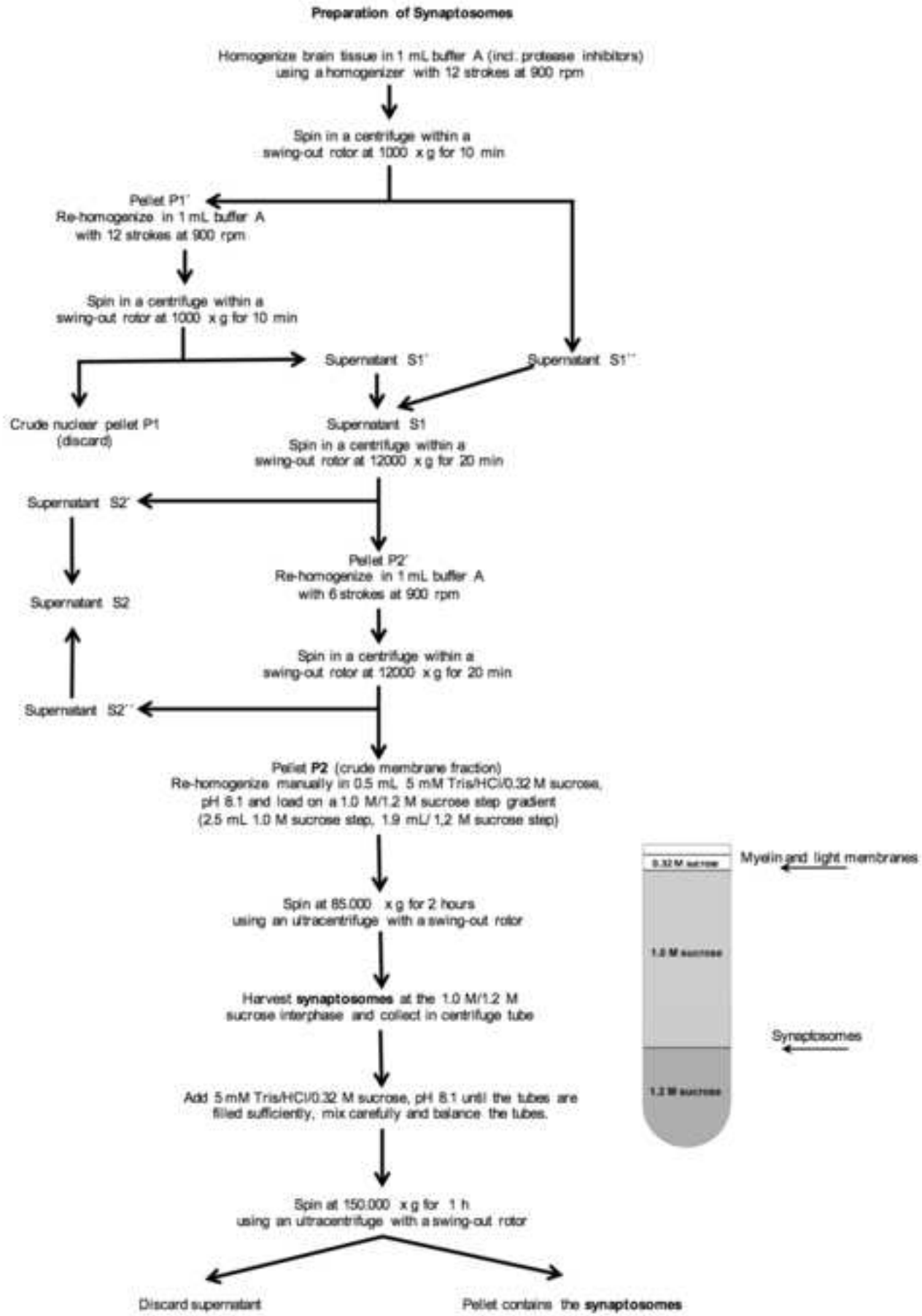
Figure

Click here to download Figure Figure2.tif



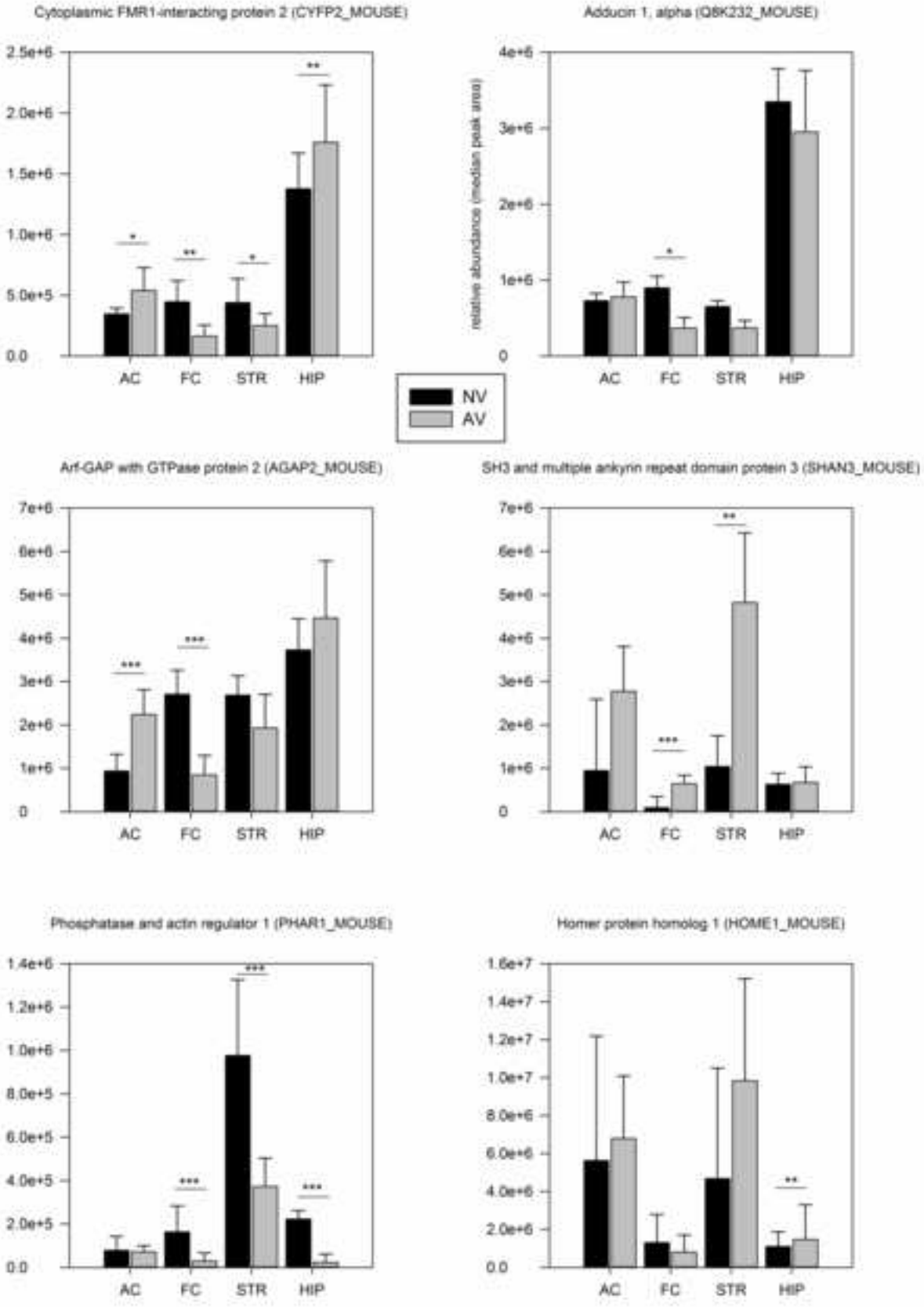
Figure

Click here to download Figure Figure3.tif



Figure

Click here to download Figure Figure4.TIF



Figure

Click here to download Figure Figure5.tiff

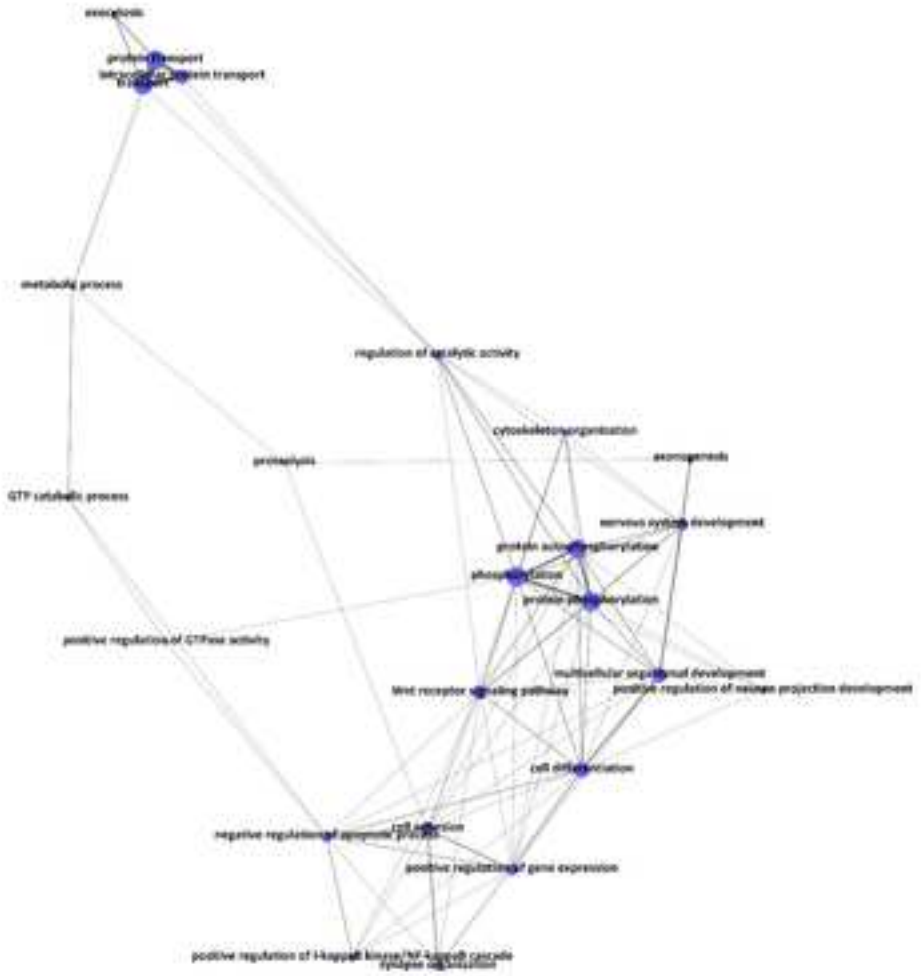


Table 1

Brain region	AC	FC	HIP	STR	∑
identified proteins	1435	1758	1572	1507	6272
regulated proteins (p<0.05)	59	130	162	108	459
↑ AV/NV	8	4	76	35	123
↓ AV/NV	51	126	86	73	336
identified phosphomotifs	197	361	273	278	1109
regulated phosphomotifs (p<0.05)	8	22	21	14	65
↑ AV/NV	4	17	5	9	35
↓ AV/NV	4	5	16	5	30

Table 2 Click here to download Table Table Settings 4.2.2.xlsx

Error tolerances

precursor mass (fourier transformation mass spectrometry)
fragment ion mass (linear ion trap)

Maximum missed cleavages per peptide

Fixed modifications

for in-gel-digested samples
for in-solution-digested samples

Variable modifications

Database

Taxonomy

Statistical identification-acceptance settings

de novo average local confidence (ALC)
Peptide-false discovery rate (FDR, based on est. decoy-fusion)
Protein significance (-10logP, based on modified T-test)
unique peptides / protein

Quantification settings:

Peptides used for quantification if:
Peptide significance (-10logP)
Peptide identification in
Peptide signal quality
Peptide average area

Peptide retention time tolerance
Normalization

10 ppm
0.6 Da

3

Carbamidomethylation of Serine
Methylthiolation of Serine

Oxidation of Methionine
Deamidations of Asparagin and/or Glutamine

Uniprot/Sprot
mouse

> 50%
< 1%
> 20
≥ 1

> 30
≥ 50% of samples
>1
> 1E5

< 5 min
by total ion current (TIC)

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment Table of Specific Materials.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
3M Empore Solid Phase Extraction- Filter	3M Bioanalytical Technologies	4245SD
Acclaim PepMap 100	Dionex/Thermo Scientific	164564
Acclaim PepMap 100	Dionex/Thermo Scientific	164569
Acetic acid	Carl Roth GmbH	3738.1
Acetonitrile (ACN)	Carl Roth GmbH	AE70.2
Acrylamide (30%)	AppliChem	A0951
Ammonium hydrogen carbonate	Fluka	9830
Ammonium hydroxide	Fluka	44273
Ammonium persulfate (APS)	AppliChem	A2941
Biofuge *pico*	Heraeus GmbH	75003280
Blue R-250	SERVA Electrophoresis GmbH	17525
Bromophenol Blue	Pharmacia Biotech	17132901
C57BL/6J mice	Charles River	
Cantharidin	Carl Roth GmbH	3322.1
Centrifuge tubes for MLS-50	Beckman Coulter	344057
Centrifuge tubes for TLA 100.1 rotor	Beckman Coulter	343776
Dithiothreitol (DTT)	AppliChem	A1101
Eppendorf 5417R centrifuge	VWR	22636138
Eppendorf A-8-11 rotor	VWR	5407000317
Formic acid	Fluka	14265
GeneCodis	http://genecodis.cnb.csic.es/	
Gephi	https://gephi.org/	
Glycerol	AppliChem	A1123
Glycine	AppliChem	A1067
HALT Phosphatase Inhibitor Cocktail	Pierce /Thermo Scientific	78420
HEPES Buffer solution	PAA Laboratories GmbH	S11-001
Homogenization vessel 2 mL	Sartorius AG	854 2252
Hydrochloric acid	Sigma-Aldrich	H1758
Imidazole	Sigma-Aldrich	I2399
Ingenuity Pathway Analysis	Qiagen	
Iodoacetamide (IAA)	Sigma-Aldrich	I1149
Laboratory drilling drive K-ControlTLC 4957	Kaltenbach & Vogt GmbH	182997
LTQ Tune Plus 2.7.0.1112 SP2	Thermo Scientific	
LTQ Orbitrap Velos Pro	Thermo Scientific	
Macs-mix tube rotator	Miltenyi Biotech	130-090-753
Magic Scan 4.71	UMAX	
Methanol	Carl Roth GmbH	AE71.2
MLS-50 rotor	Beckman Coulter	367280
Optima MAX Ultracentrifuge	Beckman Coulter	364300
PageRuler Prestained Protein Ladder	Thermo Scientific	26616
PEAKS 7.5	Bioinformatic Solutions	
Phosphatase Inhibitor Cocktail 3	Sigma-Aldrich	P0044
PhosphoRS 3.1	IMP/IMBA/GMI	

PhosSTOP	Roche	4906845001
Plunger/pestle made of PTFE	Sartorius AG	854 2651
PotterS homogenizer	Sartorius AG	853 3024
Protease Inhibitor complete mini	Roche	4693159001
Quantity One 4.5.1	BioRad	
RapiGest	Waters	186002122
Shuttle box	Coulbourne Instruments	
Sodium dodecylsulfate (SDS)	AppliChem	A1112
Sodium molybdate	Carl Roth GmbH	274.2
Sodium tartrate dihydrate	Sigma-Aldrich	228729
SONOREX RK 156 Ultrasonic Bath	BANDELIN electronic GmbH & Co. KG	305
Soundproof chamber	Industrial Acoustics Company	
Sucrose	Carl Roth GmbH	4621.2
Tetramethyl ethylene -1,2-diamine (TEMED)	Sigma-Aldrich	T9281
Thermomixer *basic*	CallMedia	111000
Titansphere TiO 5µm	GL Sciences Inc. Japan	502075000
TLA 100.1 rotor	Beckman Coulter	343840
Trifluoro acetic acid (TFA)	Sigma-Aldrich	T6508
Tris (hydroxymethyl) aminomethane (TRIS)	AppliChem	A1086
Triton X-100	Sigma-Aldrich	T8532
Trypsin Gold	Promega	V5280
Ultimate 3000 Ultra HPLC	Dionex/Thermo Scientific	
Ultracentrifuge tube	Beckman Coulter	343776
Unijet II Refrigerated Aspirator	Uniequip Laborgeräte- und Vertriebs GmbH	
UNIVAPO 100 H Concentrator Centrifuge	Uniequip Laborgeräte- und Vertriebs GmbH	
Urea	AppliChem	A1049
Water (high quality purifed)		
Xcalibur 3.0.63	Thermo Scientific	
ZipTip$_{C18}$ Pipette Tips	MILLIPORE	ZTC18S960

Comments/Description
7 mm/3 ml
100 µm x 2 cm, C18
75 µm x 25 cm, C18

Resistivity: > 18.2 MΩ*cm at 25 °C
Pyrogens: < 0.02 EU/ml
TOC: < 10 ppb

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: High resolution quantitative synaptic proteome profiling of mouse brain regions after auditory discrimination learning.

Author(s): Kolodziej, A; Smalla, K.-H.; Richter, S.; Engler, A.; Pielot, R.; Dieterich, D.C.; Tischmeyer, W.; Naumann, M.; Kähne, T.

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Kähne, Thilo, PhD
Department:	Institute of Experimental Internal Medicine
Institution:	Medical School, Otto von Guericke University, Magdeburg, Germany
Article Title:	High resolution quantitative synaptic proteome profiling of mouse brain regions after auditory discrimination learning.
Signature:	
Date:	12/05/2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

List of changes addressing editorial and peer review comments:

1. Formatting:
-All figure legends should have a title and a brief description.
All figures have a title and a brief description (line 667 et sqq).

2. Length warning: The protocol is at the maximum for highlighted material, so if any material is added during revisions or as a result of peer review it may be necessary to cut from the existing highlighting.
Highlighting of new material according to the comments and cutting of other material was balanced, resulting in the same amount of highlighted material as in the last version.

3. Grammar: Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance, and some errors are listed below.
-1.2.1 – Please use “euthanize” rather than “kill”.
-2.3.2, 3.2.2.3, 3.2.2.4, 5.1.2 – Please correct the run-on sentence.
-3.2.1.2 – “Trench the lanes within the gel in areas” – please clarify what is meant here. It is not clear what action would be performed or how the “areas” are defined.
-4.1.2 – Should be “as follows:”
-4.3.1 – “keep samples dissolve”
Grammatical errors were eliminated.

-Line 475 – “Permitted”
We are not sure what is meant here.

4. Visualization: Protocol is highly discontinuous. All steps essential to complete the protocol should be highlighted in a filmed section, including centrifugation steps, for example. For example, reduction and carbamidomethylation of cysteines prior to digest should be highlighted in these sections, and only one type of digest (probably in-solution) should be shown. In addition, not enough steps are highlighted if the behavioral aspect is to be shown. Please readdress the highlighting of the protocol to make sure continuity is maintained and all required material for each procedure is included.
Reduction and carbamidomethylation of cysteines prior to digest was highlighted in these sections, and only the in-solution digest is supposed to be shown in the film. Additional behavioral steps are highlighted as well.

5. Additional detail is required:
-1.2.3 – Is the brain placed in any solution during dissection? Is a stereomicroscope used for dissection?
A stereomicroscope is used (this was added to the revised manuscript, line 190). During dissection, the brain is not kept in any solution.
-2.2.2 – How many strokes are required for homogenization?
Six strokes are required (added to the manuscript, line 245).
-3.3.1.1 – How much sample is required for a replicate?
We addressed this in the revised version of the manuscript. (line 383 et sqq.)

6. Branding must be removed:
-Please remove all of the trademark symbols/branded items from Figure 3. This includes Potter S, Eppendorf 5147R, Beckman Optima MAX, Beckman Optima XPN 80. There are 7 brand names mentioned in this figure alone.
A new version of this figure is provided.
-2.3.1, 2.3.3 – Teflon
'Teflon' was replaced by 'PTFE (Polytetrafluorethylene)' (line 260).
-3.1.2 – RapiGest , exchange to: removable detergent (e.g. acid cleavable)
Exchanged (Line 299).
-5.1, 5.1.1 – Ingenuity (please also remove the website)
This section was removed.

7. Results: Figure 4 – What statistical test is used?
We used a t-test (line 687).

8. Discussion: Please discuss the significance of the method with respect to alternative techniques and provide independent citations. Please also discuss the limitations and future applications of the method.
According changes were made in the manuscript (line 708 et sqq.)

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript describes a workflow to reveal the changes of synapse proteomes induced by a behavioural paradigm. This type of MS quantitative analysis is routinely used by the proteomics laboratories for years. Nevertheless, the "visualized" procedure is a welcome addition to the large number of review articles on similar topics.
There are several minor points that the authors should consider in their revised version.

(1)The quantities of tissue/protein/peptide that should be used in an experiment must be mentioned in the text. For example, 2.3.1 Homogenize tissue (from one or more animals depending on the brain regions and the preparations of PSD versus synaptosome?) in 100uL

extraction buffer; 3.1.1 Dissolve synaptosomes (from how many tissues or from X ug proteins?).....in 20-50 uL of 8M urea; 3.4.1. Dissolve peptides (how much, or referred to 3.1.1?) produced by in-gel or in-solution digest in 150 uL.
Respective information was specified in the revised manuscript.

(2)Line 123-129 highlight the usefulness of LFQ for protein quantitation. The statement however is not accurate. Of the several popular methods such as LFQ, DIA/SWATH, TMT/iTRAQ and SILAC, MS1-based LFQ has the highest sample to sample variation and high number of missing values. On the other hand, due to its ease to perform it remains the most used approach for global protein/peptide quantitation. The authors should add a few sentences to explain a bit what LFQ is, and tune down its "advantage" above the other methods. A few references on LFQ may be added here.
We addressed this point in line 126 et sqq.

(3)The reason that the authors dissolve synaptosome in 8M urea and then dilute it to 2 M urea in 1% RapiGest (3.1.1 and 3.1.2) is that (2/3 of) the sample is eventually digested in-solution. For in-gel digest synaptosome can be dissolved in SDS sample buffer and directly run on the gel. The authors should mention this option in case in-solution digestion is not performed; bear in mind that RipiGest is very expensive.
We mention this opportunity in our revised manuscript (line 294 et sqq.).

(4)It seems that the authors used 8 fractions per sample from the in-gel digested protocol (8x2=16 hours MS run time), but a single fraction for in-solution digestion (2 hours MS run time?). For fair comparison it would be better to fractionate the in-solution digested sample with SCX or highPH c18 into say 8 fractions before LC-MS analysis. The authors should at least discuss this option. The discussion from line 662- should be changed accordingly.
We clarified this point in line 378.

(5)4.1.3. Run three technical replicates for all samples. Why technical replicates? It is better to have at least 4 biological replicates for statistical analysis.
Three technical replicates are required to "compensate" run to run variations in label free quantification. For statistical analysis we recommend at least 6 biological replicates.

(6)4.2. Protein identification. The authors used PEAKS. They should also mention (in the discussion section) that there are other software such as the very popular Mascot and the freeware Maxquant.
We included this in our revised manuscript. (lines 496, 727)

(7)It will be nice if the authors can include in their video the analyses using IPA etc.
We had to remove the section mentioning IPA according to editorial comments, but added steps 5.2.1.1., 5.2.3.1. and 5.2.3.2. to the highlighted material.

(8)Table 1. The identified proteins from different brain regions are expected to have high percentages of overlap (I suppose 60-70%?) The sum of identified proteins of 6272 is probably misleading because it does not take into account of the overlapping identified proteins in the 4 brain regions. The same holds for regulated proteins etc.
We addresses this point in the description of table 1 (line 700 et sqq.).

Reviewer #2:
Manuscript Summary:
I have no major concerns about the proposed manuscript - the authors know the methods proposed, the tools and supplies, as well as the limitations and the reach of the method. It is good to see a paper on preparation and MS-based analysis of pre- and post-synaptic densities. For years we have limited ourselves to in-house protocols from several laboratories, a publication such as the one proposed here should help the scientific community unify these important methods for neuro-proteomics studies.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
This manuscript deals with methodology of comprehensive and systematic analysis of synaptic proteins related to memory formation and synaptic plasticity. The method enables identification of certain synaptic proteins from a single mice and very useful for researchers in wide range of field. The manuscript is clearly written and example of the experiment is appropriate. The description covers detailed conditions and scientific quality is enough.

Major Concerns:
N/A

Minor Concerns:
Minor point:
1) It may be greatly helpful to show a list for abbreviated words somewhere in the text.

We decided to not include such a list in our manuscript, as all abbreviations are explained when mentioned the first time in the text, which is according to the editorial instructions.

2) Please consider to add g(max) or g(average) for all centrifugation conditions.
All g-values are given as g(average) (compare line 213).

3) This reviewer is wonder how enriched the PSD is in the "PSD-enriched fraction". It is more scientific to describe about the extent of enrichment of PSD in the fraction.
We adressed this point in line 283.

4) Line 266: This fraction may contain "DRM" (detergent-resistant membrane) as well as other components described by the authors.
We addressed this point in line 276.

Additional Comments to Authors:
N/A