**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