nature portfolio

Peer Review File

Using In Vivo Intact Structure for System-wide Quantitative Analysis of Changes in Proteins

Corresponding Author: Professor John Yates III

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors report a novel ex vivo Lys labeling method to investigate potential structural protein changes in mice. This method has been applied to study multi-tissue structural changes in an AD mouse model. The novelty of the method includes its potential for broad implementation to study Lys reactivity ex vivo, offering a unique proteomic dimension beyond protein levels and modifications. The experiments are well designed, incorporating suitable controls, time courses, and biological replicates. yielding robust data. The data appears to be solid, but the biological implications are still not clear.
Major concerns include:
1. The manuscript includes a section of inherent limitations of the method towards the end, noting that changes in Lys reactivity can be influenced by structural changes, and PTMs. In addition, biomolecule interactions, and any other factors affecting Lys accessibility, may be accounted for. These confounding factors, which could lead to alternative interpretations of the data, should be clearly outlined early in the manuscript.
2. The technique described is an ex vivo method rather than an in vivo approach, which might limit its applicability under natural biological conditions.
3. The manuscript lacks validation analyses. For technological emphasis, a comparison of ex vivo results with in vitro analyses (post-brain lysis) could prove insightful, as many protein conformations might be maintained following tissue lysis. If focusing on the biological implications, applying the same ex vivo technique to a different AD mouse model could validate the findings.
4. The coverage of the proteome by this method is not comprehensive. Discussion of possible reasons for this limitation would be beneficial.
5. In statistical analyses, the authors selected the Mann-Whitney U test for pairwise comparisons and the Kruskal-Wallis H test for multi-group comparisons, presupposing non-normal data distribution. The rationale for selecting nonparametric tests over parametric ones warrants further justification, potentially through normality testing.
6. Minor points include: typos in the manuscript, such as "biolodical" in the title of Table S1. In the introduction, while the authors reference their previous work, they neglect to mention other relevant amino acid labeling methods to study possible protein changes (PMID: 33988997; PMID:33683887; PMID: 37307028; PMID: 36108266).

Reviewer #2

(Remarks to the Author)

This manuscript describes the application of CPP to whole organ systems in a mouse model of AD. The CPP technique, which was originally developed by the authors several years ago, has been demonstrated and proven useful for the analysis of proteins in cells and cell lysates. It is now one of a handful of structural proteomic methods for characterizing the conformational changes of proteins induced by disease. While some previously reported approaches (e.g, FPOP) have shown promise for in vivo analyses, none have so far been extended to in vivo analyses of mammalian tissue samples. In this regard the current work represents a significant advance in the field that warrants publication in a high impact journal such as Nature Communications. Overall, the work is well done and well-presented (with a few notable exceptions outlined below). The scope of the work is large (proteomics data is presented on 7 tissue samples from multiple NC and AD mice at multiple age-points). However, the authors do a nice job distilling down the key results of the paper (e.g., the detection of many AD associated conformational changes in brain proteins that are highly enriched in this organ compared to other

organs).
The described approach stands to be an important and general method for in vivo studies of the conformational of proteins in mouse tissues. This reviewer is supportive of publication in Nature Communications provided the points outlined below can be addressed.

More Major points:

1. The paper talks about expression level analyses (e.g., p8 lines 40-50), but there is no discussion in the Methods section as to how such expression level analyses were conducted. For example, how was the mass spectral data used/analyzed to compare the relative amount of a protein in one organ compared to another organ. Along these lines, it is unclear what actual values (i.e., spectral counts, ion intensities, etc) are used to generated the fold change values on the Y-axis in Figure 3C, which seems to talk about Cnp overexpression. Maybe this information is embedded in the SI somewhere (e.g.,Fig S7)? Regardless, this should be clarified/discussed either in the main text or SI text. Similarly, it is not clear how the "value of protein abundance" was arrived at to the generate the data in Fig. S16
2. The wording in the conclusion on P8 lines 52-56 is maybe not quite right. The protein expression level data the authors seem to have is the relative amount of a given protein in the brain as opposed to in other organs for the NC and AD mice. Not necessarily the relative expression level of a given protein in NC and AD mice. For example, in the case the author's call out with Cnp, is this protein not present at about the same levels in both NC and AD mice, but just significantly over expressed in the brain samples of each? Again, given point (1) above, it is unclear how the described approached provides information protein expression level differences across the NC and AD mice. That said, the author's conclusion about the 62 brain proteins showing the most significant accessibility differences in AD vs. NC mice coming from highly enriched brain proteins (i.e., protein that are highly expressed in the brain compared to other organs) does seem to be supported by the data.
3. The MudPit paragraph on p. 11 lines 43-51 seems a little out of place/unnecessary. Is this the first time MudPit was used with CPP? Even so, it is not clear that such a widely used fractionation strategy warrants such an extended discussion. The last sentence in this paragraph also seems unnecessary. Is not MudPit always done using cation exchange followed by C18.
4. The ability to study the conformational changes of proteins directly in mouse tissues is exciting. But it begs the question, what, if anything is different from analyses performed in tissue cell lysates? If you took a mouse brain tissue cell lysate and compared the dimethyl labeling properties of protein in this sample to a mouse brain tissue analysis, would the conformational properties of all the proteins in these two samples be similar? Or would there be differences, and would those differences be relevant to the characterization of AD? This may be beyond the scope of this work, but data/comments to this point would certainly amp up the work's impact. Can any comparisons of this work be made to their 2021 JPR paper describing CPP on human AD brain tissue lysates?

More Minor points:

P.2; Line 18: "rapamycin" should be "cyclosporin."
P7; Line 9: The confounding factors that were investigated in the SI should be summarized here.
P7;Line 35: Would be useful to say in the text that the major finding from the SI that the labelling efficiency was 91.5-97.5% depending on the organ.
P7;Line 42: change "were" to "was"
P8; Line17: should be Bai et al
P8;Line 19: should be Seyfried et al
P10; line 27: The phrase, "..showed an increased but insignificant pattern.." is odd. If the "increase" is not significant, then it probably doesn't deserve mention.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This reviewer appreciates the thorough revision of the manuscript, including the additional mouse experiments in 5xFAD and the extensive, informative discussion. The manuscript is nearly ready for acceptance; however, a few typos still remain, such as "uring" in line 654.

Reviewer #2

(Remarks to the Author)
The concerns of this reviewer have been adequately addressed in the revised manuscript.

Response to the reviewers

We appreciate the reviewers' valuable feedback on the manuscript. Their insights have been instrumental in enhancing the overall quality of the paper. We have addressed the suggestions and corrections provided by the reviewers. Detailed responses to the comments are listed below, with the authors' response shown in blue font. The revised content in the manuscript has been marked in blue.

REVIEWER COMMENTS

Reviewer #1:

In this manuscript, the authors report a novel ex vivo Lys labeling method to investigate potential structural protein changes in mice. This method has been applied to study multi-tissue structural changes in an AD mouse model. The novelty of the method includes its potential for broad implementation to study Lys reactivity ex vivo, offering a unique proteomic dimension beyond protein levels and modifications. The experiments are well designed, incorporating suitable controls, time courses, and biological replicates. yielding robust data. The data appears to be solid, but the biological implications are still not clear. Major concerns include:

1. The manuscript includes a section of inherent limitations of the method towards the end, noting that changes in Lys reactivity can be influenced by structural changes, and PTMs. In addition, biomolecule interactions, and any other factors affecting Lys accessibility, may be accounted for. These confounding factors, which could lead to alternative interpretations of the data, should be clearly outlined early in the manuscript.

Authors response:

As per the reviewer's suggestion, we have outlined the limitations of our approach in the early part of the Results section to enhance the clarity of our interpretations. This will help prevent potential confusion in the results interpretation. This content is described on page 8, lines 26-40, as follows: *Prior to presenting the results, it is important to outline the scope and objectives of the CPP method to prevent potential misinterpretation. Dimethylation by the CPP method does not occur on lysines that are already modified (i.g. acetylation or ubiquitination). Even for lysines at the same position, the accessibility can vary depending on whether they have undergone prior modifications. However, it is not possible to determine which specific modification hinders the dimethyl labeling. The goal of this method is simply to measure surface accessibility of lysine residues and the labeling process can be effected by PTMs, mutations, or protein-protein interactions that block a site of labeling. It is important to note that while this may influence accessibility results, the overall purpose of the method remains to detect structural changes in proteins, rather than pinpointing the specific causes of these structural changes. Furthermore, since non-natural isotopic dimethylation was used in both initial and secondary dimethylation, naturally occurring dimethylation can be distinguished from dimethylation that occurred during the experiment. This approach aims to provide a quantification of protein structural alterations, regardless of the specific type of PTM or modification involved.*

2. The technique described is an ex vivo method rather than an in vivo approach, which might limit its applicability under natural biological conditions.

Authors response:

Ex vivo (Latin: "out of the living") means that which takes place outside an organism. In science, ex vivo refers to experimentation or measurements done in or on tissue in an artificial environment outside the organism with the minimum alteration of natural conditions. Ex vivo conditions allow experimentation under more controlled conditions than is possible in in vivo experiments (in the intact organism), at the expense of altering the "natural" environment. (https://www.genemedi.net/i/ex-vivo-in-vivo-in-vitro)

Respectfully, we disagree with the characterization of dimethylation through perfusion as an *ex vivo* method. In our approach, labeling proteins *in situ* within the tissue without organ extraction or external intervention allows preserving the native protein conformations at most. This minimizes protein denaturation or structural alterations common in *ex vivo* methods. Our approach maintains the structural and functional integrity of tissues by labeling them in their original location, better reflecting *in vivo* conditions.

We argue that our approach more closely aligns with *in vivo* labeling, based on several key considerations and studies. Recent research has significantly expanded our understanding of cellular viability and function in the post-mortem period, challenging conventional definitions of death at the cellular level. Studies have demonstrated that viable cells can be collected from multiple organs after cardiac arrest and successfully maintained in culture[1, 2]. This finding is further supported by research showing the extraction and cultivation of stem cells from post-mortem tissues, indicating that tissues retain a 'living' state in the early post-mortem period. Moreover, the boundaries of post-mortem cellular function have been pushed even further. A study has shown the restoration and maintenance of microcirculation and molecular and cellular functions in intact porcine brains for up to 4 hours postmortem[3]. This research suggests that cellular viability and function can persist for extended periods beyond what was previously thought possible. Additionally, researchers have successfully recovered cells and tissues through systemic perfusion in porcine models, even one hour after the cessation of blood flow[4]. These collective findings indicate that cellular functions can be preserved and even restored for significant periods post-mortem, fundamentally altering our understanding of the transition between life and death at the cellular level. The viability of organs for transplantation hours after donor death underscores that cellular death is not immediate following organismal death. For instance, hearts and lungs remain viable for transplantation up to four hours post-extraction, while kidneys can be preserved for 24-48 hours (https://www.nyp.org/transplant/organ-donation/organ-transplant-process).

Our method is performed within 30 minutes of cardiac arrest, a timeframe in which cells maintain significant viability and metabolic activity. In our case, performing dimethylation within 30 minutes of cardiac arrest places our method squarely within a window where cellular processes are still active and responsive. This suggests that our dimethylation process occurs in an environment that closely mimics the *in vivo* state, capturing proteins in their native conformations and interactions.

In conclusion, we propose that our method better resembles *in vivo* conditions than traditional *ex vivo* approaches. Critically, protein labeling occurs while cellular biological functions are maintained, allowing us to capture the *in vivo* environment as closely as possible.

3. The manuscript lacks validation analyses. For technological emphasis, a comparison of ex vivo results with in vitro analyses (post-brain lysis) could prove

insightful, as many protein conformations might be maintained following tissue lysis. If focusing on the biological implications, applying the same ex vivo technique to a different AD mouse model could validate the findings.

Authors response:

We appreciate your valuable feedback. We would like to respectfully note that the validation analyses you suggested have already been conducted and are presented in Figures S4 and S5 of the supplementary data. Additionally, we conducted further validation experiments to examine various other conditions that could potentially affect dimethylation (Figures S1-S3). These analyses are described in detail under the section titled "Validation for the utility of CPP via perfusion" in the supplementary materials. These analyses addressed multiple aspects of the CPP methodology combined with perfusion, examining its robustness and reliability across various biological and experimental conditions. The investigations encompassed the impact of aging on vascular permeability, the influence of protein abundance on labeling efficiency, the consistency of labeling patterns across different approaches for the initial labeling, and the correlation between CPP and the traditional crystal analyses. Collectively, these studies validated the CPP method's consistency and reliability, demonstrating its resilience to potential confounding factors and its agreement with established structural biology techniques. To explain based on the considerations we took into account, we would like to outline the following points:

i) Would the distribution of CPP reagents be influenced by vasculature, with a variability in vascular permeability in aging? We determined the proportion of labeled peptides within the population of lysine-containing peptides, encompassing both labeled and unlabeled variants per time point from 6 months to 15 months, for all organs. No difference in the proportion of the labeled peptides was observed across age groups. Despite the potential for age-related changes in vascular structure, we observed no significant impact on the distribution of the reagent during the perfusion process.

ii) Would labeling efficiency be affected by protein abundance level? We considered the issue if highly abundant proteins are more efficiently labeled by CPP reagents. We categorized proteins into three groups (low, medium, and high) based on their abundance levels and analyzed the accessibility distribution within each group. As results across all seven organs were examined, we observed consistent accessibility distributions of labeled peptides, regardless of their abundance levels.

iii) Would the pattern of dimethyl labeling be consistent among different initial labeling approaches? To address this question, we compared dimethyl labeling patterns using three different initial labeling approaches: 1) perfusion labeling, the original method, 2) tissue block labeling, which is to label with small tissue block after extracting the organ and 3) tissue lysate labeling, which is to label with tissue lysate. We selected the labeled peptides quantified in 3 out of 4 measurements from two replicates with two biological replicates. Compared to two different approaches for dimethyl labeling, the original method was significantly correlated with R values over 0.6. Additionally, the original method was not influenced by the protein abundance, while the other methods were impacted on the labeling efficiency depending on the abundance of protein, showing different distributions of accessibility across the low, medium, and high abundant protein groups.

iv) To what extent do structural measurements from CPP labeling show agreement with conventional approaches using crystal structures? We assessed the concordance between accessibility, as determined by dimethyl labeling exposure, and Solvent Accessible Surface Area (SASA) derived from crystal

structures. This analysis focused on housekeeping proteins whose labeled lysine sites were quantified across all seven tissues. Our results showed a strong concordance between Lys residues shown to be surface accessible by X-ray crystallography and CPP. We have included a brief summary of these validation experiments on page 8 line 10–15 of the main text as follows: *We validated the methodology's capacity to discern structural changes in proteins by evaluating the presence of potential confounding factors that might influence the interpretation of the results. These factors include the impact of age-related changes in vascular structure on the permeability of reagents, the potential influence of protein abundance on labeling efficiency, and the correlation between accessibility obtained from tissue blocks and tissue lysates post-organ extraction and accessibility obtained from labeling via perfusion.*

In response to the reviewer's suggestion about using different AD mouse models for validation, we employed the 5XFAD model, an alternative to the initially used APP NL-F model. This model, expressing multiple AD-linked mutations, allowed us to track similar structural changes in protein communities related to carbon metabolism and metal ion homeostasis. Despite the statistical insignificance in the p-values, the substantial effect sizes measured indicated meaningful differences in protein accessibility between the groups at different ages, supporting the validity of our findings. The limited sample size might have reduced the power of our statistical tests. The relevant content is represented in Figure S22, and is described under the section titled "Validation with different AD mouse model" in the supplementary text as follows: *To validate the findings of this study, we used a different AD mouse model (5XFAD) at 2 and 12 months of age. This mouse model expresses human APP and PSEN1 transgenes with a total of five AD-linked mutation and rapidly develops amyloid pathology, with extracelluar amyloid deposition beginning around 2 months. With the APP NL-F model, we highlighted the structural changes of protein communities enriched in carbon metabolism, and metal ion homeostatsis. These proteins exhibited a decreased pattern in the accessibility as AD progressed (Fig. 5). With the 5XFAD model, we focused on the same peptides identified in the APP NL-F model. Proteins enriched in carbon metabolism and metal ion homeostasis showed a different trend in the accessibility between two-month and twelve-month old mice; the accessibility on average decreased by 2.7% for carbon metabolism and 4.6% metal ion homeostasis as AD progressed (Supplementary Fig. 22A and 22B). Although the P-value did not reach statistical significance (P-value = 0.2287 for carbon metabolism, P-value = 0.0775 for metal ion homeostasis), the effect size as measured by Cohen's d were large (d = 0.30211 for carbon metabolism and d = 0.720492 for metal ion homeostasis). Additionally, Mag and Map1a, which were highlighted in the APP NL-F model as they are highly expressed in neuronal cells and showed accessibility changes for the multiple peptides (Fig. 4F and 4H), exhibited decreased accessibility by an average of 5.8% and 4.3%, respectively (Supplementary Fig.22C and 22D). For Plp1, the lysine residue of FSKNYQDY showed a greater decrease in the accessiblity in AD compared to normal control in the APP NL-F model (Fig. 6C). The 5XFAD model also showed a similar pattern for this labeled peptide, with the accessibility in 12-months-old mice decreased by 7.4% (Supplementary Fig. 22E). While the p-value did not reach statistical significance (P-value > 0.05), indicating no observable difference between the groups based on traditional hypothesis testing, the effect sizes measured by Cohen's d were substantial: 0.6 (Mag), 0.8 (Map1a), and 2.1 (Plp1). The discrepancy between statistical significance and effect size suggests that the limited sample size may have reduced the statistical power of the test, preventing the detection of a significant difference. Despite the non-significant p-value, the large effect sizes*

highlight potentially meaningful differences between the groups. This underscores that traditional significance testing may not capture substantial differences when sample sizes are limited. Therefore, while the statistical evidence based on P-value for a difference is weak, the magnitude of the effects indicates that the observed differences might be practically meaningful.

4. The coverage of the proteome by this method is not comprehensive. Discussion of possible reasons for this limitation would be beneficial.

Authors' response:

We have addressed this limitation in the discussion section on page 16 line 30–37 as follows: *An additional limitation of this method is its reliance on lysine residues as conformational reporters. Lysines typically comprise only 5-7% of amino acids in proteins, which constrains the amount of structural information that can be obtained. The quality and comprehensiveness of the analysis therefore scales with the sequence coverage of individual proteins. Higher sequence coverage generates more MS/MS data from lysine-containing peptides, thereby increasing the completeness of the structural analysis. However, this selective focus on lysine residues means that significant portions of the protein structure remain unexamined, potentially limiting the overall structural insights.*

5. In statistical analyses, the authors selected the Mann-Whitney U test for pairwise comparisons and the Kruskal-Wallis H test for multi-group comparisons, presupposing non-normal data distribution. The rationale for selecting nonparametric tests over parametric ones warrants further justification, potentially through normality testing.

Authors' response:

We added the rationale for the non-parametric tests. The reason we chose a non-parametric test is that the sample size of the data we analyze is small. Three mice per group were analyzed as biological replicates; no technical replicates were analyzed. For small sample size, normality tests have little power to reject the null hypothesis that the population is normally distributed and therefore small samples most often pass normality tests[5, 6]. Generally, when the sample size is 30 or more, the Central Limit Theorem can be applied, and it can be assumed that the distribution of the sample mean approximates a normal distribution. However, with a sample size of 3, such an assumption cannot be made. According to these rationales, a non-parametric test was chosen. We have described the rationale on page 10 line 24–28 as follows: *Given the limited sample size (3) in our study, it is challenging to assume or test "normality". As a result, we chose to employ a non-parametric test to minimize the likelihood of Type I errors, or false positive results. Non-parametric tests apply relatively flexible assumptions to small sample sizes, potentially yielding more reliable results.*

6. Minor points include: typos in the manuscript, such as "biolodical" in the title of Table S1. In the introduction, while the authors reference their previous work, they neglect to mention other relevant amino acid labeling methods to study possible protein changes (PMID: 33988997; PMID:33683887; PMID: 37307028; PMID: 36108266).

Authors' response:

Thank you for the thorough review. We have corrected the typo. The references have been added accordingly.

Reviewer #2 (Remarks to the Author):

This manuscript describes the application of CPP to whole organ systems in a mouse model of AD. The CPP technique, which was originally developed by the authors several years ago, has been demonstrated and proven useful for the analysis of proteins in cells and cell lysates. It is now one of a handful of structural proteomic methods for characterizing the conformational changes of proteins induced by disease. While some previously reported approaches (e.g, FPOP) have shown promise for in vivo analyses, none have so far been extended to in vivo analyses of mammalian tissue samples. In this regard the current work represents a significant advance in the field that warrants publication in a high impact journal such as Nature Communications. Overall, the work is well done and well-presented (with a few notable exceptions outlined below). The scope of the work is large (proteomics data is presented on 7 tissue samples from multiple NC and AD mice at multiple age-points). However, the authors do a nice job distilling down the key results of the paper (e.g., the detection of many AD associated conformational changes in brain proteins that are highly enriched in this organ compared to other organs).
The described approach stands to be an important and general method for in vivo studies of the conformational of proteins in mouse tissues. This reviewer is supportive of publication in Nature Communications provided the points outlined below can be addressed.

More Major points:

1. The paper talks about expression level analyses (e.g., p8 lines 40-50), but there is no discussion in the Methods section as to how such expression level analyses were conducted. For example, how was the mass spectral data used/analyzed to compare the relative amount of a protein in one organ compared to another organ. Along these lines, it is unclear what actual values (i.e., spectral counts, ion intensities, etc) are used to generated the fold change values on the Y-axis in Figure 3C, which seems to talk about Cnp overexpression. Maybe this information is embedded in the SI somewhere (e.g.,Fig S7)? Regardless, this should be clarified/discussed either in the main text or SI text. Similarly, it is not clear how the “value of protein abundance” was arrived at to the generate the data in Fig. S16

Authors’ reseponse:

The various points the reviewer has mentioned converge on the question of how protein abundance was derived. To provide more details on the protein abundance quantification process, we have added further explanations under the sub-title of Determination of accessibility of lysine sites and protein expression in the Methods section on page 6 line 21 – 23 as follows: *The level of protein abundance was determined by summing the intensities of the top 3 unique peptides. Peptide abundances were summed together regardless of their labeling status.*

Concerning Fig. 3C (now Supplementary Figure 9B), we have described details on page 10, line 48 – 50 to clarify how the relative protein expression levels in the brain compared to other organs were calculated as follows: *The relative abundance of protein in the brain was determined by dividing the protein abundance in brain tissue by the average protein abundance of six other tissues for each age group.*

Figure S16 (now Supplementary Figure 17) focuses exclusively on brain proteins. The "fold-change of abundance" referred to here represents the ratio of protein abundance in the AD group to that in the NC group. The detailed method has been added on page 13 line 11 – 15 as follows: *To isolate protein expression changes*

specifically attributed to AD progression while excluding effects of normal aging, we calculated the fold-change of abundance by dividing the protein abundance in the AD group by that in the NC group. This approach allowed us to analyze changes in protein expression levels as time progressed from 6 to 15 months.

2. The wording in the conclusion on P8 lines 52-56 is maybe not quite right. The protein expression level data the authors seem to have is the relative amount of a given protein in the brain as opposed to in other organs for the NC and AD mice. Not necessarily the relative expression level of a given protein in NC and AD mice. For example, in the case the author's call out with Cnp, is this protein not present at about the same levels in both NC and AD mice, but just significantly over expressed in the brain samples of each? Again, given point (1) above, it is unclear how the described approached provides information protein expression level differences across the NC and AD mice. That said, the author's conclusion about the 62 brain proteins showing the most significant accessibility differences in AD vs. NC mice coming from highly enriched brain proteins (i.e., protein that are highly expressed in the brain compared to other organs) does seem to be supported by the data.

Authors' response:

Thank you for the valuable review. We have updated the data accordingly. The relative abundance of proteins in brain was calculated to show which proteins were enriched in mouse brain, as the selected 62 proteins were human homologues known to be enriched in human brain. We sought to determine how much the abundance of these proteins was altered in mouse brain, the organ most closely associated with AD, compared to other organs.

While our goal was to demonstrate the complementary relationship between protein abundance and structure, we recognized the need for a more detailed description of how we selected the 62 proteins and analyzed their abundance. Additionally, we have added data (Fig. S9A and S9C) on the absolute abundance of 62 proteins in brain, not just the relative protein abundance in brain compared to those in the other 6 organs. The approach to determine a level of protein abundance was described in point #1 (above). Most of the 62 proteins that exhibited AD-specific structural alterations did not show significant changes in their abundance levels during either AD progression or normal aging. This observation suggests that while these proteins undergo structural modifications characteristic of AD, their expression levels remain relatively stable throughout the disease course and aging process. Specifically, the abundance of Cnp in brain was not altered during normal aging, but exhibited a statistically significant change during AD progression. The change between 6 month and 15 month was significant with P-value <0.05 (Dunn's test). Also, the change of abundances of Cnp in brain between NC and AD at the same age were not significantly different.

We have elaborated the above contents from page 10, line 28 to page 11, line 15, as follows: *We first used a Kruskal-Wallis test to compare lysine site accessibility among the four age groups (6, 9, 12, and 15 months) of AD (P-value ≤ 0.05), and then we used a Mann-Whitney test to compare lysine site accessibility between AD and NC per age (P-value ≤ 0.05). This approach allowed us to select labeled peptides that showed significant changes in the accessibility throughout disease progression (6–15 months), while also exhibiting significant differences from NC at each age point. Of the 3,456 peptides that were tested, 83 peptides corresponding to 62 proteins showed a significant difference in accessibility in all tests. Among 83 peptides, the lysine position in four pairs of peptides was the same: APVISAEKAY and APVISAEKAYHEQL for*

Tuba1, HPEQLITGKEDAANNY and ITGKEDAANNY for Tuba1, QVVLVEPKTAW and QYQVVLVEPKTAW for Cnp, and RYLSEVASGENKQTTVSNSQQAY and SEVASGENKQTTVSNSQQAY for Ywhab. Most peptides exhibited a tendency for decreased accessibility with aging. Accessibility in the peptides from the AD group demonstrated a distinctive pattern characterized by a precipitous decline at the 9-month time point. (Fig. 3A, 3B). These results suggest that the structural changes in these proteins are more influenced by AD pathology than by the normal aging process. Furthermore, it suggests that AD may not only alter the structures of individual proteins, it may also produce disease-related physiological changes that modify communities of multiple proteins acting as networks.

We examined 62 mouse proteins that have human homologs known to be highly enriched in the human brain to confirm that they are more abundant in the mouse brain than in the other six organs. The relative abundance of protein in the brain was determined by dividing the protein abundance in brain tissue by the average protein abundance of six other tissues for each age group. Of the 62 proteins, 60 (excluding two brain-specific proteins) exhibited a range of enrichment factor from 0.13-fold (Eef2) to 1,232-fold (Tuba1b) when compared to their expression in other tissues (Fig. 3C). On average, 51 of these proteins were expressed at higher levels in the brain than in other tissues, while 9 proteins were expressed at lower levels in the brain than in other tissues. Expression of a total 60 proteins was also enriched 21.7-fold in NC and 20-fold in AD on average. No tendency in expression was observed in either aging or AD status (Supplementary Fig. 9A). The abundances of most of the 62 proteins in brain was not significantly different between NC and AD nor did they show a consistent pattern that correlated with AD. For example, 2',3'-cyclic-nucleotide 3'-phosphodiesterase (Cnp), which is associated with neuronal cells and glial cells, was found to be enriched in the brain. Cnp in the brain was relatively highly, with levels ranging from 13.4- to 23.9-fold in the NC and from 17.0- to 27.7-fold in the AD across four ages (Supplementary Fig. 9B). The accessibility of 2 peptides of Cnp decreased in progressing AD but remained unchanged in the aging NC (Fig. 3D and 3E). Additionally, differences in expression levels of Cnp in brain were not statistically significant between NC and AD, and they did not change significantly with AD progression (Supplementary Fig. 9C).

3. The MudPit paragraph on p. 11 lines 43-51 seems a little out of place/unnecessary. Is this the first time MudPit was used with CPP? Even so, it is not clear that such a widely used fractionation strategy warrants such an extended discussion. The last sentence in this paragraph also seems unnecessary. Is not MudPit always done using cation exchange followed by C18.

Author's response:

MudPit was not used in this study, and this paragraph had been written to convey a suggestion that it could be used instead of SCX. However, we agree with your opinion that this paragraph is unnecessary, and have removed it.

4. The ability to study the conformational changes of proteins directly in mouse tissues is exciting. But it begs the question, what, if anything is different from analyses performed in tissue cell lysates? If you took a mouse brain tissue cell lysate and compared the dimethyl labeling properties of protein in this sample to a mouse brain tissue analysis, would the conformational properties of all the proteins in these two samples be similar? Or would there be differences, and would those differences be relevant to the characterization of AD? This may be beyond the scope of this work, but

data/comments to this point would certainly amp up the work's impact. Can any comparisons of this work be made to their 2021 JPR paper describing CPP on human AD brain tissue lysates?

Authors' reseponse:

We appreciate your insightful feedback and your thorough review but would like to respectfully draw your attention to the supplementary data, where the comparative analysis with tissue lysate you suggested has already been included in Figure S4 and S5.

We compared dimethyl labeling patterns using three different initial labeling approaches: 1) perfusion labeling, the original method, 2) tissue block labeling, which is to label with small tissue block after extracting the organ and 3) tissue lysate labeling, which is to label with tissue lysate. We selected the labeled peptides quantified in 3 out of 4 measurements from two replicates with two biological replicates. Compared to each of two the other different approaches (approach #1 vs. approach #2, approach #1 vs. approach #3), the original method was significantly correlated with R values over 0.6, for both comparisons.

We observed distinct patterns of accessibility distribution based on protein abundance for each of the approaches #2 and #3 (Fig. S5). When using the perfusion labeling technique, we found no significant variations in accessibility distribution across the three groups based on the protein abundance. However, when labeling was performed using tissue blocks, a notable difference in accessibility emerged between the low and high groups. The most pronounced differences were observed in the tissue lysate labeling method, where the high group exhibited significant variations in accessibility compared to both the medium and low groups. These findings suggest that the effectiveness of perfusion labeling is not dependent on protein concentration, whereas the efficiency of labeling in tissue lysate and tissue blocks is influenced by protein levels. The data implies that utilizing the mouse's circulatory system for reagent dispersion offers benefits over labeling in tissue blocks or lysate. Furthermore, these analyses serve to confirm the efficacy of our methodology in mice. The detailed information is provided in the Supplementary text under the section titled 'Validation for the utility of CPP via perfusion'.

As per the reviewer's feedback, we compared our findings with the findings from the previous study. In the previous study, mitochondrial succinate dehydrogenase B was noted to exhibit changes in the accessibility of lysine #137, which was less exposed in AD patients than in normal subjects. In this study, we found similar structural changes in mitochondrial succinate dehydrogenase B, which is unsurprising given the sequences of these proteins showed 91% identity. Lysine #139 showed a decreasing pattern in the accessibility as AD progressed, starting at 9-month. This demonstrates that the novel dimethyl labeling approach combined with perfusion can be used as a viable alternative to tissue lysate-labeling. Along with this comparison, we also detailed how our findings align with recent research in the discussion section on page 16, line 4 – 24 as follows: *In our previous study, we observed structural changes in mitochondrial succinate dehydrogenase B in brain lysates of AD patients. We highlighted that lysine #137 of succinate dehydrogenase B was less accessible in AD patients than in control subjects. Interestingly, in this study we have found that mitochondrial succinate dehydrogenase B in mice is also affected by AD (Supplementary Fig. 21). In AD mouse samples, the accessibility of lysine #139 in succinate dehydrogenase B changed significantly from 6 to 15 months of age, with a decrease in exposure beginning at 9 months. In contrast, the accessibility of this residue remained consistent across all four time points in the NC group. The high*

sequence homology of these proteins (92%) between mouse and human species, coupled with the observed decrease in accessibility of lysine residues at positions 139 and 137, respectively, as AD progresses, suggests that this protein is structurally and functionally conserved across species. Furthermore, this result demonstrates that the novel dimethyl labeling approach combined with perfusion can be used as a viable alternative to tissue lysate-labeling. Interestingly, changes in the activity of tricarboxylic acid (TCA) cycle enzymes and the electron transport chain (ETC), have recently been reported to affect energy production and thus synapse loss in AD models. Given that succinate dehydrogenase represents both a critical enzyme in the TCA cycle and complex II of the ETC, our new findings further support the presence of structural changes in the proteins underlying mitochondrial energy metabolism in AD. Furthermore, we observed that our findings from the APPNL-F model were reproducible uring 5XFAD model (See Supplementary text under subheading 'Validation with different AD mouse model' and Supplementary Fig. 22).

More Minor points:

P.2; Line 18: "rapamycin" should be "cyclosporin."
Author's response:
Thank you for the thorough review. We have corrected the term to cyclosporin.

P7; Line 9: The confounding factors that were investigated in the SI should be summarized here.
Author's response:
We have briefly described what we evaluated for presenting the validity of our method at line 10-15 on page 9 as follows: *We validated the methodology's capacity to discern structural changes in proteins by evaluating the presence of potential confounding factors that might influence the interpretation of the results. These factors include the impact of age-related changes in vascular structure on the permeability of reagents, the potential influence of protein abundance on labeling efficiency, and the correlation between accessibility obtained from tissue blocks and tissue lysates post-organ extraction and accessibility obtained from labeling via perfusion.*

P7;Line 35: Would be useful to say in the text that the major finding from the SI that the labelling efficiency was 91.5-97.5% depending on the organ.
Author's response:
We have revised the sentence as reviewer's suggestion.

P7;Line 42: change "were" to "was"
Author's response:
We have corrected this typo.

P8; Line17: should be Bai et al
Author's response:
We have corrected this typo.

P8;Line 19: should be Seyfried et al
Author's response:
We have corrected this typo.

P10; line 27: The phrase, “..showed an increased but insignificant pattern..” is odd. If the “increase” is not significant, then it probably doesn’t deserve mention.

Author’s response:

As per the reviewer’s suggestion, we remove this sentence and revised the content as follows on page 13, line 31 – 33: *The enriched proteins in metal ion homeostasis showed no significant change in expression but a significant decrease in accessibility from 9 months*.

References

1. Latil M*, et al.* Skeletal muscle stem cells adopt a dormant cell state post mortem and retain regenerative capacity. *Nat Commun* **3**, 903 (2012).

2. Porretti L*, et al.* Animal model for liver cell banking from non-heart beating donors after prolonged ischaemia time. *Dig Liver Dis* **38**, 905-911 (2006).

3. Vrselja Z*, et al.* Restoration of brain circulation and cellular functions hours post-mortem. *Nature* **568**, 336-343 (2019).

4. Andrijevic D*, et al.* Cellular recovery after prolonged warm ischaemia of the whole body. *Nature* **608**, 405-412 (2022).

5. ÖZTUNA D, ELHAN AH, TÜCCAR E. Investigation of Four Different Normality Tests in Terms of Type 1 Error Rate and Power under Different Distributions. *Turkish Journal of Medical Sciences* **36**.

6. Ghasemi A, Zahediasl S. Normality tests for statistical analysis: a guide for non-statisticians. *Int J Endocrinol Metab* **10**, 486-489 (2012).