nature portfolio

Peer Review File

Multi-omic analysis of Huntington disease reveals a compensatory astrocyte state

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Paryani et al. performed both bulk and single-nuclei transcriptomic and lipidomic analyses of multiple brain regions using HD patient postmortem tissues. The study provides rich datasets informing the brain regional and cell-type specific transcriptomic changes that are associated with disease severity, and specific lipid changes (e.g. long chain fatty acids) that are altered in HD. The most mechanistically informative finding is the discovery of a neuroprotective protoplasmic astrocyte cluster (P1) that is associated with protection of vulnerable MSNs against neurodegeneration in HD, and it is upregulated in cingulate cortex and nucleus accumbens while depleted in the caudate nucleus. Interestingly, GWAS analysis with combined GeM-HD and Venezuelan Kindreds reveals 3 SNPs that are significantly associated with delayed age at onset. The study also presents a rich set of in vitro experiments to show the benefit of human astrocytes overexpressing MT3: (i). increasing glutamate buffering capacity and protecting against rotenone-induced cytotoxicity; (ii). protecting iPSC-derived HD MSNs from expressing cell death markers in a co-culture; and (iii); increasing microglial phagocytic capacity in a co-culture.

Overall, this is a comprehensive multi-omic survey of the molecular changes in both vulnerable and resistant brain regions in HD postmortem brains. The study provides a multitude of molecular insights into selective neuronal vulnerability and resilience in HD, including novel human genetic and cell biological findings to support a role of MTs (particularly MT3) in astrocytes as a key neuroprotective mechanism in HD. The manuscript could be suitable for publication in Nature Communications if the authors can address the minor issues listed below.

Minor points.

• The CAG repeat in various brain tissues ranges from 40 to 71. Is there any evidence of somatic repeat expansion in tissues that are more vulnerable in HD, e.g., caudate, compared to those that are more resistant, e.g., cingulate cortex?

• What is the reference for the “striatal identity gene PCP4”? I am not aware of this claim.

• Lines 947-955. The detailed methods for snRNA-seq QC steps and DE analysis for various cell clusters (e.g. Lines 248-249) or brain regions (Lines 283-285) are missing and should be provided in more detail. The current description is not sufficient: "Depending on the cell type quality and whether pseudo bulking was applied, the counts filter criteria varied."

• Figure 3C should also include neuronal markers. What is the reason for their exclusion?

• Fig. 4. Please add a supplemental figure to show the distribution of different fibrous and protoplasmic astrocyte cluster cells across individual control and HD patient samples. The aim is to rule out that a particular type is overrepresented by a few samples.

• Fig. 4. Could you show the distribution of different astrocyte types by BOTH brain regions and HD stages or WT? I'm interested in whether the enrichment of “neuroprotective” F1 clusters or any other clusters could be brain region- and HD stage-specific?

• Line 289. The writing style should be more consistent and formal throughout the manuscript. Please remove the casual term such as “For starters,...”

• Both “medium spiny neuron” and “spiny projection neuron” are used in the text to refer to the same neuronal types. Please use one term consistently.

• Lines 432-438. Please provide references for your selection of striatal and cortical neuronal cell type marker genes.

Reviewer #2 (Remarks to the Author):

The paper by Paryani et al., provides multiple integrated multiomic analyses of post mortem HD tissue and concludes with the finding that astrocytes display a neuroprotective state, which was explored with in vitro models. Furthermore, the study contains human validations for the key protein of focus, MT3. Overall, I think the study is valuable and the data seem of high quality. The paper is largely an omic analyses and lacks any real physiology or pathological work in vivo, but this is the nature of this type of work and should not be used to adversely judge the analyses the authors did do. I have several changes to suggest

1. The paper is written poorly in my view and contains long sections and paragraphs that are hard to follow. As such, the paper was dense and very hard to read. I think they should consider separating out the major sections of the results with smaller subheadings for the data related to different brain areas, those related to protoplasmic and fibrous astrocytes etc. In the current format all of these are lumped together and this makes it a very difficult paper to understand. Therefore, I suggest some major edits to enhance the clarity of the presentation. For example, the section starting at line 198 is nearly three pages of single spaced text with no subheadings!

2. The abstract is too long. The authors should try and distill the key take way message into a shorter abstract.

3. The figures contain multiple panels that have font sizes that are too small to be read. The authors should make a better effort to increase the visibility and clarity of the figures.

4. I would caution the authors against using the word neuroprotective, because this has become a loaded term owing to the now defunct neurotoxic and neuroprotective A1 and A2 nomenclature proposed by Barres/Liddelow. Perhaps the authors would consider some subtle edits to the way these particular data are presented. Perhaps they should just discuss “viability”, which is what was measured in Fig 8.

5. Given that the authors propose astrocytes assume neuroprotective roles, the authors should provide a plot of the previously reported pan, A1 and A2 astrocyte reactivity genes. This will allow the reader to easily see if any of these change. How do the authors interpret their findings with respect to the Barres/Liddelow Nature paper arguing astrocytes in HD were neurotoxic? They should place those studies into context with the benefit of the more in depth analyses shown here.

6. Can the authors provide a few more introductory sentences for the pseudotime analysis section and what it means.

7. A specific section of the methods on “Data analysis and statistics” is needed, reporting n numbers, statistical tests and how they were chosen etc. This is particularly relevant for Fig 7 and 8, which contain cellular assessments.

8. Fig 8A was impossible to see and must be changed to be able to interpret it.

9. Bar graphs in Figures 7 and 8 must show n numbers.

Overall, a potentially valuable study that needs another round of hard work to tidy it up and make it more digestible. In the current format, my fear is that few people will actually read or follow the study. With improved presentation this could be a much stronger paper.

Reviewer #3 (Remarks to the Author):

The authors have made commendable efforts to collect multiple samples and to perform multi-omic analyses. This study aims to highlight a cluster of astrocytes that may exhibit neuroprotective qualities in Huntington's disease (HD). While the authors identified that metallothionein-3 (MT3) in astrocytes confers neuroprotection, extending previous observations of MT1 and MT2, the potential functional redundancy or compensatory mechanisms within the MT family remain unclear. When comparing regions of the brain that are more severely affected by HD to those less impacted, it is evident that many cells, genes, and proteins can differ. Logically, one would expect fewer disease-related changes in regions less affected by HD. However, attributing this difference directly to neuroprotection requires experimental validation. The bulk RNA-seq and lipidomic analyses of brain regions present intriguing findings that call for more in-depth analysis and experimental validation. Though the title suggests a multi-omic approach, there seems to be a disconnect between the bulk RNA-seq and lipidomic analyses, and the snRNA-seq analyses. With the data provided in the current version of the manuscript, several conclusions drawn by the authors may require further evidence for validation.
1. Lines 20-21: "Huntington disease (HD) is an incurable neurodegenerative disease characterized by neuronal loss and astrogliosis." The indicated sentence is not entirely accurate. More cell types are involved in HD pathogenesis. As a pathological hallmark of HD, the formation of inclusion bodies in neurons should also be mentioned.

2. Lines 152-153 and Supplementary Figure 4: Both DPP10 and CD44 are not astrocyte-specific genes. Although the expression of CD44 is higher in astrocytes, it is also expressed in other brain cells. DPP10, on the other hand, is expressed mainly in neurons (as also mentioned by the author; Supp Line 58). From the snRNA-seq data, what are the expression patterns of CD44 and DPP10 in other brain cells? It is challenging to discern the co-expression of CD44 and DPP10 in astrocytes from the provided images. Would it be possible to co-stain with a general astrocyte marker to strengthen this assertion?

3. Figures 1F & 1G: Could the author clarify whether the DEGs depicted are separated into upregulated and downregulated DEGs, or if they encompass all DEGs? This distinction would aid in understanding.

4. Lines 160-162: Could the author provide more details on the criteria or methods used to determine that the modules are enriched in genes involved in DNA damage response and loss of connectivity of key astrocytes? Additionally, as mentioned above, when analyzing these genes, are they categorized as upregulated DEGs, downregulated DEGs, or are they a combination of both?

5. Lines 169-172: The rationale presented is unclear. One might expect that analyzing a region more severely affected by HD would yield a stronger correlation with HD progression when compared to a less affected region. How is a less affected region a good candidate to correlate with HD progression? Please elaborate or provide further justification for the chosen region (i.e., the cingulate cortex).

6. Lines 188-189: The statement 'Sex was correlated with the integrated x-y variate-2 dimension'

requires further clarification. It is not immediately evident how this conclusion was drawn. Please elucidate the relationship or provide additional context to support this assertion.

7. Lines 196-197: Similar concerns arise for the statement "… implicating the unfolded protein response as a central pathology that correlates with lipidomic pathology in HD". Please provide more detailed reasoning or evidence to support this statement.

8. Figures 3B and S5C: It would be more informative to include the difference in proportions between HD and Con.

9. Lines 232-234: From Figure 3E, CD44 is highly expressed in Cluster 0, and a portion of Cluster 2, while WIF1 is highly expressed in Cluster 1 and a portion of Cluster 2. Cluster 2 seems to share features of fibrous-like astrocytes and protoplasmic astrocytes. Please further comment on categorizing Cluster 2 as protoplasmic astrocytes?

10. Figure 3F' & 3F'': While fibrous astrocytes appear to exhibit region specificity, there seems to be less distinction in terms of HD grade specificity. Notably, HD grade 4 appears to have a higher representation of the F2 cluster and a reduced F0 cluster. Could the authors elaborate on this observation and discuss its potential implications or associations with the progression or characteristics of HD?

11. Figures 4D & 4H: Although the astrocytes have been classified into 4 fibrous-like and 7 protoplasmic categories, the DEGs analysis seems to treat them as a collective group. It may be more informative to conduct DEGs analysis at the cluster level. This could be especially relevant for the CD44-high cluster F2, which could offer deeper insights.

12. Lines 331-333: The authors have highlighted the capability of pseudotime analysis to uncover and detail the dynamics of branched trajectories in gene expression in an unsupervised manner. Therefore, it might be more informative to first conduct pseudotime analysis on all astrocytes prior to segregating them into fibrous-like and protoplasmic categories. Could this approach potentially illuminate the branching trajectories that differentiate fibrous-like from protoplasmic astrocytes?

13. Lines 410-411: GFAP and Aldh1L1 were used as the markers for astrocytes in Figure S6A-D. With regard to FABP5 and FABP7, is their observed downregulation consistent across all clusters of astrocytes, or is it specific to certain clusters?

14. Figures 6A, 6B, 6G: It would be insightful to understand if there are any changes in cell-cell communications between the caudate neurons and both the fibrous-like and protoplasmic astrocytes. Specifically, are there discernible alterations when considering the PPP1R1B-enriched neuronal clusters?

15. Line 447: It is interesting to see there are loss of LGR5+ interneurons. Based on the marker list in Supplementary Table-8, this cluster may generate ambiguous annotation. Please provide additional analysis/evidence to strengthen the annotation of this cell cluster.

16. Lines 465-466 & Figure 7: Same as above, the author can include the difference in proportions between HD and Con directly from the snRNA-seq analysis. Is the snRNA-seq analysis data consistent with the staining data?

17. Lines 471-472 & Figure 7: The author mentioned "We were intrigued by this finding because it was most prominent in protoplasmic rather than fibrous-like astrocytes", while GFAP was used as the

marker for astrocytes. Is GFAP sufficient to differentiate fibrous-like and protoplasmic astrocytes?

18. Figure 7C: A noticeable difference in the appearance of GFAP-positive cells in the Control group, as compared to the Control groups in 7A and 7E.

19. Figures 7, 8F & Figure S8: Unpaired one-tailed T-test was used for the statistical analysis. Please provide the rationale for selecting a one-tailed test over the more commonly used unpaired two-tailed T-test.

20. Lines 525-526 & Figure S9D: Is the finding on higher levels of SLC1A2 and GLUL consistent with snRNA-seq analysis for the MT3-high astrocytes?

21. Figures 8C-E: Heavy metal (Cadmium) and Rotenone were used as neurodegeneration stimuli. However, given that the focus of this study is HD, using mutant Huntingtin protein as the stressor for such experiments would offer insights more closely aligned with the context of HD.

22. Lines 545-551: To enhance clarity and aid readers in locating relevant data, the author could specify Supplementary Figures 10 and 11 directly in the main text, rather than directing readers to 'see supplementary results' at the end of the paragraph. This paragraph is oversimplified. Please elaborate on the findings with a proper conclusion, or remove the paragraph. In addition, the supplementary results did not provide sufficient details. It is strongly recommended to rearrange the text so that readers can follow more easily.

23. The lipidomic analysis identified elevated levels of long-chain fatty acids in HD. When combined with the transcriptomic data, a set of unfolded protein response genes emerged. When projecting this set of unfolded protein response genes onto the snRNA-seq data, which cell cluster exhibits the highest enrichment? Clarification on this would offer deeper insights into the cellular implications of these findings and would provide a better link between the bulk RNA-seq, lipidomic, and snRNA-seq analyses.

24. Given that metallothionein proteins are highly emphasized in the study, have any members of the metallothionein family (especially MT3) been implicated within the identified unfolded protein response genes? Furthermore, please elaborate on the potential associations between metallothionein proteins and the unfolded protein response genes, specifically in the context of HD.

25. The lipidomic analysis of the HD brain is intriguing. The part that highlights the significant correlation between lipidomic data and HD grade requires further elaboration. Are those long-chain fatty acids protective or detrimental to HD progression? Could the authors conduct functional analyses to validate their hypothesis based on this lipidomic data?

26. Please provide data on the expression levels of MT1, MT2, and MT3 across the three brain regions in different cell types, and highlight the differences between HD and control samples.

27. Lines 612-613: The author claimed that the neuropathological examination using IHC for CD44 suggests a transition from protoplasmic to CD44+ fibrous-like astrocytes. Nonetheless, no supporting data for this assertion were found. Could the authors please explicitly point out which IHC data provide compelling evidence for such a transition?

28. Lines 619-620 and Figure 5D: “This suggested that trajectory 2 represents a conversion of protoplasmic astrocytes to fibrous-like.” To support this claim convincingly, it would be prudent for the authors to perform the pseudotime analysis using all cells before separating them into fibrous-like and

protoplasmic astrocytes.

29. Lines 627-630: “This suggests that the CD44+astrocytes that are intrinsic to the normal CNS do not respond to HD in this way. The protoplasmic astrocytes that have become CD44+, more fibrous-like, also do not protect neurons in this way either.” The basis for this inference is not readily apparent in the presented data.

30. Lines 853-854: The authors mention the profiling of mouse plasma and tissue samples. Could the authors please explicitly indicate where these data are presented in the current study?

31. Supplementary result, lines 158-159 and Figures S11D-E: Given that a human microglial cell line (HMC3) was used for this study, the authors might consider selecting specific genes from this observation for further validation.

32. Supplementary result, lines 160-161 and Figures S11G: The authors mentioned that “microglia co-cultured with MT3 but not CLU overexpressing astrocytes increased their phagocytic activity.” Considering that the RNA-seq analysis is presented in Figures S11D-E, were there any genes or pathways identified that are specifically associated with this increase in phagocytic activity?

33. Supplementary results, lines 161-163: The claim that the increased phagocytic activity in HMC3 is a “compensatory positive phenomenon” appears unsupported by the provided data. Could the authors clarify the connection between this observed increase in phagocytosis and its implications for HD

Minor comments:
1. Line 133: The statement mentions 'HD samples'. For clarity, could the authors please specify whether you are referring to HD samples in general or specifically to HD caudate samples?

2. Line 700: In the phrase 'DEGs shared that are shared…', please revise for clarity.

3. Line 150: for gene name, P2RY1?

4. Figure 4I: Is that minus log10(P-value)?

5. Lines 266-267, 301: For clarity, could you specify the cut-off for the cell number below which they are excluded from further analysis?

6. Line 475: The phrase '...and the found no significant differences...' contains an extra 'the'.

7. Figure 8G: It is unclear why there is a labeling of “Control P2A vs HD P2A”.

8. Supplementary results, line 168: Typo for Figure S11F.

9. Figure S11G: Why was a paired two-tailed t-test used for the statistical analysis?

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Paryani et al. performed both bulk and single-nuclei transcriptomic and lipidomic analyses of multiple brain regions using HD patient postmortem tissues. The study provides rich datasets informing the brain regional and cell-type specific transcriptomic changes that are associated with disease severity, and specific lipid changes (e.g. long chain fatty acids) that are altered in HD. The most mechanistically informative finding is the discovery of a neuroprotective protoplasmic astrocyte cluster (P1) that is associated with protection of vulnerable MSNs against neurodegeneration in HD, and it is upregulated in cingulate cortex and nucleus accumbens while depleted in the caudate nucleus. Interestingly, GWAS analysis with combined GeM-HD and Venezuelan Kindreds reveals 3 SNPs that are significantly associated with delayed age at onset. The study also presents a rich set of in vitro experiments to show the benefit of human astrocytes overexpressing MT3: (i). increasing glutamate buffering capacity and protecting against rotenone-induced cytotoxicity; (ii). protecting iPSC-derived HD MSNs from expressing cell death markers in a co-culture; and (iii); increasing microglial phagocytic capacity in a co-culture.

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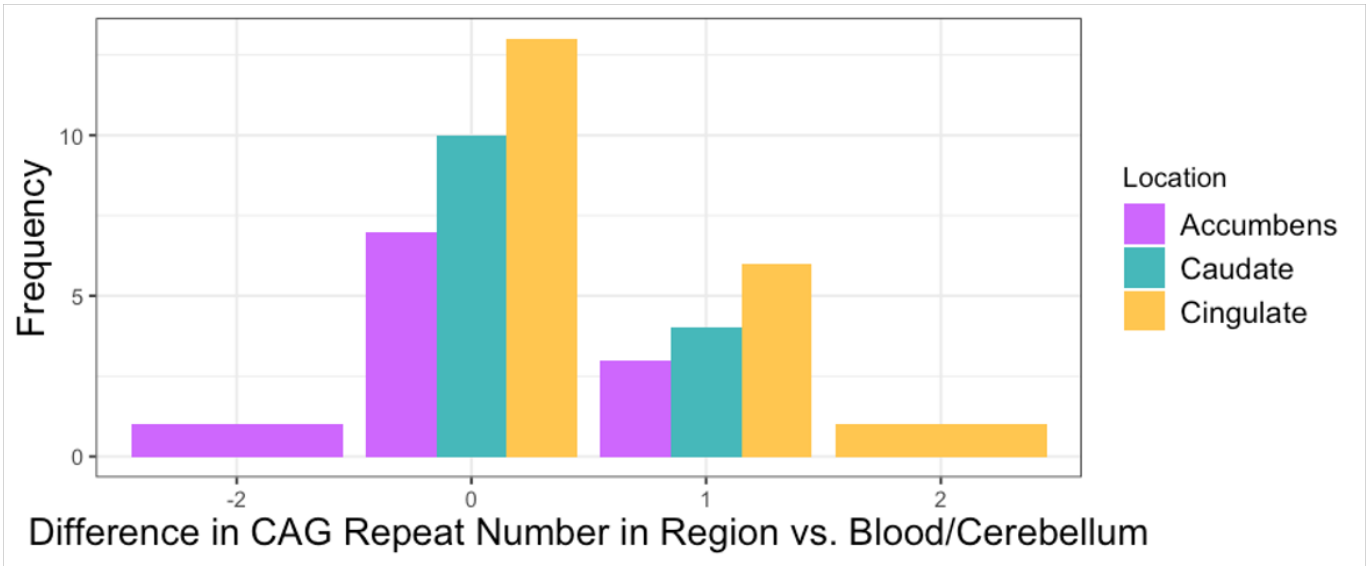
We thank the reviewer for the constructive remarks. We addressed the minor points as indicated below.

Minor points.

• The CAG repeat in various brain tissues ranges from 40 to 71. Is there any evidence of somatic repeat expansion in tissues that are more vulnerable in HD, e.g., caudate, compared to those that are more resistant, e.g., cingulate cortex?

We find that there is very little somatic expansion when we compare the modal peaks within each region per donor. We measured the CAG modal peaks (repeat length) directly from the brain tissue and compared that to the modal peaks derived from the blood or cerebellar tissue. We provided this data in supplementary table-1. We provide a plot of these results below, and in **Figure S1C**. Generally, the most common change in the mode of the CAG repeat is no change or +1 change (see additional figure 1). The caveat is that we do not measure repeat instability, or an expansion index the way Dr. Wheeler outlines (10.1186/1752-0509-4-29). We have noted that as a limitation of our study in the discussion section.



Additional Figure 1. CAG Repeats in Brain vs. Blood. A) Barplot of the difference in CAG repeats in each brain region (purple for accumbens, green for caudate, yellow for cingulate) compared to the blood/cerebellum. Negative x axis values represent fewer CAG repeats in the brain, positive values represent increased CAG repeats in the brain, and 0 represents no change. The y axis shows the number of samples which are represented by the x axis value.

• What is the reference for the “striatal identity gene PCP4”? I am not aware of this claim.
According to the human protein atlas, PCP4 is most highly expressed in the basal ganglia (Brain tissue expression of PCP4 - Summary - The Human Protein Atlas). PCP4 has been previously reported to be highly expressed in the caudate and putamen as well (10.1016/j.acthis.2014.04.012). We included this reference in the main text, and modified this sentence to reflect that PCP4 expression is highest in the basal ganglia rather than it being an identity gene.

• Lines 947-955. The detailed methods for snRNA-seq QC steps and DE analysis for various cell clusters (e.g. Lines 248-249) or brain regions (Lines 283-285) are missing and should be provided in more detail. The current description is not sufficient: "Depending on the cell type quality and whether pseudo bulking was applied, the counts filter criteria varied."
As suggested, we have clarified the methods and provided additional details on QC steps and DE analysis.

• Figure 3C should also include neuronal markers. What is the reason for their exclusion?
We have modified Figure 3C to include an expanded selection of neuronal markers, which now includes *GAD1, SNAP25, CAMK2A, SYT1, RBFOX1,* and *KCNQ5.*

• Fig. 4. Please add a supplemental figure to show the distribution of different fibrous and protoplasmic astrocyte cluster cells across individual control and HD patient samples. The aim is to rule out that a particular type is overrepresented by a few samples.
We have added panel F to Figure S5, which shows the distribution of the clusters by sample as requested by the reviewer. The small clusters P4, P5 and P6, as well as F3, were contributed to by few samples despite aggressive data integration. We did not focus on these clusters in our analysis.

• Fig. 4. Could you show the distribution of different astrocyte types by BOTH brain regions and HD stages or WT? I'm interested in whether the enrichment of “neuroprotective” F1 clusters or any other clusters could be brain region- and HD stage-specific?

We now show the proportions of each cluster in each brain region in Figure S5 (related to Figure 4), panel G, by HD grade and region. Indeed, Cluster F1 is enriched in the cingulate cortex. However, we did not find significant differences between HD and controls in the frequencies of F1 astrocytes in different regions through differential abundance analysis, the results of which are outlined in the table below.

	Caudate	Accumbens	Cingulate
HD mean +/- StDev	83.3+/-84.8	121+/-150.0	106.3+/-73.6
Control mean +/- StDev	32+/-20.6	31.3+/-27.4	56.8+/143.7
logFC	-0.13	0.22	-0.12
P value	0.78	0.4	0.68

• Line 289. The writing style should be more consistent and formal throughout the manuscript. Please remove the casual term such as “For starters,...”

We have edited the text to remove any informalities.

• Both “medium spiny neuron” and “spiny projection neuron” are used in the text to refer to the same neuronal types. Please use one term consistently.

We have edited the text and figures and now only refer to spiny projection neurons (SPNs).

• Lines 432-438. Please provide references for your selection of striatal and cortical neuronal cell type marker genes.

We provided references as suggested by the reviewer.

Reviewer #2 (Remarks to the Author):

The paper by Paryani et al., provides multiple integrated multiomic analyses of post mortem HD tissue and concludes with the finding that astrocytes display a neuroprotective state, which was explored with in vitro models. Furthermore, the study contains human validations for the key protein of focus, MT3. Overall, I think the study is valuable and the data seem of high quality. The paper is largely an omic analyses and lacks any real physiology or pathological work in vivo, but this is the nature of this type of work and should not be used to adversely judge the analyses the authors did do. I have several changes to suggest

We thank the reviewer for the constructive critique. We recognize that the lack of in vivo work is a limitation of our study. We addressed the points raised by the reviewer as indicated below.

1. The paper is written poorly in my view and contains long sections and paragraphs that are hard to follow. As such, the paper was dense and very hard to read. I think they should consider separating out the major sections of the results with smaller subheadings for the data related to different brain areas, those related to protoplasmic and fibrous astrocytes etc. In the current format all of these are lumped together and this makes it a very difficult paper to understand. Therefore, I suggest some major edits to enhance the clarity of the presentation. For example, the section starting at line 198 is nearly three pages of single spaced text with no subheadings!

We apologize for the inconvenience related to formatting. We have re-written the paper and edited the text to make it clearer and added more headings/subheadings as suggested by the reviewer. We have also simplified the analyses to make the results easier to follow.

2. The abstract is too long. The authors should try and distill the key take way message into a shorter abstract.
We have shortened the abstract and made it more concise.

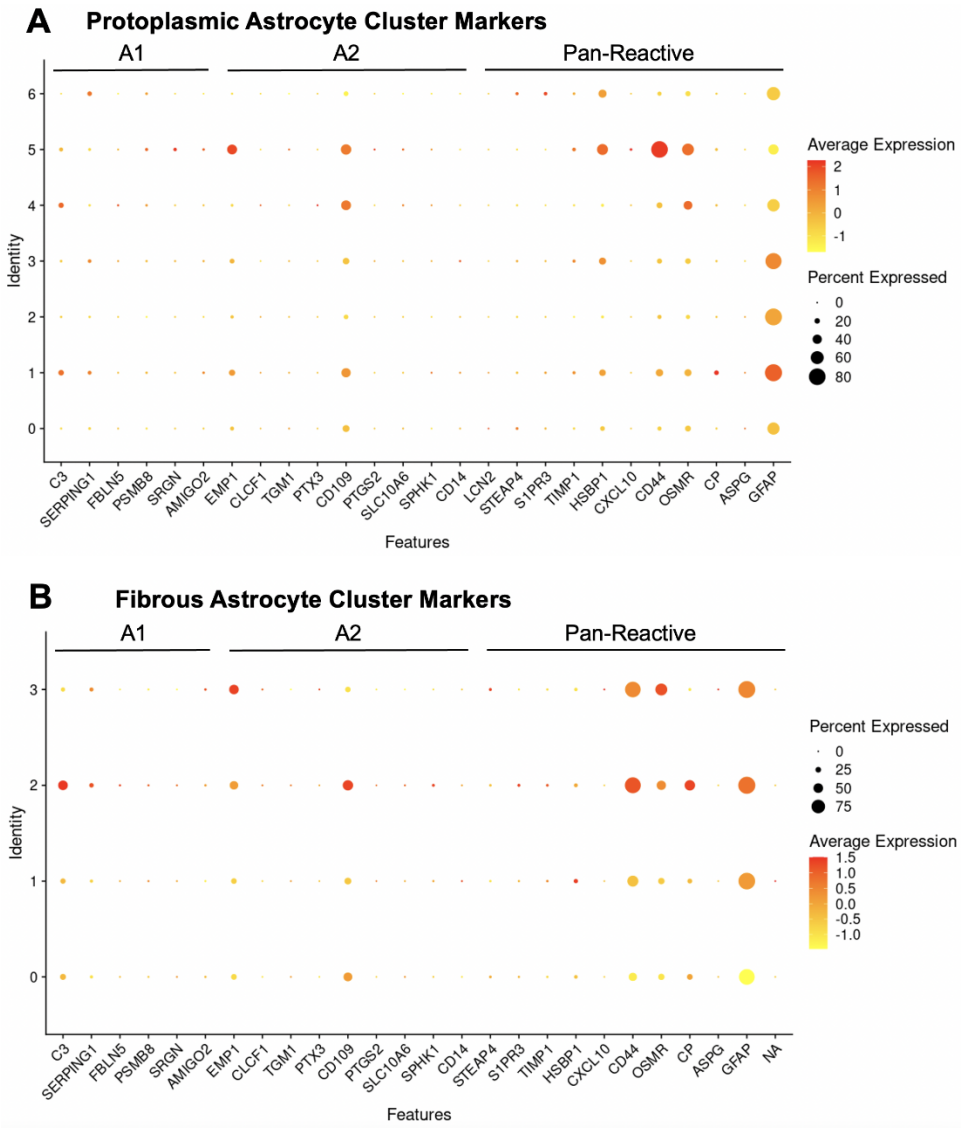
3. The figures contain multiple panels that have font sizes that are too small to be read. The authors should make a better effort to increase the visibility and clarity of the figures.

We have made sure all figures are legible when printed in standard A4 format.

4. I would caution the authors against using the word neuroprotective, because this has become a loaded term owing to the now defunct neurotoxic and neuroprotective A1 and A2 nomenclature proposed by Barres/Liddelow. Perhaps the authors would consider some subtle edits to the way these particular data are presented. Perhaps they should just discuss “viability”, which is what was measured in Fig 8.

We agree with the reviewer. We edited the abstract, sections headings, and the result section to emphasize that what we measure is viability. Accordingly, we discuss that our interpretation of MT3-high astrocytes as neuroprotective requires further validation. We note that in the discussion section.

5. Given that the authors propose astrocytes assume neuroprotective roles, the authors should provide a plot of the previously reported pan, A1 and A2 astrocyte reactivity genes. This will allow the reader to easily see if any of these change. How do the authors interpret their findings with respect to the Barres/Liddelow Nature paper arguing astrocytes in HD were neurotoxic? They should place those studies into context with the benefit of the more in depth analyses shown here.
We provide panels below to show the expression of A1, A2, and pan-reactive genes in astrocytic clusters as suggested by the reviewer. However, we believe referring to the now-defunct A1/A2 states is distracting. The Liddlelow/Barres’ paper only measured expression of C3 in the HD caudate astrocytes as a marker of “toxic A1” astrocytes, and there was no functional validation of whether the HD C3-positive astrocytes were indeed toxic to neurons. We reported previously that C3 was expressed in caudate but not cingulate astrocytes (PMC7029580), and although this finding is consistent with Barres/Liddlelow’s Nature paper, we found before (PMC7029580) and report in this document that the A1 and A2 genes are often co-expressed in the same cells/clusters, or not expressed at all. The fact that a recent Neuron paper (PMC9167747) by the Liddlelow group makes no mention of A1 or A2 states in human AD or control cortex is consistent with what we and others report: that there is no strong support for the existence of pure A1 and A2 signatures in human tissue. Instead, we found that the signature reported by Diaz-Castro et al. (Khakh group 2019 sci tranls med.) is indeed present in our dataset and represents a distinct protoplasmic cluster (P2). We now show this data in Figure 4, panels B and E. We feel that it is more useful for the field to use reproducible signatures and not dwell over ones we cannot reproduce. If the reviewer would like us to include the A1 A2 pan-reactive signature panel in the supplement, we will be happy to oblige.



Additional Figure 2. A) Dot plot of A1, A2, and pan-reactive astrocytic markers in protoplasmic astrocyte clusters. Red dots indicate high average expression, yellow dots indicate low average expression. Larger dots represent a high percentage of expression. B) Same as A but for fibrous-like astrocyte clusters.

6. Can the authors provide a few more introductory sentences for the pseudotime analysis section and what it means.

We have edited the text accordingly and added more introductory sentences to pseudotime analysis.

7. A specific section of the methods on “Data analysis and statistics” is needed, reporting n numbers, statistical tests and how they were chosen etc. This is particularly relevant for Fig 7 and 8, which contain cellular assessments.

We added this section to the methods and explicitly indicated the n numbers and statistics in the legends and figures.

8. Fig 8A was impossible to see and must be changed to be able to interpret it.

We apologize for this inconvenience. We have now improved the quality of figure 8 (now Figure 7) and confirmed its legibility in printed A4 format.

9. Bar graphs in Figures 7 and 8 must show n numbers.
We now explicitly indicate the n numbers in both figures.

Overall, a potentially valuable study that needs another round of hard work to tidy it up and make it more digestible. In the current format, my fear is that few people will actually read or follow the study. With improved presentation this could be a much stronger paper.

We thank the reviewer for the constructive critique. We performed all suggested edits as indicated by the reviewer.

Reviewer #3 (Remarks to the Author):

The authors have made commendable efforts to collect multiple samples and to perform multi-omic analyses. This study aims to highlight a cluster of astrocytes that may exhibit neuroprotective qualities in Huntington's disease (HD). While the authors identified that metallothionein-3 (MT3) in astrocytes confers neuroprotection, extending previous observations of MT1 and MT2, the potential functional redundancy or compensatory mechanisms within the MT family remain unclear. When comparing regions of the brain that are more severely affected by HD to those less impacted, it is evident that many cells, genes, and proteins can differ. Logically, one would expect fewer disease-related changes in regions less affected by HD. However, attributing this difference directly to neuroprotection requires experimental validation. The bulk RNA-seq and lipidomic analyses of brain regions present intriguing findings that call for more in-depth analysis and experimental validation. Though the title suggests a multi-omic approach, there seems to be a disconnect between the bulk RNA-seq and lipidomic analyses, and the snRNA-seq analyses. With the data provided in the current version of the manuscript, several conclusions drawn by the authors may require further evidence for validation.

We thank the reviewer for the constructive critique. We sincerely appreciate the thoughtful comments and suggested edits. We addressed the comments and performed all the suggested experiments that are within the scope of this work. In the main body of the critique, the reviewer refers to three major points that we would like to clarify:

1- "the potential functional redundancy or compensatory mechanisms within the MT family remain unclear"

We do not fully understand the functional redundancy or compensatory between different metallothionein proteins. Dissecting this biological process is an important question, however given the breadth of the study, we think this endeavor is beyond the scope of our study. We now present this as a limitation of the current study in the discussion section.

2- "When comparing regions of the brain that are more severely affected by HD to those less impacted, it is evident that many cells, genes, and proteins can differ. Logically, one would expect fewer disease-related changes in regions less affected by HD. However, attributing this difference directly to neuroprotection requires experimental validation"

We have now made it clear in the text that we do not causally attribute differential neuronal vulnerability to astrocytic phenotypes. Instead, we make the correlations between astrocytic phenotypes that we tested experimentally and neuronal viability. This is a limitation of the study which we now include in the discussion section.

We agree that one would logically expect fewer disease-related changes in less affected brain regions. However, examining the numbers of DEGs in astrocytes tells a different story (see Venn diagrams in Figure 4). See the additional table for a summary:

Astrocyte type	DEG direction (in HD)	Cingulate	Caudate
Protoplasmic	Increased	234	529
Protoplasmic	Decreased	766	471
Fibrous	Increased	499	498
Fibrous	Decreased	501	502

For fibrous astrocytes, the number of DEGs that increased or decreased in HD cingulate and caudate is equivalent. For protoplasmic astrocytes, more DEGs decrease in the cingulate and increase in the caudate. The point we are making is that the disease-related changes differ by brain region and by cell-type. One such difference is the upregulation in MTs in cingulate and accumbens protoplasmic astrocytes. We provided experimental support that MTs in astrocytes enhanced neuronal viability in two models of neurodegeneration – one of which is directly converted HD-patient derived neurons.

3- "The bulk RNA-seq and lipidomic analyses of brain regions present intriguing findings that call for more in-depth analysis and experimental validation. Though the title suggests a multi-omic approach, there seems to be a disconnect between the bulk RNA-seq and lipidomic analyses, and the snRNA-seq analyses"

Our way to connect the lipidomic and transcriptomic analysis was via computational data integration and by deriving a lipidomic gene signature that we directly measure in snRNAseq. This signature was enriched in ontologies related to cell death. We performed functional studies to determine if lipids increased in HD are neurotoxic. One such lipid is a poly-unsaturated fatty acid (20 carbon and 3 unsaturated chains – di-homo gamma linolenic acid). We found DGLA sensitized neurons to death by Rotenone (**Figure 2F-G**). We believe the addition of this functional experiment better connects the different datasets.

We address the major and minor points as indicated below:

1. Lines 20-21: "Huntington disease (HD) is an incurable neurodegenerative disease characterized by neuronal loss and astrogliosis." The indicated sentence is not entirely accurate. More cell types are involved in HD pathogenesis. As a pathological hallmark of HD, the formation of inclusion bodies in neurons should also be mentioned.

We have adjusted the text to include inclusion body formation in the lines specified by the reviewer.

2. Lines 152-153 and Supplementary Figure 4: Both DPP10 and CD44 are not astrocyte-specific genes. Although the expression of CD44 is higher in astrocytes, it is also expressed in other brain cells. DPP10, on the other hand, is expressed mainly in neurons (as also mentioned by the author; Supp Line 58). From the snRNA-seq data, what are the expression patterns of CD44 and DPP10 in other brain cells? It is challenging to discern the co-expression of CD44 and DPP10 in astrocytes from the provided images. Would it be possible to co-stain with a general astrocyte marker to strengthen this assertion

We have both added CD44 and DPP10 to the dot plot in Figure 3C to show the expression patterns in snRNAseq data, and included the co-expression and expression patterns of CD44 and DPP10 in Figure S4. We co-labeled brain sections with GFAP and ALDH1A1 as general astrocyte markers, which can also be seen in Figure S4H-I. We note that in the brain, CD44 is largely specific to astrocytes in the normal brain. We have published a paper supporting this assertion. The only time we see CD44 in cells other than astrocytes, is in the setting of ischemia and infiltration of the brain by T cells and monocytes (10.3390/cells13020129). Normal or activated microglia do not express CD44.

3. Figures 1F & 1G: Could the author clarify whether the DEGs depicted are separated into upregulated and downregulated DEGs, or if they encompass all DEGs? This distinction would aid in understanding.
As indicated in the legend, these are genes both increased and decreased. This is indicated in the figure legend, and we now make this clear in the main text.

4. Lines 160-162: Could the author provide more details on the criteria or methods used to determine that the modules are enriched in genes involved in DNA damage response and loss of connectivity of key astrocytes? Additionally, as mentioned above, when analyzing these genes, are they categorized as upregulated DEGs, downregulated DEGs, or are they a combination of both?
As requested, we clarified the WGCNA methods in method sections. We provided all the enrichment results including p values, geneset size, and overlap size in table S3. The enrichment analysis was performed using a hypergeometric test on module genes – no differentially expressed genes were used here. We clarified this point in the methods.

5. Lines 169-172: The rationale presented is unclear. One might expect that analyzing a region more severely affected by HD would yield a stronger correlation with HD progression when compared to a less affected region. How is a less affected region a good candidate to correlate with HD progression? Please elaborate or provide further justification for the chosen region (i.e., the cingulate cortex).
As suggested by the reviewer, we have elaborated on our rationale for selecting this brain region in the text. We note that the caudate is severely degenerated in post-mortem human brains. Most of our cases were grade 2 and grade 3, where neuronal loss and astrogliosis is advanced (~50-75% of all neurons are lost at grade 2 and grade 3, respectively). We reasoned that at this advanced stage of degeneration, any lipidomic findings may represent an "end stage" phenotype. We wanted to understand the changes that occur earlier in the course of neurodegeneration. So, we sought to uncover transcriptomic and lipidomic abnormalities in a brain region that is not burnt-out and correlate it to the Vonsattel grade of neurodegeneration. The cingulate cortex, as we and others have shown before, exhibits neurodegeneration. Indeed, we show that a specific population of neurons is depleted in layer 5 (Figure 5F). Cingulate involvement is known to correlate with symptomatology, especially psychiatric and mood disorder manifestations, which represent key clinical findings in HD (10.1136/jnnp.2009.181149). Therefore, the cingulate cortex is as suitable a brain region as any other that exhibits neurodegeneration (such as the motor cortex or BA9) and is not end-stage. Our results directly show that we can predict Vonsattel grade which based on striatal neurodegeneration, from the lipidomics applied to the cingulate cortex. Having established this correlation, we can use the cingulate cortex as a window to understand earlier events in neurodegeneration. We now clarified this rational further in the text.

6. Lines 188-189: The statement 'Sex was correlated with the integrated x-y variate-2 dimension' requires further clarification. It is not immediately evident how this conclusion was drawn. Please elucidate the relationship or provide additional context to support this assertion.
This statement was made based on visual inspection of supplemental panel 3C. As the reviewer suggests, we tried to elucidate this relationship. Thus, we performed a statistical test to measure the correlation between sex and x-y vartiate-2 loadings. While we note that average loading of Female and Male sex were -2.06 and 2.27, respectively, sex was not significantly correlated with the loadings (Kendall's correlation Tau coefficient = 0.13 p value = 0.144). Based on this observation, we have now removed this statement.

7. Lines 196-197: Similar concerns arise for the statement "… implicating the unfolded protein response as a central pathology that correlates with lipidomic pathology in HD". Please provide more detailed reasoning or evidence to support this statement.
We thank the reviewer for making this point. The output of integrating lipidomics and transcriptomics was a gene signature enriched in ontologies related to the response to unfolded proteins GO:0006986. These genes include *ATF4, DNAJB1, HSPA1A, HSPA1B, HSPB1,* and *HSF1* genes. We recognize that the response to unfolded protein is not the same as unfolded protein response. We now therefore replace instances of unfolded protein response with the appropriate GO term – response to unfolded protein.

8. Figures 3B and S5C: It would be more informative to include the difference in proportions between HD and Con.

As suggested by the reviewer, we have included differences in proportions between HD and Con in Figure S5F-G.

9. Lines 232-234: From Figure 3E, CD44 is highly expressed in Cluster 0, and a portion of Cluster 2, while WIF1 is highly expressed in Cluster 1 and a portion of Cluster 2. Cluster 2 seems to share features of fibrous-like astrocytes and protoplasmic astrocytes. Please further comment on categorizing Cluster 2 as protoplasmic astrocytes?
As noted by the reviewer, Cluster 2 is a protoplasmic astrocytic cluster. We categorized all cells of cluster 0 as fibrous-like, and all other nuclei, including cluster 2, as protoplasmic. After combining clusters 1, 2, and 3, and then performing sub-clustering of all these astrocytes which we considered protoplasmic, cluster P5 showed high *CD44* expression and contained almost all of the original cluster 2 – supporting the reviewer's point that the original cluster 2 (and subsequently subcluster P5) has mixed protoplasmic and fibrous-like features. Since it was a small cluster, we did not carry it forward for additional analysis.

10. Figure 3F' & 3F'': While fibrous astrocytes appear to exhibit region specificity, there seems to be less distinction in terms of HD grade specificity. Notably, HD grade 4 appears to have a higher representation of the F2 cluster and a reduced F0 cluster. Could the authors elaborate on this observation and discuss its potential implications or associations with the progression or characteristics of HD?

We thank the reviewer for this important point. As the reviewer correctly points out, cluster F2 has more nuclei from HD4 in the caudate nucleus compared to cluster F0 – this is now better shown in updated Figure S5E and denoted in the main text. Unfortunately, our samples are not equally represented across the HD grades and brain regions. This precludes us from elaborating the relationships between clusters and HD grade. Our results also did not identify significant correlations. Accordingly, we presented this limitation in the discussion section.

11. Figures 4D & 4H: Although the astrocytes have been classified into 4 fibrous-like and 7 protoplasmic categories, the DEGs analysis seems to treat them as a collective group. It may be more informative to conduct DEGs analysis at the cluster level. This could be especially relevant for the CD44-high cluster F2, which could offer deeper insights.

As the reviewer suggests, we performed cluster level DEGs as well as gene set enrichment analysis, and provided the results in figure S6A-B as well as updated supplementary table 6.

Regarding cluster F2, we note that it is enriched in HD (updated Figure S5F) and under-represented in control samples. In fact, when pooling all HD grades together, F2 was enriched in HD cingulate and accumbens fibrous-like astrocytes (see below table and Table S5). As noted above by the reviewer, this cluster is also more enriched in HD grade 4. There was an imbalance in its abundance between control and HD, which precluded meaningful DEG analysis. Accordingly, the DEG analysis on cluster F2 was not revealing. The results showed only 5 DEGs, which were not informative. We have now added the results to the supplement.

	Caudate	Accumbens	Cingulate
HD mean +/- StDev	84.00+/-88.34	72.58+/-66.28	7.67+/-10.31
Control mean +/- StDev	30.6+/-24.95	15.50+/-19.67	4.11+/-6.64
logFC	0.009	1.49	0.474
P value	0.9822	0.00082	0.2105

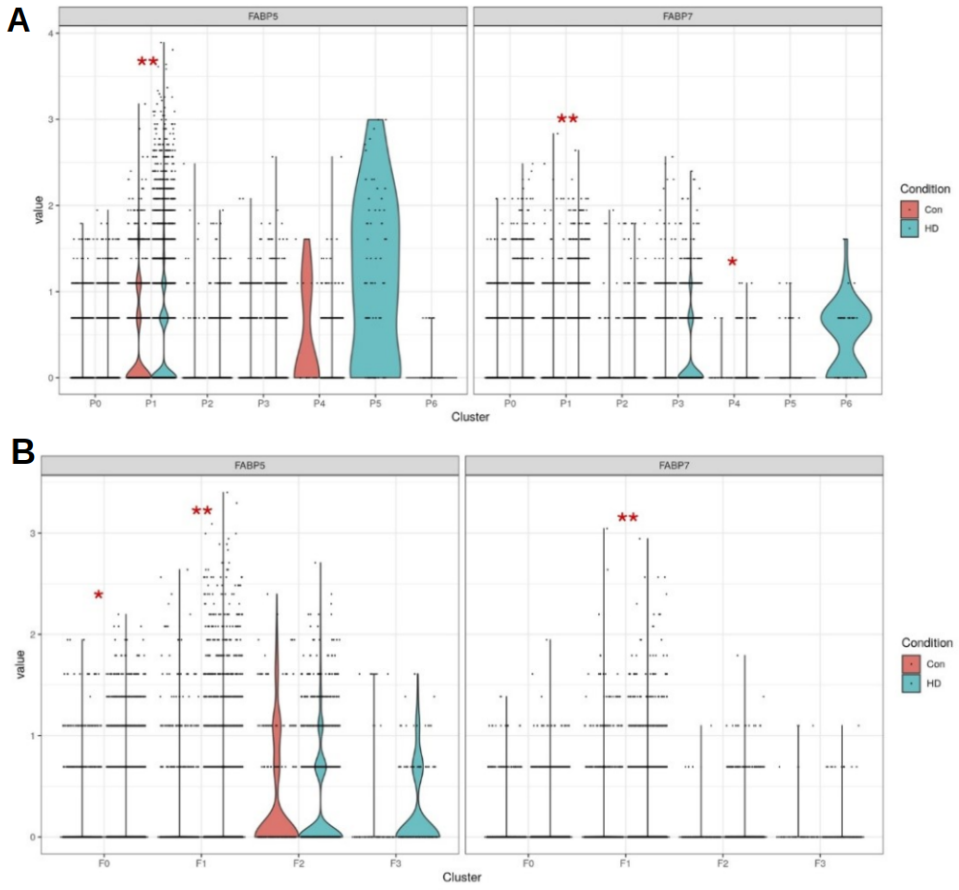
12. Lines 331-333: The authors have highlighted the capability of pseudotime analysis to uncover and detail the dynamics of branched trajectories in gene expression in an unsupervised manner. Therefore, it might be more

informative to first conduct pseudotime analysis on all astrocytes prior to segregating them into fibrous-like and protoplasmic categories. Could this approach potentially illuminate the branching trajectories that differentiate fibrous-like from protoplasmic astrocytes?

We agree with the reviewer. The proposed analysis was informative and more revealing than what we have previously presented. As the reviewer suggested, we have performed pseudotime analysis on all astrocytes together. Because fibrous-like and protoplasmic astrocytes are transcriptionally distinct, we expected the two types to fall on either end of the pseudotime axis, which are defined by high SLC1A2 and high CD44 expression. These results are shown in figure S5D, where trajectory 1 represents this transition from protoplasmic to fibrous-like astrocytes. GO analysis shows that across this trajectory, as astrocytes transition, metallothionein related ontologies are depleted. The results of this analysis are shown in Figure S5E.

13. Lines 410-411: GFAP and Aldh1L1 were used as the markers for astrocytes in Figure S6A-D. With regard to FABP5 and FABP7, is their observed downregulation consistent across all clusters of astrocytes, or is it specific to certain clusters?

We performed cluster level differential gene expression analysis and found that there were only minor reductions in FABP5 and FABP7, mainly in cluster P1 and F1 - see additional figure 3. Given that these changes were not compelling, we chose to remove this data point (previous Figure S6A-D). We think this simplifies the story especially that we do not build on this finding.



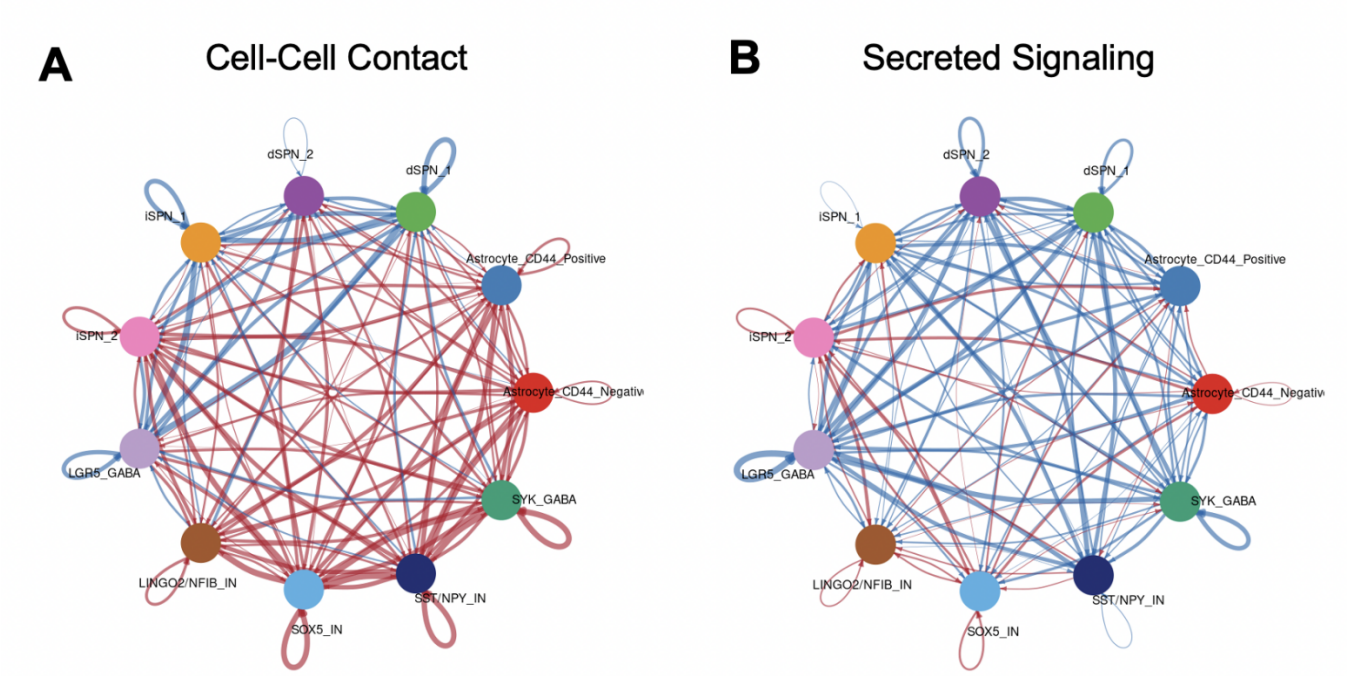
Additional Figure 3. Fatty Acid Binding Protein Expression. A) Violin plots of FABP5 (left) and FABP7 (right) in each of the protoplasmic astrocyte clusters. Orange represents gene expression in control astrocytes, blue represents that in HD. B) Same as A, but for fibrous astrocyte clusters. P values: * <0.05, ** <0.01.

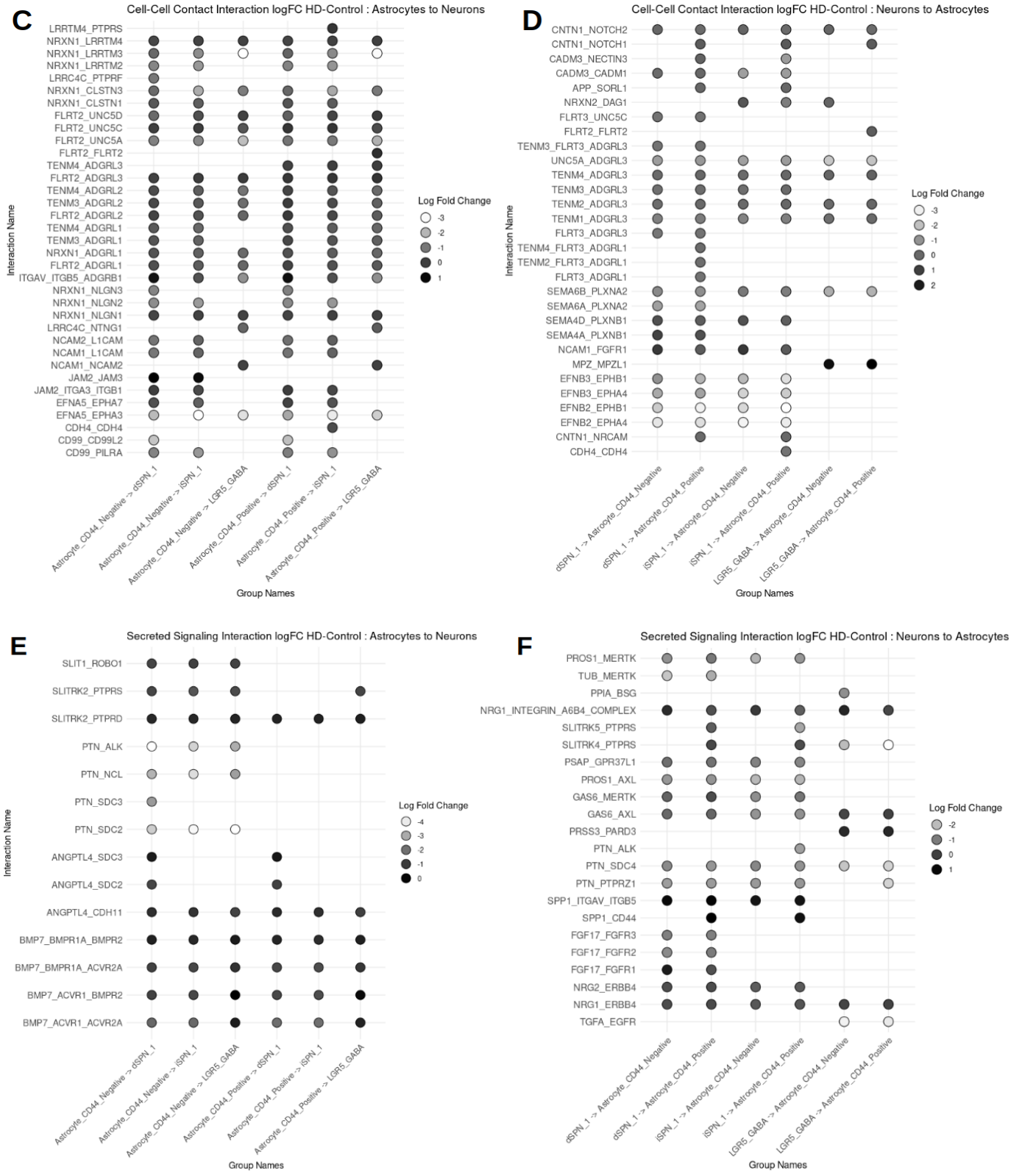
14. Figures 6A, 6B, 6G: It would be insightful to understand if there are any changes in cell-cell communications between the caudate neurons and both the fibrous-like and protoplasmic astrocytes. Specifically, are there

discernible alterations when considering the PPP1R1B-enriched neuronal clusters?

We performed cell-cell interaction analysis using the CellChat R package, and evaluated differences in cell-cell contact and secreted signaling between neurons and astrocytes in the caudate. The results showed few signaling pathways with increased probabilities of interaction, while there were many pathways with decreased probability of interaction. To the point of this manuscript, a few pathways with decreased probability of interaction were relevant to our work. Additional Figure 4A shows diagrams of the number of interactions between all PPP1R1B+ neurons (iSPN's, dSPN's, and LGR5+ neurons) and astrocytes – CD44+ (fibrous-like) and CD44- (protoplasmic). Overall, there was a decrease in probability of interaction between astrocytes and neurons via secreted ligand – receptor pathways, but an increase in probability of interaction between astrocytes and neurons via cell-cell contact (additional figure 4A-B). Also, there was decreased probability of ephrin signaling both from astrocytes to neurons and neurons to astrocytes (Additional Figure 4C-D), decreased BMP signaling from astrocytes to neurons (Additional Figure 4E), and decreased MERTK signaling from neurons to astrocytes (Additional Figure 4F) in the HD caudate compared to control.

While these results are intriguing, further validation is required to ascertain the significance of these findings. We are happy to include these results in the supplementary results section if the reviewer wants us to. At this point, we think that the statements made from cell-cell interaction analysis will remain preliminary without further validation, which is beyond the scope of this manuscript. Thus, we would like to limit the analysis to this document and not include the data in the manuscript.



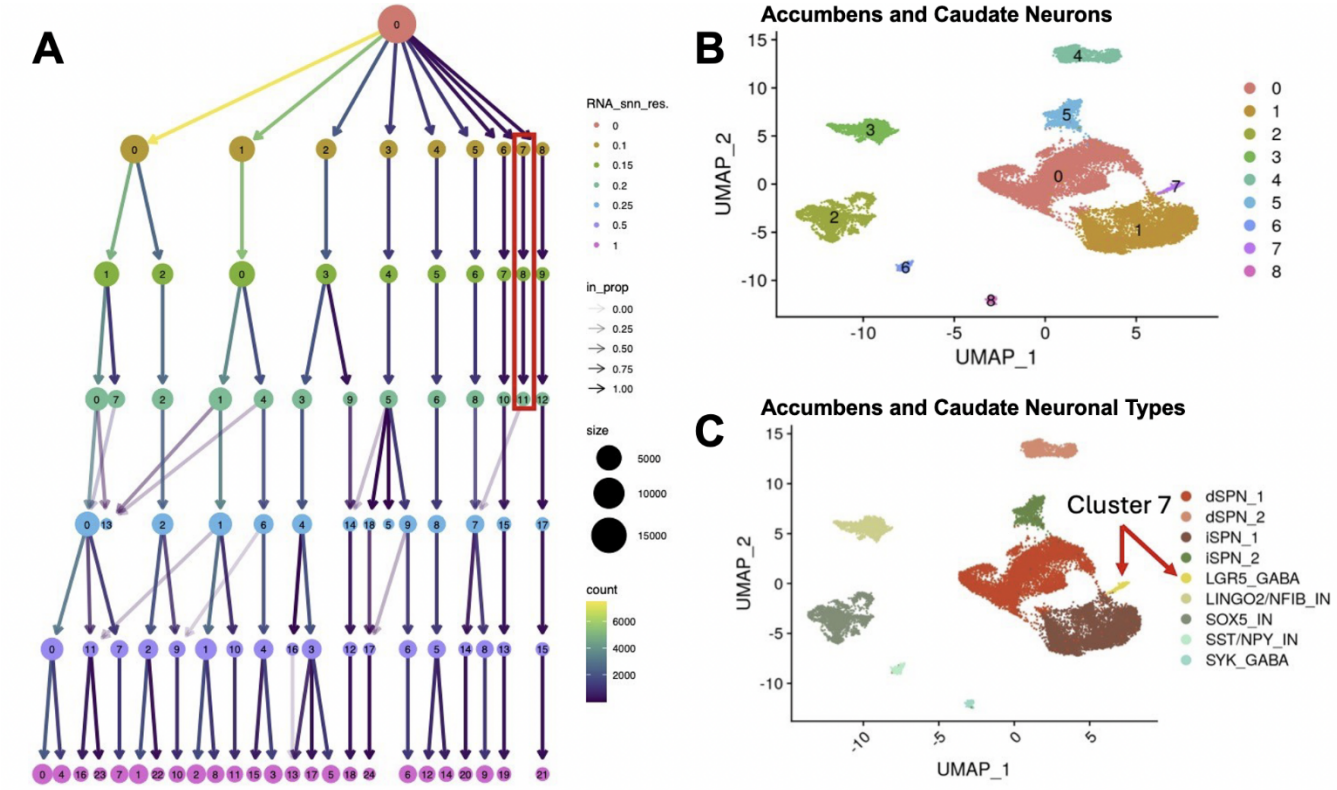


Additional Figure 4. Cell-Cell Communication Analysis.

A-B) Circle plots depicting the difference in the number of interactions between HD and control across all cell types in cell-cell contact (A) and secreted signaling (B). Red lines indicate an increased number of interactions (pathways) in HD and blue lines represent a decreased number of interactions (pathways) in HD. C-D) Dot plots indicating the log (base 2) fold change in cell-cell contact signaling probabilities in HD compared to control in astrocyte to neuron signaling (C) and neuron to astrocyte signaling (D). Darker circles represent more positive logFC values, lighter circles more negative. Signaling pathways are depicted on the y axis, and sub-clusters combinations (represented as origin -> target) on the x axis. E-F) Same as C-D but for secreted signaling.

15. Line 447: It is interesting to see there are loss of LGR5+ interneurons. Based on the marker list in Supplementary Table-8, this cluster may generate ambiguous annotation. Please provide additional analysis/evidence to strengthen the annotation of this cell cluster.

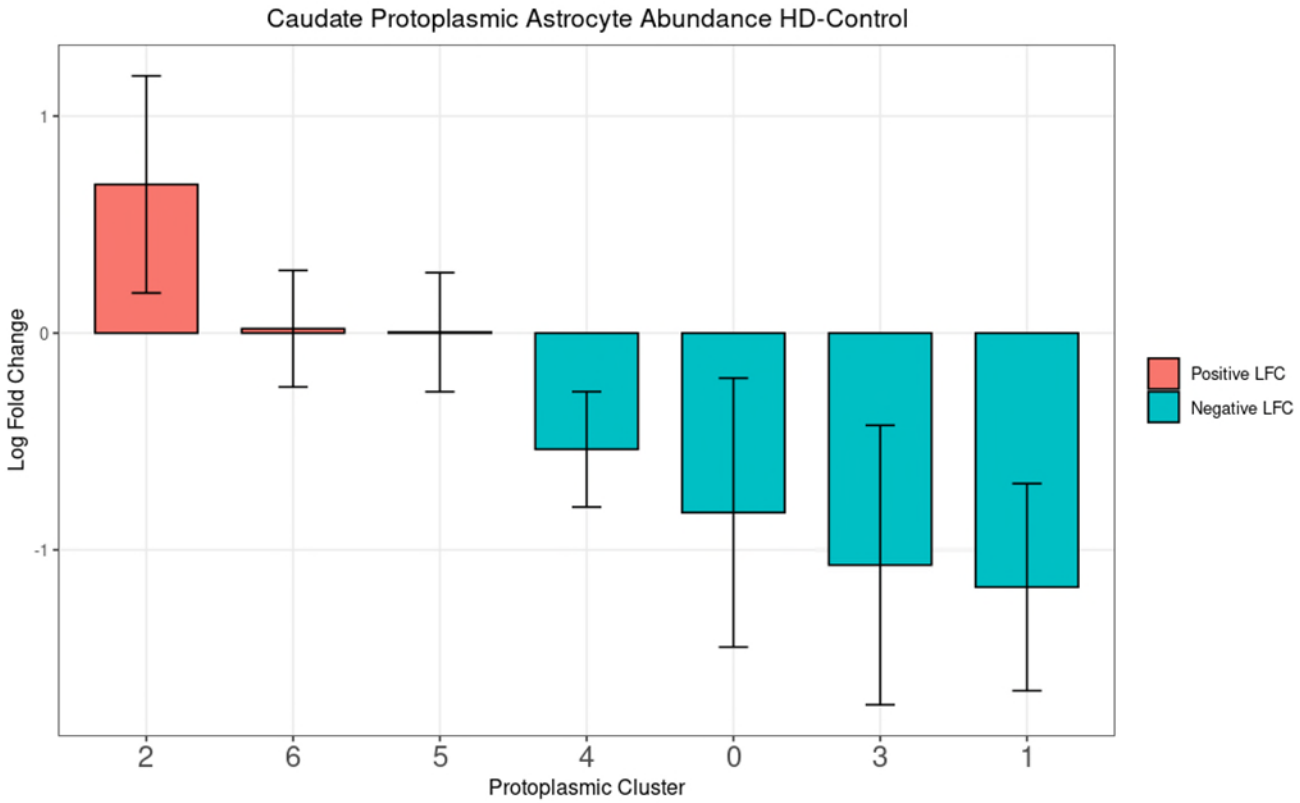
We provided additional analysis to confirm the identity/annotation of this cluster. See cluster stability plot below (Additional Figure 5). Briefly, the clustree package allows the user to determine how stable a cluster is on iterative clustering with different resolutions. The LGR5 cluster (labeled as cluster 7) is stable throughout the clustering process at different resolutions 0-0.2. We chose resolution 0.1 for our analysis . We also performed IHC to validate the loss of LGR5+ neurons in the HD caudate (Supplementary figure 7F).



Additional Figure 5. Cluster Resolution Determination. A) Clustree diagram for accumbens and caudate neurons. Each level, from top to bottom, represents the number of clusters output by different resolutions, with the lowest resolution (0) shown at the top, and the highest resolution (1) shown at the bottom, represented by the color of the dot. The red box highlights the stability of cluster 7 through multiple resolution levels. B) UMAP of accumbens and caudate neurons showing the number of clusters resulting from the selected resolution (0.1). C) Same as B, but displaying the lineages assigned to each cluster.

16. Lines 465-466 & Figure 7: Same as above, the author can include the difference in proportions between HD and Con directly from the snRNA-seq analysis. Is the snRNA-seq analysis data consistent with the staining data?

As suggested by the reviewer, we performed differential abundance analysis on the snRNAseq. We found that, consistent with our IHC data, protoplasmic clusters P1 and P3 which are enriched in metallothionein genes, show decreased abundance in the HD caudate. Cluster P1 decreases in abundance with log fold change of –1.17 with significant p value 0.014 and standard error 0.48, and cluster P3 returns logFC value –1.07, with insignificant P value 0.097 and standard error 0.64 (Additional Figure 6). We present these results below and in the main text.



Additional Figure 6. Differential abundance analysis of caudate protoplasmic astrocyte sub-clusters. Bar plots depicting the log (base e) fold change in abundance for each protoplasmic astrocyte sub-cluster in HD vs control. Orange represents a positive logFC, or increase in abundance in HD, and blue represents a negative logFC, or decrease in abundance in HD. Error bars indicate standard error of the mean. See above text for p values.

17. Lines 471-472 & Figure 7: The author mentioned "We were intrigued by this finding because it was most prominent in protoplasmic rather than fibrous-like astrocytes", while GFAP was used as the marker for astrocytes. Is GFAP sufficient to differentiate fibrous-like and protoplasmic astrocytes?

We thank the reviewer for this point. GFAP is not sufficient to distinguish protoplasmic from fibrous-like astrocytes. CD44, on the other hand, is. We have published a paper recently detailing the expression of CD44 in astrocytes and find no evidence of expression in protoplasmic astrocytes in normal conditions. Instead, we find CD44 expressed in fibrous-like astrocytes in different brain regions (10.3390/cells13020129). Accordingly, we performed additional multiplex immunofluorescence with GFAP, CD44, and MT3 to determine if MT3 is altered in CD44 fibrous-like astrocytes in the caudate and found that MT3 was not increased in fibrous astrocytes, rather it was decreased in CD44- protoplasmic astrocytes (figure S4A, D-E).

18. Figure 7C: A noticeable difference in the appearance of GFAP-positive cells in the Control group, as compared to the Control groups in 7A and 7E.

We appreciate the reviewer's brilliant neuropathologic observation. We agree – the morphologic difference is quite prominent. We have noted that in the results section and provided additional quantifications of astrocytic morphology (as quantified by average astrocyte process length a) in Figure S4C – which show reduction in the average length of astrocytic processes in HD.

19. Figures 7, 8F & Figure S8: Unpaired one-tailed T-test was used for the statistical analysis. Please provide the rationale for selecting a one-tailed test over the more commonly used unpaired two-tailed T-test.

We performed unpaired one-tailed t-test because our transcriptomic analysis allowed us to hypothesize that MT3 was increased in HD in the cingulate in figure 7E-F (now figure 6E-F), decreased in the caudate in figure 7C-D now

(6C-D), and neuroprotective in 8F (Now 7F). We did not have a strong hypothesis on CLU levels (Figure S8), so we now changed the test into a two-tailed t-test. This is a well-accepted concept in statistics (10.1111/j.2041-210x.2010.00014.x). Per the guidelines outlined in the paper referred to, we now elaborate in the methods (data analysis and statistics section) that the selection of a one-tailed t-test was justified because: A) Our hypothesis is driven by transcriptomic data, so we have a priori grounds to predict the direction of the change; and B) We weigh the two potential outcomes in the alternative hypothesis, namely, that there exist no difference between the groups and that the difference between the groups is opposite to our prediction, equally. The reason for this equal treatment of the two outcomes of the alternative hypothesis lies in the goal of the experiment – to validate a predicted finding. Both outcomes of the alternative hypothesis equally invalidate prediction, and the paradoxical outcome of finding a change in the opposite direction of that predicted will not drive future exploration of the original hypotheses. Thus, we surmise that the gain in power attained using the one-tailed t-test justifies the inability to discover findings opposite to what is predicted.

We have now simplified the manuscript and removed data that we do not build on. Accordingly, we removed the data in figure S8 regarding YKL40 results.

20. Lines 525-526 & Figure S9D: Is the finding on higher levels of SLC1A2 and GLUL consistent with snRNA-seq analysis for the MT3-high astrocytes?

We now provide violin plots for *SLC1A2* and *GLUL* expression in MT-high astrocyte clusters in Figure S9E-G to allow the reader to compare the results. We found that *GLUL* expression was higher in *MT3*-high astrocytes. Also, the levels of *MT3* and *GLUL* were correlated. We did not find the same to apply to *SLC1A2* at the snRNAseq results, however, finding reduced glutamate levels in the media of MT3-astrocytes are consistent with increased levels of *SLC1A2*. This may be due to differences between astrocytes *in vivo* and *in vitro*.

21. Figures 8C-E: Heavy metal (Cadmium) and Rotenone were used as neurodegeneration stimuli. However, given that the focus of this study is HD, using mutant Huntingtin protein as the stressor for such experiments would offer insights more closely aligned with the context of HD.

We agree with the reviewer. We used Cadmium because MT3 is known to bind Cadmium (see for review 10.3390/ijms18061117). We expected MT3 overexpression to protect against Cadmium in astrocytes. In essence that showed that MT3 overexpression does what we expect it to. We used Rotenone because it is known to induce neurodegeneration (10.1016/j.neulet.2011.03.036), that would serve as one model of neurodegeneration. Finally, in Figure 7E (previously 8E), we measured HD neuronal apoptosis markers in neurons directly converted from HD patient fibroblasts, co-cultured with astrocytes with either GFP control expression, or MT3 expression. This latter point speaks directly to the reviewer's point.

22. Lines 545-551: To enhance clarity and aid readers in locating relevant data, the author could specify Supplementary Figures 10 and 11 directly in the main text, rather than directing readers to 'see supplementary results' at the end of the paragraph. This paragraph is oversimplified. Please elaborate on the findings with a proper conclusion, or remove the paragraph. In addition, the supplementary results did not provide sufficient details. It is strongly recommended to rearrange the text so that readers can follow more easily.

As the reviewer suggests, we have now removed this paragraph, and instead discuss the results in the supplementary results section. We also re-wrote the section on astrocyte-microglia cross-talk in the supplementary results to provide a clearer and more comprehensive description of the data with additional details as suggested.

23. The lipidomic analysis identified elevated levels of long-chain fatty acids in HD. When combined with the transcriptomic data, a set of unfolded protein response genes emerged. When projecting this set of unfolded protein response genes onto the snRNA-seq data, which cell cluster exhibits the highest enrichment? Clarification on this would offer deeper insights into the cellular implications of these findings and would provide a better link between the bulk RNA-seq, lipidomic, and snRNA-seq analyses.

This visualization was originally provided as heatmaps for protoplasmic and fibrous astrocytes separately across regions in original Figure 4C and 4G. Per the reviewer's suggestions, we now provide a clearer projection of the enrichment of the unfolded protein response geneset on the snRNAseq clusters in now updated Figure 4B and 4E.

24. Given that metallothionein proteins are highly emphasized in the study, have any members of the metallothionein family (especially MT3) been implicated within the identified unfolded protein response genes? Furthermore, please elaborate on the potential associations between metallothionein proteins and the unfolded protein response genes, specifically in the context of HD.

Metallothioneins are not part of the lipidomics-correlated gene set, which we highlighted as enriched in genes involved in response to unfolded protein. Examination of figure 4 reveals no consistent associations between the metallothionein signature and the genes correlated with the lipidomic signature or response to unfolded protein. In the discussion section, we now refer to a report whereby MT3 has been noted to block mutant Htt aggregation in vitro (10.1042/BST0380552), and indicate that metallothioneins may block neurodegeneration via multiple mechanisms. This is a potential link between metallothioneins and protein folding.

25. The lipidomic analysis of the HD brain is intriguing. The part that highlights the significant correlation between lipidomic data and HD grade requires further elaboration. Are those long-chain fatty acids protective or detrimental to HD progression? Could the authors conduct functional analyses to validate their hypothesis based on this lipidomic data?

As the reviewer suggested, we have now performed functional studies on the effects of a long chain fatty acid species correlated with HD grade on neuronal viability. Our data show that at least a subset of these long-chain fatty acids is deleterious.

We tested the effects of a poly-unsaturated fatty acid (di-homo-gamma linolenic acid - 20:3) on neuronal viability and found that it sensitized murine neurons to cell death induced by Rotenone. The results are provided in main Figure 2F-G.

26. Please provide data on the expression levels of MT1, MT2, and MT3 across the three brain regions in different cell types, and highlight the differences between HD and control samples.

As suggested by the reviewer, we provide this data. MT genes were mainly expressed in astrocytes (Figure 3C). We also show the expression of MT genes in the protoplasmic and fibrous-like astrocytic clusters across the three brain regions in Figure S9E-F.

27. Lines 612-613: The author claimed that the neuropathological examination using IHC for CD44 suggests a transition from protoplasmic to CD44+ fibrous-like astrocytes. Nonetheless, no supporting data for this assertion were found. Could the authors please explicitly point out which IHC data provide compelling evidence for such a transition?

This statement was made based on quantifications (previous Figure S4A-B now presented as Figure S4F) and the neuropathological assessment of CD44 in the caudate. In the control brain, CD44 is limited to the subependymal zone, around large vessels, and in white matter tracts. Protoplasmic astrocytes in caudate parenchyma do not express CD44. We report that in this body of work (0.3390/cells13020129).

We have now performed additional IHC for GFAP and CD44 and provided quantification of CD44 in the parenchymal GFAP+ astrocyte in controls and HD in the updated Figure S4A-B. Our results show an increase in the proportion of parenchymal GFAP+ cells that are CD44+ in HD versus control. These GFAP+CD44+ astrocytes were parenchymal astrocytes not associated with large vessels, subependymal zone, or white matter (Figure S4F). These data support that protoplasmic astrocytes increase CD44 in HD, and by definition, become fibrous-like, i.e., CD44+.

28. Lines 619-620 and Figure 5D: "This suggested that trajectory 2 represents a conversion of protoplasmic astrocytes to fibrous-like." To support this claim convincingly, it would be prudent for the authors to perform the pseudotime analysis using all cells before separating them into fibrous-like and protoplasmic astrocytes.

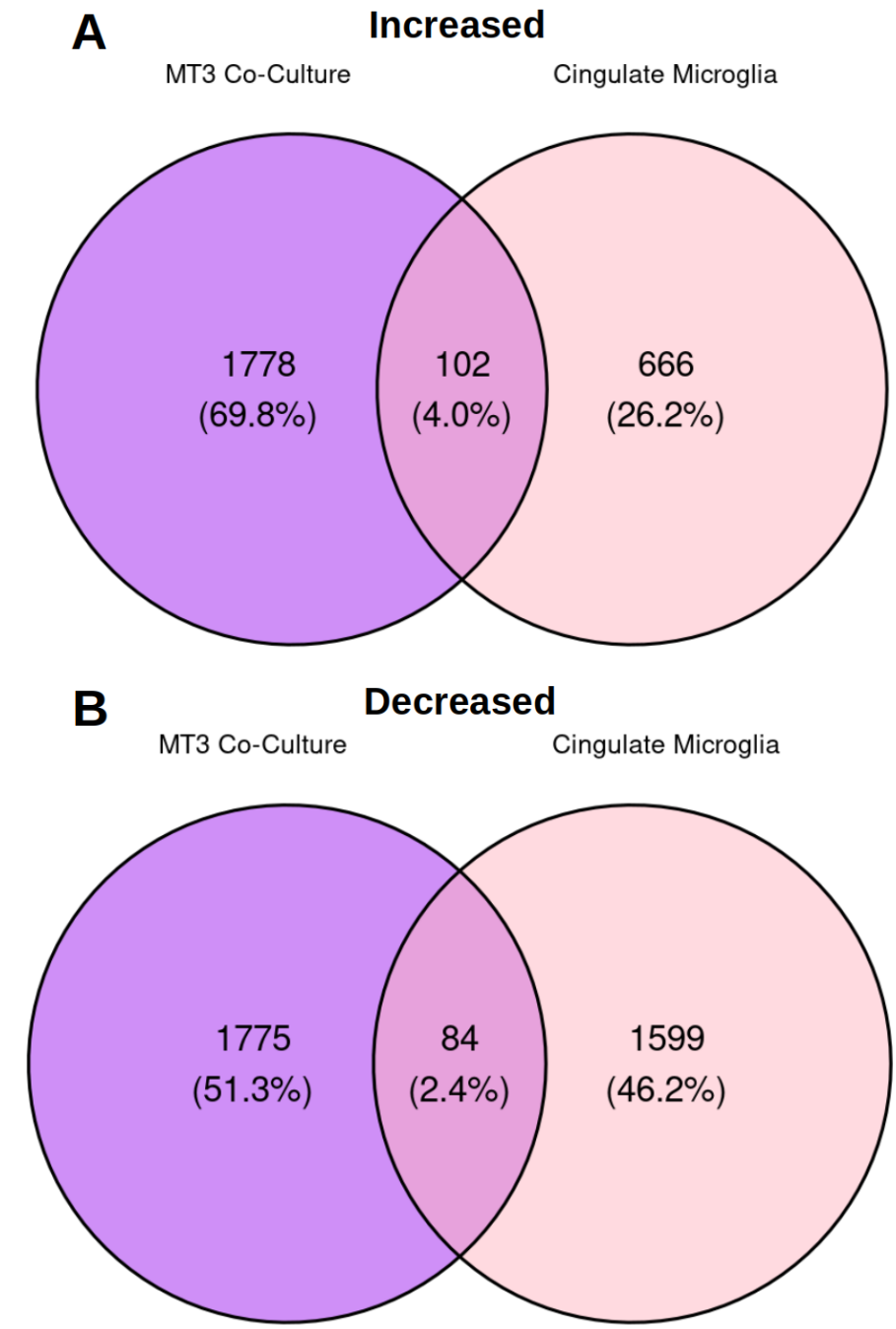
As suggested by the reviewer, we performed this analysis and report the results in Figure S5D-E. Based on these results, it was clear that the analysis proposed by the reviewer is more compelling and relevant than the one we had previously presented in Figure-5. We therefore removed the previous figure 5 and replaced it with the simpler Figure S5D-E. We believe this provides a clearer picture of astrocyte state transitions and relates it to metallothionein levels – which is relevant to the message this manuscript makes.

29. Lines 627-630: "This suggests that the CD44+astrocytes that are intrinsic to the normal CNS do not respond to HD in this way. The protoplasmic astrocytes that have become CD44+, more fibrous-like, also do not protect neurons in this way either." The basis for this inference is not readily apparent in the presented data.
We have removed this statement.

30. Lines 853-854: The authors mention the profiling of mouse plasma and tissue samples. Could the authors please explicitly indicate where these data are presented in the current study?
We apologize for this mistake. This is not accurate. We have corrected the text. No mouse serum or tissue was used in this study.

31. Supplementary result, lines 158-159 and Figures S11D-E: Given that a human microglial cell line (HMC3) was used for this study, the authors might consider selecting specific genes from this observation for further validation.
Before we attempt to validate microglial gene expression changes human brain tissue, we examined the overlap between DEGs in HD vs control microglia in the cingulate cortex (where the MT3 upregulation in astrocytes was confirmed), and the DEGs in HMC3 co-cultured with MT3 vs control astrocytes. Overall, there were few shared genes (Additional Figure 7A-B). And the LFC's were small in the snRNAseq. We decided to first perform qPCR validation studies on a subset of genes increased in HMC3 co-cultured with MT3 astrocytes to confirm the increase in the genes prior to further validation (See table). One such gene was shared with DEGs from HD cingulate microglia (*SLC5A3* – we chose this gene because we have an antibody for it). Our results showed no significant increase in RNA levels as determined by qPCR (for example *ITGB6* – see Additional Figure 7C below). It is unlikely to identify changes in protein levels with such small logFC's. This could be related to the difference between microglia in situ in the brain and HMC3 cells cultured *in vitro*. We therefore decided that this experiment is not feasible and will not be the best use of valuable human patient material.

Name	Cingulate Microglia log2FC	MT3 Co-Culture log2FC
ITGB6	Not DE	1.21 (adj. p value 0.013)
ELOVL4	-0.04 (adj. P value 0.480)	0.87 (adj. p value 0.010)
NACA2	Not DE	2.15 (adj. p value 0.001)
SLC5A3	0.3 (adj. P value 5.3e-6)	0.61 (adj. p value 0.030)


A
Increased
MT3 Co-Culture
Cingulate Microglia
1778
(69.8%)
102
(4.0%)
666
(26.2%)
B
Decreased
MT3 Co-Culture
Cingulate Microglia
1775
(51.3%)
84
(2.4%)
1599
(46.2%)
C
20240118_HAcoCultureMT3
Fold change
1.5
1.0
0.5
0.0
ITGB6
ELOVL4
NACA2
SLC5A3
HMC3-P2A
HMC3-MT3

Additional Figure 7. A) Venn diagram of shared increased DEGs between cingulate microglia, HD vs. control, and HMC3 co-cultured with MT3 astrocytes vs. P2A (control) astrocytes. B) Same as A, but for decreased DEGs. C) Barplots indicating the normalized expression of specified genes (normalized to GAPDH). Lighter bars represent expression in HMC3 co-cultured with control GFP (P2A) astrocytes, dark bars represent HMC3 co-cultured with MT3 astrocytes. Data is shown as mean +/- SEM. N = 3 three experiments. No significant changes were detected.

32. Supplementary result, lines 160-161 and Figures S11G: The authors mentioned that "microglia co-cultured with MT3 but not CLU overexpressing astrocytes increased their phagocytic activity." Considering that the RNA-seq analysis is presented in Figures S11D-E, were there any genes or pathways identified that are specifically associated with this increase in phagocytic activity?
We did not find any differentially expressed genes that were associated with phagocytosis. We now offer potential explanations for this discrepancy. We specifically write: "... This may be explained by the fact that the percentage of microglial cells with increased phagocytosis was relatively low in our assay, thus, gene expression changes may not have been large enough to be detected by bulk RNAseq. It is also possible that the changes in phagocytosis induced by MT3 astrocytes occur on the protein level without durable gene expression changes in phagocytosis-related pathways."
33. Supplementary results, lines 161-163: The claim that the increased phagocytic activity in HMC3 is a "compensatory positive phenomenon" appears unsupported by the provided data. Could the authors clarify the connection between this observed increase in phagocytosis and its implications for HD
We had prefaced the statement by "we posit that" to indicate that the statement was speculative. We have now clearly indicated that the functional implications of this phenomenon, whether it is compensatory or deleterious, remains to be determined.

Minor comments:
1. Line 133: The statement mentions 'HD samples'. For clarity, could the authors please specify whether you are referring to HD samples in general or specifically to HD caudate samples?
We refer to all the samples we analyzed from the 3 brain regions - we have now clarified this statement in the text.

2. Line 700: In the phrase 'DEGs shared that are shared…', please revise for clarity.
We have now clarified this statement.

3. Line 150: for gene name, P2RY1?
We have fixed the gene name in the text.

4. Figure 4I: Is that minus log10(P-value)?
Yes- we have clarified the legend accordingly.

5. Lines 266-267, 301: For clarity, could you specify the cut-off for the cell number below which they are excluded from further analysis?
We clarified the cut-off for excluding cells from analysis. We excluded any cluster with less than 200 nuclei - (Cluster F3, P5, and P6). A total of 278 cells were excluded. We note these details in the methods section and in the related results section.

6. Line 475: The phrase '...and the found no significant differences...' contains an extra 'the'.
We have addressed this typo.

7. Figure 8G: It is unclear why there is a labeling of "Control P2A vs HD P2A".
We clarified this labeling – P2A represents GFP transduced astrocytes. We removed this confusing nomenclature.

8. Supplementary results, line 168: Typo for Figure S11F.
We have fixed the typo.

9. Figure S11G: Why was a paired two-tailed t-test used for the statistical analysis?
Paired testing was used because the data is derived from 4 paired (condition vs control) biological replicates, which exhibit batch effects because the experiments were done on separate plates and separate days. The batch effects are controlled for by pairing. A two-tailed test was used because we did not have a prior hypothesis on the effect of MT3 on phagocytosis. We now clarify these points in the methods section – data analysis and statistics.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this revision, the authors have fully addressed the minor issues raised by this reviewer. I consider the manuscript suitable for publication in Nature Communications.

Reviewer #2 (Remarks to the Author):

The authors responses have addressed the previous comments and the manuscript is improved. Unfortunately, the authors did not highlight the parts of the text that they changed, which made identifying the changes difficult. The following remaining comments should be addressed.

1. In Fig 2F, why is viability greater than 100%? Some clarification needed.

2. The manuscript frequently mixes singular and plural. This needs to be corrected.

3. In one of my major comments I cautioned against using the word "neuroprotective" when referring to the astrocyte signatures. In their rebuttal, the authors agreed and said they made changes accordingly. However, the title still declares astrocytes as neuroprotective in HD and this is repeatedly stated in the text. In fact, there is no evidence to support this for HD. The in vitro experiments are far removed from HD and either involve toxins (cadmium, rotenonone) or induced neurons. Given the simplistic nature of these evaluations, I again recommend that the authors tone down their assertions that astrocytes are neuroprotective. If they wish to use this description they should provide stronger supporting evidence.

4. In Fig 6 the authors should clarify in the text that they are plotting the % of GFAP+ astrocytes that also express MT. This is because GFAP does not label all astrocytes. This could be done by editing the Y-axis labels of the bar graphs in Fig 6.

5. Graphs in Fig 2F, 7D, 7E and 7G all assess viability. But, the Y-axes are different for Fig 2, Fig 7D,E and Fig 7G. The data should be shown in a manner that reports cell viability as a percent as in Fig 2F. The current presentation of the data is confusing.

6. The writing has improved, but parts were still hard to follow, and the manuscript appeared rushed (some titles of sections are in blue, other are not and the relevance is unclear). This could be fixed at the editorial stage with professional editorial help.

7. The title starts with the word "Multi-OMIC". Why is OMIC in capitals? I suspect "Multi-omic" is sufficient.

Hopefully the authors can address these comments at the next stage without the need for another round of review. They should check the text for clarity.

Reviewer #3 (Remarks to the Author):

Although this revision significantly improves the manuscript, there are still concerns to be addressed and typos to be corrected as follows. In addition, the manuscript remains hard to follow. Further editing to make it flow better would be helpful.

1. Figure S4H-I: It is difficult to observe cells with triple signals (DPP10 and CD44, plus ALDH1L1 or GFAP). The regions indicated by white arrows fail to confirm the observation suggested by the authors. Please improve the image quality.
2. Figure S6B: The title of the Figure legend is “Heatmap showing GO enrichment analysis of the DEGs”. However, the data presented seem to be a combination of GO, KEGG, and Reactome. Please verify and revise the legend, if necessary. In addition, in the main text (Lines 399-400), the authors suggest that “… glutamate receptor activity term was most significantly enriched in 400 genes with lower expression in accumbens fibrous-like astrocytes in HD. (Figure S6B)”. However, it is unclear how the results in Figure S6B could reflect the abovementioned statement. Please verify.
3. Figure 7F: Please include the HD grade in the legend.
4. Figure S9E-F: The violin plots show the expression of a few genes of interest. Were they plotted using normalized or unnormalized data? Please verify.
5. Figure 4B, 4E: The authors have shown that a combination of lipidomic and transcriptomic data yielded “unfolded protein response genes”. It is worth noting that these two data sets (lipidomic and transcriptomic) were derived from bulk tissues, not exclusively from astrocytes. Thus, when projecting these “unfolded protein response genes” onto snRNA-seq data (not just astrocytes), what is the expression pattern of this set of genes among brain cells? The author please verify.
6. Figure 4B, 4E: What is “Diaz Castro”? Please clarify.
7. Please clarify whether the enrichment analyses presented in figures throughout the manuscripts are a combination of GO, KEGG, and Reactome. If so, proper legends and text should be provided, rather than simply referring to them as “GO analysis”.
8. Line 461: Please note that there is no Figure 4I in Figure 4. Is it a typo?
9. Line 462: It is unclear why (Figure 4G) is assigned here. Please clarify.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this revision, the authors have fully addressed the minor issues raised by this reviewer. I consider the manuscript suitable for publication in Nature Communications.

Reviewer #2 (Remarks to the Author):

The authors responses have addressed the previous comments and the manuscript is improved. Unfortunately, the authors did not highlight the parts of the text that they changed, which made identifying the changes difficult. The following remaining comments should be addressed.

We followed the reviewer's suggestions in round-1 comments and edited/re-wrote almost every paragraph in the manuscript. Thus, highlighting the edited text would have not been helpful.

1. In Fig 2F, why is viability greater than 100%? Some clarification needed.

This is explained by well-to-well variability in fluorescence measurement, which are normalized to the control wells. In fact, a one-sample t-test of the viability in each group shows none are significantly different from 100% (two tailed p values: 0.9994, 0.9332, 0.6901, 0.1100 for the groups shown in figure 2F, respectively). We included this clarification in the methods.

2. The manuscript frequently mixes singular and plural. This needs to be corrected.

We edited the text to improve clarity. We will make further editorial changes as necessary and as suggested by the editorial team to address this point.

3. In one of my major comments I cautioned against using the word "neuroprotective" when referring to the astrocyte signatures. In their rebuttal, the authors agreed and said they made changes accordingly. However, the title still declares astrocytes as neuroprotective in HD and this is repeatedly stated in the text. In fact, there is no evidence to support this for HD. The in vitro experiments are far removed from HD and either involve toxins (cadmium, rotenonone) or induced neurons. Given the simplistic nature of these evaluations, I again recommend that the authors tone down their assertions that astrocytes are neuroprotective. If they wish to use this description they should provide stronger supporting evidence.

We changed the title and the throughout the text to switch the word "neuroprotective" for "compensatory", or prefaced the word "neuroprotective" with "putative".

4. In Fig 6 the authors should clarify in the text that they are plotting the % of GFAP+ astrocytes that

also express MT. This is because GFAP does not label all astrocytes. This could be done by editing the Y-axis labels of the bar graphs in Fig 6.

We edited the y-axis label in figure 6 accordingly.

5. Graphs in Fig 2F, 7D, 7E and 7G all assess viability. But, the Y-axes are different for Fig 2, Fig 7D,E and Fig 7G. The data should be shown in a manner that reports cell viability as a percent as in Fig 2F. The current presentation of the data is confusing.
We changed the y-axis of the bar graphs in figure 7 D, E, and F to show percent viability rather than proportion.

6. The writing has improved, but parts were still hard to follow, and the manuscript appeared rushed (some titles of sections are in blue, other are not and the relevance is unclear). This could be fixed at the editorial stage with professional editorial help.

We will editorial changes as suggested by the editorial team to improve clarity and consistency in formatting.

7. The title starts with the word "Multi-OMIC". Why is OMIC in capitals? I suspect "Multi-omic" is sufficient.

We changed the title accordingly.

Hopefully the authors can address these comments at the next stage without the need for another round of review. They should check the text for clarity.

Reviewer #3 (Remarks to the Author):

Although this revision significantly improves the manuscript, there are still concerns to be addressed and typos to be corrected as follows. In addition, the manuscript remains hard to follow. Further editing to make it flow better would be helpful.

We edited the text to improve clarity. We will make further editorial changes as necessary and as suggested by the editorial team to address this point.

1. Figure S4H-I: It is difficult to observe cells with triple signals (DPP10 and CD44, plus ALDH1L1 or GFAP). The regions indicated by white arrows fail to confirm the observation suggested by the authors. Please improve the image quality.

Since the reviewer is not convinced of the co-labeling of DPP10 and astrocyte markers, we removed the data in Figure S4G-I, and removed the supplementary section entitled “A subset of fibrous-like astrocytes expresses DPP10” – lines 68-77 supplementary results. This does not change any of the other findings in the manuscript.

2. Figure S6B: The title of the Figure legend is “Heatmap showing GO enrichment analysis of the DEGs”. However, the data presented seem to be a combination of GO, KEGG, and Reactome. Please verify and revise the legend, if necessary.

We modified the legend accordingly to indicate GO, KEGG and Reactome pathways.

In addition, in the main text (Lines 399-400), the authors suggest that “… glutamate receptor activity term was most significantly enriched in 400 genes with lower expression in accumbens fibrous-like astrocytes in HD. (Figure S6B)”. However, it is unclear how the results in Figure S6B could reflect the abovementioned statement. Please verify.

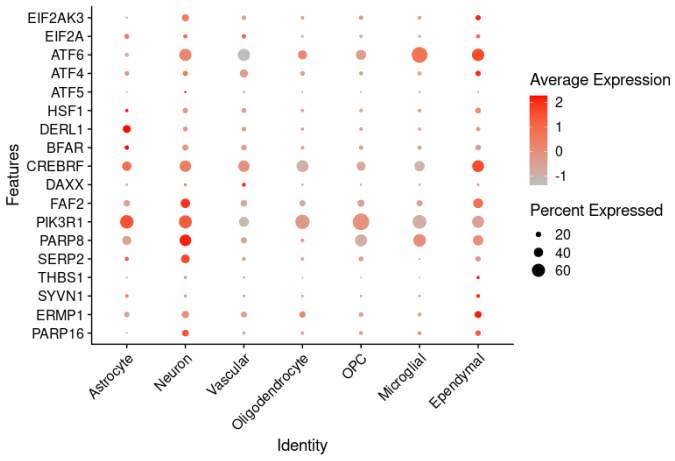
This is a typo. We deleted the reference to Figure S6B.

3. Figure 7F: Please include the HD grade in the legend.
These cell lines were derived from live patients – a formal Vonsattel grade is not available or applicable. We now provide information on CAG repeats of these lines in the methods.

4. Figure S9E-F: The violin plots show the expression of a few genes of interest. Were they plotted using normalized or unnormalized data? Please verify.
These are normalized expression counts. We included this in the legend.

5. Figure 4B, 4E: The authors have shown that a combination of lipidomic and transcriptomic data yielded “unfolded protein response genes”. It is worth noting that these two data sets (lipidomic and transcriptomic) were derived from bulk tissues, not exclusively from astrocytes. Thus, when projecting these “unfolded protein response genes” onto snRNA-seq data (not just astrocytes), what is the expression pattern of this set of genes among brain cells? The author please verify.

We provide a dot plot showing the expression of a subset of these genes.



We do not claim that the enrichment of unfolded response genes is specific to astrocytes. Unfolded protein response gene can be increased in a multitude of cell types. We believe that adding this panel to the paper does not advance our narrative or help the reader understand the results. Therefore, we would like to exclude this panel from the manuscript.

6. Figure 4B, 4E: What is “Diaz Castro”? Please clarify.

Diaz-Castro refers to the gene set indicated in the figure panels is referred to in the legend and the main text. It is also provided in the supplementary datasets – table 2.

7. Please clarify whether the enrichment analyses presented in figures throughout the manuscripts are a combination of GO, KEGG, and Reactome. If so, proper legends and text should be provided, rather than simply referring to them as “GO analysis”.

Each figure legend clarifies whether enrichment included GO versus KEGG and/or Reactome.

8. Line 461: Please note that there is no Figure 4I in Figure 4. Is it a typo?

This is a typo – we corrected it. It is Figure 4.

9. Line 462: It is unclear why (Figure 4G) is assigned here. Please clarify.

Figure 4G clearly shows enrichment of the ontology “Metallothionein binds ions”, which harbors many MT genes, in protoplasmic versus fibrous astrocyte differentially expressed genes. This is the statement we make in lines 461-462.