nature portfolio

Peer Review File

A Human Brain Map of Mitochondrial Respiratory Capacity and Diversity

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
The manuscript introduces MitoBrainMap, a new brain-wide multi-scale map of mitochondrial distribution and specialization. This new resource provides a foundation for exploring the molecular energetic landscape that enables normal brain functions, and presents a novel physical voxelization approach to establish the spatial distribution of mitochondrial diversity within the human brain.

The results of the study are novel and highly valuable for the neuroscience community. The conclusions are sound and supported by the data, which are a unique resource. The methodology used is described in great details, with honest and rigorous acknowledgment of the limitations and potential improvements. Particular care has been taken to make all the data, procedures and codes publicly available.

The manuscript is of the highest quality and presents unique and precious new information mapping inter-individual differences in the sub-cellular energetic architecture (specific cell types, among different brain regions), which is crucial to advance our understanding of the large-scale brain dynamics.

Before suggesting publication, I would like the Authors to address the following specific minor points:

1. The first introductory part could benefit from the inclusion of a brief description of the neuroimaging techniques mostly used for the large-scale mapping of brain metabolism, such as PET but also CEST MRI and metabolite functional MRS (e.g., at line 60 and 73). What are the limitations of these techniques and how the large-scale mapping proposed in this work complement the information provided by such techniques and could eventually help improving their specificity?

2. The poor performance of the prediction of voxel origin from UMAP clustering in gray matter is concerning. Could the Authors clarify the origin and consequences of it with respect to their conclusions?

3. It is really interesting to see strong correlations between some MRI metrics/indices and mitochondrial features. However, only a brief comment to the orientation dispersion index is included. Could the Authors comment on the others, for example FA being very strongly correlated to CIV and MitoD, or the T1w/T2w ratio being strongly correlated to all the features? These are two imaging indices mostly linked to myelin; can the Authors discuss how these could support their conclusions on the mitochondrial underpinnings of human brain evolution?

4. Related to the MRI analysis, the Authors used three different sources of information from MRI (structural, functional and diffusion). Why treating these different MRI-derived information in the same way, i.e. why not using a different approach, e.g. for fMRI and diffusion-weighted MRI? Most likely, the metrics/indices derived by these two techniques may not have linear relationship with the mitochondrial features. Can the Authors provide evidence that this is not the case?

5. Related to point 4, why only testing linear modelling? Did the Authors try also non-linear modelling or other approaches?

6. How confident can the Authors be that the performance of the learnt mapping (from MRI to mitochondrial features) may generalize to other subjects of different age and gender and, most importantly, in pathology? The training set was very small and heterogeneous; the model arguably simplistic; hence generalizability may not be guaranteed a priori.

7. The MRI data used in this study were from two different open source datasets. Was any harmonization between the two datasets necessary and, if so, performed?

8. The statement: '[…] which differ in their abundance among different brain areas (WM vs. GM; cortical vs. subcortical).' at lines 223-224 needs reference to supporting works.

Referee #2

(Remarks to the Author)
The manuscript titled "A Human Brain Map of Mitochondrial Respiratory Capacity and Diversity" by Mosharov et al. introduces a particularly intriguing conceptual and experimental approach for defining mitochondrial respiratory capacity in the human brain, offering regional specificity down to a volume of 3mm^3. The approach relies on a multiparameter assessment of various mitochondrial variables, ranging from the number of mitochondrial genomes per voxel to the enzymatic activity of different complexes of the respiratory chain within each voxel. These parameters are precisely assigned to anatomical coordinates within each tissue voxel and subsequently mapped into a reference neuroanatomical space for MRI research, the Montreal Neurological Institute Brain Reference. This voxelization of human brain tissue for the study of its biochemical and molecular features, followed by their mapping into a standardized 3D brain cartography, represents a uniquely novel and impactful approach.

Taking this methodology a step further, the authors leverage their datasets and spatial information to train a machine learning algorithm. This algorithm demonstrates the ability to predict de novo mitochondrial parameters, which, in a 'post-hoc' test with previously untested tissue, exhibits exceptional correlation. While the biological insights into mitochondria from these studies largely corroborate findings in other vertebrates, the experimental design itself stands out as groundbreaking, with profound implications for any biochemical or molecular study of the human brain.

While some may argue that the study's sample size of one individual is insufficient for drawing robust conclusions, the study's value lies in the extraordinary novelty of its approach and the remarkable predictive power achieved through a machine learning strategy. The potential for these studies to become paradigm shifting in the way we approach human omic studies is uniquely strong.

The paper is well-presented, with rigorous experimental design evident throughout. Attention to redundancy in experimental approaches, multiplicity of internal controls and calibrators, as well as a blind approach to data collection, instills great confidence in the data. Overall, I find this study exciting and I'm supportive of its findings.

However, I would like to offer some suggestions to further enhance these studies:

1. The use of snRNA seq in Figure 3 provides informative insights, yet the true value of the RNAseq approach would be realized if the mitochondrial annotated RNAseq per voxel showed a strong correlation with mitochondrial enzymatic parameters. This mitochondrial annotated RNAseq should then behave similarly to the mitochondrial enzymatic parameters in the machine learning approach. The data presented in Fig. 3D are insufficient to judge the authors' statement regarding the correlation between overall relative gene expression of OxPhos complex subunits and measured enzymatic activities of CI, II, and IV in all brain regions ("overall, relative gene expression of OxPhos complex subunits corresponded well with measured enzymatic activities of CI, II and IV in all brain regions"). Furthermore, there is limited evidence on how mitochondrial annotated RNAseq behaves when mapped to the reference neuroanatomical space for MRI research and its predictive value in machine learning. This comment is inspired by the poor correlation between RNAseq and proteomes, particularly evident in brains, suggesting that RNAseq strategies may have limited predictive value in brain studies (see for example PMID: 27104977- 30777892- 33483739- 35115731).

2. The authors should consider collecting proteomic data on voxelized human brain samples and subjecting those data to the same analysis presented in Figures 3 and 4. While this may require significant financial and time resources, the rationale for sample selection for snRNAseq could be applied here as well. Proteomics data would offer exceptional confirmatory evidence and could potentially reveal more granularity in mitochondrial phenotypes, such as mitochondrial protein synthesis, the types of carbon substrates likely used by each region, expression of neurotransmitter transporters of the SLC25 family in brain. Pairing such proteomic data with annotations in Mitocarta 3.0 could provide deeper insights into the diversity of mitochondrial function in the human brain. Furthermore, demonstrating the validity of data analyses in Figures 3 and 4 for any 'omics' data would have a significant impact on the research community as an experimental strategy model to be emulated. This comment should not be viewed as binding and the authors should feel free to not address it.

3. The authors should consider the utilization of PET maps of human brain glucose consumption and PET imaging of synaptic vesicle glycoprotein 2A (SV2A) to correlate their mitochondrial parameters with functional measures of metabolic activity and synaptic density. Additionally, the authors should comment on the poor and negative beta correlation between entropy (a proxy for synapse function) and mitochondrial parameters in Extended Table 1.

4. Providing Excel matrices containing all presented data and the anatomical location of the voxel of origin would enable others to process and use the data in further studies.

5. Analyses in Figures 3G-H may need to be reexplored, categorizing distinct types of neurons. For example, inhibitory neurons could be further divided into Parv, Sst, and others, considering reports indicating heightened mitochondrial numbers and respiratory chain enzymatic activity in GABA neurons as well as mRNAs (see for example PMID: 24896567).

6. The authors should consider and comment on the possibility that while these studies were carried out with one human brain, the machine learning algorithm allowed them to use statistically curated brain imaging data to predict mitochondrial parameters in other brain regions previously untested. Could mitochondrial data from one new brain region, lets say the cerebellar cortex (not yet tested in this manuscript), back-predict using the same AI algorithm the measured mitochondrial status of the cortex already presented in this manuscript. If this were to be the case, this would be revolutionary as small tissue samples precisely mapped to the anatomy of the human brain could generate predictions and hypotheses for the whole brain.

7. Workflow schematics in Figure 1 and Figure 2 are a little lost in the figure because of segmentation with letter notation for each step. There might be a better way to show the schematic, like the whole schematic represented with one letter (the whole flow as “a” instead of a-g). This or some other formatting adjustment might help differentiate the schematic from the subsequent data in the same figure.

Referee #3

(Remarks to the Author)
This is a well written paper and represents a massive effort to generate an atlas of mitochondrial content, mitochondrial functional activities, diversity of mitochondrial phenotypes at a resolution comparable to MRI. In general, the data are of high quality and state of the art methodology is used.
There are several important conclusions, and one of the major ones is that the major driver of
the differences in OxPhos transcriptome for each cell type is the brain region in which it resides. This is very well documented in this study, but somewhat to be expected based on the current status of this field, where it also has become evident that mitochondrial deficiencies are central in neurodegeneration.
A severe limitation, as they point out is that this work represents one individual brain 8 hrs postmortem. As they also point out, the entire dynamics might be very different if they had another postmortem time interval, as this is critical for enzymatic function. 8 hrs is not a short postmortem, they could have found 4 hr samples.
It is a lot to ask, but studies in a diseased brain or attempt to relate this to neurodegenerative disease and thus to actual MRI results would verify that this approach has clinical value.
While it is an excellent study, the scientific advance is limited, it is more of a methods advance

Referee #4

(Remarks to the Author)
This manuscript details a work-intensive, brute-force effort by the authors to characterize/describe mitochondrial profiles across the “pixels” of a grid-delineated human brain. One reason for doing this is to create a novel, anatomically well-defined anatomical characterization of human brain mitochondria across the brain, which itself provides insight into mitochondria-neuroanatomical correlations and can inform questions of mitochondria in white matter vs gray matter, by cell type, etc. The authors further propose a more conceptual utility, which is perhaps their map at some point may inform translational questions. Specifically, our ability to characterize mitochondria in living brains is currently quite limited. There are current neuroimaging techniques many believe at least indirectly reflect mitochondrial function, but we really aren’t that sure how to extrapolate data from such studies to infer a territory’s underlying mitochondrial status. The “map” the authors generate can potentially serve as a bridging step to better link data from clinical neuroimaging studies to brain mitochondria status; hence the plan to characterize a brain in voxels that are comparable in size to achievable MRI scan voxels. From a significance perspective, these are important questions to address and the rationale underlying the study is strong.

The work-intensive, brute force approach essentially adapts existing mitochondrial assessment procedures for high-throughput applications. In some cases, some procedures were already suited for medium-throughput studies, but in this case the authors take the throughput issue to a whole other level. There are not existential concerns about the mitochondrial assessments themselves, which are essentially state of the art. For example, the Shirihai group applied their oxygen consumption methods for analyzing electron transport chain enzyme activities in an extraordinary number of small tissue samples. In some cases, redundant techniques were applied, which is a strength rather than a limitation. The authors define terms such as “mitochondria density” as a value defined by mtDNA copy number and citrate synthase, and one can argue about the pros and cons of assumptions such as this, but it is acknowledged one must start from somewhere. Work of his nature is going to require some assumptions.

The results essentially confirm what is already known at a broad level, which is that mitochondria in the brain function differently and are indeed structured differently in different brain cell types and brain regions. It certainly, though, is worthwhile to see this type of analysis conducted as such a refined anatomic level. The authors further use bioinformatics approaches to provide cell/regional specificity insight, which nicely complements the mitochondrial function data form those regions.

No fatal flaws are identified. It’s hard to say how useful this map will ultimately prove to be, and perhaps by the time its translational utility and limitations are worked out the field will have imaging techniques that more directly interrogate mitochondria at neuroanatomically and cell-type discrete levels, but this current study is perceived as an ambitious study that (1) advances our understanding of neuroanatomical differences in brain mitochondria at a higher than before spatial resolution level, and (2) moves to create a new resource that could enter the public domain, and (3) at some point may have the ability to inform brain imaging-based translational studies.

If terms of points to address, really the only one that perhaps worth mentioning is that the text initially gives the impression that this study is really focused on using MRI to inform the status of brain mitochondrial. One might argue that is more of a potential future application.

(Remarks on code availability)
No further comments

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The Authors have addressed extensively and with rigour all my comments and I do not have any further concerns or feedback. I am happy to recommend the acceptance of this work in its revised form.

Referee #2

(Remarks to the Author)
The authors have satisfactorily addressed all my comments. This was an outstanding manuscript that now is even better.

Referee #3

(Remarks to the Author)
None

Referee #4

(Remarks to the Author)
No further comments

Editorial comments

Your manuscript, "A Human Brain Map of Mitochondrial Respiratory Capacity and Diversity", has now been seen by 4 referees, whose comments are attached below. While they find your work of potential interest, as do we, they have raised important concerns that in our view need to be addressed before we can consider publication in Nature. … I feel that generally the reviews are constructive and thoughtful. I do think we can have a discussion about reviewer 2's ask for a proteomic study. I think that might be beyond the scope unless you have it already available. We are also willing to consider it without any additional brains as there is a clear methodological advance and we see it as a solid foundation for future studies.

We thank the Editorial Board for considering our work and for the opportunity to resubmit a revised, improved manuscript. We hope that the additional analyses, discussion and clarification of specific Results revision satisfactorily address all specific points raised by the referees.

Referee #1

The manuscript introduces MitoBrainMap, a new brain-wide multi-scale map of mitochondrial distribution and specialization. This new resource provides a foundation for exploring the molecular energetic landscape that enables normal brain functions, and presents a novel physical voxelization approach to establish the spatial distribution of mitochondrial diversity within the human brain.

The results of the study are novel and highly valuable for the neuroscience community. The conclusions are sound and supported by the data, which are a unique resource. The methodology used is described in great details, with honest and rigorous acknowledgment of the limitations and potential improvements. Particular care has been taken to make all the data, procedures and codes publicly available.

The manuscript is of the highest quality and presents unique and precious new information mapping inter-individual differences in the sub-cellular energetic architecture (specific cell types, among different brain regions), which is crucial to advance our understanding of the large-scale brain dynamics.

We thank the reviewer for the positive comments, and for the thoughtful suggestions below.

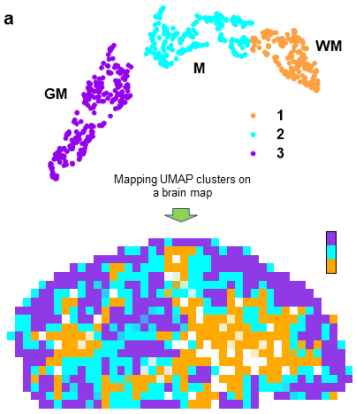
1.1. The first introductory part could benefit from the inclusion of a brief description of the neuroimaging techniques mostly used for the large-scale mapping of brain metabolism, such as PET but also CEST MRI and metabolite functional MRS (e.g., at line 60 and 73). What are the limitations of these techniques and how the large-scale mapping proposed in this work complement the information provided by such techniques and could eventually help improving their specificity?

Response 1: Excellent question. We have added a section about how the new data adds on to what exists with neuroimaging in the introduction: "Neuroimaging techniques are invaluable for mapping brain metabolism on a large scale. Positron emission tomography (PET) provides quantitative measurements of metabolic processes including glucose metabolism using [18F]-FDG, but requires invasive radioactive

tracers and has limited spatial resolution[29]. Blood oxygen level dependent (BOLD) functional MRI captures variations in hemoglobin oxygenation at millimeter- and second-resolution, but it only indirectly reflects the mixed contribution of metabolic (aerobic and anaerobic) responses to brain tissue activity[30]. Chemical exchange saturation transfer magnetic resonance imaging (CEST MRI) maps metabolites by detecting exchangeable biomolecule protons with relatively high spatial resolution, but faces limits to quantify absolute metabolite concentrations[31]. Functional magnetic resonance spectroscopy (fMRS) quantifies neurotransmitter and metabolite concentrations such as glutamate and lactate that offer unique insights into in vivo brain metabolism, but low signal-to-noise ratio limits temporal and spatial resolution, requiring large voxel sizes during acquisition[32]. While these and the integration of other techniques (e.g., CMRO2, CBV[9,33,34]) provide a broad view of brain metabolism in relation to function, they call for a high-resolution, brain-wide map of the molecular energetic landscape crucial for understanding regional metabolic properties and energy transformation capacity. Such a map would allow to integrate mitochondrial bioenergetics with macroscopic neuroimaging data, potentially enhancing the specificity of metabolic assessments from PET, fMRI-BOLD, CEST MRI, and fMRS by linking them to mitochondrial biology."

1.2. The poor performance of the prediction of voxel origin from UMAP clustering in gray matter is concerning. Could the Authors clarify the origin and consequences of it with respect to their conclusions?
Response 2: We now provide additional details clarifying the ability of the UMAP analysis to predict anatomical voxel localization. Extended Data Fig 9a (also copied below) now shows how voxels identified based on UMAP clusters were mapped as grey matter (GM), mixed (M) or white matter (WM). The revised table now clearly shows that most of the identification errors occurred due to expected edge effects. For example, 110/172 (64%) of the voxels from UMAP cluster #1 were correctly anatomically identified as WM, 53 as mixed, and only 9 (5%, true misidentification rate) as GM. Similarly, 2/251 (>1%) of voxels from UMAP cluster #3 were misidentified as WM.

Thus, the prediction accuracy in this UMAP model establishes the accuracy that can be obtained with only 6 mitochondrial features, using a relatively poor "classifier" (UMAP algorithm). Although UMAP is not a robust test of "prediction accuracy" per se, we opted to include this result in the manuscript because UMAP is increasingly used across several fields, giving investigators a clear point of reference for the accuracy, or the lack of thereof, using a well-known method. We have clarified these results on page 7: "Conversely, when UMAP clusters were used to predict the origin of the voxels, they mapped to GM and WM voxel locations with 82% and 64% accuracy, respectively (chance level = 51% [GM] and 21% [WM], Extended Data Fig. 9a). Remaining voxels were assigned to the "mixed" category containing both WM and GM tissue, making the misclassification rate 1-5% (Extended Data Fig. 9a)."



	Cluster	1	2	3	Correct
Maps to	WM	110	20	2	64 %
	M	53	81	42	39 %
	GM	9	109	207	82%
	Total	172	210	251	

Extended Data Fig. 9a: Three visually defined UMAP clusters obtained after dimensionality reduction of mitochondrial features of all brain voxels (Fig. 2e) were used to predict whether voxels originated from GM, WM or a mixed sample. The table lists prediction accuracy with the number of voxels identified as each type and the fractions that were identified correctly. Note that most localization errors occurred due to the presence of voxels with mixed composition, which complicates their anatomical mapping. $X^2(2, N=1031)=10.3$, $p<0.01$.

1.3. It is really interesting to see strong correlations between some MRI metrics/indices and mitochondrial features. However, only a brief comment to the orientation dispersion index is included. Could the Authors comment on the others, for example FA being very strongly correlated to CIV and MitoD, or the T1w/T2w ratio being strongly correlated to all the features? These are two imaging indices mostly linked to myelin; can the Authors discuss how these could support their conclusions on the mitochondrial underpinnings of human brain evolution?

Response 3: We thank the reviewer for bringing an important discussion point. First, we want to address a potential misunderstanding regarding the statistical analyses presented. The beta values reported in our hierarchical linear regression models are not Pearson correlation. Instead, they reflect the standardized regression coefficients (β), indicating the unique contribution of each predictor to the outcome variable *while accounting for the influence of all other variables* in the model. Specifically, hierarchical linear regression with backward elimination identifies variables that have significant independent associations with the outcome. This approach systematically eliminates variables that do not significantly contribute to the model or are redundant with other variables, ensuring that the remaining predictors explain distinct variance. The following sentence was added to Methods to clarify this point: "The model produces beta (β) values, which should not be mistaken with Pearson correlation coefficients. Instead, they reflect the regression coefficients, indicating the unique contribution of each predictor to the outcome variable after accounting for the influence of all other variables in the model."

As noted by the reviewer, both Fractional Anisotropy (FA) and the T1w/T2w ratio are commonly associated with myelin integrity and microstructural organization at the histological level. FA is an indicator of fiber tract integrity and is highly dependent on axonal myelination and the organization of the white matter[1]. FA's positive beta value for CIV and MitoD in Extended Data Table 1 indicates that, after accounting for other factors (such as main variation between the grey and the white matter), FA

independently predicts higher values for these mitochondrial parameters. This may suggest that mitochondria are at least partially detected by diffusion-weighted imaging metrics. Mitochondria support the metabolic demands of myelinated axons and signal transduction[2,3]. The source of this signal could include astrocytes, which contain 70% of mitochondria in the optic nerve for example (a myelinated axonal tract)[4]. In the optic nerve, mitochondria occupy a constant fraction of axonal volume, such that mitochondrial volume increases with power 2 (squared) as the axonal diameter increases[4]. The positioning and molecular specialization of mitochondria in myelinated axons, where they sit mostly at the nodes of Ranvier (at least in the optic nerve axons[5]) could contribute to this association. In axon-rich regions in general, higher mitochondrial density and OxPhos capacity could therefore support the energetic demands of highly organized and myelinated fiber tracts. Accordingly, relative to mitochondria in dendrites, the smaller axonal mitochondria[6] would allow the maintenance of the structural integrity and efficient conduction properties of white matter, which are critical for fast and efficient brain connectivity – a key element in humans' evolution of complex cognitive functions. Why FA has a strong loading specifically for CIV but not for other ETC components (CI, CII) is unclear.

We speculate that other aspects of mitochondrial biology not yet fully understood could contribute to the link between the mitochondrial parameters and FA and T1w/T2w imaging. For example, recent work demonstrated that mitochondria unexpectedly interact with the visible portion of the electromagnetic spectrum – demonstrating that mitochondria in retinal cells act as microlenses that focus the flow of photons (i.e., visible light)[7]. We also previously found microstructural alignment of cristae inside mitochondria at the site of contact of other mitochondria, suggesting that forces – most likely electromagnetic – act upon the iron-rich electron transport chain complexes[8]. While not directly applicable to FA, we note, in principle, the ability of variations in mitochondrial ultrastructure and morphology to influence the diffusion/linear translation of molecular elements. Perhaps this principle applies beyond photons to oscillating water molecules, or that the minute paramagnetic elements of the mitochondrial OxPhos system interact with specific MRI sequences. It also is possible that the unique electrochemical and proteinaceous properties of mitochondria could contribute to other biophysical states relevant to FA, such as water liquid-liquid phase separation[9,10], the process by which intracellular spaces condense into, or dissolve gel-like properties[11]. By changing the physical properties of water, sub-cellular biochemical processes could alter the FA signal[12,13].

The *T1w/T2w ratio* is often used as a proxy for myelin content[14] and exhibits strong negative β coefficients for all brain mitochondrial features in the MitoBrainMap. This relationship could suggest that mitochondria that are essential for myelin synthesis and maintenance are less concentrated in myelin than in other brain tissues leading to this negative relationship.

To avoid excessively lengthening the discussion while sharing these potentially useful reflections, we have added an edited version of this discussion as Supplemental Text (Supplemental File 6) to the revised manuscript. We defer to the Reviewer and Editor about whether this should be included in the final article and would be happy to modify or edit as needed.

1.4. Related to the MRI analysis, the Authors used three different sources of information from MRI (structural, functional and diffusion). Why treating these different MRI-derived information in the same way, i.e. why not using a different approach, e.g. for fMRI and diffusion-weighted MRI? Most likely, the metrics/indices derived by these two techniques may not have linear relationship with the mitochondrial features. Can the Authors provide evidence that this is not the case?

Response 4: We initially treated the structural, functional, and diffusion-weighted MRI data uniformly, assuming they would exhibit comparable linear relationships with mitochondrial features. However, we recognize that this assumption may not fully capture the complexities of fMRI and dMRI, which measure different biological processes and structures such as neural activity and water diffusion along white matter tracts, respectively. These processes might not have linear correlations with mitochondrial bioenergetics.

To address this point, we have now performed additional analyses to explore non-linear relationships, incorporating both exponential and logarithmic models. These analyses revealed some improvement in the fit between mitochondrial features and neuroimaging metrics, particularly for diffusion-weighted imaging. However, when we evaluated the models' performance on out-of-sample predictions, the non-linear transformations did not consistently improve prediction accuracy (paired sample t test < 1, p=0.675) and in some cases led to overfitting. This occurs when the model captures noise or specific idiosyncrasies of the training data rather than generalizable patterns. The out of sample prediction accuracy for the model combining all imaging modalities was 29.5% for the linear model compared to 27.9% for the nonlinear model.

In light of these results, we decided to retain the simpler linear model, which balances fit quality and reproducibility. We believe that this simpler approach will facilitate replication by other research groups and ensure more robust, uniform models in the present and future studies. Below, as suggested, we provide the detailed results of our non-linear exploration, including their impact on out-of-sample predictions. The best fit is highlighted in bold. In all cases, the differences between linear, exponential and logarithmic goodness of fit were within 1-3%.

Diffusion-weighted imaging fitted models (R^2)

	Linear	Exponential	Logarithmic
CI	.293	**.299**	.297
CII	.314	**.325**	.305
CIV	.324	**.337**	.313
MitoD	.352	**.366**	.341
TRC	.344	**.353**	.338
MRC	.271	**.273**	.263

Structural imaging fitted models (R^2)

	Linear	Exponential	Logarithmic
CI	.330	.315	**.337**
CII	.320	.301	**.324**
CIV	**.335**	.318	.328
MitoD	.347	.344	**.360**
TRC	**.366**	.346	.365
MRC	**.290**	.278	.288

Functional imaging fitted models (R^2)

	Linear	Exponential	Logarithmic
CI	**.240**	.223	.240
CII	**.237**	.225	.230
CIV	.241	.221	**.245**
MitoD	.243	.212	**.254**
TRC	.261	.243	**.263**
MRC	**.206**	.199	.203

All imaging modalities fitted models (R^2)

	Linear model	Nonlinear model
CI	.374	**.381**
CII	.351	**.366**
CIV	.381	**.388**
MitoD	.403	**.417**
TRC	.406	**.413**
MRC	**.326**	**.326**

All imaging modalities out of sample prediction (R^2)

	Linear model	Nonlinear model
CI	**.366**	.361
CII	.266	**.296**
CIV	.315	**.320**
MitoD	**.228**	.218
TRC	**.346**	.321
MRC	**.295**	.279

In summary, while non-linear transformations slightly improved model fits, they did not significantly enhance out-of-sample prediction accuracy. Therefore, we opted to retain the linear model that avoid the risk of overfitting, and are more likely to lead to robust and more generalizable results in future applications. In the revised manuscript, we address this with the following statement on Page 11: "Additional exploration of non-linear transformations of the variables did not significantly enhance out-of-sample prediction accuracy (see *Methods* for details)."

Also, in Methods (Page 23) we write: "Additional analyses investigated whether non-linear relationships, incorporating both exponential and logarithmic models could improve predictions. While non-linear model yielded marginally (~1-3%) better fits between the 80% observed and predicted mitochondrial features during the training phase, when we evaluated the models' performance on the remaining 20% of out-of-sample predictions (testing phase), the non-linear transformations did not consistently improve prediction accuracy (paired sample t test < 1, p=0.68). Considering these results, the simpler linear model was retained, balancing fit quality and reproducibility."

1.5. Related to point 4, why only testing linear modelling? Did the Authors try also non-linear modelling or other approaches?

Response 5: As mentioned in Response 4, we explored the Reviewer's excellent suggestion to explore non-linear relationships using exponential and logarithmic models. Although these non-linear models showed a marginally improved fit for the training data, they did not improve the accuracy of out-of-sample predictions. This indicates that the added complexity of the non-linear models may have caused overfitting, capturing noise rather than improving generalizability. Therefore, to strike a balance between model interpretability, reproducibility, and prediction performance we decided to use the simpler linear model in our manuscript. This simpler approach should help other research teams to reproduce or extend upon these findings.

1.6. How confident can the Authors be that the performance of the learnt mapping (from MRI to mitochondrial features) may generalize to other subjects of different age and gender and, most importantly, in pathology? The training set was very small and heterogeneous; the model arguably simplistic; hence generalizability may not be guaranteed a priori.

Response 6: We fully agree. This study provides proof-of-concept for this approach in one human brain. Future work will need to examine this critical point, which unfortunately is beyond our ability to address at the moment.

In the revised manuscript, we now clarified this limitation in the discussion (page 12) as follows: "The reliance on a single neurotypical human brain sample, akin to other whole-brain microscopic initiatives[35-37], underscores the necessity for additional postmortem data sets to ensure generalizability of our neuroimaging model to other healthy and pathological subjects of different age and gender."

1.7. The MRI data used in this study were from two different open-source datasets. Was any harmonization between the two datasets necessary and, if so, performed?

Response 7: The MRI data used in this study were derived from two different and complementary datasets, both normalized to the MNI152 space. Given that the datasets shared modalities of comparable resolution, we assessed the correlations between these modalities. They were highly correlated (for example, T1-weighted imaging was correlated at r=0.83; and FA at r=0.92), indicating consistency across the datasets. Based on these results, we determined that additional harmonization was not necessary, as the inherent variability between the datasets was minimal and would not impact the validity of our results.

We have added these details in the *Methods* section (page 22): "To enable comparisons between these values and neuroimaging data, we used averaged neuroimaging values derived from a sample of 1870 university students[11] and the human connectome project[68]. Both datasets were normalized to the MNI152 template space[78] (Extended Data Table 1). The two databases offered a robust array of neuroimaging metrics with highly correlated values for overlapping data. For example, T1-weighted and fractional anisotropy imaging data from the two datasets correlated with r=0.83 and 0.92, correspondingly, supporting their interchangeable use."

1.8. The statement: '[…] which differ in their abundance among different brain areas (WM vs. GM; cortical vs. subcortical).' at lines 223-224 needs reference to supporting works.

Response 8: References were added to support this statement: "Specialized mitochondrial phenotypes (i.e., mitotypes) map either to specific brain areas[5] or to specific cell types[6,49], which differ in their abundance among different brain areas (WM *vs*. GM; cortical *vs*. subcortical differ in their cytoarchitecture[35,50,51]).

Referee #2

The manuscript titled "A Human Brain Map of Mitochondrial Respiratory Capacity and Diversity" by Mosharov et al. introduces a particularly intriguing conceptual and experimental approach for defining mitochondrial respiratory capacity in the human brain, offering regional specificity down to a volume of 3mm^3. The approach relies on a multiparameter assessment of various mitochondrial variables, ranging from the number of mitochondrial genomes per voxel to the enzymatic activity of different complexes of the respiratory chain within each voxel. These parameters are precisely assigned to anatomical coordinates within each tissue voxel and subsequently mapped into a reference neuroanatomical space for MRI research, the Montreal Neurological Institute Brain Reference. This voxelization of human brain tissue for the study of its biochemical and molecular features, followed by their mapping into a standardized 3D brain cartography, represents a uniquely novel and impactful approach.

Taking this methodology a step further, the authors leverage their datasets and spatial information to train a machine learning algorithm. This algorithm demonstrates the ability to predict de novo mitochondrial parameters, which, in a 'post-hoc' test with previously untested tissue, exhibits exceptional correlation. While the biological insights into mitochondria from these studies largely corroborate findings in other vertebrates, the experimental design itself stands out as groundbreaking, with profound implications for any biochemical or molecular study of the human brain.

While some may argue that the study's sample size of one individual is insufficient for drawing robust conclusions, the study's value lies in the extraordinary novelty of its approach and the remarkable predictive power achieved through a machine learning strategy. The potential for these studies to become paradigm shifting in the way we approach human omic studies is uniquely strong.

The paper is well-presented, with rigorous experimental design evident throughout. Attention to redundancy in experimental approaches, multiplicity of internal controls and calibrators, as well as a blind approach to data collection, instills great confidence in the data. Overall, I find this study exciting and I'm supportive of its findings.

Thank you for the positive comments and for the astute suggestions below.

2.1. The use of snRNA seq in Figure 3 provides informative insights, yet the true value of the RNAseq approach would be realized if the mitochondrial annotated RNAseq per voxel showed a strong correlation with mitochondrial enzymatic parameters. This mitochondrial annotated RNAseq should then behave similarly to the mitochondrial enzymatic parameters in the machine learning approach. The data presented in Fig. 3D are insufficient to judge the authors' statement regarding the correlation between overall relative gene expression of OxPhos complex subunits and measured enzymatic activities of CI, II, and IV in all brain regions ("overall, relative gene expression of OxPhos complex subunits corresponded well with measured enzymatic activities of CI, II and IV in all brain regions").

Response 1: Thank you. We agree that the data across only 4 voxels are insufficient to draw definitive conclusions about the correlation between RNAseq and biochemistry. While this data rules out a potential striking divergence between RNA and activities, we keep ourselves from making strong quantitative claims from this limited dataset. More extensive studies, ideally hundreds of brains, would be required to properly assess the RNA, protein, enzyme activity relationships. We have toned down the corresponding statement in the text on page 9: "Overall, although data from only 4 voxels is insufficient to draw definitive conclusions, the relative gene expression of OxPhos complex subunits were proportional to the measured enzymatic activities of CI, II and IV in the Putamen, Hippocampus and WM (Fig 3d), providing partial convergent evidence for the observed enzymatic data that requires additional validation in larger datasets."

2.2. Furthermore, there is limited evidence on how mitochondrial annotated RNAseq behaves when mapped to the reference neuroanatomical space for MRI research and its predictive value in machine learning. This comment is inspired by the poor correlation between RNAseq and proteomes, particularly evident in brains, suggesting that RNAseq strategies may have limited predictive value in brain studies (see for example PMID: 27104977- 30777892- 33483739- 35115731).

Response 2: We entirely agree with the general lack of robust alignment between RNAseq and proteomes. With RNAseq in only 4 voxels this study is not sufficiently powered to attempt this mapping into the MNI neuroanatomical space. More extensive studies, ideally in hundreds of brains with RNAseq and proteomic data across multiple regions, would be required to properly address this question. These datasets are being developed by multiple groups/consortia and should eventually be amenable to the type of rigorous studies the reviewer is calling for.

2.3. The authors should consider collecting proteomic data on voxelized human brain samples and subjecting those data to the same analysis presented in Figures 3 and 4. While this may require significant financial and time resources, the rationale for sample selection for snRNAseq could be applied here as well. Proteomics data would offer exceptional confirmatory evidence and could potentially reveal more granularity in mitochondrial phenotypes, such as mitochondrial protein synthesis, the types of carbon substrates likely used by each region, expression of neurotransmitter transporters of the SLC25

family in brain. Pairing such proteomic data with annotations in Mitocarta 3.0 could provide deeper insights into the diversity of mitochondrial function in the human brain. Furthermore, demonstrating the validity of data analyses in Figures 3 and 4 for any 'omics' data would have a significant impact on the research community as an experimental strategy model to be emulated. This comment should not be viewed as binding and the authors should feel free to not address it.

Response 3: We agree with the reviewer – a proteomic-based MitoBrainMap would be exciting and a valuable extension of the current enzyme/molecular map. We are not in a position to extend our analyses at this time, but will attempt to generate and harmonize protein, enzyme, and mRNA in the future.

2.4. The authors should consider the utilization of PET maps of human brain glucose consumption and PET imaging of synaptic vesicle glycoprotein 2A (SV2A) to correlate their mitochondrial parameters with functional measures of metabolic activity and synaptic density. Additionally, the authors should comment on the poor and negative beta correlation between entropy (a proxy for synapse function) and mitochondrial parameters in Extended Table 1.

Response 4: We appreciate the reviewer's suggestion to consider correlations with PET maps of human brain glucose consumption and synaptic density. As recommended, we explored additional correlations with available PET metrics derived from existing SPM templates[15] and recent work by[16]. The results show, as expected, that many of these molecular features correlate with higher mitochondrial content (MitoD), as well as other mitochondrial features. This is the case for glucose consumption (FDG), and also interestingly for the cannabinoid receptor CB1, the metabotropic glutamate receptor 5 (mGluR5). These results are in line with the higher metabolic costs among brain regions more heavily decorated with neuromodulatory receptors, as recently described by Riedl and colleagues[17]. We were not able to access PET imaging data of synaptic vesicle glycoprotein 2A (SV2A), but, based on the results below, there is no reason to suspect that it would not confirm that greater synaptic and neuromodulatory components correlate with higher mitochondrial content.

The table below lists the linear associations (Pearson R^2) between raw mitochondrial measures and available PET metrics (ligands):

PET metrics	CI	CII	CIV	MITO	TRC	MRC
FDG (fluorodeoxyglucose)	0.163	0.191	0.196	0.246	0.200	0.131
5HT1a_cumi_hc8_beliveau	0.065	0.066	0.070	0.058	0.072	0.065
5HT1a_way_hc36_savli	0.078	0.079	0.091	0.070	0.092	0.083
5HT1b_az_hc36_beliveau	0.054	0.056	0.059	0.055	0.061	0.051
5HT1b_p943_hc22_savli	0.152	0.136	0.156	0.163	0.163	0.118
5HT1b_p943_hc65_gallezot	0.042	0.054	0.064	0.081	0.057	0.035
5HT2a_alt_hc19_savli	0.148	0.126	0.165	0.132	0.161	0.135
5HT2a_cimbi_hc29_beliveau	0.090	0.069	0.089	0.072	0.090	0.078
5HT2a_mdl_hc3_talbot	0.090	0.081	0.118	0.091	0.106	0.086
5HT4_sb20_hc59_beliveau	0.040	0.053	0.040	0.054	0.047	0.032
5HT6_gsk_hc30_radhakrishnan	0.052	0.062	0.058	0.082	0.063	0.038

5HTT_dasb_hc100_beliveau	0.008	0.031	0.013	0.028	0.016	0.010
5HTT_dasb_hc30_savli	0.000	0.014	0.003	0.017	0.004	0.001
5HTT_madam_hc10_fazio	0.020	0.048	0.026	0.054	0.032	0.018
A4B2_flubatine_hc30_hillmer	0.000	0.000	0.001	0.014	0.000	0.001
CB1_FMPEPd2_hc22_laurikainen	0.194	0.190	0.216	0.249	0.219	0.146
CB1_omar_hc77_normandin	0.114	0.142	0.132	0.122	0.141	0.118
D1_SCH23390_hc13_kaller	0.031	0.052	0.032	0.069	0.041	0.019
D2_fallypride_hc49_jaworska	0.002	0.012	0.003	0.019	0.005	0.001
D2_flb457_hc37_smith	0.017	0.034	0.016	0.046	0.023	0.009
D2_flb457_hc55_sandiego	0.005	0.020	0.008	0.031	0.010	0.002
D2_raclopride_hc7_alakurtti	0.003	0.005	0.001	0.007	0.003	0.001
DAT_fepe2i_hc6_sasaki	0.001	0.006	0.001	0.011	0.002	0.000
DAT_fpcit_hc174_dukart_spect	0.021	0.002	0.010	0.000	0.011	0.019
FDOPA_fluorodopa_hc12_gomez	0.002	0.012	0.003	0.016	0.005	0.001
GABAa-bz_flumazenil_hc16_norgaard	0.071	0.061	0.080	0.101	0.078	0.051
GABAa_flumazenil_hc6_dukart	0.204	0.197	0.227	0.207	0.230	0.183
H3_cban_hc8_gallezot	0.031	0.055	0.036	0.071	0.043	0.023
M1_lsn_hc24_naganawa	0.225	0.232	0.215	0.249	0.247	0.179
MU_carfentanil_hc204_kantonen	0.127	0.174	0.142	0.202	0.159	0.108
MU_carfentanil_hc39_turtonen	0.072	0.121	0.100	0.171	0.103	0.059
NAT_MRB_hc10_hesse	0.000	0.000	0.001	0.002	0.000	0.000
NAT_MRB_hc77_ding	0.055	0.050	0.074	0.070	0.065	0.047
NMDAR_mean	0.193	0.190	0.191	0.220	0.209	0.146
VAChT_feobv_hc18_aghourian_sum	0.024	0.036	0.021	0.052	0.029	0.012
VAChT_feobv_hc4_tuominen	0.004	0.014	0.005	0.024	0.007	0.001
VAChT_feobv_hc5_bedard_sum	0.009	0.022	0.010	0.031	0.014	0.004
mGluR5_abp_hc22_rosaneto	0.222	0.199	0.208	0.217	0.232	0.167
mGluR5_abp_hc28_dubois	0.258	0.252	0.273	0.267	0.287	0.218
mGluR5_abp_hc73_smart	0.191	0.197	0.224	0.224	0.223	0.167

The complete dataset for our study, including the metrics in the table above, are available to readers to perform analyses of interest in ‘Supplementary File5 - Excel spreadsheet with MRI data correlations’.

Regarding the poor and negative β coefficients between entropy (a proxy for synapse function) and mitochondrial parameters, we hypothesize that entropy may reflect distinct aspects of synaptic dynamics that do not align straightforwardly with mitochondrial metrics, particularly those related to OxPhos capacity. It is possible that higher mitochondrial features are related to lower entropy due to the differential energetic demands of highly organized versus more variable synaptic networks. Empirically, we put less confidence in the mathematical operationalization of “entropy” derived from MRI data than onto more direct observations from the PET ligands binding data above. Nevertheless, this observation warrants further investigation to clarify the relationship between mitochondrial function and synaptic disorder.

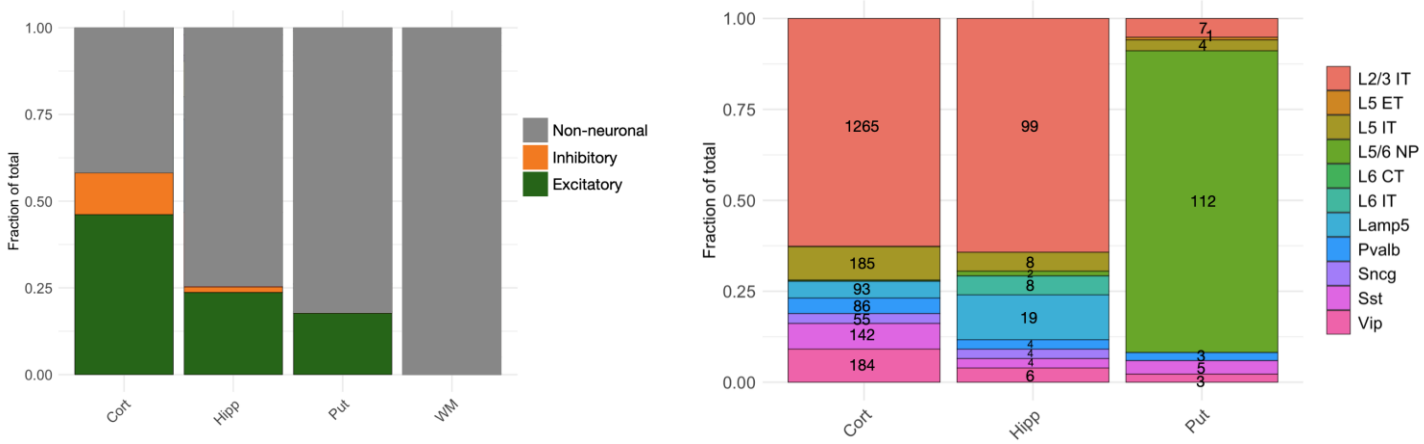
2.5. Providing Excel matrices containing all presented data and the anatomical location of the voxel of origin would enable others to process and use the data in further studies.

Response 5: Thank you for the suggestion. The entire dataset, including the observed and predicted mitochondrial values and the corresponding dMRI, sMRI, fMRI and PET values are now provided in a separate Excel file (Supplemental File 5). This file also includes formulas for the predicted mitochondrial features, including coefficients that match those listed in Supplemental Table 1. Each voxel coordinates in NIfTI format and the Python code for their conversion to MNI space are in Supplementary File 4.

2.6. Analyses in Figures 3G-H may need to be reexplored, categorizing distinct types of neurons. For example, inhibitory neurons could be further divided into Parv, Sst, and others, considering reports indicating heightened mitochondrial numbers and respiratory chain enzymatic activity in GABA neurons as well as mRNAs (see for example PMID: 24896567).

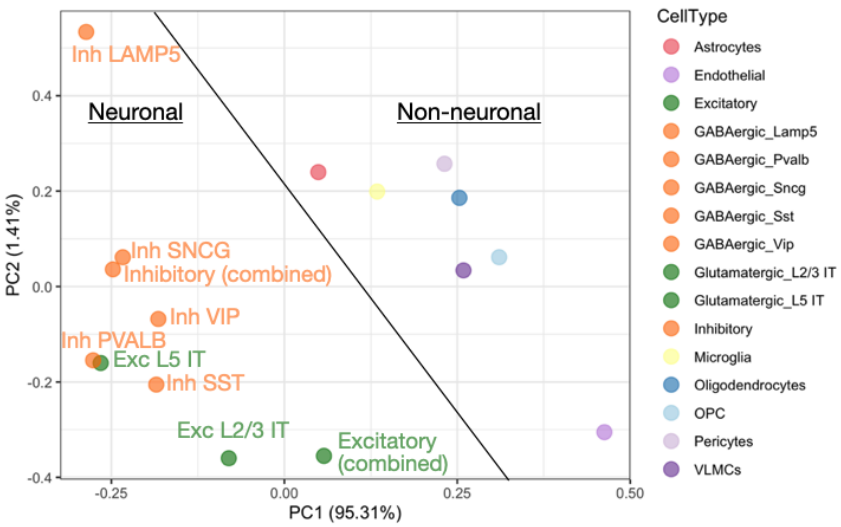
Response 6: Indeed, as the reviewer knows well there is substantially more mitochondrial biology to be discovered from single-cell RNAseq data from brain tissue. The snRNAseq analysis in this project was performed as a proof-of-concept study that this sort of molecular approach is feasible on physical voxels from a frozen brain section kept at -80^{o}C for an extended period of time, and to validate the expected cell composition of distinct anatomical regions. However, as stated in the manuscript, the harsh homogenization methods required for enzyme activities are poorly compatible with gentle single nuclei isolation approaches typically required to obtain high-quality nuclei with intact RNA. As a result, although the n=4 voxel dataset achieves the two primary objectives initially intended – a compelling demonstration of the feasibility of snRNAseq on frozen human brain voxels, and a validation of the expected cellular populations in each sample – the coverage is admittedly inferior to studies specifically designed to profile single cell transcriptomes.

The figure below shows cell type composition (left) and exact numbers of extracted nuclei of neuronal sub-classes (right) in each voxel. There is a negligible number of neurons in the white matter (WM) voxel, so we focused our analysis of neuronal subtypes on the gray matter (right panel).



This subtyping analysis demonstrates that the coverage of neuronal subtypes is expectedly poor, and too low to provide reliable transcriptome analysis, particularly for mitochondrial genes. Nevertheless, in the voxel from the cortex, which has better neuronal sub-type coverage including dozens to hundreds of

cells per subtype, principal component analysis (PCA) using only the 1,118 mitochondrial genes demonstrates that neurons reliably cluster separately from all non-neuronal cells:



Thus, as the reviewer suspected, transcriptome analysis of neuronal sub-populations will likely provide important additional information. However, satisfactorily addressing this question will require i) a larger number of cells, ii) many more brains samples to examine the stability/variability of neuronal subtype mitochondrial profiles across individuals, and iii) data from samples processed specifically for single nuclei extraction. Thus, we agree with the reviewer that this is an interesting point, and our data support the reviewer's intuition that different subtypes of neurons likely have different mitochondrial phenotypes. Given the limitations outlined here and in the manuscript, we guard ourselves from drawing conclusions on the analysis of neuronal subtypes. For the same reasons, we would not include the preliminary data above, unless the reviewer and editor feel that this would meaningfully contribute to the paper.

2.7. The authors should consider and comment on the possibility that while these studies were carried out with one human brain, the machine learning algorithm allowed them to use statistically curated brain imaging data to predict mitochondrial parameters in other brain regions previously untested. Could mitochondrial data from one new brain region, lets say the cerebellar cortex (not yet tested in this manuscript), back-predict using the same AI algorithm the measured mitochondrial status of the cortex already presented in this manuscript. If this were to be the case, this would be revolutionary as small tissue samples precisely mapped to the anatomy of the human brain could generate predictions and hypotheses for the whole brain.

Response 7: Excellent question. Testing both the specificity and accuracy of such model will require further studies in multiple areas from multiple brains, in individuals whose brains were scanned prior to death. Ongoing studies should provide such data in the coming years, which will be a superb opportunity to further develop and refine the MitoBrainMap. To help readers appreciate the possible outcomes of such efforts, the revised discussion addresses the possibility and promise, while outlining the current gap in knowledge and limitation of the current study:

"[…] The reliance on a single neurotypical human brain sample, akin to other whole-brain microscopic initiatives[35-37], underscores the necessity for additional postmortem data sets to determine the generalizability of our neuroimaging model to other healthy and pathological subjects of different age and gender. A generalizable model would mean that mitochondrial assays in a small tissue sample(s) precisely mapped to the anatomy of the human brain could generate predictions and hypotheses for the whole brain. The scale of this task promises to be technically challenging. […]"

2.8. Workflow schematics in Figure 1 and Figure 2 are a little lost in the figure because of segmentation with letter notation for each step. There might be a better way to show the schematic, like the whole schematic represented with one letter (the whole flow as "a" instead of a-g). This or some other formatting adjustment might help differentiate the schematic from the subsequent data in the same figure.

Response 8. The requested adjustments have been made to Fig 1a and Fig 2a, separating the schematics from the data.

Referee #3

This is a well written paper and represents a massive effort to generate an atlas of mitochondrial content, mitochondrial functional activities, diversity of mitochondrial phenotypes at a resolution comparable to MRI. In general, the data are of high quality and state of the art methodology is used.

There are several important conclusions, and one of the major ones is that the major driver of the differences in OxPhos transcriptome for each cell type is the brain region in which it resides. This is very well documented in this study, but somewhat to be expected based on the current status of this field, where it also has become evident that mitochondrial deficiencies are central in neurodegeneration.

Thank you

3.1. A severe limitation, as they point out is that this work represents one individual brain 8 hrs postmortem. As they also point out, the entire dynamics might be very different if they had another postmortem time interval, as this is critical for enzymatic function. 8 hrs is not a short postmortem, they could have found 4 hr samples.

Response 1: The choice of this specific brain was based on several criteria, including donor's age, general health condition, psychological autopsy (i.e., absence of mental illness), negative toxicology, cause of death, storage time and post-mortem interval. From the brains available in our brain bank, we selected the best-suited for this project, although we agree that brains with shorter postmortem interval could be found.

Previous studies in mouse brains found a decline in mitochondrial activities over 0-12 hours PMI (https://www.sciencedirect.com/science/article/pii/0022510X9090056S). Note, however, that there is no statistically significant difference between OxPhos enzymes activities at 4h and 8h PMI (highlighted regions). Some enzymes appear to show mild-to-moderate decline (complex IV most, complex I less)

while complex II enzyme activity at 8h appears slightly higher than in fresh tissue. Overall, although somewhat surprising, statistical analyses in this paper shows no consistent decline in enzymatic activities from brain tissue within 8 hours (we highlighted the ROIs on the graphs below).

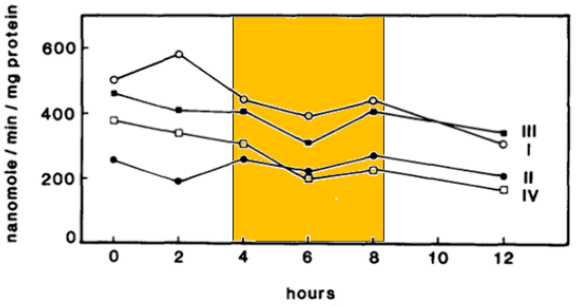


Fig. 1. Postmortem changes in activities of Complex I, II, III and IV in the electron transport system in mouse brains. Means of duplicate assays on duplicate samples. The ordinate represents the specific activity, and the abscissa duration between the cervical dislocation and the freezing of the brain expressed in hours.

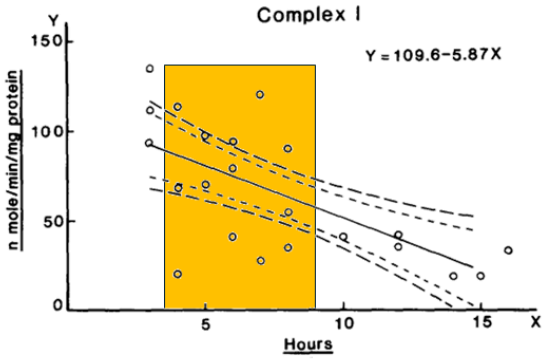


Fig. 3. A representative example of the least square regression analysis on the activity of Complex I with respect to the postmortem delay. The ordinate represents the specific activity, and the abscissa the duration between the patient's death and the freezing of the brain expressed in hours. Dotted lines represent the confidence bands at 95% and 99% levels, respectively. A significant negative correlation was noted. $R = 0.68$, $r^2 = 0.46$, $P < 0.01$.

Another study also concluded that postmortem mouse brain cortical mitochondria preserve OxPhos activity up to 24 hours postmortem. Across several parameters, including the generation of mitochondrial membrane potential, the authors reported no significant changes between freshly frozen brains and 10-hour PMI mouse brains (https://faseb.onlinelibrary.wiley.com/doi/epdf/10.1096/fj.09-152108).

Based on these data, it is unlikely that a donor brain with a PMI < 8h – a task that is challenging by itself – would influence the results, especially since our study focuses on the relative differences between brain regions (i.e. all mitochondrial characteristics are z-scored across the brain), and these relative differences are expected not to change during brain storage before or after cryopreservation. We agree however that future work comparing brain tissue from donors with different storage intervals is required to quantitatively address this question.

3.2. It is a lot to ask, but studies in a diseased brain or attempt to relate this to neurodegenerative disease and thus to actual MRI results would verify that this approach has clinical value. While it is an excellent study, the scientific advance is limited, it is more of a methods advance.

Response 2: We agree that extending this biochemical and neuroimaging approach to diseased brains would be a superb way to provide further scientific advance. This is currently not possible given the time and resource constraints that such studies would require.

Referee #4

This manuscript details a work-intensive, brute-force effort by the authors to characterize/describe mitochondrial profiles across the “pixels” of a grid-delineated human brain. One reason for doing this is to create a novel, anatomically well-defined anatomical characterization of human brain mitochondria across the brain, which itself provides insight into mitochondria-neuroanatomical correlations and can inform questions of mitochondria in white matter vs gray matter, by cell type, etc. The authors further

propose a more conceptual utility, which is perhaps their map at some point may inform translational questions. Specifically, our ability to characterize mitochondria in living brains is currently quite limited. There are current neuroimaging techniques many believe at least indirectly reflect mitochondrial function, but we really aren't that sure how to extrapolate data from such studies to infer a territory's underlying mitochondrial status. The "map" the authors generate can potentially serve as a bridging step to better link data from clinical neuroimaging studies to brain mitochondria status; hence the plan to characterize a brain in voxels that are comparable in size to achievable MRI scan voxels. From a significance perspective, these are important questions to address and the rationale underlying the study is strong.

The work-intensive, brute force approach essentially adapts existing mitochondrial assessment procedures for high-throughput applications. In some cases, some procedures were already suited for medium-throughput studies, but in this case the authors take the throughput issue to a whole other level. There are not existential concerns about the mitochondrial assessments themselves, which are essentially state of the art. For example, the Shirihai group applied their oxygen consumption methods for analyzing electron transport chain enzyme activities in an extraordinary number of small tissue samples. In some cases, redundant techniques were applied, which is a strength rather than a limitation. The authors define terms such as "mitochondria density" as a value defined by mtDNA copy number and citrate synthase, and one can argue about the pros and cons of assumptions such as this, but it is acknowledged one must start from somewhere. Work of his nature is going to require some assumptions.

The results essentially confirm what is already known at a broad level, which is that mitochondria in the brain function differently and are indeed structured differently in different brain cell types and brain regions. It certainly, though, is worthwhile to see this type of analysis conducted as such a refined anatomic level. The authors further use bioinformatics approaches to provide cell/regional specificity insight, which nicely complements the mitochondrial function data form those regions.

No fatal flaws are identified. It's hard to say how useful this map will ultimately prove to be, and perhaps by the time its translational utility and limitations are worked out the field will have imaging techniques that more directly interrogate mitochondria at neuroanatomically and cell-type discrete levels, but this current study is perceived as an ambitious study that (1) advances our understanding of neuroanatomical differences in brain mitochondria at a higher than before spatial resolution level, and (2) moves to create a new resource that could enter the public domain, and (3) at some point may have the ability to inform brain imaging-based translational studies.

4.1. If terms of points to address, really the only one that perhaps worth mentioning is that the text initially gives the impression that this study is really focused on using MRI to inform the status of brain mitochondrial. One might argue that is more of a potential future application.

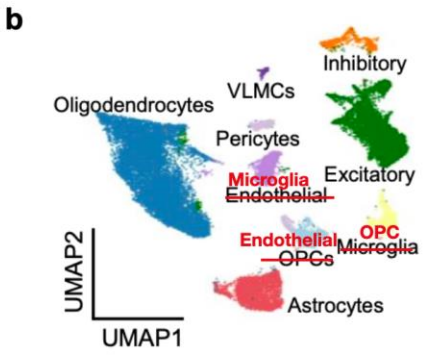
Response 1: We thank the reviewer for the positive comments and assessment of the work and its translational potential. We agree that using MRI to indirectly measure brain mitochondria is a future

application. We have revised the introduction to better align the reader's expectations with what the manuscript provides.

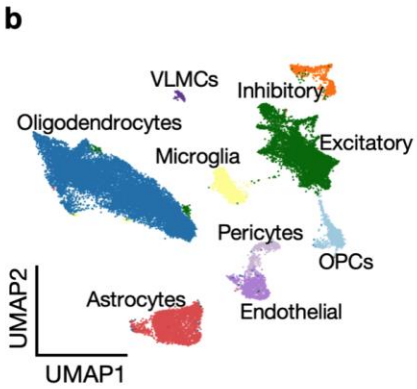
To all Referees and Editor:

In the original submitted manuscript, we noticed a small but meaningful error in single nuclei clusters annotation, affecting the microglia, endothelial, and oligodendrocyte precursor cell clusters (Figure 3 and Supplementary File 1). This mistake resulted from building the R Markdown file, as cluster IDs/numbers change stochastically with their assignment. This correction does not change the results discussed in the paper. Given that the cluster (mis)identification affected only these three poorly represented cell populations, the quantified cell type proportions are still similarly low. Stochasticity is a normal part of single cell RNA sequencing analysis and small changes occur with different package versions. This explains the visual differences in the UMAP presentation and new clusters in the corrected version of the UMAP plot. The revised manuscript includes a new R Markdown file and correspondingly updated figures. For transparency, we present below the previous (left) and corrected (right) versions of the figures, including the mis-identifications, and list below the minor yet meaningful effects that this correction has on the results in Figure 3.

Old version (wrong annotation)

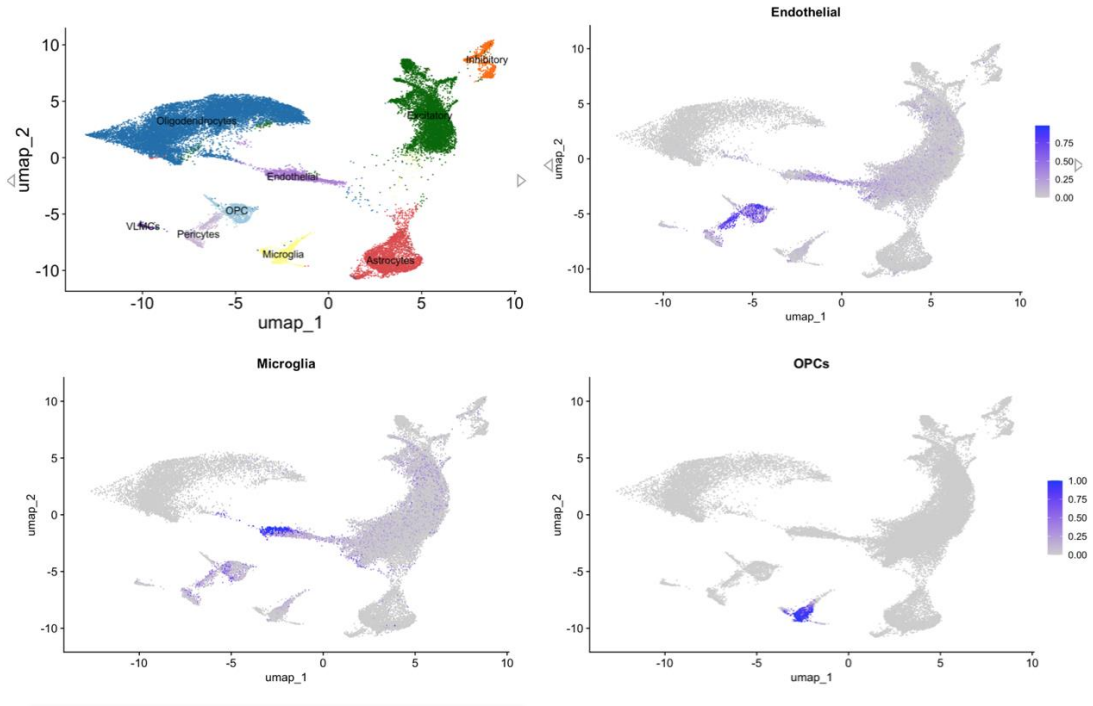


New version (corrected annotation)*

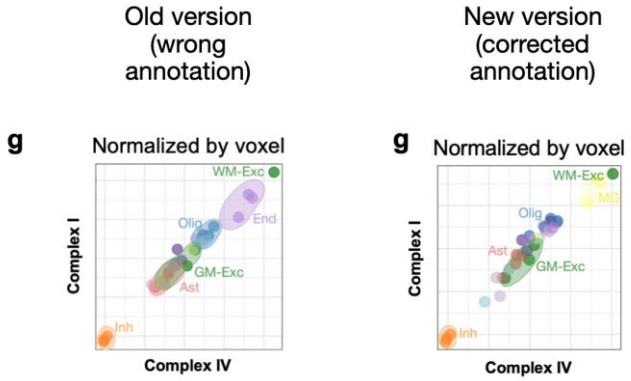


**Note that Seurat package was updated, hence the UMAP presentation looks slightly different (proportions and clustering are the same)*

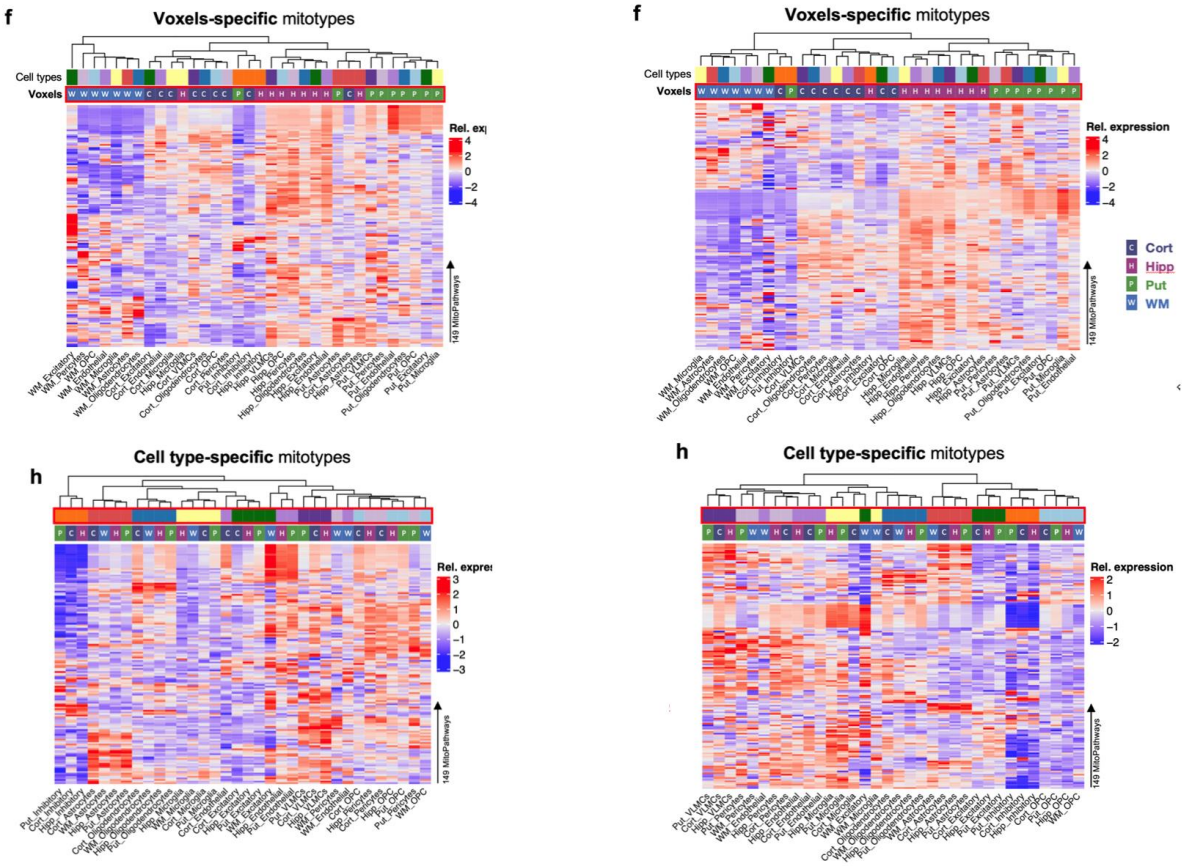
The figure below (from Supplementary file1) confirms the previously erroneous cluster identification (top left) and predicted cluster identity (top right, bottom), revealing that microglia were assigned as endothelial cells, that endothelial cells were assigned as OPCs, and that OPCs were assigned as microglia. As shown above, these have now been corrected.



The correct cluster assignment causes small changes in Figure 3g (previously labeled endothelial cells are microglia – top right).



The previous (left) and corrected (right) panels f and h in Figure 3, showing that the main findings were not affected by the mis-annotation. In fact, the correct annotation in 3f (where we compare mitotype signatures across the 4 voxels and all cell types) and in 3h (where we compare cell types normalized within each voxel by Z-scores, to remove the main effect of voxels) shows even more robust clustering by cell type. Using the correct annotations, all vascular cells cluster together, in proximity to microglia and oligodendrocytes; while neurons, astrocytes, and oligodendrocyte precursors cluster together to form a second, more coherent main cluster than in the previous version. These minor changes to not affect the main conclusions, and in fact reinforce our confidence in the presence of both voxel- and cell type-specific mitochondrial phenotypes that contribute to the diversity of mitochondria in the human brain.



We apologize for this error and have made sure that all aspects of the new version are correct.

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4. Perge, J. A., Koch, K., Miller, R., Sterling, P. & Balasubramanian, V. How the Optic Nerve Allocates Space, Energy Capacity, and Information. *J. Neurosci.* **29**, 7917–7928 (2009).
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