nature portfolio

# Peer Review File

## Multimodal gradients of basal forebrain connectivity across the neocortex

Reviewer #1 (Remarks to the Author):

Overall Summary of reviewer comments:

[The present study is primarily a meta-analysis of data that was acquired from several previous studies. It also appears to include some new mouse FEOBV data acquired for this study per se, The reviewers found it somewhat disconcerting that the data resources from which the study has been drawn are not presented in a transparent manner. ]

Chakraborty et al. have evaluated the relationship between the structural organization of the basal forebrain cholinergic system and its functional integration as assessed by resting state MRI from human subjects. The authors analyzed previously collected 7T diffusion and resting state MRI datasets to examine BF connectivity across the cortical mantle. In accordance with the literature, the authors observed variability in the structure-function coupling across the brain. Specifically, they found reduced tethering in transmodal (association) cortices as compared to unimodal (primary sensory) cortices. Using previously acquired FEOBV datasets in human subjects, the authors determined that those cortices with higher cholinergic innervation exhibited lower correspondence between structure and function. Moving on to mouse data, the authors compared anterograde tracing experiments with some newly acquired mouse FEOBV microPET data. From these data the authors conclude that terminal field density varies across cortical domains with differences in how these branches are functionally integrated.

Review text:
Overall, the manuscript presents a timely evaluation of the structure-function relationship across the brain, with respect to a modulatory system worthy of high profile attention. In addition, the idea that association cortices---areas that are known to integrate multiple sensory modalities---receive heterogenous cholinergic input (i.e. input from multiple populations) and are likely to have a more diffuse structure-function relationship, as compared to primary cortices, is a reasonable notion.
The major issue is that the paper in its current form lacks transparency on the sources of the data that were used and presented. The abstract does not appropriately account for the datasets that were used nor does it highlight the subset of new data that was actually acquired and presented for the first time in this study. The introduction includes citations for the human connectome project dataset but does not appropriately cite or account for the human FEOBV dataset nor the mouse anterograde tracing dataset. It is also unclear if optogenetic tracing data was acquired from another study (or Allen Brain Datasets) or incorrectly referred to as such. The origins of the multiple datasets are buried in the Methods section rather than presented up front. (In addition, all data sets should also be detailed at the end of the manuscript, where dataset/code availability is listed).The manuscript requires substantial revision for transparency, prior to one being able to conduct a properly detailed consideration of the work for publication. As such we recommend rejection. We hope that the associated comments are helpful in clarifying the presentation if the manuscript is rewritten.

Major Points for Revision:
1. Transparency - The authors should clarify up front which analyses included are essentially meta-analyses of existing datasets. It is particularly important to reference the precise resources used as these datasets in the main text and to precisely specify the availability of and access links to the datasets at the end of the manuscript. From the methods it appears as

though the human DTI and rsfMRI data were analyzed from the Human Connectome Project dataset. Human FEOBV data seems to be obtained primarily from Kanel et al. 2022, but appears to also have been supplemented by additional datasets for support/replication. These must be explicitly noted.
a. The Mouse anterograde tracing data was analyzed from the Allen Brain dataset (?) and from Li et al. 2017. Mouse FEOBV data seems to be newly acquired for this manuscript. In the results section it is written as if all the data assessed were newly "acquired" rather than obtained from other resources. Please make it clear where the data can be accessed. In addition:
b. If any Allen brain datasets were combined to evaluate cholinergic terminal density in cortex, they should be listed with the experiment ID in a designated methods section for reproducibility.
c. From Li et al. 2017, it seems like the projection data is derived from AAV-CAG-FLEX-GFP injections that were primarily targeted to MS/DB cholinergic neuron projections, which do not project as widely and readily to cortex as the NBM region which presents a challenge to the interpretation of the mouse data and its use for evaluation of sparse vs. densely innervated cortical targets. Furthermore, the human data suggests the posteriomedial regions (defined as NBM), are more likely to exhibit low tethering but the structural components of the NBM projections cannot be well evaluated with this dataset. The evaluation of unimodal vs. transmodal tethering cannot be evaluated appropriately in the mouse dataset without NBM projections.
2. Quantification: The human and mouse FEOBV data seem to be quantified with different methods. Mouse BPnd (and corresponding DVR) metrics were acquired from a dynamic scan using an image-derived input function with the blood activity quantified from a voxel placed in the heart. The Human BPnd (and corresponding DVR) metrics were acquired from late static scan imaging using the cerebellar region for reference quantification. Have the authors evaluated using IDIF vs. reference region approaches in the mouse data? In general, it would be best quantify the FEOBV in a comparable manner between the mouse and human FEOBV data. The limitations of the comparisons with the different methods used should be delineated
3. Conclusions drawn: Its not clear what the functional measure is in the mouse datasets. FEOBV provides a metric of cholinergic synaptic integrity or distribution of the vesicular acetylcholine transporter. The anterograde tracing data also provides a structural measure of the density of cholinergic terminals in a cortical region. Did the authors acquire rsfMRI data with the rodent MRI scans? Without this, I think that the claims about human and mouse data supporting a gradient of tethering cannot be made.
4. Accessibility of Study: Nature Communication is a journal with broad readership. While we agree that this manuscript could be of interest to the readership of Nat Com, the introduction does little to ease the reader into this complicated set of analyses and this field that is newly emerging. The readers would benefit from the authors adding more of an introduction of what has been done to evaluate the structure function relationship across the brain (e.g. Yang et al. 2023 Nat Com, Paquola et al. 2019), the metrics, and the vocabulary (e.g. tethering, geodesic distance). The writing as it stands it not accessible to a broad readership without a better crafted introduction.
5. Interpretation/Study Design: The structural measures as evaluated by DTI are not specific to cholinergic projections and the cholinergic system. These fiber tracts encompass connectivity from many neuronal types and as such cannot provide an accurate assessment of cholinergic structure per se. The authors should consider evaluating the relationship between the FEOBV and the resting state in cortical regions. This ensures at least one of these metrics is a specific measure of the cholinergic system. This could also be why the relationship between structural connectivity and FEOBV was poor.

Minor Comments:
6. The mouse projection data that was analyzed does not seem to be from optogenetic data, but from anterograde tracing experiments. If a different dataset was used, please note that and clarify. Otherwise, this should be amended throughout to say anterograde tracing dataset.
7. This might be an accessibility issue on our end, but the figure legends do not appear to be present for the main figures in the manuscript file OR on the figure files themselves. Figure legends are going to be critical to follow all of these complicated analyses.
8. Please include a demographics/sample characteristics table for all the different datasets that were included in the study for human (1) and mouse (2). This would help to evaluate strain, age, sex etc. of the cross evaluated populations.

Reviewer #2 (Remarks to the Author):

The topic is of considerable interest – cholinergic basal forebrain connectivity remains uncertain and is only being examined in detail since single-cell tracing in mice and whole-brain reconstruction techniques became available. It is of particular significance since acetylcholine release plays important roles in local cortical network integration for both input and output of thalamic or inter/intra cortical information, with demonstrated roles in attention, decision-making, learning, and memory – particularly all higher-order brain computations. The loss of cholinergic basal forebrain function underpins loss of cognitive function resulting in, at least contributing to, dementia.
The combination of human and mouse studies for the validation of certain conclusions is particularly innovative and significant.
I also find the way the authors have proceeded through this study to be logical and the experimental design to be imaginative. The background is clear and the authors clearly state the predicted outcomes of the assessments as they relate to the hypotheses to be tested, which are in turn based on evidence/references, including for the assumptions in the underlying methods ( e.g. diffusion MRI = streamlines = white matter = axonal connections between regions of interest), albeit with varying degrees of caveats ( as per the Discussion).
Furthermore, I find the authors' conclusions and inferences to be stimulating. They agree with fledgling ideas but the current results are leading the way in testing models using human multi-modal imaging.
The Methods often outstrip my statistical expertise but are written in a way that are reproducible and ‘open’ as to what has been performed.
I have minor issues, with my only real hesitations being that
(i) The assumptions underlying the method-biology may require further justification, at least in the results sections,
(ii) in some instances it feels like a circular argument.
(iii) changes to terminology are likely to assist the above; the flow of what is being undertaken is why is typically clear, but I suggest it will be less accessible to biologists, and thus the robustness of the results are/will be harder to judge. For some instances in Results, I was unsure what values of the data were being used, so I suggested some modifications to the text for reader understanding.

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(my internal dialogue is below to assist in identifying what I struggled to comprehend – when I

read it, it makes sense but when I try to explain it I can't confidently). I'm sorry it is so long!
1. PDF pg last paragraph of 6, top 7: For each 'modality'; does modality refer to each of spatial and functional 'projectome or connectome'? I associate modality with sMRI or fMRI
2. 'Interregional similarity of features… eigenvectors of BF connectivity axes'. I'm trying to grasp what the data, once it has undertaken the computational gymnastics, represents: firstly it is not fully clear that the two matrices are being processed separately.
3. Then, do you mean you computed the similarity/non-similarity of [the strength of structural/diffusion (streamline) connections from/with the BF] in one matrix and [the correlational strength of (brain areas in functional networks)] in another? And from this, you generate the eigen vectors projected onto the BF (sup fig 2)? For sMRI this is a straightforward/reasonably direct measure (assuming streamlines = actual axons and thus the direction (of the final target, not of the start of axons). For fMRI the voxels that fire together BF and cortex are linked 'as the crow flies' in a similar way.
4. Fig 2 abc: Then, because there is a lot of variability (in the strength or confidence?) in the computed directionality for each voxel, they were 'ranked' to represent a gradient of brain areas from strong-week connections to/with the BF (and between BF voxels?) (sG1) and have strong-week connections each other (and between locally close voxels)(fG1)?
I can't quite grasp that there is more than one gradient: are there just many alternative ways of ranking each connection matrix?
5. 30% variance: does 'variance' refer to how variable the eigen vector directions are in surrounding voxels and/or between subjects/people? (or something else altogether?) Note: typo in methods 'litter' for 'little'
6. 'Followed by a reduction in explained variance by 50% of the second gradient': does this mean the next gradient explained less than 15% of the variance? if so it is oddly put.
7. Pg 8 The use/introduction of the word tethering = magnitude of the share variance. Tethering evokes for me a boat or horse tied up. Essentially (I think) you computed the similarity/non-similarity of [the strength of structural/diffusion (streamline) connections ( or projected eigen vector) from/with the BF] with [the correlational strength of (brain areas in functional networks)] using sG1 X fG1. So does "tethering" represent a value of statistical or computational strength per voxel between structure measures/gradients and functional/gradients measures? with that value suggesting - assumed to be a representation of the strength of the computed relationship between the two? Is this assumption justified? Shared variance following data gymnastics could represent associations that are not causal and might even be both functionally and structurally unrelated.
8. Fig 3A Because I'm unclear as to what the residual measure is (is this the computed 'tethering' value) I worry that it could be circular to argue that because Ch2 projects to the hippocampus/ entorhinal cortex which are in relatively close proximity and in function, but that the Ch4 projects to the entire cortex which is has a myriad of functions and more projection routes, that any 'variability' difference between the 2 nuclei is evidence of something more interesting as opposed to an interesting observation but biologically meaningless. This is a spot where the terminology could be changed/toned down – albeit the subsequent experiments support the conclusion, they also rely on the assumption that this observation is biological.
9. Fig 3BC Pg 9 I would expect the tethering values to align with some predefined networks e.g. resting state, given they are derived at least partially from resting state MRI. If you multiply X by Y and then divide by a factor of Y the result (Zi) is a multiple of X – if the networks are not based on Y ( actually the opposite (ie attention e.g. divide by a prime number)), the result of Zii might be quite unlike Zi; So of course the networks differ- am I missing something here? However, I would not predict that the uni-modal areas would be more similar than the multi-modal cortical areas

– unless this happens to correlate with the structural eigen vectors (ie X). Can this be checked and clarified?
10. Glad to see the cross-validation, but didn't follow how it was performed
11. FEOVB: great way to test some of the conclusions/hypotheses from the previous results and be specific for cholinergic not just basal forebrain. However Vchat could be lower/higher for functional reasons not related to structure – so nice to use the mouse neurons to directly link these findings together and, to some extent, the consistency with your prior assumptions partly alleviates my hesitancy in conclusions evident in my previous points.
12. Concerning the wiring cost reasoning – is the initial matrix for sMRI based on actual length or only directionality? If only directionality then mixing in actual length is an independent variable, if not it's a bit circular again.
13. How different are the salience and the attentional networks?
14. Can you conclude "further translational evidence that BF cholinergic neurons exhibit an arborization gradient which is shaped by the function 'and' physical distance of their cortical targets." Or would 'and/or' be more accurate?
15. I found the discussion illuminating and well-reasoned (assuming my issues were unfounded)

Reviewer #3 (Remarks to the Author):

This study provides a comprehensive examination of the cholinergic innervation of the cortex, originating from the basal forebrain (BF). Through the use of high-resolution 7T diffusion and resting-state functional MRI in humans, the authors investigate the multimodal gradients of BF cholinergic connectivity with the cortex, elucidating a complex structural and functional relationship. Notably, the study identifies a gradient of reduced tethering between structural and functional connectivity, and further demonstrates that cortical areas with higher concentrations of cholinergic innervation exhibit lower tethering, suggesting patterns of diffuse axonal arborization. The authors extend their findings to a rodent model, providing a cross-species replication that underscores the generality of their observations.

The methodology and statistical approaches employed are sound, reflecting a rigorous and multidimensional exploration of the subject matter. I appreciate the substantial amount of work behind this study, which considers the topic from multiple angles and presents replication analyses where necessary. However, I have one comment and a request before I can recommend this for publication:

In reviewing the methodology, particularly the structural connectivity reconstruction technique, I find it noteworthy and innovative. The challenges of conducting seed-to-cortex tractography from subcortical areas like the BF are known, given that most connections terminate near the seed region, and only a few pathlines continue, often reflecting the brain's geometry more than the true topography of white matter pathways, influenced significantly by the partial volume effect. To ensure a thorough understanding of what we are observing, I kindly request an addition to your manuscript: an image similar to those depicting fiber length and wiring cost, specifically detailing the total number of streamlines received from the BF for each cortical parcel. This seems to be partially addressed in Supplementary Fig 1C, however, the scale is absent. Providing this would greatly enhance the clarity and completeness of your findings,

ensuring the observed connectivity patterns accurately reflect the underlying anatomical architecture. I would also suggest considering the inclusion of such a figure in the supplementary materials to further increase the persuasiveness of your methodology.

Reviewer #3 (Remarks on code availability):

After a brief review of the code repository associated with this publication, I can confirm the inclusion of a succinct README file, as well as the provision of input data, source Python scripts, and the output dataset. The README file offers clear instructions for setting up and running the application, contributing significantly to the usability of the code for the wider community. From my assessment, these resources are well-organized and should indeed facilitate replication efforts considerably.

**REVIEWER COMMENTS**

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Overall Summary of reviewer comments:

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We thank the reviewer for providing in-depth feedback on our manuscript. In our revision, we have taken multiple steps to make the sources of data used for our analyses more transparent to the reader. We detail these steps in our responses interspersed below.

Chakraborty et al. have evaluated the relationship between the structural organization of the basal forebrain cholinergic system and its functional integration as assessed by resting state MRI from human subjects. The authors analyzed previously collected 7T diffusion and resting state MRI datasets to examine BF connectivity across the cortical mantle. In accordance with the literature, the authors observed variability in the structure-function coupling across the brain. Specifically, they found reduced tethering in transmodal (association) cortices as compared to unimodal (primary sensory) cortices. Using previously acquired FEOBV datasets in human subjects, the authors determined that those cortices with higher cholinergic innervation exhibited lower correspondence between structure and function. Moving on to mouse data, the authors compared anterograde tracing experiments with some newly acquired mouse FEOBV microPET data. From these data the authors conclude that terminal field density varies across cortical domains with differences in how these branches are functionally integrated.

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We thank the reviewer for considering our work “worthy of high profile attention.”

The major issue is that the paper in its current form lacks transparency on the sources of the data that were used and presented. The abstract does not appropriately account for the datasets that were used nor does it highlight the subset of new data that was actually acquired and presented for the first time in this study. The introduction includes citations for the human connectome project dataset but does not appropriately cite or account for the human FEOBV dataset nor the mouse anterograde tracing dataset. It is also unclear if optogenetic tracing data was acquired from another study (or Allen Brain Datasets) or incorrectly referred to as such. The origins of the multiple datasets are buried in the Methods section rather than presented up front. (In addition,

all data sets should also be detailed at the end of the manuscript, where dataset/code availability is listed). The manuscript requires substantial revision for transparency, prior to one being able to conduct a properly detailed consideration of the work for publication. As such we recommend rejection. We hope that the associated comments are helpful in clarifying the presentation if the manuscript is rewritten.

We acknowledge that the origins of the datasets used in this manuscript should be more accessible to the reader. Following from the reviewer's suggestions above, we have added systematic references to the datasets in the following sections of the paper in addition to Methods (line 576-788): (1) in the Introduction (line 95-123) we have citations for all retrospective datasets used in this manuscript; (2) in Supplemental Table 1 where we provide references and brief descriptions of each dataset, and (3) in the Data/Code Availability section (line 776-788) at the end of the manuscript we provide links to all retrospective and prospective datasets.

Major Points for Revision:

1. Transparency - The authors should clarify up front which analyses included are essentially meta-analyses of existing datasets. It is particularly important to reference the precise resources used as these datasets in the main text and to precisely specify the availability of and access links to the datasets at the end of the manuscript. From the methods it appears as though the human DTI and rsfMRI data were analyzed from the Human Connectome Project dataset. Human FEOBV data seems to be obtained primarily from Kanel et al. 2022, but appears to also have been supplemented by additional datasets for support/replication. These must be explicitly noted.

In the Introduction (line 95-123), Supplemental Table 1, Methods (line 576-788) and Data/Code Availability (line 776-788) sections, we now cite the origin of each dataset used, provide links to the dataset where applicable, and differentiate between whether the data is retrospective or was collected prospectively.

a. The Mouse anterograde tracing data was analyzed from the Allen Brain dataset (?) and from Li et al. 2017. Mouse FEOBV data seems to be newly acquired for this manuscript. In the results section it is written as if all the data assessed were newly "acquired" rather than obtained from other resources. Please make it clear where the data can be accessed.

In the Methods (line 681-736) section, we now explicitly state whether the data is retrospective (e.g. Li et al. 2017) or was collected prospectively (mouse [$^{18}$F]FEOBV PET). No raw data from the Allen Mouse Brain dataset was used in this study. However, the mouse tracing data from the Li et al. 2017 study and the mouse [$^{18}$F]FEOBV PET data from our experiment were registered to the Allen Mouse Brain anatomical reference space (common coordinate framework) so that we could use systematic annotations for labeling brain regions. We now make this more explicit in the Results as well.

In addition:
b. If any Allen brain datasets were combined to evaluate cholinergic terminal density in cortex, they should be listed with the experiment ID in a designated methods section for reproducibility.

No raw data from the Allen Mouse Brain dataset was used in this study. We used the Allen Mouse Brain (common coordinate framework) as an anatomical reference space. We now make this more explicit for the mouse tracing data from the Li et al. 2017 study and the mouse [$^{18}$F]FEOBV PET data from our experiment.

c. From Li et al. 2017, it seems like the projection data is derived from AAV-CAG-FLEX-GFP injections that were primarily targeted to MS/DB cholinergic neuron projections, which do not project as widely and readily to cortex as the NBM region which presents a challenge to the interpretation of the mouse data and its use for evaluation of sparse vs. densely innervated cortical targets. Furthermore, the human data suggests the posteriomedial regions (defined as NBM), are more likely to exhibit low tethering but the structural components of the NBM projections cannot be well evaluated with this dataset. The evaluation of unimodal vs. transmodal tethering cannot be evaluated appropriately in the mouse dataset without NBM projections.

We agree that our findings in humans using the HCP dataset would be more translatable with Li et al. 2017 if their sample of labeled neurons extended to include populations from the NbM. However, we note that multiple MS/DB cholinergic neurons in the Li et al. sample do in fact project to the transmodal regions overlapping the mouse salience network, including cingulate and infralimbic cortex. Moreover, this subset of neurons exhibited significantly higher arborization than neurons which do not project to transmodal cortical regions, consistent with the arborization gradient model. Our results in humans imply that the likelihood of capturing more neurons exhibiting a highly arborized projection pattern targeting salience network regions would be higher in NbM than in MS/DB, but they do not preclude the existence of such neurons in the MS/DB subregions. We have clarified in the limitations subsection of the Discussion (line 562-567) that further cell type specific tracing work is needed to capture the full profile of projection patterns spanning the BF nuclei in mice.

2. Quantification: The human and mouse FEOBV data seem to be quantified with different methods. Mouse BPnd (and corresponding DVR) metrics were acquired from a dynamic scan using an image-derived input function with the blood activity quantified from a voxel placed in the heart. The Human BPnd (and corresponding DVR) metrics were acquired from late static scan imaging using the cerebellar region for reference quantification. Have the authors evaluated using IDIF vs. reference region approaches in the mouse data? In general, it would be best quantify the FEOBV in a comparable manner between the mouse and human FEOBV data. The limitations of the comparisons with the different methods used should be delineated

We thank the reviewer for their question regarding the normalization approaches used for [$^{18}$F]FEOBV brain PET data in humans versus mice. We agree that discussing the differences between the approaches should be more clearly articulated and we have updated the Methods to include this information. Below we summarize our rationale for using an anatomical brain reference region for the human [$^{18}$F]FEOBV and image-derived input function for mouse [$^{18}$F]FEOBV.

“The Human BPnd (and corresponding DVR) metrics were acquired from late static scan imaging using the cerebellar region for reference quantification”

The Kanel [$^{18}$F]FEOBV human dataset (see Kanel et al. 2022, Aging Brain) presented in the main results, as well as the Aghourian (see Aghourian et al. 2017, Molecular Psychiatry) and Bedard (see Bedard et al. 2019, Sleep Medicine), and unpublished data provided by Tuominen (see Markello et al. 2022, Nature Methods) human datasets used in the supplementary replication analyses, all employed the global cerebral or supratentorial white matter as the reference region for DVR and/or SUVr image calculation. The cerebellum exhibits specific uptake of [$^{18}$F]FEOBV (see Albin et al. 2018, Journal of Comparative Neurology; Okkels et al. 2023, Neuroimage) that can be affected by both aging and disease (see Albin et al. 2017, eNeuro; Mazere et al. 2021, Brain; Kanel et al. 2022, Aging Brain). Given the *ex vivo* and *in vivo* evidence of cholinergic innervation to the cerebellum, this region is no longer considered to provide a valid measure of reference uptake for [$^{18}$F]FEOBV brain PET quantification.

"Mouse BPnd (and corresponding DVR) metrics were acquired from a dynamic scan using an image-derived input function with the blood activity quantified from a voxel placed in the heart. […] Have the authors evaluated using IDIF vs. reference region approaches in the mouse data?"

There are important differences in mouse neuroanatomy and mouse imaging parameters that create major obstacles to using a white matter reference region for [$^{18}$F]FEOBV normalization. Unlike in the human brain where the ratio of the total surface area of gray matter to white matter is approximately 2:3, the mouse brain exhibits an almost 9:1 difference (see Krafft et al. 2012, International Journal of Stroke). This disparity in tissue type surface area substantially limits the availability of white matter regions for estimating reference [$^{18}$F]FEOBV uptake. Additionally, reliable delineation of white matter structures in the mouse brain requires objectively higher spatial resolutions (<0.1 mm$^3$) than what can be achieved with microPET (>0.7 mm$^3$). This is in comparison to humans where white matter structures and PET imaging are on a more similar spatial scale (>1 mm$^3$). Intensity measures from such regions in the mouse brain will thus yield poor estimates for normalization of [$^{18}$F]FEOBV uptake.

One alternative approach could then be to perform a multi-time point graphical analysis of the human [$^{18}$F]FEOBV brain PET data as was performed in mice. In this instance, the [$^{18}$F]FEOBV data in humans would need to have been collected from the time of injection onwards to most correctly estimate [$^{18}$F]FEOBV concentration in different brain regions. However, given that prior work in humans (see Petrou et al. 2014, Journal of Nuclear Medicine) has demonstrated that DVR/SUVr values from [$^{18}$F]FEOBV images acquired over a late static scan period are strongly positively correlated with $BP_{ND}$/DVR values from dynamic [$^{18}$F]FEOBV PET imaging with subsequent kinetic modeling, late static [$^{18}$F]FEOBV scans are conducted in place of dynamic imaging experiments for clinical feasibility.

Overall, we believe that the methods we have employed for human and mouse [$^{18}$F]FEOBV brain PET normalization provide the most accurate and reliable measure of [$^{18}$F]FEOBV brain uptake in these two species. The methods for normalization were conducted after taking into careful consideration the known profiles of cholinergic innervation in the mouse and human brain, as well as important differences in both the neuroanatomy of the two species and inherent limitations of imaging resolution.

3. Conclusions drawn: Its not clear what the functional measure is in the mouse datasets. FEOBV provides a metric of cholinergic synaptic integrity or distribution of the vesicular acetylcholine transporter. The anterograde tracing data also provides a structural measure of the density of cholinergic terminals in a cortical region. Did the authors acquire rsfMRI data with the rodent MRI scans? Without this, I think that the claims about human and mouse data supporting a gradient of tethering cannot be made.

We agree with the reviewer that a combined dataset in mice integrating *in vivo* resting state fMRI and diffusion MRI to study BF connectivity would be ideal for cross-species translation. Unfortunately, the technical challenges of acquiring such a dataset in mice make it a near impossibility with currently available MRI instruments. The single greatest challenge to such a study is the spatial resolution of *in vivo* mouse MRI, which at present is ~100 micron isotropic. The volume of the mouse BF (all nuclei) is 2.68 uL (Supplemental Table 4 in Wang et al., 2020 Cell). Hence, even at 100 micron isotropic resolution, the number of voxels one could claim overlap the BF nuclei would be very small. This precludes calculating a gradient of connectivity, which requires a substantial number of voxels to obtain reliable patterns. We have clarified this rationale in the results by removing "human" from this sentence: *"However, a limitation of in vivo dMRI and rs-fMRI techniques is that neither can resolve single cell axonal branching of cholinergic neurons,"* (line 379-380) because these limitations apply to both mouse and human MRI.

4. Accessibility of Study: Nature Communication is a journal with broad readership. While we agree that this manuscript could be of interest to the readership of Nat Com, the introduction does little to ease the reader into this complicated set of analyses and this field that is newly emerging. The readers would benefit from the authors adding more of an introduction of what has been done to evaluate the structure function relationship across the brain (e.g. Yang et al. 2023 Nat Com, Paquola et al. 2019), the metrics, and the vocabulary (e.g. tethering, geodesic distance). The writing as it stands it not accessible to a broad readership without a better crafted introduction.

We thank the reviewer for considering our work "of interest to the readership of Nat Com." We agree the introduction introduced several key terms before properly defining them, most critically, the term "gradient" and "geodesic distance". To improve the accessibility of the manuscript to a broader readership, we have adjusted the introduction so that when novel technical terms like "gradient" and "geodesic distance" are first introduced they are defined and references are provided. We believe these improvements will help better guide the readers through the different metrics and vocabulary. (lines 55-123)

5. Interpretation/Study Design: The structural measures as evaluated by DTI are not specific to cholinergic projections and the cholinergic system. These fiber tracts encompass connectivity from many neuronal types and as such cannot provide an accurate assessment of cholinergic structure per se. The authors should consider evaluating the relationship between the FEOBV and the resting state in cortical regions. This ensures at least one of these metrics is a specific measure of the cholinergic system. This could also be why the relationship between structural connectivity and FEOBV was poor.

We thank the reviewer for highlighting the importance of analyzing the relationship between cortical [$^{18}$F]FEOBV and cortical resting state connectivity with BF. This finding is provided in Figure 4C (line 305). Consistent with predictions there is a significant positive relationship, where cortical regions exhibiting higher [$^{18}$F]FEOBV intensity tend to also exhibit stronger BF resting state connectivity. We have edited the Results section where we describe Figure 4 to clarify these findings.

Minor Comments:

6. The mouse projection data that was analyzed does not seem to be from optogenetic data, but from anterograde tracing experiments. If a different dataset was used, please note that and clarify. Otherwise, this should be amended throughout to say anterograde tracing dataset.

We thank the reviewer for pointing out this error in terminology. We have replaced 'optogenetic' with 'anterograde viral tracing' throughout the manuscript. (line 47, 117, 383, 416)

7. This might be an accessibility issue on our end, but the figure legends do not appear to be present for the main figures in the manuscript file OR on the figure files themselves. Figure legends are going to be critical to follow all of these complicated analyses.

We sincerely apologize for this oversight. In the revised manuscript the legends for Main Figures (interspersed) and Supplemental Figures (after References) are included as part of the merged article file.

8. Please include a demographics/sample characteristics table for all the different datasets that were included in the study for human (1) and mouse (2). This would help to evaluate strain, age, sex etc. of the cross evaluated populations.

We have added the following table (Supplemental Table 1) to summarize all the datasets used in this study.

**Supplemental Table 1. Summary of the datasets.** Demographic details of all the datasets used in this study. Ages presented as mean(standard deviation), where applicable.

| Species | Reference | Imaging | Imaging materials | Use in Chakraborty et al. | Strain | Age (y or m) | Sex (M:F) |
|---|---|---|---|---|---|---|---|
| Human | HCP | 7 Tesla MRI | dMRI<br>rs-fMRI | gradient calculation, residual analysis | N/A | 22-35 y | 69:104 |
| Human | Kanel | PET | [$^{18}$F]FEOBV | principal correlation analysis of structure-function tethering and cholinergic innervation | N/A | 24.5(4.9) y | 10:3 |
| Human | Aghourian | PET | [$^{18}$F]FEOBV | replication correlation analysis of structure-function tethering and cholinergic innervation | N/A | 66.8(6.8) y | 5:13 |
| Human | Bedard | PET | [$^{18}$F]FEOBV | replication analysis of | N/A | 68.3(3.1) y | 4:1 |

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|---|---|---|---|---|---|---|---|
| | | | | structure-function tethering and cholinergic innervation | | | |
| Human | Tuominen | PET | [$^{18}$F]FEOBV | replication analysis of structure-function tethering and cholinergic innervation | N/A | 37(10.2) y | 3:1 |
| Mouse | Li | SIM | AAV-CAG-flex-GFP | unimodal versus transmodal cholinergic neuron branch counts with retrospective mouse data | *ChAT*-ires-Cre | 3-6 m | N/A |
| Mouse | N/A | microPET | [$^{18}$F]FEOBV | cross validation of human cortical cholinergic innervation with prospective mouse data | *VAChT*$^{flox/flox}$ | 6 m | 3:3 |
| | | | | cross validation of human cortical cholinergic innervation with prospective mouse data | C57BL/6J | | 2:3 |

**Reviewer #2 (Remarks to the Author):**

The topic is of considerable interest – cholinergic basal forebrain connectivity remains uncertain and is only being examined in detail since single-cell tracing in mice and whole-brain reconstruction techniques became available. It is of particular significance since acetylcholine release plays important roles in local cortical network integration for both input and output of thalamic or inter/intra cortical information, with demonstrated roles in attention, decision-making, learning, and memory – particularly all higher-order brain computations. The loss of cholinergic basal forebrain function underpins loss of cognitive function resulting in, at least contributing to, dementia.

The combination of human and mouse studies for the validation of certain conclusions is particularly innovative and significant.

I also find the way the authors have proceeded through this study to be logical and the experimental design to be imaginative. The background is clear and the authors clearly state the predicted outcomes of the assessments as they relate to the hypotheses to be tested, which are in turn based on evidence/references, including for the assumptions in the underlying methods ( e.g. diffusion MRI = streamlines = white matter = axonal connections between regions of interest), albeit with varying degrees of caveats ( as per the Discussion).

Furthermore, I find the authors' conclusions and inferences to be stimulating. They agree with fledgling ideas but the current results are leading the way in testing models using human multi-modal imaging.

The Methods often outstrip my statistical expertise but are written in a way that are reproducible and ‘open’ as to what has been performed.

I have minor issues, with my only real hesitations being that

1. The assumptions underlying the method-biology may require further justification, at least in the results sections,
2. In some instances it feels like a circular argument.
3. Changes to terminology are likely to assist the above; the flow of what is being undertaken is why is typically clear, but I suggest it will be less accessible to biologists, and thus the robustness of the results are/will be harder to judge. For some instances in Results, I was unsure what values of the data were being used, so I suggested some modifications to the text for reader understanding.

We respond to these three points below, where the reviewer has elaborated on them.

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(my internal dialogue is below to assist in identifying what I struggled to comprehend – when I read it, it makes sense but when I try to explain it I can't confidently). I’m sorry it is so long!

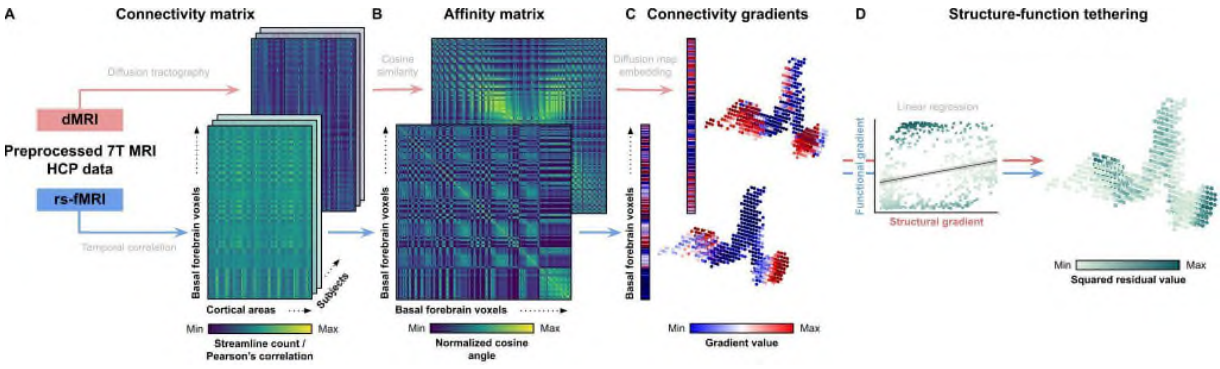
We value the reviewer's positive evaluation of our work. In the following, we detail the steps we have taken to improve the clarity of the analytical techniques.

1. PDF pg last paragraph of 6, top 7: For each 'modality'; does modality refer to each of spatial and functional 'projectome or connectome'? I associate modality with sMRI or fMRI

We understand the confusion and agree that the term 'modality' is more commonly used to distinguish different types of imaging acquisition techniques. To this end, we have removed all instances of the word "modality" and instead indicate the specific imaging dataset (i.e., dMRI or rsfMRI) that is being used in an analysis or retrospectively discussed. We have also removed the word "projectome" and replaced it with "basal forebrain cortical cholinergic innervation." For consistency, "connectome" always refers to the independent structural or functional associations (i.e. connectivity matrices) among basal forebrain subregions and their cortical targets. (line 143, 152-153, 258-259, 294, Supplemental Figure 4 legends, line 456: Fig.8 legend, line 275, 137)

2. 'Interregional similarity of features… eigenvectors of BF connectivity axes'. I'm trying to grasp what the data, once it has undertaken the computational gymnastics, represents: firstly it is not fully clear that the two matrices are being processed separately.

The dMRI and rsfMRI data undergo separate processing to extract the structural and functional connectivity matrices. The dMRI data are processed to reconstruct streamlines (indeed, representing axonal connections between regions of interest) via diffusion tractography, while rsfMRI data are analyzed for temporal correlation between BF voxels and cortical regions. In the original version of Supplemental Figure 2, which detailed our analytical workflow, we acknowledge that these separate analytical steps were not clearly differentiated. We have therefore substantially modified Supplemental Figure 2 to clarify the distinct processing paths for the dMRI and rsfMRI (snapshot of panels A-D below).




3. Then, do you mean you computed the similarity/non-similarity of [the strength of structural/diffusion (streamline) connections from/with the BF] in one matrix and [the correlational strength of (brain areas in functional networks)] in another? And from this, you generate the eigen vectors projected onto the BF (sup fig 2)? For sMRI this is a straightforward/reasonably direct measure (assuming streamlines = actual axons and thus the direction (of the final target, not of the start of axons). For fMRI the voxels that fire together BF and cortex are linked 'as the crow flies' in a similar way.

Once we established the structural and functional connectomes between BF voxels and cortical areas (Supplemental Figure 2A above), we first computed their respective BF gradients (i.e., eigenvectors) that demonstrate their principal axes of connectivity variability (i.e., topographies) among BF voxels (Supplemental Figure 2B-C). We then computed the similarity or 'tethering' using a linear regression analysis between the structural and functional connectivity BF gradients (Supplemental Figure 2D). Hence, the resulting (squared) residual values quantify the degree of tethering (lower residuals values = closer tethering).

The reviewer is right that the structural and functional connectomes represent two different aspects of BF connectivity (e.g., mono- vs. polysynaptic, respectively), and that their *in vivo* measurement relies on specific assumptions and challenges (see Discussion). Nonetheless, the depiction of structural and functional connectomes along a low-dimensional and continuous coordinate system has been demonstrated to be well suited for the comparison of essential, spatial-dependent features (Mars et al. 2021 Annu Rev Neurosci) derived from different, sometimes complex brain data.

4. Fig 2 abc: Then, because there is a lot of variability (in the strength or confidence?) in the computed directionality for each voxel, they were 'ranked' to represent a gradient of brain areas from strong-weak connections to/with the BF (and between BF voxels?) (sG1) and have strong-weak connections each other (and between locally close voxels)(fG1)?

I can't quite grasp that there is more than one gradient: are there just many alternative ways of ranking each connection matrix?

Core to calculation of the gradients is the computation of an affinity matrix (Supplemental Figure 2B) that captures inter-voxel similarity of their structural or functional connectivity, followed by the application of diffusion map embedding, a dimensionality reduction technique, to identify a gradual ordering of the affinity matrix in a lower dimensional space, i.e., the gradients. Figure 2A shows how well each of the resulting gradients explain the variance within the structural and functional connectomes. It is important to note that repeating this analysis will yield consistent gradient orderings. Given that the initial gradients accounted for a significant portion of the voxel-wise variance (approximately 30%), we concentrated on the first structural and functional gradients for further analysis. Moreover, Supplemental Figure 4 demonstrates the robustness of the structural-functional tethering pattern across various gradient combinations, ultimately converging on a single dominant profile. These text-based clarifications are part of Supplemental Figure 2 caption.

5. 30% variance: does 'variance' refer to how variable the eigen vector directions are in surrounding voxels and/or between subjects/people? (or something else altogether?) Note: typo in methods 'litter' for 'little'

Following our previous response, gradients help us simplify the complexity in the structural and functional connectomes by highlighting their most significant patterns across BF voxels based on the inter-voxel similarity. When we talk about the explained variance (or 'eigenvalues'), we are essentially referring to the 'importance' of these gradients. These text-based clarifications are part

of Supplemental Figure 2 caption. As for the typo, thank you for catching that, this has been corrected in the main manuscript now. (line 657)

6. 'Followed by a reduction in explained variance by 50% of the second gradient': does this mean the next gradient explained less than 15% of the variance? if so it is oddly put.

The reviewer's interpretation is accurate. The second gradient (component) explained less than 15%. As we recognize that this is phrased oddly, we have now revised it as follows:

*"The first gradient for both BF structural and functional connectivity data explained the most (30%) variance, with a drop to 15% explained variance for the second gradient (Fig. 2A)."*(line 150-151)

7. Pg 8 The use/introduction of the word tethering = magnitude of the share variance. Tethering evokes for me a boat or horse tied up. Essentially (I think) you computed the similarity/non-similarity of [the strength of structural/diffusion (streamline) connections ( or projected eigen vector) from/with the BF] with [the correlational strength of (brain areas in functional networks)] using sG1 X fG1. So does "tethering" represent a value of statistical or computational strength per voxel between structure measures/gradients and functional/gradients measures? with that value suggesting - assumed to be a representation of the strength of the computed relationship between the two?

Yes, the reviewer is correct in understanding our approach. Following the calculation of the gradients, we run a linear regression analysis between the first structural (derived from streamline counts) and functional gradients (derived from Pearson's correlation strength) to derive voxel-wise residual values (Supplemental Figure 2D). In this context, the residual values thus gauges how well a voxel's functional gradient value can be explained by its structural gradient value (i.e., share variance), referred to in the manuscript as 'tethering', and a lower residual value indicates a higher tethering. As such, the (squared) value indeed represents the strength of the computed relationship between the structural and functional gradient. These text-based clarifications are part of Supplemental Figure 2 caption.

Is this assumption justified? Shared variance following data gymnastics could represent associations that are not causal and might even be both functionally and structurally unrelated.

We concur with the reviewer's perspective regarding the limitations of inferring causality from shared variance, particularly following multiple data transformations. However, it is important to note that our primary aim was not to establish causal relationships. Rather, our analysis focused on two key objectives: firstly, evaluating the association ('tethering') between BF structural and functional connectivity patterns, guided by prior knowledge of BF white matter tracts and existing rs-fMRI studies. Secondly, we aimed to explore the validity of our arborization hypothesis, leveraging human PET and cross-validation mice PET and anterograde tracing data to elucidate the observed tethering differences.

8. Fig 3A Because I'm unclear as to what the residual measure is (is this the computed 'tethering' value) I worry that it could be circular to argue that because Ch2 projects to the

hippocampus/ entorhinal cortex which are in relatively close proximity and in function, but that the Ch4 projects to the entire cortex which is has a myriad of functions and more projection routes, that any 'variability' difference between the 2 nuclei is evidence of something more interesting as opposed to an interesting observation but biologically meaningless. This is a spot where the terminology could be changed/toned down – albeit the subsequent experiments support the conclusion, they also rely on the assumption that this observation is biological.

We hope that the improvements to Supplemental Figure 2 and the introduction have clarified the definition of the tethering value. We agree that we cannot infer any biological significance from the observed differences in the distributions of residuals captured by the Ch123 and Ch4 compartments of the basal forebrain, e.g, how differences in tethering may relate to cognitive functions of their cortical targets. Our aim in Figure 3 is to point out that there are differences in the concentration and spread of residuals depending on different a priori divisions of the basal forebrain and cortex. We have therefore switched from histogram plot in Figure 3A and 3B to a rug plot format. Now, each line in these plots represents the residual value captured by a BF voxel (Figure 3A) or the mean residual value captured by a cortical parcel (Figure 3B). Solid black lines represent the mean and dotted black lines represent 1 standard deviation. We believe the rug plot more accurately reflects the quantitative analyses of mean and CoV we performed on these regions and also better captures differences in the density and spread of residuals between different regions. (line 208 - 219)

9. Fig 3BC Pg 9 I would expect the tethering values to align with some predefined networks e.g. resting state, given they are derived at least partially from resting state MRI.

There are three main patterns that could emerge in Figure 3B. In pattern 1, the distributions of residuals in each of the 7 rug plots (corresponding to each of the 7 predefined cortico-cortical networks from Yeo et al.) would be very tight with little overlap with one another vertically moving from top to bottom rug plot. In this case, one could infer that the gradient of structure-function tethering follows closely with the borders of each cortico-cortical network, e.g. with lowest residuals concentrated all in the visual network and highest residuals concentrated in the ventral attention network. By contrast, in pattern 2, the distributions of residuals in each plot would be broad and highly overlapping vertically moving from top to bottom plots, indicating that the gradient of structure-function tethering is more or less evenly distributed within and across cortico-cortical networks. What we actually observe is a mixture of pattern 1 and pattern 2; there is differentiation at the extremes of the sensory-fugal cortical hierarchy, e.g. visual network versus ventral attention, yet there is still considerable overlap and spread in other networks. We believe the modified rug plots better convey this effect.

If you multiply X by Y and then divide by a factor of Y the result (Zi) is a multiple of X – if the networks are not based on Y ( actually the opposite (ie attention e.g. divide by a prime number)), the result of Zii might be quite unlike Zi; So of course the networks differ- am I missing something here? However, I would not predict that the uni-modal areas would be more similar than the multi-modal cortical areas – unless this happens to correlate with the structural eigen vectors (ie X). Can this be checked and clarified?

We hope that the modifications to the introduction and Supplemental Figure 2 help clarify the computation of the basal forebrain gradients and cortical gradient-weighted surfaces. It is true that because there is a strong spatial gradient of structure-function tethering observed in the basal forebrain, it follows that the cortical expression of these tethering values should also exhibit some spatial differentiation. The interesting observation is that the spatial profile of this differentiation tracks with the sensory-fugal cortical hierarchy and highlights core hubs of a well validated ventral attention network. These observations are non-trivial because the spatial profile of cortical structure-function tethering correlates with the spatial profile of cortical VAChT.

11. Glad to see the cross-validation, but didn't follow how it was performed

In Supplemental Figures 3 and 4 (now included!), we detail how the cross-validations were conducted for both the stability of the gradients and tethering across individuals (Supplemental Figure 3) and for the stability of the spatial pattern of tethering across different gradient pairs (Supplemental Figure 4).

11. FEOVB: great way to test some of the conclusions/hypotheses from the previous results and be specific for cholinergic not just basal forebrain. However Vchat could be lower/higher for functional reasons not related to structure – so nice to use the mouse neurons to directly link these findings together and, to some extent, the consistency with your prior assumptions partly alleviates my hesitancy in conclusions evident in my previous points.

We agree with the reviewer that, by itself, the *in vivo* [$^{18}$F]FEOBV PET data in humans does not necessarily indicate more structural connections. For instance, it could be the case that in areas of higher binding concentration, presynaptic cholinergic terminals express more VAChT per terminal, as opposed to more terminals overall. However, the mouse data provide evidence that this alternate explanation is unlikely.

12. Concerning the wiring cost reasoning – is the initial matrix for sMRI based on actual length or only directionality? If only directionality then mixing in actual length is an independent variable, if not it's a bit circular again.

The initial structural connectivity matrix is constructed based on streamline counts, as detailed in Fig.6A, Supplemental Table 2 and Supplemental Figure 2A. This matrix records the number of streamlines reaching each HCP-MMP cortical parcel (columns) from individual BF voxels (rows). Consequently, the consideration of wiring cost, which involves actual fiber length, remains independent of the structural connectivity derived from streamline counts.

13. How different are the salience and the attentional networks?

This is a point of ongoing debate in the field. The ventral attention and salience networks have distinct and overlapping cortical hubs: The key regions of the salience network include the anterior insula and the anterior/mid cingulate cortex. The ventral attention network also involves the anterior insula and anterior./mid cingulate cortex, but extends to include right temporoparietal junction (TPJ), the inferior frontal gyrus (IFG), and the middle frontal gyrus (MFG). Uddin et al 2019 argue that the mid-cingulate and insular hubs of these networks form a unique core set

which they refer to as the midcingulo-insular (M-CIN) network. In our study, the areas we find to exhibit the highest disagreement between basal forebrain structure and functional connectivity (lowest tethering) are in the M-CIN. However, the TPJ and some frontal areas associated with the VAN also exhibit relatively low tethering. We elected to use the boundaries of the VAN in Figure 8 to illustrate these overlaps (they are projected as white boundaries on the cortical surface), and the particular concentration in the more anterior hubs.

14. Can you conclude "further translational evidence that BF cholinergic neurons exhibit an arborization gradient which is shaped by the function 'and' physical distance of their cortical targets." Or would 'and/or' be more accurate?

The reviewer makes a good point. We cannot distinguish whether cortical function and distance are two distinct additive properties. We have modified the text from 'and' to 'and/or'. (line 379)

15. I found the discussion illuminating and well-reasoned (assuming my issues were unfounded)

We thank the reviewer for their positive assessment of the discussion!

**Reviewer #3 (Remarks to the Author):**

This study provides a comprehensive examination of the cholinergic innervation of the cortex, originating from the basal forebrain (BF). Through the use of high-resolution 7T diffusion and resting-state functional MRI in humans, the authors investigate the multimodal gradients of BF cholinergic connectivity with the cortex, elucidating a complex structural and functional relationship. Notably, the study identifies a gradient of reduced tethering between structural and functional connectivity, and further demonstrates that cortical areas with higher concentrations of cholinergic innervation exhibit lower tethering, suggesting patterns of diffuse axonal arborization. The authors extend their findings to a rodent model, providing a cross-species replication that underscores the generality of their observations.

The methodology and statistical approaches employed are sound, reflecting a rigorous and multidimensional exploration of the subject matter. I appreciate the substantial amount of work behind this study, which considers the topic from multiple angles and presents replication analyses where necessary. However, I have one comment and a request before I can recommend this for publication:

In reviewing the methodology, particularly the structural connectivity reconstruction technique, I find it noteworthy and innovative. The challenges of conducting seed-to-cortex tractography from subcortical areas like the BF are known, given that most connections terminate near the seed region, and only a few pathlines continue, often reflecting the brain's geometry more than the true topography of white matter pathways, influenced significantly by the partial volume effect. To ensure a thorough understanding of what we are observing, I kindly request an addition to your manuscript: an image similar to those depicting fiber length and wiring cost, specifically detailing the total number of streamlines received from the BF for each cortical parcel. This seems to be partially addressed in Supplemental Fig 1C, however, the scale is absent. Providing this would greatly enhance the clarity and completeness of your findings, ensuring the observed connectivity patterns accurately reflect the underlying anatomical architecture. I would also suggest considering the inclusion of such a figure in the supplementary materials to further increase the persuasiveness of your methodology.

We thank the reviewer for the positive comment. We have added an image of streamline counts similar to fiber length and wiring cost (Fig. 6A) and have also added an Excel file (Supplemental Table 2) detailing the total number of streamlines received from the BF (all voxels) for each cortical parcel (Left and Right combined) to the GitHub repository here.

Reviewer #3 (Remarks on code availability):

After a brief review of the code repository associated with this publication, I can confirm the inclusion of a succinct README file, as well as the provision of input data, source Python scripts, and the output dataset. The README file offers clear instructions for setting up and running the application, contributing significantly to the usability of the code for the wider community. From my assessment, these resources are well-organized and should indeed facilitate replication efforts considerably.

We thank the reviewer for looking into our github repository. We have updated the repository with the Tables and Figures requested by the reviewers.

Reviewer #1 (Remarks to the Author):

Nat Communications Review
Chakraborty et al. Multimodal gradients of human basal forebrain connectivity

Overall Summary:

Chakraborty et al. present a META DATA analysis that re-evaluates the relationship between the structural organization of the basal forebrain cholinergic system largely based on previously published data, and without clearly acknowledging the original data sources.

Specifically, this includes
(a) a meta -analysis of resting state MRI and diffusion MRI data from the human connectome project,
(b) a re-analysis of [18F]FEOBV PET data from several previous studies* and
(c) a re-evaluation of mouse anterograde tracing data, also from a previously published tracing study.
There does appear to one new addition of data – these are from a newly acquired mouse [18F]FEOBV microPE dataset.

* are the human data presented from other studies properly coded, acknowledged?

Outcome of Re-Review:
The revisions have NOT at all addressed the essential problem previously identified by the reviewers. The work is NOT presented in a transparent manner, at all (see below).

The manuscript is written in a manner that the reader would not know that – in fact- the data resources used in this study largely derive from other prior publications and databases: i.e. it is essentially a META DATA analysis.
This reviewer had to search through the methods section to find any acknowledgement of the data from others that are the major basis for this report. Although the manuscript may provide a thoughtful evaluation of the structure-function relationship of the cholinergic system and an innovative way to combine previously published datasets for a different perspective on the organization and heterogeneity of the cholinergic system across species, it is not presented in sufficiently transparent manner. The reader can easily miss the important fact that the current paper is in fact a meta-data analysis.
In addition, the mouse anterograde tracing dataset used does not equivalently assess all BF cholinergic nuclei and, as such, underestimates the number X branch complexity/arborization of cortical regions. As such, the interpretation of the analyses using this dataset, is challenging.

Major issues:

1. Insufficient Transparency throughout the presentation:

The authors must be more transparent about the sources of datasets used for the meta data analysis that they have presented in this manuscript. Specifically, they must distinguish which subset of the data are their own new data as opposed to the bulk of the data presented that are from previously published datasets /contributions.

Although the combination of these particular datasets in a single analyses is novel, the study does not properly acknowledge that it is largely a meta-data analysis. Specifically the authors must clarify the original sources of the data and refer to the published reports from which they derive. More precisely,
- The title should include the proper descriptor that the paper is largely a meta-data analysis. ( eg “A meta-data analysis supporting multimodal gradients of human basal forebrain connectivity”)
- The abstract must be explicit in the role of primarily meta data analysis in this paper . It would be best if the abstract referred to the specific previously published/acquired datasets that were used.
- The introduction should state that the current study is a meta-analysis of multiple studies in both humans and mouse and acknowledge all of the studies from which they have drawn data.
- At each point where the data is first introduced in the results section and with each figure, in the legend, there must be a citation to the dataset(s) used and a link and/or reference included. In addition, for each figure that includes any previously published data there should be a reference to supplemental table 1 with all additional details and links.

- Although Supplemental Table 1 is a valuable new addition to the manuscript, it is lacking in detail and is buried in reference to its content ( in addition to being in a Supplemental Table – unlikely to gain the proper acknowledgement for the work of the many authors and references that were used) In terms of detail of supplemental Table 1:. Under the “reference” column, a full reference should be given rather than just an abbreviated reference. Wherein possible, a link to the dataset should be provided. An additional column should be added to include the figure numbers in the current manuscript that use the specific datasets in the analysis. These additions are critical to aid in reproducibility of the results and must be reported.

- At each point at the beginning of the methods section for each dataset, please mention and reference the manuscripts from which the datasets were pulled. This is clear in some cases but not others.
- Methods are missing for the data that were pulled from Li et al. 2017 (mouse anterograde tracing dataset). A section of the methods should exist for each bit of data that is included and analyzed in the manuscript. This is important for reproducibility. Please include a reference to the manuscript where the dataset was acquired and include details as how the data was selected for use in the current meta data analysis presented in this manuscript.
o Minor Point: The methods heading “Human Data Acquisition” should be revised to “Human MRI Data” to parallel the “Human FEOBV PET” section later

2. Potentially deceptive phrasing.
We provide just one of many, many examples of the potentially deceptive nature of the wording used throughout this manuscript:
--- line 521, “To mitigate this issue, we used optimized MRI protocols to acquire high spatial resolution dMRI (1.05 mm3) and rsfMRI (1.6 mm3) at 7T” should be revised to “To mitigate this issue, we analyzed datasets that were acquired…” to appropriately acknowledge the source. These changes should be made throughout the manuscript.

In brief, -- the authors must carefully read through the manuscript and correct all such statements that may lead to confusion as to the original source of the data used.

3. Incomplete Mouse Tracing Dataset used for meta data analysis without acknowledgment of limitations:
The human MRI and PET datasets and the mouse microPET datasets all include analysis that evaluate all basal forebrain cholinergic nuclei. These datasets also offer an unbiased estimation of the VAChT density of the cortical regions (coming from all projecting populations).
As previously mentioned, the mouse anterograde tracing dataset that was acquired in Li et al. 2017 primarily targets anteriorly positioned cholinergic neurons (MS/DB). While it is known that this cluster of neurons does have some cortical projections, they do not project as widely to cortex as posterior and laterally located cholinergic neurons. Both unimodal and transmodal regions that are being evaluated in this study receive innervation from these posteriorly located neurons. An evaluation of only anteriorly located cholinergic neurons vastly underestimates any assessment of the number of neurons, their branch complexity, or their extent of arborization to any cortical region.
This incomplete evaluation of cholinergic neurons that innervate cortex impacts the analysis and interpretation of the structure to function relationship of the cholinergic system.
The brief mention in line 554-557 of a need for larger samples with greater coverage is not sufficient to describe the impact of this limitation. The discussion on branch complexity beginning at line 520 focuses heavily on the NBM which is not assessed in the current study or the source dataset and is misleading.
The authors should consider removing these analyses from the manuscript as the comparisons are not equivalent. Alternatively, authors must make it far more explicit that only a subpopulation of cholinergic neurons that project to these regions are being evaluated and as such the estimation of number X branch complexity/arborization is an underestimation and is incomplete.

Reviewer #2 (Remarks to the Author):

What are the noteworthy results?
As previously stated, the topic is of considerable interest – cholinergic basal forebrain connectivity remains uncertain. Only several studies have examined it in detail since single-cell tracing and whole-brain reconstruction techniques became available in mice. It is of particular significance since acetylcholine release plays important roles in local cortical network integration for both input and output of cortical information, with demonstrated roles in attention, decision-making, learning, and memory – particularly all higher-order brain computations. The loss of cholinergic basal forebrain function underpins the loss of cognitive function resulting in, at least contributing to, dementia.
The combination of human and mouse studies for the validation of certain conclusions is particularly innovative and significant.
I also find the way the authors have proceeded through this study is logical and the experimental design is imaginative. The background is clear and the authors clearly state the predicted outcomes of the assessments as they relate to the hypotheses to be tested, which are in
turn based on evidence/references, including for the assumptions in the underlying methods ( e.g.
diffusion MRI = streamlines = white matter = axonal connections between regions of interest),

albeit with varying degrees of caveats ( as per the Discussion).
The revision has further clarified the terminology and rationale and data sources used.

Will the work be of significance to the field and related fields? How does it compare to the established literature?
Yes - it is field-leading. Conclusions are robust and the less certain nuances will be teased out with future work by others (and the authors) as the field develops additional methods. Several different analyses help to answer any of my hesitations.

Is the methodology sound? Does the work meet the expected standards in your field?
Is there enough detail in the methods for the work to be reproduced?
Yes. The clarity for non-experts has improved significantly. Understanding the methods still requires certain assumptions (and advanced statistical knowledge) to independently 'reinterpret' the data and thus concur fully with the conclusions. However, these are discussed in the limitations section, are justified, or the conclusions have been tempered appropriately. The author's conclusions are readily accessible for nonexperts in the methods to appreciate.

Reviewer #3 (Remarks to the Author):

Dear authors, thank you for including the additional figure and the table as requested. Based on the information presented in the new figure, my reservations about the technical validity of the method have been addressed. I now have confidence in the robustness of your methodology and am pleased to recommend the manuscript for publication.

**Response to REVIEWERS' COMMENTS**

We thank the Reviewers for their helpful feedback. Our responses to the remaining comments are in blue fonts below:

Reviewer #1 (Remarks to the Author):

*Chakraborty et al. present a META DATA analysis that re-evaluates the relationship between the structural organization of the basal forebrain cholinergic system largely based on previously published data, and without clearly acknowledging the original data sources.*

*Specifically, this includes*

A. *A meta -analysis of resting state MRI and diffusion MRI data from the human connectome project*
B. *A re-analysis of [18F]FEOBV PET data from several previous studies* and*
C. *A re-evaluation of mouse anterograde tracing data, also from a previously published tracing study.*

*There does appear to one new addition of data – these are from a newly acquired mouse [18F]FEOBV microPE dataset.*

** are the human data presented from other studies properly coded, acknowledged?*

*Outcome of Re-Review:*

*The revisions have NOT at all addressed the essential problem previously identified by the reviewers. The work is NOT presented in a transparent manner, at all (see below).*

*The manuscript is written in a manner that the reader would not know that – in fact- the data resources used in this study largely derive from other prior publications and databases: i.e. it is essentially a META DATA analysis.*

We thank the reviewer again for the feedback on our manuscript. While we appreciate the request to emphasize the “meta-analytic” aspect of our set of analyses, we would like to stress the fact that our study goes beyond a typical meta-[data] analysis which would involve the statistical combination of results from two or more separate studies. While we combine data from published and open-source datasets, a significant portion of our work involves novel analyses of source data from these datasets in ways that provide new insights into the organization and function of the basal forebrain.

*This reviewer had to search through the methods section to find any acknowledgement of the data from others that are the major basis for this report. Although the manuscript may provide a thoughtful evaluation of the structure-function relationship of the cholinergic system and an innovative way to combine previously published datasets for a different perspective on the organization and heterogeneity of the cholinergic system across species, it is not presented in sufficiently transparent manner. The reader can easily miss the important fact that the current paper is in fact a meta-data analysis.*

To acknowledge the different data sources in a more transparent manner, we have moved the Supplemental Table 1 to the main text, which is now labeled as Table 1. As outlined below per section, several other textual changes have been implemented as well to improve transparency.

*In addition, the mouse anterograde tracing dataset used does not equivalently assess all BF cholinergic nuclei and, as such, underestimates the number X branch complexity/arborization of cortical regions. As such, the interpretation of the analyses using this dataset, is challenging.*

In the human data, we observe a continuous gradient of structure-function tethering that traverses the anteromedial and posterolateral subregions of the BF. The anteromedial nuclei thus represent a component within this continuum, not an exception to it. Another way to think about this is as follows: in humans, the structure-function tethering restricted to the anteromedial MS/DBB still captures a gradient of residuals (see Figure 3A). Similarly, the Li et al. dataset, where viral tracing is restricted to cholinergic projections emanating from MS/DBB, captures a subset of this gradient, as evidenced by the variance in branch counts. The main novel point here is that there is a gradient in structure-function tethering in BF connectivity (human), and this gradient may reflect cholinergic branch complexity (mouse). We acknowledge that the upper bound of this branch complexity in mice remains to be determined, and would likely be reflected by cholinergic neurons in NbM. Per the reviewer's suggestion, we have further highlighted this point in the Results by adding this sentence: "*Note that the upper bound of this branch count likely reflects an underestimate[2-4], given that cholinergic neurons originating in the NbM were not labeled in this study and the technique for quantifying the terminal fields was conducted at mesoscopic resolution.*" (Lines 316-319)

*Major issues:*

*1. Insufficient Transparency throughout the presentation:*

*The authors must be more transparent about the sources of datasets used for the meta data analysis that they have presented in this manuscript. Specifically, they must distinguish which subset of the data are their own new data as opposed to the bulk of the data presented that are from previously published datasets /contributions.*

*Although the combination of these particular datasets in a single analyses is novel, the study does not properly acknowledge that it is largely a meta-data analysis. Specifically the authors must clarify the original sources of the data and refer to the published reports from which they derive.*

*More precisely,*

- *The title should include the proper descriptor that the paper is largely a meta-data analysis. (eg "A meta-data analysis supporting multimodal gradients of human basal forebrain connectivity")*
- *The abstract must be explicit in the role of primarily meta data analysis in this paper . It would be best if the abstract referred to the specific previously published/acquired datasets that were used.*
- *The introduction should state that the current study is a meta-analysis of multiple studies in both humans and mouse and acknowledge all of the studies from which they have drawn data.*

In line with our aforementioned reasoning concerning our manuscript being considered a “meta [data] analysis”, and as per editorial requests, we have kept the manuscript title, abstract and introduction as they were.

- *At each point where the data is first introduced in the results section and with each figure, in the legend, there must be a citation to the dataset(s) used and a link and/or reference included. In addition, for each figure that includes any previously published data there should be a reference to supplemental table 1 with all additional details and links.*

As requested by the reviewer, we have modified the Results and Methods section to facilitate transparency of the origin of data:

- We have moved Supplemental Table 1 to the main text article (now referenced Table 1)
- Per the editor’s suggestions:
    - Line 476 “Human Data Acquisition” has been rephrased to “Human MRI Datasets”.
    - Line 562 has “dataset” appended to it.
    - Line 618 has the word “acquisition” appended to it.
    - We reference (i) the relevant sources/manuscripts and (ii) Table 1 when a public dataset is first mentioned in the results section as well as in the figure captions.

- *Although Supplemental Table 1 is a valuable new addition to the manuscript, it is lacking in detail and is buried in reference to its content ( in addition to being in a Supplemental Table –unlikely to gain the proper acknowledgement for the work of the many authors and references that were used) In terms of detail of supplemental Table 1:. Under the “reference” column, a full reference should be given rather than just an abbreviated reference. Wherein possible, a link to the dataset should be provided. An additional column should be added to include the figure numbers in the current manuscript that use the specific datasets in the analysis. These additions are critical to aid in reproducibility of the results and must be reported.*

We have moved Supplemental Table 1 to the main text (reference as Table 1) and have modified it to include the additional details requested by the reviewer, namely: direct hyperlinks to the papers from which data were drawn and explicit listing of the figures in the current paper where these data were used for analysis.

- *At each point at the beginning of the methods section for each dataset, please mention and reference the manuscripts from which the datasets were pulled. This is clear in some cases but not others.*

We have ensured that each subsection of the Methods mentions the source dataset used.

- *Methods are missing for the data that were pulled from Li et al. 2017 (mouse anterograde tracing dataset). A section of the methods should exist for each bit of data that is included and analyzed in the manuscript. This is important for reproducibility. Please include a reference to the manuscript where the dataset was acquired and include details as how the data was selected for use in the current meta data analysis presented in this manuscript.*

We have added in a subsection to the Methods describing the data used from Li et al and how it was leveraged in the current paper. This can be found under the section header "*Mouse viral tracing dataset*"(Line 577) as follows:

"*Viral tracing of mouse cholinergic BF neurons is described in-depth by Li et al[2] . Briefly, a Cre dependent adeno-associated virus (AAV) expressing FLEX-GFP under the CAG promoter (Serotype 9;UNC Gene Therapy Center Vector Core, Chapel Hill, NC) was injected into the Diagonal Band of Broca of Chat-ires-Cre mice to label cholinergic neurons. Whole-brain tissue was imaged using fMOST and the 3D data was aligned to the Allen Mouse Brain Atlas[55]. Finally, reconstruction of 50 cholinergic Diagonal Band of Broca neurons was performed, and the axon-targeted areas were determined using the atlas-based region labels. The branch counts and Allen Mouse Brain Atlas annotations for areas targeted by each branch are provided by Li et al[2] in their Supplemental Appendix Figure 7.*

*To investigate the relationship between cholinergic branch complexity and the cortical area each branch targets, we first sorted the Allen Mouse Brain Atlas annotations for the 50 labeled cholinergic Diagonal Band of Broca neurons according to whether they targeted unimodal sensory cortical and subcortical regions (group 1) or transmodal cortical areas (group 2). To assess the statistical significance of the mean difference in branch counts between the two groups, a permutation test with 10k iterations was conducted by randomly shuffling the combined data and recalculating the mean difference between the two groups for each permutation. The p-value was then computed as the proportion of permuted mean differences that were equal to or exceeded the observed mean difference in absolute value.*

*For the distributions of branch counts derived from (i) the 50 labeled cholinergic Diagonal Band of Broca neurons in mice and (ii) the residuals encoding tethering between BF structural and functional connectivity in humans, we divided each dataset into three equal parts (tertiles) based on a fixed-interval approach that uses the maximum value of the dataset as a reference point. This strategy groups the range of data in each distribution into lower, middle, and upper tertiles, where the size of each bin depends on the distribution of the data.*

*To assess the similarity between the distributions of branch counts derived from the 50 labeled cholinergic Diagonal Band of Broca neurons in mice and the residuals encoding tethering between BF structural and functional connectivity in humans, we first z-scored each dataset. To ensure both datasets had the same length, the human dataset was interpolated and downsampled from 599 to 50 points using linear interpolation. This procedure involved generating an interpolation vector that evenly spanned the original index range of the dataset, and then estimating the values at these new points, effectively reducing the dataset's size while preserving its overall shape and trends. We then performed a Spearman's rank correlation analysis to evaluate the monotonic relationship between the two datasets. A permutation test with 10k iterations was conducted to assess the statistical significance of the observed correlation by randomly shuffling the data and recalculating the correlation coefficient for each permutation.*" (Lines 578-617)

*o Minor Point: The methods heading "Human Data Acquisition" should be revised to "Human MRI Data" to parallel the "Human FEOBV PET" section later*

We have revised the section headers to "Human MRI Datasets"(Line 476) and "Human FEOBV PET Dataset" (Line 562). See editor's comments.

*2. Potentially deceptive phrasing.*

*We provide just one of many, many examples of the potentially deceptive nature of the wording used throughout this manuscript:*

- *Line 521, "To mitigate this issue, we used optimized MRI protocols to acquire high spatial resolution dMRI (1.05 mm3) and rsfMRI (1.6 mm3) at 7T" should be revised to "To mitigate this issue, we analyzed datasets that were acquired…" to appropriately acknowledge the source. These changes should be made throughout the manuscript.*

*In brief, the authors must carefully read through the manuscript and correct all such statements that may lead to confusion as to the original source of the data used.*

We agree with the reviewer and have changed the respective sentence as suggested. We have gone through the manuscript to correct all such statements.

*3. Incomplete Mouse Tracing Dataset used for meta data analysis without acknowledgment of limitations:*

*The human MRI and PET datasets and the mouse microPET datasets all include analysis that evaluate all basal forebrain cholinergic nuclei. These datasets also offer an unbiased estimation of the VAChT density of the cortical regions (coming from all projecting populations).*

*As previously mentioned, the mouse anterograde tracing dataset that was acquired in Li et al. 2017 primarily targets anteriorly positioned cholinergic neurons (MS/DB). While it is known that this cluster of neurons does have some cortical projections, they do not project as widely to cortex as posterior and laterally located cholinergic neurons. Both unimodal and transmodal regions that are being evaluated in this study receive innervation from these posteriorly located neurons. An evaluation of only anteriorly located cholinergic neurons vastly underestimates any assessment of the number of neurons, their branch complexity, or their extent of arborization to any cortical region.*

*This incomplete evaluation of cholinergic neurons that innervate cortex impacts the analysis and interpretation of the structure to function relationship of the cholinergic system.*

*The brief mention in line 554-557 of a need for larger samples with greater coverage is not sufficient to describe the impact of this limitation. The discussion on branch complexity beginning at line 520 focuses heavily on the NBM which is not assessed in the current study or the source dataset and is misleading.*

*The authors should consider removing these analyses from the manuscript as the comparisons are not equivalent. Alternatively, authors must make it far more explicit that only a subpopulation of cholinergic neurons that project to these regions are being evaluated and as such the estimation of number X branch complexity/arborization is an underestimation and is incomplete.*

See previous response above. The main novel point in this paper is that there is a gradient in structure-function tethering in BF connectivity (human), and this gradient may reflect cholinergic branch complexity (mouse). We acknowledge that the upper bound of this branch complexity in mice remains to be determined. We have further highlighted this point in the Results by adding this sentence: "*Note that the upper bound of this branch count likely reflects an underestimate[4], given that cholinergic neurons originating in the NbM were not labeled in this study and the*

*technique for quantifying the terminal fields was conducted at mesoscopic resolution.*" (Lines 316-319) We think this set of analyses should serve to motivate further work in this area as this intriguing pattern has not been documented previously.

Reviewer #2 (Remarks to the Author):

*What are the noteworthy results?*

*As previously stated, the topic is of considerable interest – cholinergic basal forebrain connectivity remains uncertain. Only several studies have examined it in detail since single-cell tracing and whole-brain reconstruction techniques became available in mice. It is of particular significance since acetylcholine release plays important roles in local cortical network integration for both input and output of cortical information, with demonstrated roles in attention, decision-making, learning, and memory – particularly all higher-order brain computations. The loss of cholinergic basal forebrain function underpins the loss of cognitive function resulting in, at least contributing to, dementia.*

*The combination of human and mouse studies for the validation of certain conclusions is particularly innovative and significant.*

*I also find the way the authors have proceeded through this study is logical and the experimental design is imaginative. The background is clear and the authors clearly state the predicted outcomes of the assessments as they relate to the hypotheses to be tested, which are in turn based on evidence/references, including for the assumptions in the underlying methods (e.g. diffusion MRI = streamlines = white matter = axonal connections between regions of interest), albeit with varying degrees of caveats ( as per the Discussion). The revision has further clarified the terminology and rationale and data sources used.*

*Will the work be of significance to the field and related fields? How does it compare to the established literature?*

*Yes - it is field-leading. Conclusions are robust and the less certain nuances will be teased out with future work by others (and the authors) as the field develops additional methods. Several different analyses help to answer any of my hesitations.*

*Is the methodology sound? Does the work meet the expected standards in your field?*

*Is there enough detail in the methods for the work to be reproduced?*

*Yes. The clarity for non-experts has improved significantly. Understanding the methods still requires certain assumptions (and advanced statistical knowledge) to independently 'reinterpret' the data and thus concur fully with the conclusions. However, these are discussed in the limitations section, are justified, or the conclusions have been tempered appropriately. The author's conclusions are readily accessible for nonexperts in the methods to appreciate.*

Thank you for your valuable input and time invested while reviewing our work. We are grateful for your recognition of the study's significance.

Reviewer #3 (Remarks to the Author):

*Dear authors, thank you for including the additional figure and the table as requested. Based on the information presented in the new figure, my reservations about the technical validity of the method have been addressed. I now have confidence in the robustness of your methodology and am pleased to recommend the manuscript for publication.*

Thank you for your thorough review and for giving us the opportunity to address your concerns. We are pleased that the additional figure and table we provided have successfully addressed your reservations about our methodology. Your recommendation for publication is greatly appreciated, and we are excited to contribute this work to the field.