nature portfolio

Peer Review File

Lithium Deficiency and the Onset of Alzheimer's Disease

Corresponding Author: Dr Bruce Yankner

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this manuscript, Aron et al. examine the role of lithium in the etiology and progression of Alzheimer's disease. Using a combination of human post-mortem brain samples and transgenic mouse models, the authors find that lithium bioavailability is decreased in individuals with Mild Cognitive Impairment or Alzheimer's disease; that lithium deficiency exacerbates clinical manifestations of Alzheimer's, likely by overactive GSK3-beta; and that supplementation of lithium salts reduces pathology and cognitive impairments in mouse model.
The authors present a significant contribution to the field by demonstrating the role of physiological lithium levels in Alzheimer's disease progression at the cellular, pathological and cognitive levels. Lithium at pharmacological doses has been used to treat bipolar disorder, but its effects on AD-related pathologies and cognitive function have yielded mixed results. GSK3-beta has previously been identified as a lithium target, which is upregulated in AD, contributes to tau phosphorylation, neurofibrillary tangles and amyloid plaques. GSK3-beta inhibition has been shown to play a role in slowing progression in AD clinically and in mouse models. Building on this, this study uniquely demonstrates that lithium depletion triggers multicellular effects in the brain in AD and aging, with GSK3-beta activity driving these changes.
Of particular importance, the authors identify a novel organic lithium salt, lithium orotate, which unlike the clinical standard lithium carbonate, is not sequestered by Amyloid beta. This distinction is crucial, as it enhances lithium's therapeutic potential in AD. Impressively, the study shows that submicromolar doses of lithium orotate can both prevent and reverse AD pathology, neuroinflammation, and cognitive decline in AD mouse models and aged mice, presenting an exciting advancement in the treatment of neurodegenerative disease. Together, this is a very impressive set of findings, and highlights novel pathways for therapeutic intervention. Nonetheless, the authors should provide additional insight on the mechanistic link of dysregulated GSK3-beta activity in different cell types (excitatory neurons, microglia, oligodendrocytes) that potentiates AD pathogenesis.

Points to be addressed:

1. The finding that TCF3 target genes are dysregulated by Lithium deficiency should be clarified in the text. The manuscript states that "TCF transcription factors are activated by beta-catenin in the nucleus." However, this statement is slightly misleading. The canonical TCF transcription factors activated by beta-catenin are TCF7, TCF7L1, and TCF7L2. TCF7L1 (UniProt ID Q9HCS4) was previously named TCF3, but TCF3 now refers to "Transcription Factor 3" (UniProt ID P15923), which is not related to or regulated by beta-catenin signaling. If the authors are referring to the beta-catenin dependent transcription factor, they should update the text to read "TCF7L1" to avoid confusion. If the authors are referring to TCF3, they should show that this transcription factor is indeed regulated by beta-catenin.
2. The sample sizes of three n per group in some experiments (Figure 1, h, and I; Figure 4e) are not large enough to allow for well-powered statistical comparisons. Can the authors increase their sample sizes? It is hard to interpret these results with such few replicates.
3. In Figure 2, the authors test the effects of lithium deficiency on cognition in both 3xTg mice and wildtype mice. Why are the same analyses not applied to both groups? For example, the 3xTg mice are analyzed for latency to target area, but the wildtype mice are analyzed for time in target area. What is the justification for different analyses on presumably the experiment (Morris Water Maze)? When applying the same analyses to both groups, what are the results (what was the latency to target area for the WT mice, and what was the %time in target area for the 3xTg mice?)
4. In Figure 3-4 the authors display the GO terms for the DE expressed genes in the Li-deficient versus control mice and list relevant DE gene names in the text. However, can the authors show a volcano plot or a heatmap of relevant DE genes in the main figure or extended figures?
5. Could the authors include visualizations for their transcription factor prediction results in a main figure instead of listing the

results in the supplemental tables? It would be helpful to display the TFs (TCF3, TCF4 and TCF12) and the enriched DEGs targeted by those TFs in a cell type specific manner (excitatory neurons vs. oligodendrocyte vs. microglia) in the Li-deficient versus control mice.
6. The rationale to investigate myelination in the context of lithium deficiency is sound, however the authors need to expand on their evaluation of myelin integrity and effects on oligodendrocytes in the mouse brains. Could the authors please perform transmission electron microscopy to evaluate ultrastructural soundness of the myelin sheath, and use immunohistochemistry to detect myelin basic protein and oligodendrocyte marker.
7. The authors demonstrate that GSK3-beta and its active auto-phosphorylated state are increased, and beta-catenin is decreased in Li deficient mice (Extended Figure 5). However, they only show colocalization of beta-catenin in Iba1 cells but make the claim that GSK3-beta activity is altered in oligodendrocytes and neurons as well. Can the authors co-stain for the above proteins with a mature oligodendrocyte marker and neuronal marker?
8. The authors demonstrate how lithium deficiency affects the transcriptomes of oligodendrocytes, microglia, and excitatory neurons, as well as the related functional consequences. Their TF target analysis shows that that this occurs through GSK3-beta activity in each cell type independently, but they do not address how dysregulation of GSK3-beta activity in these cell types may work jointly to potentiate AD pathogenesis. Could the authors please provide more mechanistic insight into how Li deficiency through GSK3-beta induces functional deficits in oligodendrocytes, microglia and neurons?
9. The authors demonstrate the effects GSK3-beta inhibition and LiO supplementation on microglia, astrocytes, pathological markers of AD (p-Tau and Amyloid plaques) and synaptic density. Can the authors assess for myelin integrity after these treatments? Additionally, can the authors conduct RNA-seq after LiO and LiC treatment to better understand the molecular changes in response to these two treatments, which different effects in the brain?
10. Throughout the manuscript, representative images are presented very small and appear to have low resolution, making it difficult to appreciate the observed effects. Can the authors try to increase the size and resolution of these images?

Referee #2

(Remarks to the Author)
Ms 2024-09-18526

A. Yankner et al report that endogenous lithium protects against memory loss in the presence of Alzheimer (AD)-type pathology and during aging.
They describe that submicromolar levels of endogenous Li preserve cognitive function, reduce inflammation, and suppress Aβ generation in aging wild-type mice.
They observe that the transcriptome of Li deficiency overlapped with that of AD by comparison to a recent snRNA-seq study of cortical biopsy samples from living individuals with a range of AD pathology.
They present data that endogenous lithium contributes to synapse maintenance in the aging mouse brain, and that deficiency activates microglia and impairs Aβ clearance.

They reported that microglia from Li-deficient WT mice showed significantly elevated release of the proinflammatory cytokines and chemokines. Li deficiency alters the microglial transcriptome, leading to a reactive proinflammatory state with an impaired ability to clear Aβ. Li deficiency induces GSK3β expression. Lithium deficiency alters microglial function and augments the accumulation of Aβ and phospho-tau through activation of GSK3β.
They claim that lithium orotate, effectively bypasses Aβ sequestration, compared to the pharmacologically-available lithium carbonate. This is argued to be due to its ionization properties.
They describe that Li deficiency results in loss of synaptic integrity, a pathological change that appears early in AD. They report that submicromolar levels of endogenous Li preserve cognitive function, reduce inflammation, and suppress Aβ generation in aging wild-type mice. Remarkably, the decline in learning and memory associated with aging was largely reversed by LiO.
Administration of LiO to 3xTg mice from 9-18 months of age significantly reduced Aβ plaque burden and the density of phospho-tau-positive NFT-like structures in the hippocampus, while LiC had no significant effect at the same dose. LiO also reduced Aβ plaque burden by ~70% in older J20 mice. So, they argue that LiO is highly effective in reducing Aβ deposition and phospho-tau accumulation.

I found this to be an intriguing, comprehensive and well conducted project, which introduces a new mechanism for the pathogenesis of AD, as well as an important new potential therapeutic for this incurable disorder.

Lithium has not really been regarded a biological (yet alone neurobiological) salt of any physiological importance. Curiously, the late Avi Friedlich noted that Li levels were increased in the brains of untreated bipolar patients (doi:10.1038/mp.2011.90), which is a clue to a biological role that has been unappreciated (and might deserve to be cited).

B. This is highly original data and ideas.

D-H.
I have the following concerns:

1. Introduction: Zn, Cu, Fe are referred to as “heavy metals”, but that is not accurate. These could be referred to as “transition metals” or “biometals” (since they participate in biological processes. Heavy metals are Pb, Hg etc
2. The authors mention that Zn,Cu and Fe can promote Abeta aggregation. They cite modern references, but it would be appropriate to cite the original report: PMID: 8073293

3. Para 3 “We performed an unbiased analysis of metals in the brains of”. It would be better to say that they did the analysis of frontal cortex i.e. specify the region analysed.
4. RESULTS, Para 1: “Of all the metals surveyed, only one metal, lithium (Li), was significantly altered”. This is inaccurate. It was the brain:serum RATIO that was altered.
5. Supp Table 1 only reports % changes in metal levels. The actual means +/- error data should be reported so that we can judge how close each reported value is to the Limit of Quantification (LoQ). Also, the concentrations of Li in the brain and serum are of interest and should be reported since these have not been well studied, and would be a valuable dataset.
6. The authors use the term “Uptake” when, I think, they are referring to brain:serum metal concentration ratios. This is colloquial since so little is understood about the dynamics of the metal influx and efflux at the BBB for each of the several metals assayed. But it might be okay to use “Uptake” if it is defined somewhere. I prefer brain:serum ratio, so that it is clear. This also applies to the titles in Supp Fig. 1.
7. Page 4: “The statistical significance for the other metal changes in AD was considerably less than that of Li (Fig. 1b).” I don't think it is valid to compare the dimension of significances. This seems to be a strange way to present the surveyed data. It would be better to present a Cohen's d of the effect size of the change in brain:serum ratio, and then highlight the significantly altered values. An effect size survey could easily be computed and could replace if not complement the graph of the p values. Or, even better, maybe create a mini-volcano plot. The p values alone don't give us a comparative estimation of the dimension of changes that occur for each metal. Comparing the P values does not give information about the effect size or the size of the departure in the brain:serum ratios. It is better to compare effect sizes, or present a mini volcano plot for the reader to judge the prominence of the changes in metal levels.
8. Fig 1 f. For the ICP-MS methods, it is essential that the level of detection (LoD) and LoQ be presented for each metal assayed. We need to know whether any data points are outside of the LoQ.
9. Fig 1f. It would make it clearer to the reader if it were mentioned in the legend that the metal measurements were taken from an unfixed section adjacent to the plaque-imaged section (if I’ve understood correctly).
10. Page 5: “Li in the non-plaque fraction was inversely correlated with plaque density in AD samples as indicated by CERAD score (Supplementary Table 2)”.
11. Suppl Table 2: The legend needs to specify that the analysis is of Li in the non-plaque brain homogenate fraction. It is currently labelled “Cortical Li”, which could be interpreted as Li in the total homogenate.
12. The data in Fig 1 and elsewhere are interpreted as Li being sequestered by Abeta. However, this is never directly shown i.e. that synthetic Abeta binds Li. This is a big omission in the basis of the argument. It should be easy enough e.g. with equilibrium dialysis, to demonstrate that Li saturably binds to Abeta42, and determine whether the affinity is biologically compatible. This would enable a direct comparison of the affinity of LiO vs LiC, to test the conjecture that LiC allows more Li to bind to Abeta. As the data now lie, the Li might be trapped by the myriad other non-Abeta components of amyloid plaques. We can’t be certain that Abeta itself is trapping Li.
13. Page 6: “This resulted in a significant elevation of cortical and hippocampal Aβ42, the major pathogenic Aβ species in AD, and a trend toward an increase in Aβ40 (Fig. 2c and Extended Data Fig. 2e).” I can't find the methods for the ELISA of endogenous mouse Abeta40 or42. These are notoriously difficult to assay. What species were detected? x-40, x-42? What was the LoQ?
14. Page 13, second para. Please clarify that the 4.3 µEq/L is in drinking water. What was the background concentration of Li in the water?
15. Page 13, Fig. 5a. The conductivity data were achieved at only one, supraphysiological (430 uM), concentration. The relationship between conductivity and compound concentrations is frequently non-linear, and indeed often inverted U shaped. So we have no idea whether these conductivity comparisons are relevant in the physiological, nM- uM, range. Since these data were the premise for electing LiO as the species for non-Abeta interaction, the premise could be flawed. The conductivity curves over a broad range of Li compound concentrations are needed to feel confident that the premise for choosing LiO is correct. Such data should be quite easy to achieve. Reporting the relative conductivities at a much lower concentration would be reassuring that the premise is correct, and that there are not better Li-compound choices in the list tested.
16. Extended Data 1c.: The human serum concs are reported but the mouse values are not-- just relative to the WT mean. The authors need to have the actual concentrations reported eg in Supp Fig 5.
17. Extended Data Fig. 10a. Data should be reported as actual concentrations rather than fold.
18. Page 15: “…LiO prevents age-related synapse loss and cognitive decline in mice without evidence of renal or thyroid toxicity typically associated with Li treatment at pharmacologic doses.” IN all fairness, you need to say LiO “at this dose”, because if it were used as a treatment for bipolar disorder for example, at concentration in the serum in the low mM range, it may indeed cause toxicity. We don’t know that it is safer toxicologically.
19. Page 18. Last para: “Li deficiency provides a link between Aβ accumulation and…” Not just Abeta accumulation but also tau-hyperphsophorylation.
20. Methods, Isolation of plaque-enriched and non-plaque fractions: I found the following unclear- “The samples were passed through the sieve by gravity and then centrifuged (300xg, 30 mins).”, It would be clearer to state, “The samples were passed through the sieve by gravity and the filtrate then centrifuged (300xg, 30 mins).”.
21. Methods, Isolation of plaque-enriched and non-plaque fractions: “The pellet was resuspended in 5 volumes of water”. How did the authors measure the volume of the pellet?
22. Methods, Isolation of plaque-enriched and non-plaque fractions: “10 µl of the freshly collected plaque-enriched and non-plaque fractions were layered onto albumin-coated glass slides and allowed to dry overnight. They were then washed with PBS”. Was the concentration of Li in the PBS checked as a potential source of contamination?
23.

Minor:
1. Methods, Inductively-coupled plasma mass spectrometry (ICP-MS): “Finally, consistent with previous studies, we observed significantly … reduced copper33 levels, in the AD frontal cortex”. The earliest reference for decreased copper

levels in AD frontal cortex was PMID: 22080049 DOI: 10.1016/j.freeradbiomed.2011.10.446. Also Page 16: Discussion, 2 nd para. The original report of lowered Cu in the brain in AD was not ref 33 but the same.
2. Page 24: Triton X-100 is termed Tritonx 100
3. Page 37. Microglial Aβ uptake and degradation assays. Human Abeta 1-42 was studied. But through the text, the authors refer to Abeta42, which I gather is 1-42, but they never define this. ABeta42 could refer to x-42. Important to be precise to leave no ambiguity.

I congratulate the authors on a highly original and important study.
Signed
Ashley I Bush, MD PhD

Referee #3

(Remarks to the Author)
In this manuscript, the authors present a comprehensive study on the potential role of lithium deficiency in the development of Alzheimer's disease (AD) with robust evidence. The findings are intriguing and suggest a novel approach to AD treatment. The study includes: (1) a systematic metallomic analysis of 27 abundant and trace metals across two cohorts, (2) subproteome and spatial analyses highlighting lithium enrichment/sequestration by plaques, (3) studies of how lithium deficiency exacerbates AD pathology and cognitive deficits in J20 and 3xTg mouse models, (4) multi-omics studies single-cell RNA sequencing and proteomic analyses, (5) functional studies exploring the roles of synapses, microglia, and GSK3β in lithium deficiency, and (6) the selection and testing of a plaque-evading lithium salt (LiO), examining its impact on AD-related pathologies, cognition, and age-related inflammation and cognitive decline. This comprehensive and solid work effectively links lithium deficiency to AD pathogenesis and proposes a promising treatment strategy. It is a highly significant discovery. However, there are some concerns that need addressing:
1. As Lithium treatment has been used in psychiatric medication, and Lithium treatment has been explored as a potential therapeutic approach for AD. Clinical trials in AD have shown mixed results. Could metallomic profiling help categorize MCI and AD into subtypes with minor, moderate, or severe lithium deficiency? Are there any biomarkers of lithium deficiency in plasma or CSF that could clarify these clinical inconsistencies and enhance trial design in the future?
2. The manuscript lacks in vitro strong biochemical data supporting the Li-Aβ interaction hypothesis. Data on different lithium salts binding to Aβ monomers/polymers would strengthen this hypothesis.
3. Are there specific lithium-binding biomolecules (e.g., proteins, lipids, etc.) in the brain that are concentrated in plaque areas?
4. The omics analysis, while routine, lacks advanced network analysis and prioritization of key protein/gene hubs or drivers. he identification of central lithium targets remains unclear, although the extensive data provided hold the potential to fully characterize lithium pathways.
5. It is crucial to detail the coverage and depth of omics studies in the main text, including the number of transcripts and proteins analyzed. For instance, only about 3,400 proteins are listed in supplemental table 7, which seems low.
6. The manuscript provides only processed omics data (e.g., fold changes and p-values). A detailed description of quality control, data normalization, statistical analysis, and cutoff determination is necessary for reproducibility and transparency.
7. The individual case/animal data from omics studies are not provided, limiting the opportunity for independent re-analysis.
8. The MS raw data from omics analyses are not released.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
In our initial review of this manuscript, we appreciated the novel insights Aron et al provided, but we requested additional lines of evidence to support their main claims, increased sample sizes, and more insight into potential downstream mechanisms. In their revised manuscript, the authors have now provided detailed responses to each of our points, and have satisfactorily addressed all of our original concerns. We have no further comments, and would recommend this article for publication.

Referee #2

(Remarks to the Author)
A-H I have commented in depth in my previous review, and my enthusiasm remains high for this work.
Ms 2024-09-18526A

The revised manuscript by Yankner et al is greatly improved, and they have added additional data that has greatly strengthened their conclusions.

I still have some minor concerns:

1. In the results and supplementary tables they refer in several places to Li (and other metal) “uptake” into brain tissue. I

know that I mentioned this in the first review, and they have provided a definition for “uptake”. But, “uptake” has a very specific meaning, and they implying it inaccurately by measuring brain:serum ratios. They really have not measured uptake, but rather steady state levels, which may reflect changes in efflux (that they cannot exclude) as well as true uptake. A more precise term than uptake would be “retention”.

2. They comment that “The statistical significance of the other metal changes in AD was considerably less than that of Li (Fig. 1b).”. Comparing levels of significance is not a valid way of illustrating the magnitude of any change. A result is or is not significant. The fold change is the only valid comparator. The volcano plots are welcome extra figures, and convey much of that information, but my point is that you really can’t compare size of p values. That not how p values work. The metal with the biggest change is Lead, which is way down in AD vs NCI. Not that I’m suggesting pursuing this. I think that the pursuit of Li was justified because it was the only significant change in the MCI v NCI comparison and because, unlike lead, it has pharmacological connotations.

3. Fig 5-- The legend states, “b,c, Lithium binds to Aβ42 fibrils (b) and oligomers (c) in the physiological concentration range.” Do you mean the lithium is in the physiological range? According to Supp Table 1, the normal concentration of Li in the brain is about 330 nM. The EC50 for fibrils is 3.7 uM (Supp Table 13) and for Abeta Oligomers is 35 uM (Supp Table 13). So, binding would be MINIMAL at physiological concentrations. It would only be possibly meaningful at pharmacological concentrations. So, you can't say that Li binds to Abeta fibrils and oligomers in the "physiological Li concentration range". Maybe it does in the PHARMACOLOGICAL concentration range. Throughout the text the reference is to Li supplementation in the PHYSIOLOGICAL range, But this is misleading. There is no known physiological function for Li. So you must be careful about these statements. Ideally, and maybe necessarily for a paper in Nature, you would measure the Li concentration in the brains of animals treated with LiO to ensure that the concentrations of LiO-treated animals are within the range that comports to the IC50’s that you now report.

4. Page 16: “LiO at a physiological dose prevents…” I accept the attempt to revise this concern, but in all fairness, you cannot refer to LiO at a “physiological dose”. We do not know whether LiO exists physiologically.

5. “Li deficiency also promotes phospho-tau accumulation, inflammation, and the loss of synapses, axons and myelin. The progression of this neurodegenerative process may be modulated by genetic risk variants, many of which are in Li-regulated genes, as well as environmental factors and dietary Li intake. Li deficiency is therefore a potential common mechanism for the multisystem degeneration of the brain that leads to onset of AD.”
This is a very brave statement. To my knowledge, lithium has not yet been demonstrated to have ANY physiological or biochemical role in normal metabolism. Name ONE authentically Li-regulated gene product?? This statement needs to be carefully titrated back to facts that are defended by prior literature. Even more abundant metal ions like Cu+ are only just being accepted as having signalling roles. The authors are trying to introduce Li as a regulatory metal ion, but there are not enough data to support this idea yet, although it is a noble conjecture. It needs to be more qualified and its speculative nature made explicit.

Referee #3

(Remarks to the Author)
All of my concerns have been fully addressed through additional experiments, inclusion of experimental details, and implementation of quality control in the omics analysis. The manuscript is acceptable for publication. Congratulations to the authors!

Response to Referees

We thank the referees for careful review of the manuscript. We have addressed the issues raised with additional experimental data and analysis. The response to each reviewer concern is described below.

Referee #1

1. The finding that TCF3 target genes are dysregulated by Lithium deficiency should be clarified in the text. The manuscript states that "TCF transcription factors are activated by beta-catenin in the nucleus." However, this statement is slightly misleading. The canonical TCF transcription factors activated by beta-catenin are TCF7, TCF7L1, and TCF7L2. TCF7L1 (UniProt ID Q9HCS4) was previously named TCF3, but TCF3 now refers to "Transcription Factor 3" (UniProt ID P15923), which is not related to or regulated by beta-catenin signaling. If the authors are referring to the beta-catenin dependent transcription factor, they should update the text to read "TCF7L1" to avoid confusion. If the authors are referring to TCF3, they should show that this transcription factor is indeed regulated by beta-catenin.

We thank the reviewer for this observation. When the ENCODE ChIP-seq database was established, TCF3 was the gene name for the β catenin-responsive TF now known as TCF7L1, but the annotation subsequently changed. Given this ambiguity, we have elected to instead use Ingenuity/IPA gene network analysis, which is focused on identifying signaling pathways from gene expression data and is up to date and well annotated. Ingenuity/IPA pathway analysis of DEGs from Li-deficient microglia showed that Wnt/β-catenin signaling was the most significantly enriched signal transduction pathway and is predicted to be downregulated (Extended Data Fig. 4a,b). In addition, Ingenuity/IPA analysis of single nucleus RNA-seq data from excitatory neurons and oligodendrocytes also showed significant enrichment for Wnt/β-catenin signaling with predicted downregulation of the pathway (Extended Data Fig. 4c). We have followed the reviewer's advice and now show the data as visualized network diagrams with examples of targeted DEGs in the figure rather than just in a table. Importantly, the informatic prediction that Wnt/β-catenin signaling is downregulated by Li deficiency was confirmed in multiple cell types by *in situ* labeling for nuclear β-catenin and activated GSK3β, as well as by treating mice and cultured microglia with GSK3β inhibitors.

2. The sample sizes of three n per group in some experiments (Figure 1, h, and I; Figure 4e) are not large enough to allow for well-powered statistical comparisons. Can the authors increase their sample sizes? It is hard to interpret these results with such few replicates.

The sample sizes have been increased in Figures. 1h and 1i. Regarding Figure 4e, the same experiment was replicated in the context of testing GSK3β inhibitors in Figure 4g.

3. In Figure 2, the authors test the effects of lithium deficiency on cognition in both 3xTg mice and wildtype mice. Why are the same analyses not applied to both groups? For example, the 3xTg mice are analyzed for latency to target area, but the wildtype mice are analyzed for time in target area. What is the justification for different analyses on presumably the experiment (Morris Water Maze)? When applying the same analyses to both groups, what are the results (what was the latency to target area for the WT mice, and what was the %time in target area for the 3xTg mice?)

The same analyses are now shown for each group – the conclusions are unchanged.

4. In Figure 3-4 the authors display the GO terms for the DE expressed genes in the Li-deficient versus control mice and list relevant DE gene names in the text. However, can the authors show a volcano plot or a heatmap of relevant DE genes in the main figure or extended figures?

A heatmap of the relevant DEGs is now shown in Fig. 3d. We prefer a heatmap to a volcano plot in this instance because the heatmap concisely shows differential gene expression across the cell types, which would otherwise require multiple volcano plots.

5. Could the authors include visualizations for their transcription factor prediction results in a main figure instead of listing the results in the supplemental tables? It would be helpful to display the TFs (TCF3, TCF4 and TCF12) and the enriched DEGs targeted by those TFs in a cell type specific manner (excitatory neurons vs. oligodendrocyte vs. microglia) in the Li-deficient versus control mice.

Please see the response to point 1. We now show Ingenuity/IPA network analysis implicating Wnt/GSK3β/β-catenin signaling, together with targeted DEGs, in microglia, neurons and oligodendrocytes in Li-deficient versus control mice (Extended Data Fig. 4).

6. The rationale to investigate myelination in the context of lithium deficiency is sound, however the authors need to expand on their evaluation of myelin integrity and effects on oligodendrocytes in the mouse brains. Could the authors please perform transmission electron microscopy to evaluate ultrastructural soundness of the myelin sheath, and use immunohistochemistry to detect myelin basic protein and oligodendrocyte marker.

Transmission electron microscopy has been performed and shows that Li deficiency results in significant loss of myelin structural integrity (Extended Data Fig. 3e). We have also performed immunolabeling for myelin basic protein (MBP) which showed significantly reduced MBP in lithium-deficient 3xTg mice and its restoration following pharmacological inhibition of GSK3β (Extended Data Fig. 6e). In addition, there is a significant reduction in aspartoacylase-positive oligodendrocytes in lithium-deficient mice (Fig. 3j and Extended Data Fig. 3c), which is restored by treating the mice with a GSK3β inhibitor (Extended Data Fig. 6e). Furthermore, we provide evidence that lithium deficiency also results in a significant reduction in oligodendrocyte progenitor cells, as indicated by immunolabeling for PDGFRα (Extended Data Fig. 3d). Taken together with the previous fluoromyelin labeling and snRNA-seq (Fig. 3c,i), these findings provide compelling evidence that lithium deficiency disrupts oligodendrocyte function and myelin integrity.

7. The authors demonstrate that GSK3-beta and its active auto-phosphorylated state are increased, and beta-catenin is decreased in Li deficient mice (Extended Figure 5). However, they only show colocalization of beta-catenin in Iba1 cells but make the claim that GSK3-beta activity is altered in oligodendrocytes and neurons as well. Can the authors co-stain for the above proteins with a mature oligodendrocyte marker and neuronal marker?

We now show elevated total GSK3β and decreased nuclear β-catenin in neurons by co-labeling for the neuronal marker MAP2, and in oligodendrocytes by co-labeling for the oligodendrocyte marker aspartoacylase (Extended Data Fig. 5). The previous data showed elevated total GSK3β and decreased nuclear β-catenin in microglia by co-labeling for the microglial marker Iba1 (Fig. 4f and Extended Data Fig. 5d). In addition, the proteomic analysis confirmed elevated total GSK3β protein and qRT-PCR shows elevated GSK3β mRNA in Li-deficient hippocampus. For pTyr216-GSK3β, we now show double

labeling with cell type specific markers for neurons and oligodendroctyes. The double-labeling was technically difficult in microglia due to the species availability of compatible double-labeling antibodies. However, we show that Li deficiency significantly elevates total GSK3β (Fig. 4f) and reduces nuclear β-catenin in Li-deficient microglia (Extended Data Fig. 5d). Moreover, Wnt/β-catenin signaling is among the most strongly predicted signaling pathways in the RNA-seq data from Li-deficient microglia (Extended Data Fig. 4a,b). Together, these results suggest that lithium deficiency activates GSK3β across multiple cell types.

8. The authors demonstrate how lithium deficiency affects the transcriptomes of oligodendrocytes, microglia, and excitatory neurons, as well as the related functional consequences. Their TF target analysis shows that that this occurs through GSK3-beta activity in each cell type independently, but they do not address how dysregulation of GSK3-beta activity in these cell types may work jointly to potentiate AD pathogenesis. Could the authors please provide more mechanistic insight into how Li deficiency through GSK3-beta induces functional deficits in oligodendrocytes, microglia and neurons?

We now provide additional discussion of how Li deficiency and GSK3β activation in the different cell types may contribute to the pathogenesis of AD (p. 18, 1st paragraph). Specifically, we describe how GSK3β activation from Li deficiency contributes to neurofibrillary tau pathology in neurons, Aβ deposition through impaired microglial Aβ clearance, proinflammatory microglial activation, and impaired oligodendrocyte function resulting in reduced myelination. We also discuss two primary downstream mechanisms that may give rise to the various cellular consequences – direct phosphorylation of tau and activation of the β-catenin signaling pathway, which we demonstrate in Li-deficient neurons, microglia and oligodendrocytes. We anticipate that additional downstream mechanisms will be involved, as the effects of Li deficiency are widespread. For this reason, we qualify our conclusion by stating that Li deficiency is mediated, at least in part, by GSK3β activation.

9. The authors demonstrate the effects GSK3-beta inhibition and LiO supplementation on microglia, astrocytes, pathological markers of AD (p-Tau and Amyloid plaques) and synaptic density. Can the authors assess for myelin integrity after these treatments? Additionally, can the authors conduct RNA-seq after LiO and LiC treatment to better understand the molecular changes in response to these two treatments, which different effects in the brain?

The positive effects of LiO supplementation on myelin integrity and oligodendrocytes, as indicated by MBP and aspartoacylase immunoreactivity, respectively, is now shown (Extended Data Fig. 6e).

We also now provide RNA-seq analysis of the hippocampus in 3XTg mice treated with lithium orotate (Fig. 5f, Supplementary Table 15). This analysis shows that many of the GO terms enriched in the LiO treatment DEGs are similar to those enriched in the Li deficiency DEGs, but with opposite directionality of change. Interestingly, LiO downregulates genes associated with neurodegeneration, β-catenin degradation and inflammation, and upregulates genes associated with synaptic function, memory and learning (Fig. 5f, Supplementary Table 15).

A pilot RNA-seq analysis of low-dose lithium carbonate treatment in 3xTg mice showed very few DEGs relative to control that crossed the statistical threshold (FDR < 0.05). We therefore did not pursue this further.

10. Throughout the manuscript, representative images are presented very small and appear to have low resolution, making it difficult to appreciate the observed effects. Can the authors try to increase the size and resolution of these images?

Image size in the main figures is somewhat constrained by space limitations. We have now moved some of the images to supplementary data where they can be presented in a larger format, and have increased image size in the main figures. The resolution of all the images now conforms to the Nature guidelines.

Referee #2 (Remarks to the Author):

Ms 2024-09-18526

A. Yankner et al report that endogenous lithium protects against memory loss in the presence of Alzheimer (AD)-type pathology and during aging.
They describe that submicromolar levels of endogenous Li preserve cognitive function, reduce inflammation, and suppress Aβ generation in aging wild-type mice.
They observe that the transcriptome of Li deficiency overlapped with that of AD by comparison to a recent snRNA-seq study of cortical biopsy samples from living individuals with a range of AD pathology.
They present data that endogenous lithium contributes to synapse maintenance in the aging mouse brain, and that deficiency activates microglia and impairs Aβ clearance.

They reported that microglia from Li-deficient WT mice showed significantly elevated release of the proinflammatory cytokines and chemokines. Li deficiency alters the microglial transcriptome, leading to a reactive proinflammatory state with an impaired ability to clear Aβ. Li deficiency induces GSK3β expression. Lithium deficiency alters microglial function and augments the accumulation of Aβ and phospho-tau through activation of GSK3β.
They claim that lithium orotate, effectively bypasses Aβ sequestration, compared to the pharmacologically-available lithium carbonate. This is argued to be due to its ionization properties.
They describe that Li deficiency results in loss of synaptic integrity, a pathological change that appears early in AD. They report that submicromolar levels of endogenous Li preserve cognitive function, reduce inflammation, and suppress Aβ generation in aging wild-type mice. Remarkably, the decline in learning and memory associated with aging was largely reversed by LiO.
Administration of LiO to 3xTg mice from 9-18 months of age significantly reduced Aβ plaque burden and the density of phospho-tau-positive NFT-like structures in the hippocampus, while LiC had no significant effect at the same dose. LiO also reduced Aβ plaque burden by ~70% in older J20 mice. So, they argue that LiO is highly effective in reducing Aβ deposition and phospho-tau accumulation.

I found this to be an intriguing, comprehensive and well conducted project, which introduces a new mechanism for the pathogenesis of AD, as well as an important new potential therapeutic for this incurable disorder.

Lithium has not really been regarded a biological (yet alone neurobiological) salt of any physiological

importance. Curiously, the late Avi Friedlich noted that Li levels were increased in the brains of untreated bipolar patients (doi:10.1038/mp.2011.90), which is a clue to a biological role that has been unappreciated (and might deserve to be cited).

We thank the reviewer for the encouraging comments and historical perspective. We have an ongoing project on bipolar disorder and will discuss Avi Friedlich's findings in that paper. It should be noted, however, that these findings were in a letter to the editor that does not appear to have been peer reviewed. Thus far, our preliminary studies do not support an increase in Li levels in untreated bipolar patients.

B. This is highly original data and ideas.

D-H.
I have the following concerns:

1. Introduction: Zn, Cu, Fe are referred to as "heavy metals", but that is not accurate. These could be referred to as "transition metals" or "biometals" (since they participate in biological processes. Heavy metals are Pb, Hg etc

We have changed the reference to just "metals".

2. The authors mention that Zn,Cu and Fe can promote Abeta aggregation. They cite modern references, but it would be appropriate to cite the original report: PMID: 8073293

This was an oversight. The original report is now cited.

3. Para 3 "We performed an unbiased analysis of metals in the brains of". It would be better to say that they did the analysis of frontal cortex i.e. specify the region analysed.

To be more specific, we have changed the sentence to indicate that the analysis was performed on prefrontal cortex, cerebellum (a relatively unaffected region in AD) and serum.

4. RESULTS, Para 1: "Of all the metals surveyed, only one metal, lithium (Li), was significantly altered". This is inaccurate. It was the brain:serum RATIO that was altered.

We have now amended this section as follows: "Metal uptake into the brain, determined as the ratio of brain to serum concentrations in the same individuals, was determined in prefrontal cortex (PFC), a prominently affected region in AD, and cerebellum, a relatively unaffected region. Of all the metals surveyed, only one metal, lithium (Li), showed significantly altered uptake in the PFC of individuals with early-stage memory loss associated with MCI, as well as more advanced progression to AD (Fig. 1a,b and Supplementary Table 1)." This new text provides more explicit information about the measurements.

5. Supp Table 1 only reports % changes in metal levels. The actual means +/- error data should be reported so that we can judge how close each reported value is to the Limit of Quantification (LoQ). Also, the concentrations of Li in the brain and serum are of interest and should be reported since these have not been well studied, and would be a valuable dataset.

The means +/- error metal levels, as well as LOQ and LOD, are now reported for all metals (Supplementary Table 1). The concentrations of Li in the brain and serum are shown in Figure 1d,e and

Supplementary Table 1 (for prefrontal cortex), Extended Data Fig. 1b (for cerebellum) and Extended Data Figure 1c and Supplementary Table 1 for serum.

6. The authors use the term “Uptake” when, I think, they are referring to brain:serum metal concentration ratios. This is colloquial since so little is understood about the dynamics of the metal influx and efflux at the BBB for each of the several metals assayed. But it might be okay to use “Uptake” if it is defined somewhere. I prefer brain:serum ratio, so that it is clear. This also applies to the titles in Supp Fig. 1.

Please see the response to point 4 above. “Uptake” is now defined clearly at the beginning of the Results section, and the measurements are described in the Methods.

7. Page 4: “The statistical significance for the other metal changes in AD was considerably less than that of Li (Fig. 1b).” I don't think it is valid to compare the dimension of significances. This seems to be a strange way to present the surveyed data. It would be better to present a Cohen's d of the effect size of the change in brain:serum ratio, and then highlight the significantly altered values. An effect size survey could easily be computed and could replace if not complement the graph of the p values. Or, even better, maybe create a mini-volcano plot. The p values alone don't give us a comparative estimation of the dimension of changes that occur for each metal. Comparing the P values does not give information about the effect size or the size of the departure in the brain:serum ratios. It is better to compare effect sizes, or present a mini volcano plot for the reader to judge the prominence of the changes in metal levels.

We have followed the reviewer’s suggestion and now present volcano plots of the metal changes in MCI and AD in Figs 1a,b. The volcano plots clearly show the unique position of lithium. Although the volcano plots convey more experimental information than before, many of the non-significantly changed metals cluster close to the axes and are difficult to label. However, Supplementary Table 1, which is associated with Fig. 1ab, provides all the information on every metal (means +/- error of metal levels, percent change and directionality in MCI and AD, adjusted p-value, LOD and LOQ).

8. Fig 1 f. For the ICP-MS methods, it is essential that the level of detection (LoD) and LoQ be presented for each metal assayed. We need to know whether any data points are outside of the LoQ.

The LOD and LOQ values for each metal assayed are now presented in Supplementary Table 1. For all metal measurements, the means are above the LOQ.

9. Fig 1f. It would make it clearer to the reader if it were mentioned in the legend that the metal measurements were taken from an unfixed section adjacent to the plaque-imaged section (if I’ve understood correctly).

The reviewer does understand the approach correctly and this is a good suggestion for clarity. We have now indicated the adjacent section approach in the Fig. 1f legend as well as in detail in the Methods section

10. Page 5: “Li in the non-plaque fraction was inversely correlated with plaque density in AD samples as indicated by CERAD score (Supplementary Table 2)”.

There may be some confusion because the CERAD score is an inverse measure of plaque burden and the correlation in the table is positive. We have removed this sentence to avoid any ambiguity.

11. Suppl Table 2: The legend needs to specify that the analysis is of Li in the non-plaque brain homogenate fraction. It is currently labelled “Cortical Li”, which could be interpreted as Li in the total homogenate.

This is an important clarification - the legend has now been modified.

12. The data in Fig 1 and elsewhere are interpreted as Li being sequestered by Abeta. However, this is never directly shown i.e. that synthetic Abeta binds Li. This is a big omission in the basis of the argument. It should be easy enough e.g. with equilibrium dialysis, to demonstrate that Li saturably binds to Abeta42, and determine whether the affinity is biologically compatible. This would enable a direct comparison of the affinity of LiO vs LiC, to test the conjecture that LiC allows more Li to bind to Abeta. As the data now lie, the Li might be trapped by the myriad other non-Abeta components of amyloid plaques. We can’t be certain that Abeta itself is trapping Li.

We thank the reviewer for this constructive suggestion. We performed equilibrium dialysis binding assays on synthetic Aβ42 fibrils and oligomers formed *in vitro*. This analysis demonstrates that Li binds saturably to fibrillar and oligomeric Aβ (Fig. 5b,c and Extended Data Fig. 7b). Notably, Li binds to Aβ fibrils and oligomers in the physiological Li concentration range, as shown in Fig. 5b right panel for fibrils and Fig. 5c for oligomers. Moreover, LiC exhibits higher affinity for Aβ than LiO across a broad Li concentration range (Fig. 5bc, Extended Data Fig. 7b; EC50 values with 95% confidence intervals in Supplementary Table 13). These new findings are consistent with the conductivity measurements and the *in vivo* Aβ-Li sequestration data. It is worth noting that the binding of Li to oligomers as well as fibrils suggests that depletion of bioavailable Li in the AD brain is likely to be even higher than the estimate based on plaque sequestration.

Binding assays to Aβ monomers were complicated by the rapid formation of oligomeric forms during the assay incubation in the presence or absence of Li, precluding a clear result.

13. Page 6: “This resulted in a significant elevation of cortical and hippocampal Aβ42, the major pathogenic Aβ species in AD, and a trend toward an increase in Aβ40 (Fig. 2c and Extended Data Fig. 2e).” I can't find the methods for the ELISA of endogenous mouse Abeta40 or42. These are notoriously difficult to assay. What species were detected? x-40, x-42? What was the LoQ?

More detailed protocol information for the mouse Aβ ELISA assays has now been added to the Methods section and the corresponding figure legends. The protocol for extracting endogenous Aβ40 and Aβ42, as well as the selection of ELISA kits, was based on the recommendations of Teich et al. (2013), “*A Reliable Way to Detect Endogenous Murine β-Amyloid*” (PLoS One). In that study, the authors evaluated several kits and identified the Human/Rat (Mouse) Aβ42 High Sensitivity ELISA Kit (Wako, 292-64501) and the Human/Rat (Mouse) Aβ40 ELISA Kit (Wako, 294-62501) as having the highest signal-to-noise ratio for detecting endogenous mouse Aβ, with minimal background signal when tested on APP knockout tissue. Accordingly, we used these two Wako kits and followed the protocol described by Teich et al. to quantify endogenous Aβ40 and Aβ42 species in mouse brain samples. As indicated by the manufacturer, these kits detect x-42 and x-40 Aβ species in mice. The limit of quantification (LOQ) was 4.75 pmol/L for Aβ42 and 7.44 pmol/L for Aβ40. All sample measurements (Aβ42: 11.72–22.40 pmol/L; Aβ40: 26.21–52.98 pmol/L) were above the LOQ.

14. Page 13, second para. Please clarify that the 4.3 µEq/L is in drinking water. What was the background concentration of Li in the water?

Lithium salts were dissolved in distilled, deionized water used as drinking water. The background lithium concentration in this water was minimal (0.109 µg/L), especially in comparison to the experimental concentration of 4.3 µEq Li/L (~30 µg/L). We have revised the sentence on page 13, second paragraph, and noted the background Li concentration in the Methods section.

15. Page 13, Fig. 5a. The conductivity data were achieved at only one, supraphysiological (430 uM), concentration. The relationship between conductivity and compound concentrations is frequently non-linear, and indeed often inverted U shaped. So we have no idea whether these conductivity comparisons are relevant in the physiological, nM- uM, range. Since these data were the premise for electing LiO as the species for non-Abeta interaction, the premise could be flawed. The conductivity curves over a broad range of Li compound concentrations are needed to feel confident that the premise for choosing LiO is correct. Such data should be quite easy to achieve. Reporting the relative conductivities at a much lower concentration would be reassuring that the premise is correct, and that there are not better Li-compound choices in the list tested.

We now provide a complete set of conductivity measurements over a broad concentration range (Fig 5a, Extended Data Fig. 7a). As the reviewer suggested, we also report conductivity measurements at a much lower concentration (Li 21.5 µEq/L), which is the lowest concentration at which conductivity measurements were reliable above the LOQ using the most sensitive available electrode. This new data confirms the difference between organic and inorganic lithium salts over a broad concentration range and supports the choice of LiO for further evaluation. Moreover, the predictive value of this screen is supported by the Aβ binding analysis (Fig. 5b,c) and the relative efficacy of LiO versus LiC in vivo in AD mouse models (Extended Data Fig. 7c-f).

16. Extended Data 1c.: The human serum concs are reported but the mouse values are not-- just relative to the WT mean. The authors need to have the actual concentrations reported eg in Supp Fig 5.

The serum and brain Li concentrations are now reported in ng/ml and ng/g, respectively.

17. Extended Data Fig. 10a. Data should be reported as actual concentrations rather than fold.

The cortical and serum Li concentrations in Extended Data Fig. 10a are now reported in ng/g and ng/ml, respectively.

18. Page 15: "…LiO prevents age-related synapse loss and cognitive decline in mice without evidence of renal or thyroid toxicity typically associated with Li treatment at pharmacologic doses." IN all fairness, you need to say LiO "at this dose", because if it were used as a treatment for bipolar disorder for example, at concentration in the serum in the low mM range, it may indeed cause toxicity. We don't know that it is safer toxicologically.

The reviewer is correct and the sentence has been modified to "LiO at a physiological dose prevents age-related proinflammatory changes, synapse loss and cognitive decline in mice without evidence of renal or thyroid toxicity typically associated with Li treatment at higher pharmacologic doses" (p. 16, 1st paragraph, last line). What is remarkable to us is the therapeutic potency of LiO in the AD mouse models at a Li dose equivalent ~1,000-fold lower than that clinically used in humans.

19. Page 18. Last para: “Li deficiency provides a link between Aβ accumulation and…” Not just Abeta accumulation but also tau-hyperphosphorylation.

This has been revised to more broadly encompass the multisystem effects of Li as follows: “Li deficiency also promotes phospho-tau accumulation, inflammation, and the loss of synapses, axons and myelin. The progression of this neurodegenerative process may be modulated by genetic risk variants, many of which are in Li-regulated genes, as well as environmental factors and dietary Li intake. Li deficiency is therefore a potential common mechanism for the multisystem degeneration of the brain that leads to onset of AD.”

20. Methods, Isolation of plaque-enriched and non-plaque fractions: I found the following unclear- “The samples were passed through the sieve by gravity and then centrifuged (300xg, 30 mins).”, It would be clearer to state, “The samples were passed through the sieve by gravity and the filtrate then centrifuged (300xg, 30 mins).”

The sentence has been modified as suggested.

21. Methods, Isolation of plaque-enriched and non-plaque fractions: “The pellet was resuspended in 5 volumes of water”. How did the authors measure the volume of the pellet?

We determined the pellet mass by weighing the tubes before and after centrifugation. The pellet was then resuspended in water at a ratio of 5 mL per gram of pellet mass and stored at –80 °C (plaque-enriched fraction). The sentence now reads: “The pellet was resuspended in water at a ratio of 5 mL per gram of pellet mass and stored at –80 °C (plaque-enriched fraction).”

22. Methods, Isolation of plaque-enriched and non-plaque fractions: “10 µl of the freshly collected plaque-enriched and non-plaque fractions were layered onto albumin-coated glass slides and allowed to dry overnight. They were then washed with PBS”. Was the concentration of Li in the PBS checked as a potential source of contamination?

Yes, we tested several “ultrapure” PBS buffers and selected one with lithium levels below the detection limit (<0.02 µg/L).

For the buffer used to extract the plaque-enriched and non-plaque fractions, we similarly screened multiple reagents and selected only those with extremely low lithium content. The final extraction buffer contained 2% SDS (from a 10% ultrapure stock; ThermoFisher Scientific, Cat. No. 24730020), 0.1 M β-mercaptoethanol (ultrapure grade), and 50 mM Tris-HCl, pH 7.6 (Invitrogen, Cat. No. 15567-027), prepared in ultrapure water (Aristar Ultra, VWR Cat. No. 87003-236). The lithium concentration of the complete buffer was below the detection threshold (<0.02 µg/L). These details are now included in the Methods section.

Minor:

1. Methods, Inductively-coupled plasma mass spectrometry (ICP-MS): “Finally, consistent with previous studies, we observed significantly … reduced copper33 levels, in the AD frontal cortex”. The earliest reference for decreased copper levels in AD frontal cortex was PMID: 22080049 DOI: 10.1016/j.freeradbiomed.2011.10.446. Also Page 16: Discussion, 2 nd para. The original report of lowered Cu in the brain in AD was not ref 33 but the same.

Thank you for pointing this out. The reference has now been inserted.

2. Page 24: Triton X-100 is termed Tritonx 100

The reagent name has been corrected from "Tritonx 100" to "Triton X-100."

3. Page 37. Microglial Aβ uptake and degradation assays. Human Abeta 1-42 was studied. But through the text, the authors refer to Abeta42, which I gather is 1-42, but they never define this. ABeta42 could refer to x-42. Important to be precise to leave no ambiguity.

We have now specified in both the main text and the Methods section that human $A\beta_{1-42}$ was used for the microglial Aβ uptake and degradation assays, as well as for the in vitro studies examining LiO and LiC binding to human $A\beta_{1-42}$ fibrils and oligomers.

I congratulate the authors on a highly original and important study.
Signed
Ashley I Bush, MD PhD

Referee #3 (Remarks to the Author):

In this manuscript, the authors present a comprehensive study on the potential role of lithium deficiency in the development of Alzheimer's disease (AD) with robust evidence. The findings are intriguing and suggest a novel approach to AD treatment. The study includes: (1) a systematic metallomic analysis of 27 abundant and trace metals across two cohorts, (2) subproteome and spatial analyses highlighting lithium enrichment/sequestration by plaques, (3) studies of how lithium deficiency exacerbates AD pathology and cognitive deficits in J20 and 3xTg mouse models, (4) multi-omics studies single-cell RNA sequencing and proteomic analyses, (5) functional studies exploring the roles of synapses, microglia, and GSK3β in lithium deficiency, and (6) the selection and testing of a plaque-evading lithium salt (LiO), examining its impact on AD-related pathologies, cognition, and age-related inflammation and cognitive decline. This comprehensive and solid work effectively links lithium deficiency to AD pathogenesis and proposes a promising treatment strategy. It is a highly significant discovery. However, there are some concerns that need addressing:

1. As Lithium treatment has been used in psychiatric medication, and Lithium treatment has been explored as a potential therapeutic approach for AD. Clinical trials in AD have shown mixed results. Could metallomic profiling help categorize MCI and AD into subtypes with minor, moderate, or severe lithium deficiency? Are there any biomarkers of lithium deficiency in plasma or CSF that could clarify these clinical inconsistencies and enhance trial design in the future?

It should be noted that the limited clinical trials of Li in AD have utilized lithium salts that our data predicts would undergo substantial amyloid sequestration. Physiological dose lithium orotate may bypass this block. Nonetheless, the reviewer raises an important translational issue. Our metallomic profiling data suggests a range of Li levels in the human prefrontal cortex with the lowest levels in MCI and AD, as noted in the results section. In serum, a similar range is observed, raising the possibility that metallomic profiling might predict aging individuals at risk of Li deficiency. Moreover, close

examination of Extended Data Fig. 1c shows that the highest serum Li levels in aging humans (>8 ng/ml) are detected in cognitively intact individuals and not in MCI and AD. This is also observed in the brain (Fig. 1c-e, g), raising the intriguing possibility that therapeutically raising serum Li to this benchmark level, which might be achieved with low dose LiO, could indefinitely prevent the onset of AD. We have now added these interesting observations to the discussion (2nd paragraph, last sentence).

Another potential approach alluded to by the reviewer is to utilize functional biomarkers of Li status. In particular, our data suggests that GSK3β activity predicts Li deficiency. Notably, the predictive early AD plasma markers P-tau 181 and P-tau 217 are phospho-tau isoforms generated by GSK3β. In addition, GSK3β itself and the activated pTyr216 isoform, which are elevated by Li deficiency, might also serve as plasma biomarkers. Although validation of these potential biomarkers is beyond the scope of the present manuscript, it is an important area for future investigation.

2. The manuscript lacks in vitro strong biochemical data supporting the Li-Aβ interaction hypothesis. Data on different lithium salts binding to Aβ monomers/polymers would strengthen this hypothesis.

We thank the reviewer for this suggestion. Please see the response to Reviewer 2, point #12. These experiments turned out to be quite informative – they demonstrate binding of Li to Aβ fibrils and oligomers in the physiological Li concentration range with LiC exhibiting a higher affinity than LiO (Fig. 5b,c and Extended Data Fig. 7b). These new findings are consistent with the conductivity measurements (Fig. 5a and Extended Data Fig. 7a) and the in vivo Aβ-Li interaction data (Extended Data Fig. 7c-f). The Aβ42 monomer assay proved to be technically difficult because Aβ42 rapidly aggregates to oligomeric forms during the assay incubation in the presence or absence of Li, precluding a clear result.

3. Are there specific lithium-binding biomolecules (e.g., proteins, lipids, etc.) in the brain that are concentrated in plaque areas?

Our new data suggests that Aβ, particularly fibrillar and oligomeric forms that are concentrated in plaque areas, bind lithium with high affinity. Interestingly, Li is also known to bind directly to GSK3β, which is highly expressed in neurons with phospho-tau pathology. Our data shows that Li deficiency increases GSK3β expression, which might elevate Li-GSK3β binding further depleting the bioavailable pool. It will be of interest to identify other lithium-binding macromolecules in future studies of AD and other neurodegenerative disorders.

4. The omics analysis, while routine, lacks advanced network analysis and prioritization of key protein/gene hubs or drivers. he identification of central lithium targets remains unclear, although the extensive data provided hold the potential to fully characterize lithium pathways.

We have now performed additional network analysis of key gene hubs or drivers using the Ingenuity/IPA platform (Extended Data Fig. 4). This analysis highlights Wnt/GSK3β/β-catenin as one of the most affected signaling pathways in microglia (Extended Fata Fig. 4a), and among the significant pathways in excitatory neurons and oligodendrocytes (Extended Data Fig. 4b,c). The pathway is predicted to be downregulated by Li deficiency. These predictions were confirmed experimentally by *in situ* labeling for nuclear β-catenin and GSK3β, a major regulator of the pathway, in Li-deficient microglia, neurons and oligodendrocytes (Fig. 4f, Extended Data Fig. 5a-k), and by treating Li-deficient

mice and cultured microglia with GSK3β inhibitors (Fig. 4g, Extended Data Fig. 6). Moreover, Li treatment was confirmed to have an opposite effect on the pathway based on changes in GSK3β and β-catenin, as predicted (Supplementary Fig. 9a-d). This network modeling, together with the GO analyses in Figs. 3c and 4a, provides the first characterization of endogenous lithium pathways.

5. It is crucial to detail the coverage and depth of omics studies in the main text, including the number of transcripts and proteins analyzed. For instance, only about 3,400 proteins are listed in supplemental table 7, which seems low.

The coverage and depth of omics studies have been provided in the corresponding figure legends and Methods sections. Moreover, for snRNA-seq, the number of nuclei analyzed, average reads per nucleus, and features detected by cell type are provided in Supplementary Figure 3 and Supplementary Table 3, with additional methodological details described in the Methods section. For both the microglia RNA-seq and Li orotate RNA-seq, input read counts and percentages of uniquely mapped reads are reported in Supplementary Tables 9 and 14, respectively.

Proteomics data include the total number of proteins identified and the criteria used for high-confidence identification, as detailed in the Methods. A total of 13,404 proteins were detected, of which 3,392 were identified with high confidence - defined as MS2 spectrum assignments with a false discovery rate (FDR) < 0.01 at both the protein and peptide levels, using a target-decoy database search via Percolator. These high confidence proteins were included in the statistical analyses, following standard procedures at the Harvard Center for Mass Spectrometry. Supplementary Table 7 provides the number of peptide spectral matches (PSMs) and average peptides per protein. Proteins identified with lower confidence are accessible in the raw data files deposited in the ProteomeXchange Consortium (PRIDE; dataset identifier PXD063039), which include one .msf file (containing PSMs, protein groups, modifications, scores, FDR, and metadata) and 20 .raw files (containing MS1/MS2 spectra and metadata for each of the 20 analyzed fractions).

Individual animal-level data for RNA-seq and proteomics experiments, along with associated metadata, are available through the GEO repository (accession numbers GSE272344, GSE275326, and GSE295788) and the PRIDE database (PXD063039).

6. The manuscript provides only processed omics data (e.g., fold changes and p-values). A detailed description of quality control, data normalization, statistical analysis, and cutoff determination is necessary for reproducibility and transparency.

Detailed descriptions of quality control for each of the three RNA-seq experiments and the proteomics experiment are now provided in the Methods section under dedicated "quality control" subheadings (pages 48-49, 50-51, 53-54, and 56-57). Information on data normalization, statistical analyses, and cutoff criteria has also been added (pages 48-50, 51-52, 53-54, and 56-57).

7. The individual case/animal data from omics studies are not provided, limiting the opportunity for independent re-analysis.

Individual animal data, including IDs and group assignments for all omics studies, are available in the raw data files and accompanying documentation deposited in the GEO repository (GSE272344, reviewer's token: gxoncmqshnufzuh, GSE275326, token: sdodeskmhdavfqn, and GSE295788, token: mjwjcuwyzzqxvaj) and ProteomeXchange (PRIDE, PXD063039, token: YzASPFAIolvs).

8. The MS raw data from omics analyses are not released.

The mass spectrometry proteomics data have been deposited with the ProteomeXchange Consortium via the PRIDE partner repository - dataset identifier PXD063039 (https://www.ebi.ac.uk/pride/login). The reviewer token is YzASPFAIolvs.

We thank the reviewers for their work on the manuscript.

Referee #1 (Remarks to the Author):

In our initial review of this manuscript, we appreciated the novel insights Aron et al provided, but we requested additional lines of evidence to support their main claims, increased sample sizes, and more insight into potential downstream mechanisms. In their revised manuscript, the authors have now provided detailed responses to each of our points, and have satisfactorily addressed all of our original concerns. We have no further comments, and would recommend this article for publication.

Referee #2 (Remarks to the Author):

A-H I have commented in depth in my previous review, and my enthusiasm remains high for this work. Ms 2024-09-18526A

The revised manuscript by Yankner et al is greatly improved, and they have added additional data that has greatly strengthened their conclusions.

I still have some minor concerns:

1. In the results and supplementary tables they refer in several places to Li (and other metal) “uptake” into brain tissue. I know that I mentioned this in the first review, and they have provided a definition for “uptake”. But, “uptake” has a very specific meaning, and they implying it inaccurately by measuring brain:serum ratios. They really have not measured uptake, but rather steady state levels, which may reflect changes in efflux (that they cannot exclude) as well as true uptake. A more precise term than uptake would be “retention”.

We have replaced “uptake” with Li cortex-to-serum ratio to remove any ambiguity about the measurement.

2. They comment that “The statistical significance of the other metal changes in AD was considerably less than that of Li (Fig. 1b).”. Comparing levels of significance is not a valid way of illustrating the magnitude of any change. A result is or is not significant. The fold change is the only valid comparator. The volcano plots are welcome extra figures, and convey much of that information, but my point is that you really can’t compare size of p values. That not how p values work. The metal with the biggest change is Lead, which is way down in AD vs NCI. Not that I’m suggesting pursuing this. I think that the pursuit of Li was justified because it was the only significant change in the MCI v NCI comparison and because, unlike lead, it has pharmacological connotations.

We have changed the sentence “The statistical significance of the other metal changes in AD was considerably less than that of Li (Fig. 1b)” to “the change in Li showed the lowest adjusted P-value of all metals analyzed (Fig. 1b)”. This simply states the data and allows the reader to draw conclusions. We agree with the reviewer that the singular significance of Li in the MCI vs NCI comparison is worthy of note, and is now highlighted in the abstract and discussion.

3. Fig 5-- The legend states, “b,c, Lithium binds to Aβ42 fibrils (b) and oligomers (c) in the physiological concentration range.” Do you mean the lithium is in the physiological range? According to Supp Table 1, the normal concentration of Li in the brain is about 330 nM. The EC50 for fibrils is 3.7 uM (Supp Table 13) and for Abeta Oligomers is 35 uM (Supp Table 13). So, binding would be MINIMAL at physiological concentrations. It would only be possibly meaningful at pharmacological concentrations. So, you can't say that Li binds to Abeta fibrils and oligomers in the "physiological Li concentration range". Maybe it does in the PHARMACOLOGICAL concentration range. Throughout the text the reference is to Li supplementation in the PHYSIOLOGICAL range, But this is misleading. There is no known physiological function for Li. So you must be careful about these statements. Ideally, and maybe necessarily for a paper in Nature, you would measure the Li concentration in the brains of animals treated with LiO to ensure that the concentrations of LiO-treated animals are within the range that comports to the IC50’s that you now report.

We did in fact measure Li concentrations in the brains of animals treated with LiO. This data was shown for both brain and serum in LiO-treated 3xTg (Supp Fig. 8bc), J20 (Supp Fig. 8a), and wild-type mice (Extended Data Fig. 10a). These results show that treatment with the low dose of LiO results in Li levels within the endogenous range. We have changed “physiological” to “endogenous” in the text, but have maintained “physiological” in a few instances that refer to our data, namely that depleting endogenous Li has physiological effects on the brain.

The reviewer states that our binding curves suggest that Li “binding would be minimal at physiological concentrations”. However, Fig. 5bc shows that Li binding to Aβ fibrils and oligomers is clearly detected above the LOD and LOQ in the endogenous Li concentration range, which is 0-2 µM (Supp Fig. 8, Extended Data Fig. 10a). The fact that higher levels of Li binding are detected at pharmacologic concentrations, which are ~500-1,000-fold the endogenous level, does not mitigate the binding in the endogenous range. Moreover, the in vitro binding is consistent with the in vivo data showing sequestration of endogenous Li by Aβ plaques in humans with MCI and AD, as well as mouse models with amyloid deposition.

4. Page 16: “LiO at a physiological dose prevents…” I accept the attempt to revise this concern, but in all fairness, you cannot refer to LiO at a “physiological dose”. We do not know whether LiO exists physiologically.

We have changed “LiO at a physiological dose” to “low dose LiO” (p.13, line 3).

5. “Li deficiency also promotes phospho-tau accumulation, inflammation, and the loss of synapses, axons and myelin. The progression of this neurodegenerative process may be modulated by genetic risk variants, many of which are in Li-regulated genes, as well as environmental factors and dietary Li intake. Li deficiency is therefore a potential common mechanism for the multisystem degeneration of the brain that leads to onset of AD.”
This is a very brave statement. To my knowledge, lithium has not yet been demonstrated to have ANY physiological or biochemical role in normal metabolism. Name ONE authentically Li-regulated gene product?? This statement needs to be carefully titrated back to facts that are defended by prior literature. Even more abundant metal ions like Cu+ are only just being accepted as having signalling roles. The authors are trying to introduce Li as a regulatory metal ion, but there are not enough data to

support this idea yet, although it is a noble conjecture. It needs to be more qualified and its speculative nature made explicit.

"Li-regulated genes" was a reference to the data in the paper showing that a subset of AD risk genes show significantly altered expression when mice were made Li-deficient or treated with Li. It could be argued that "Li-regulated" implies a direct action of Li on the gene regulation, which is likely since we show that endogenous Li targets GSK3β, a central regulator of beta catenin-TCF mediated transcription. However, since we have not distinguished direct from indirect Li regulation of the different AD risk genes, we have deleted "Li-regulated" as a qualifier in this sentence.

Referee #3 (Remarks to the Author):

All of my concerns have been fully addressed through additional experiments, inclusion of experimental details, and implementation of quality control in the omics analysis. The manuscript is acceptable for publication. Congratulations to the authors!