nature portfolio

Peer Review File

CK2 inhibition suppresses glial inflammation in models of neuroinflammation and neurodegeneration

Corresponding Author: Professor Fred Gage

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript examines the impact of modulating CK2 activity on neuroinflammation phenotypes. I was asked to comment specifically regarding the chemical biology aspects of the manuscripts, and my comments center around figures 1-3.

Strengths of the work include that they've used a breadth of approaches to validate CK2 as a target regulating inflammatory signaling. These include multiple small-molecule probes, target engagement assays, and multiple genetic manipulations. A significant limitation, cited appropriately in the text, is that flavonoids have already been well-linked to CK2 inhibition, and CK2 inhibition has already been linked to NFkB inhibition and suppression of inflammatory signaling. While the work here does fill in some gaps, the conceptual advance relating to the chemical biology aspects of the work is limited and no novel chemical tools are reported.

Data issues:

126-127—"In astrocytes" suggests a cell-based assay; please edit to clarify that this assay takes place in astrocyte lysates

Fig. 1f: This looks nice, but it's hard to square it visually with the data shown in Supp 2a, where the bands on the edges look comparable. Likewise, how Supp 2f's blots lead to the graph at right is hard to understand. Particularly for TMF, how does the left band being strikingly brighter than all other bands support the smooth curve seen at right?

Fig 2d: hard to understand why the a1 siRNA has such a wide scatter of knockdown effect (such that it doesn't pass significance) but such a tight effect on inflammatory transcript levels, while a2 shows stronger knockdown but wide scatter on the IL8 side. Also what is Supp 4a, which presents similar but stronger knockdown data, for? It points to Fig 2b but that experiment doesn't use siRNA.

Fig 2f: These cells are so fully impaired in their NFkB response (panel directly above) that it's hard to interpret the potency of a flavonoid to block NFkB activity. If keeping the data, soften the conclusion that a2 not a1 is responsible in these cells. Better to leave it uncertain.

Fig 2g: To agree with the stated conclusion that the K68M mutant 'abrogated inflammation' would require comparison to mock transfected cells; as shown, it's possible that overexpression of WT exacerbates inflammation, and the inactive mutant unsurprisingly has no such effect.

Overall, from a chemical biology perspective, a significant strength is the breadth of approaches supporting the linkage of CK2 inhibition and suppression of NFkB/neuroinflammation. However, this link has been made previously including in related cellular contexts, and some experiments need clarification to ensure their rigor.

Reviewer #2

(Remarks to the Author)
In the manuscript of Da Silva et al. the authors convincingly show that CK2 has a central role in regulation of astrocytic

inflammation. They used a variety of models and techniques to show that natural flavonoid compounds bind to the CK2α2 isoform of the catalytic subunit of CK2 and inhibit its activity in vitro as well as in vivo. The authors clearly show that CK2 inhibition with pharmacologic as well as genetic approaches dampens inflammation in astrocytes and reduces NF-κB induced IL6 and IL8 secretion. They also show that in Alzheimer's disease human samples in comparison to controls CK2 activity is higher while this might be related in a shift in ratio of the catalytic isoforms towards the CK2α2 isoform rather than a higher protein expression of CK2 subunits in general. Finally, the authors demonstrate that inhibiting CK2 activity in the APP/PS1 AD mouse model significantly reduces astrocyte and microglial inflammation and their co-localization with Aβ plaques without affecting the Aβ plaque load in treated animals.
Over all the study is well designed performed and written and presents interesting findings about the role of CK2 and here specifically the CK2α2 isoform of the catalytic subunit in astrocytic inflammation activation. Also putting it in the context of Alzheimer's disease as well as other neurodegenerative and psychiatric disorders and demonstrating its potential relevance in this context is interesting and relevant for a broader audience and might help in expanding treatment options for these patients in the long run. The strength of this study lies clearly in showing the relevance of CK2 in astrocytic inflammation in detail, which is, as even described by the authors, not a completely new result. Nevertheless, the results shown deliver new aspects for better understanding its role in the context of Alzheimer's disease. The clear weakness of this study is that it completely lacks data about the clinical relevance of the treatment with the TAL606 inhibitor of CK2 activity that they have used in their mouse in vivo study. Showing data about cognitive and memory benefits of the inhibitor treatment in mice would clearly improve the relevance of the study. Overall, the study is well planned and presented, but has some need for major and minor improvements.
Specific comments, with recommendations addressing the comment
1) Neuroinflammation plays a role in Alzheimer's disease as well as most other neurodegenerative disorders and not as stated just in neurodegenerative disorders related to AD.
2) The last paragraph of the introduction is very similar to the abstract. We strongly recommend re-phrasing it and to re-focus
3) For assessing the anti-inflammatory activity of flavonoid compound the authors used primary human cerebellar astrocytes (HCA). We want to state that cerebellar astrocytes are different to hippocampal and cortical astrocytes and as such a better model for ataxia research than Alzheimer's disease research. Maybe the authors want to state why they did not use iPS derived astrocytes of different patients and controls in these experiments instead?
4) What is missing are behavioral mouse data showing a beneficial learning and memory effect of the TAL606 treatment. In case the authors are not able to provide such data at least they might want to emphasize in it in the discussion of the manuscript
5) In the discussion the authors describe "Recently, components of the CK2 holoenzyme have been characterized as genetic risk factors in AD as well as other neurodegenerative and psychiatric diseases, including amyotrophic lateral sclerosis, multiple sclerosis, and schizophrenia." This statement needs to be re-written as in its current form it might be misleading as ALS and MS are no psychiatric diseases.
6) Figure legend of figure 1: please describe the abbreviation iMGL or re-phrase
7) Figure 1: The axis labeling sizes are inconsistent, axis labels are inconsistent (CHR vs CHR conc.), thicknesses are inconsistent, the cytokine dot blot is to small.
8) Figure 2: The axes of the panels are misalignment, this makes the data presentation twitchy
9) Figure 3 A: The authors should quantify signals and ratios and need to show statistical analysis of the results
10) Figure legend of figure 3 C: "pIκB/IκBα levels": this is a ratio and not a level. And based on the representative blow the authors show we can assume that IκBα expression levels are raised upon treatment with CX and CHR at higher concentrations without any effect on the total phosphorylation status. The description of that in the figure legend is very superficial and needs to be revised. Also the authors should quantify signals and ratios and need to show statistical analysis of the results
11) Figure legend of figure 3 F: "gray nodes and edges unrelated to CK2, black nodes connected to CK2 via red edges" In the actual figure there are no grey nodes, but just black nodes with grey and black labels
12) Figure 4 claims that "CK2 is dysregulated in Alzheimer's disease (AD) patients and models", but what the authors show are not data from patient tissue analyses but from induced human astrocytes, which can also be considered a model as well as the mouse model shown as well. We suggest to adjust the title to be more precise and not to claim things not shown in the figure.
13) Please revise the Materials and Methods section for the correct SI unit terminology (h instead of hr, g instead of gm, µg/kg instead of ug/kg etc.)
14) Approval numbers for ethical approvals of animal work and work with human subjects/material are missing

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revisions have addressed my concerns. I'm on board with their suggestion of replacing the old data with their new Supp Fig 2f.

Apologies that I mis-read Fig. 2d initially such that my critique was off base.

Reviewer #2

(Remarks to the Author)
None

January 28, 2025

Dear reviewers,

We greatly appreciate the thoughtfulness demonstrated by the reviewers and assistance they have provided to improve this manuscript. We also appreciate your reconsideration of this manuscript with the changes below. We herewith provide a revision to our manuscript **NCOMMS-25-64617**. On the following pages we provide a response (in red) to the reviewers' comments and have submitted a revised manuscript with tracked changes.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript examines the impact of modulating CK2 activity on neuroinflammation phenotypes. I was asked to comment specifically regarding the chemical biology aspects of the manuscripts, and my comments center around figures 1-3.

Strengths of the work include that they've used a breadth of approaches to validate CK2 as a target regulating inflammatory signaling. These include multiple small-molecule probes, target engagement assays, and multiple genetic manipulations. A significant limitation, cited appropriately in the text, is that flavonoids have already been well-linked to CK2 inhibition, and CK2 inhibition has already been linked to NFkB inhibition and suppression of inflammatory signaling. While the work here does fill in some gaps, the conceptual advance relating to the chemical biology aspects of the work is limited and no novel chemical tools are reported.

Although flavonoids have been previously linked to CK2 inhibition, the literature remains murky, as flavonoids have also been linked to many other targets over the past decades (Zhao, L. *et al.* BMC 2019, Zaplatic et al, *Life Sciences* 2019). Moreover, no one has previously annotated which structural features of flavonoids correlate robustly with their biological activity. Many papers claim that flavanones, which do not possess the key C2-C3 unsaturation needed for these molecules to fit inside the small ATP binding pocket of CK2, are also anti-inflammatory. In this work, we clarify these issues with robust SAR data and take a previously unexplored unbiased chemoproteomics approach for the identification of neuroinflammation-relevant flavonoid targets. Coupled with pharmacological and genetic validation in human in vitro models, including state-of-the-art CNS models such as glia-enriched cortical organoids, our work provides new chemical biology insight into the neuroinflammatory role of CK2 in relevant cellular contexts. Overall, our work clarifies the structural features of flavonoid compounds enabling potent CK2 activity and anti-inflammatory activity.

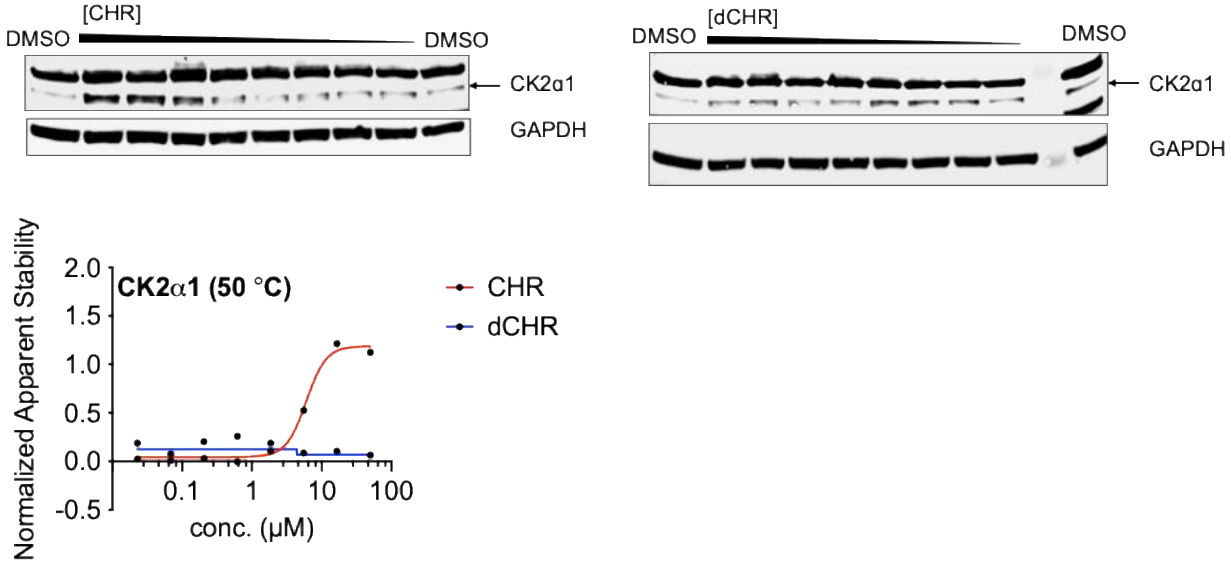
Here we report for the first time on the biological activity of TAL606, a novel chemical probe first disclosed in our accompanying medicinal chemistry paper (Tucker et al, BMC 2025). TAL606 exhibits 40 nM potency for CK2A2, is brain-penetrant, and extremely

selective both in the kinome and the larger CNS proteome (CK2A1 and CK2A2 are the only kinases inhibited >90% at 1 µM in gold-standard scanMAX-468 panel; EC_{50} > 10 µM for 45 CNS off-targets & 320 GPCRs). To our knowledge, TAL606 represents the most selective, potent, and brain-penetrant chemical probe for CK2.

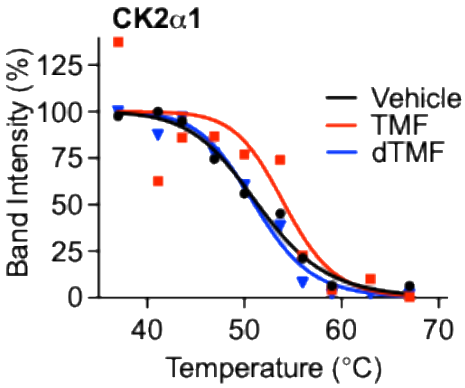
Data issues:

126-127— "In astrocytes" suggests a cell-based assay; please edit to clarify that this assay takes place in astrocyte lysates
Done, thank you.

Fig. 1f: This looks nice, but it's hard to square it visually with the data shown in Supp 2a, where the bands on the edges look comparable.
Following the TPP method, we normalize all raw densitometry values to lowest dose, then transform k' = k – 1 such that apparent stability = 0 for lowest dose (Franken, H. *et al. Nature protocols* **10**, 1567–1593 (2015).). The confusion may have arisen from the fact that the blot was a bit washed out, making it difficult to see – we've adjusted the brightness to make sure the bands are properly visible (Supp Fig 2A).
Please also see data from another experiment we performed at 50 °C, where the effect is more apparent:



Likewise, how Supp 2f's blots lead to the graph at right is hard to understand. Particularly for TMF, how does the left band being strikingly brighter than all other bands support the smooth curve seen at right?
We appreciate the reviewer's observation. The leftmost lane represents 37 °C – the blot had a lot of background in that lane (see Supp Fig. 2F for full blot), contributing to the band appearance as darker to an indeterminate degree, so we were not able to reliably quantify this lane in the blot and it was not included in the original plot. We've now gone ahead and quantified this band; we normalized the dataset such that the first two datapoints (37 °C and 41 °C) average 100% due to the seemingly high technical error for these first two datapoints, at which there should be no appreciable denaturation of CK2A1.



We'd like to propose to replace Supp Fig. 2F with the following figure, which represents 4 pooled experiments including the data above, in order to increase the robustness of the presented data. Here we show normalized stability of CK2A1 at each temperature after TMF or dTMF treatment relative to vehicle (N = 3-4 TMF, N = 2 dTMF except at 67C, where N = 2 TMF, N = 1 dTMF; the variability in sample sizes is due to lack of signal at increasing temperatures as CK2A1 becomes fully denatured). As expected, CK2A1 appears to be most stabilized by TMF around 50-55˚C. At 53.7 ˚C, CK2A1 is significantly more stabilized after TMF treatment compared to dTMF treatment (~1.4-fold, p = 0.03, mixed effects ANOVA with Tukey's post-hoc test).

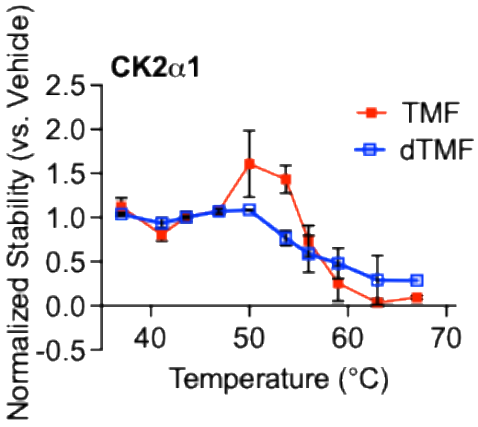


Fig 2d: hard to understand why the a1 siRNA has such a wide scatter of knockdown effect (such that it doesn't pass significance) but such a tight effect on inflammatory transcript levels, while a2 shows stronger knockdown but wide scatter on the IL8 side. Also what is Supp 4a, which presents similar but stronger knockdown data, for? It points to Fig 2b but that experiment doesn't use siRNA.

Apologies for the confusion here, there was an error in the referencing for Supp Fig 4A, which should have pointed to Fig 2D, not Fig 2B. The Fig 2D KD experiment is showing IL6 and IL8 levels in knockdown cells. The underlying knockdown efficiency for the Fig 2D KD experiment is presented in Supp Fig 4A – you can see there that the CK2A1 KD efficiency is indeed more widely distributed than CK2A2 KD efficiency, mirroring the inflammatory transcript level results.

Fig 2f: These cells are so fully impaired in their NFkB response (panel directly above) that it's hard to interpret the potency of a flavonoid to block NFkB activity. If keeping the

data, soften the conclusion that a2 not a1 is responsible in these cells. Better to leave it uncertain.
Thank you, we've rephrased this.

Fig 2g: To agree with the stated conclusion that the K68M mutant 'abrogated inflammation' would require comparison to mock transfected cells; as shown, it's possible that overexpression of WT exacerbates inflammation, and the inactive mutant unsurprisingly has no such effect.
Thank you for pointing this out, we've changed the wording of the results to clarify the comparison and what it suggests about the role of CK2's kinase activity in driving inflammation.

Overall, from a chemical biology perspective, a significant strength is the breadth of approaches supporting the linkage of CK2 inhibition and suppression of NFkB/neuroinflammation. However, this link has been made previously including in related cellular contexts, and some experiments need clarification to ensure their rigor.

Reviewer #2 (Remarks to the Author):

In the manuscript of Da Silva et al. the authors convincingly show that CK2 has a central role in regulation of astrocytic inflammation. They used a variety of models and techniques to show that natural flavonoid compounds bind to the CK2α2 isoform of the catalytic subunit of CK2 and inhibit its activity in vitro as well as in vivo. The authors clearly show that CK2 inhibition with pharmacologic as well as genetic approaches dampens inflammation in astrocytes and reduces NF-κB induced IL6 and IL8 secretion. They also show that in Alzheimer's disease human samples in comparison to controls CK2 activity is higher while this might be related in a shift in ratio of the catalytic isoforms towards the CK2α2 isoform rather than a higher protein expression of CK2 subunits in general. Finally, the authors demonstrate that inhibiting CK2 activity in the APP/PS1 AD mouse model significantly reduces astrocyte and microglial inflammation and their co-localization with Aβ plaques without affecting the Aβ plaque load in treated animals.
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Showing data about cognitive and memory benefits of the inhibitor treatment in mice would clearly improve the relevance of the study. Overall, the study is well planned and presented, but has some need for major and minor improvements.
Specific comments, with recommendations addressing the comment
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Thank you, we've rephrased this.
2) The last paragraph of the introduction is very similar to the abstract. We strongly recommend re-phrasing it and to re-focus.
Thank you, we've rephrased the last paragraph as much as possible while still keeping in line with Nature Communications formatting instructions for the introduction section.
3) For assessing the anti-inflammatory activity of flavonoid compound the authors used primary human cerebellar astrocytes (HCA). We want to state that cerebellar astrocytes are different to hippocampal and cortical astrocytes and as such a better model for ataxia research than Alzheimer's disease research. Maybe the authors want to state why they did not use iPS derived astrocytes of different patients and controls in these experiments instead?
We double checked the catalog #1800 and found that these cells are in fact primary human cortical astrocytes, not cerebellar astrocytes as was erroneously retained in our records—we apologize for the confusion and have edited the manuscript and methods to clarify this.

4) What is missing are behavioral mouse data showing a beneficial learning and memory effect of the TAL606 treatment. In case the authors are not able to provide such data at least they might want to emphasize in it in the discussion of the manuscript
We agree that including behavioral data would greatly strengthen the link between the anti-inflammatory effects of TAL606 and the potential for cognitive benefits. Unfortunately, the penetrance of the plaque phenotype in the APP/PS1 model was only around 50%, with the remaining 50% of transgenic APP/PS1 mice having few to no plaques. Given this level of penetrance and the resulting need to age large numbers of APP/PS1 mice to 9 months of age for behavioral testing, we opted to focus on biochemical and histological measures that are tractable at smaller sample sizes. We did attempt a novel object location test on one cohort of TAL606-treated APP/PS1 mice, and we've added this data in the manuscript (Supplementary Fig. 13C). However, we saw no difference between wildtype and transgenic mice in the vehicle condition (Supplementary Fig. 13D). Limiting our analysis to only those transgenic animals with a high plaque burden did not suggest any difference in performance based on plaque levels at these sample sizes (Supplementary Fig. 13D). We agree that assessing behavior in an alternative AD model is an important future direction and we have added this to the discussion.

5) In the discussion the authors describe "Recently, components of the CK2 holoenzyme have been characterized as genetic risk factors in AD as well as other neurodegenerative and psychiatric diseases, including amyotrophic lateral sclerosis,

multiple sclerosis, and schizophrenia." This statement needs to be re-written as in its current form it might be misleading as ALS and MS are no psychiatric diseases.
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Thank you, we've added quantification to the figure and rephrased the results, which is now Supplementary Fig. 4G in the manuscript.
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Thank you, we've added quantification to the figure, which is now Figure 3B in the manuscript.
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