progress in neurobiology

# Peer Review Overview

**Manuscript Title: "Inhibition of Death-associated Protein Kinase 1 Attenuates Cis P-tau and Neurodegeneration in Traumatic Brain Injury"**


| | |
|---|---|
| Received | 04-Sep-2020 |
| 1st Decision | 26-Oct-2020 |
| 1st Revision Submitted | 23-Dec-2020 |
| 2nd Decision | 07-Feb-2021 |
| 2nd Revision Submitted | 05-Apr-2021 |
| Accepted | 05-May-2021 |


---

## 1st Decision Letter

Dear Dr. Lee,

Thank you for submitting your manuscript to Progress in Neurobiology.

We have completed our evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. We invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Dec 25, 2020.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

To submit your revised manuscript, please log in as an author at https://www.editorialmanager.com/proneu/, and navigate to the "Submissions Needing Revision" folder.

Progress in Neurobiology values your contribution and we look forward to receiving your revised manuscript.

Kind regards,

Jeannie Chin
Associate Editor
Progress in Neurobiology

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Editor and Reviewer comments:

Reviewer #1: Manuscript Number: PRONEU-D-20-00367

Title: Inhibition of Death-associated Protein Kinase 1 Attenuates Cis P-tau and Neurodegeneration in Traumatic Brain Injury

This manuscript tried to combine the previously published two papers regarding DAPK1-pin1-cis-pTau-hyperphosphorylated tau pathway in TBI model.
(1) Death-associated protein kinase 1 has a critical role in aberrant tau protein regulation and function. Cell Death & disease (2014) 5, e1237.

(2) Antibody against early driver of neurodegeneration cis P-tau blocks brain injury and tauopathy. Nature (2015) 523, 431-436.
To show the role of DAPK1 on pTau (T231), cic-pTau, pPin1 (S71), GFAP, Iba1, authors used DAPK1 KO mice with TBI model as well as DAPK1 inhibitor to SY5Y cell lines with hypoxia damaged model. As summary in Figure7, they provide the hypothesis that DAPK1-pin1-cis-pTau-hyperphosphorylated tau pathway affects neurodegeneration, neuroinflammation and hyperphosphorylation of tau in TBI model. The manuscript is straightforward and hypothesis is simple and clear although it is less novel because of previous reports. There are several points to make this manuscript clearer.

1. Previous report (Cell Death & Disease, 2014) showed that DAPK1 overexpression increased total tau level, while DAPK1 KD decreased total tau level. However, in this manuscript, TBI-induced WT mice increased DAPK1 level without total tau level changes. Also, there was no difference of total tau level between DAPK1 KO mice and WT mice under TBI condition. Authors should explain what is the reason of discrepancy.
2. Does TBI induce neuronal injury? There is no data regarding this phenomenon. If it is true, TBI-induce DAPK1 KO model should be nod neuronal damage. This result should be added in the result section.
3. TBI-induced brain changes should be shown in whole brain section, not in restricted area. In this case, we can understand TBI can increase DAPK1 and cis-pTau level changes in the brain. Now, it is hard to think that these molecular changes come from TBI induction.
4. Previous report showed DAPK1 binds to NMDA receptor (NR2B) and activate it to induce calcium influx, resulting in neuronal death. Also, another report showed that DAPK1 directly binds to tau to phosphorylate it in stroke model (Cereb Cortex. 2015, 25(11):4559-4571, A novel mechanism of spine damages in stroke via DAPK1 and tau). Therefore, authors should discuss several possible mechanisms such as other kinases activation including GSK-3beta to phosphorylate tau under TBI condition.
5. Authors showed the role of pin1 on conversion of cis-pTau to trans-pTau using TBI model through the phosphorylation on ser71 pin1. But, there is no data about total pin1 level under TBI condition as well as DAPK1 KO mouse model. Authors should show these data.

Reviewer #2: Here the authors demonstrated a role for the death-associated protein kinase 1 (DAPK1) in regulating cis P-tau induction post TBI. DAPK1 was significantly up-regulated in mouse brains after TBI and promotes cis P-tau induction. DAPK1 genetic deletion significantly reduces cis P-tau expression and attenuates neuropathology onset and rescues behavioral deficits post TBI.
Phosphorylation of Pin1 at Ser71 regulates DAPK1-mediated cis P-tau induction. Pharmacological suppression of DAPK1 decreases Pin1 phosphorylated at Ser71 levels and cis P-tau after neuronal stress. The authors suggest DAPK1 as a novel modulator of the development of TBI and a therapeutic target for TBI. Although the results are intriguing there are several issues that need to be addressed by the authors:

1. The authors should add how the specificity of each antibody was determined to Table 1.
2. Although the authors state that male mice were used in the study, there is not mention of age at time of behaviorally test.
3. There is no mention of how many mice were used in each experiment.
4 What method was used to replicate positioning of the animal's head prior to TBI?
5. Was the skull exposed prior to TBI?
6. What was the room temperature and where were mice kept during recovery?
7. Since the animal exhibit seizures and other abnormality, were these variables included in the analysis of the behavioral and biological changes post TBI.
8. What was the section thickness?
9. What form of antigen retrieval was used for each antibody?
10. Was the overnight antibody incubation done at room temperature on in the cold?
11. It would be helpful to specify what the Fiji analysis includes.
11. Were the primary neurons derived from the same cortical region injured in the study and derived from the same strain obtained from Jackson Labs?
12. Add the concentration of the DAPK1 inhibitor in the methods section.
13. Since the authors state that the mice were fixed and embedded, where the animals used for immunoblotting an additional group?
14. The authors should include what was used as the control protein for calculation purposes and how many runs were performed in the immunoblotting section?
15. What do the authors mean by the ..."number of repeats was increased according to the sample conditions".
16. The authors should define ssTBI vs rmTBI the first time the abbreviations are used.
17. Images of the extent of the lesion and a demonstration that there was axonal damage related to the

injury should be included.
18. It would be helpful to the reader if the antibody used to detect a specific protein mentioned in the reslts were aded to the text.
19. The authors use descriptive term such as robustly increased in the text. Better to indicate whether the changes were significant or not.
20. Iba-1 staining shown in Figs. 3E, F do not have the morphology of microglia.
21. What does "chronic stage of TBI in DAPK1 KO mice" refer too? Does this refer to the ssTBI or rmTBI at what time point?
22. The labeling above the blots in S6 and 7 need to be done more professionally so that the information is readable.

## 1st Author Response Letter

**Responses to the Reviewers' Comments**
We deeply appreciate the careful analysis and expert advice on improving our manuscript by both the reviewers. We fully agree with the reviewers' outstanding comments and have revised our manuscript accordingly, as detailed below.

**Reviewer #1:**
**1. Previous report (Cell Death & Disease, 2014) showed that DAPKl overexpression increased total tau level, while DAPKl KD decreased total tau level. However, in this manuscript, TBI-induced WT mice increased DAPK1 level without total tau level changes. Also, there was no difference of total tau level between DAPKl KO mice and WT mice under TBI condition. Authors should explain what is the reason of discrepancy.**
There is not any discrepancy between this manuscript and the previous report (*Cell Death & Disease, 2014*) regarding total tau level in DAPK1 KO mice. In the previous paper, we showed that the total tau level didn't change in young DAPK1 KO mice, but significantly decreased in aging DAPK1 KO mice after 12-month-old, as reference paragraph described below:

"We next evaluated endogenous tau protein expression in these mice. Whereas there was no significant difference in tau expression between 1- and 6-month-old WT and KO mice (Figure 6b), it was significantly decreased in 12-and 22-month-old DAPK1 KO mice compared with WT mice of the same age (*Cell Death & Disease, 2014*)."

In this manuscript, we used young (2-8 month-old) DAPK1 KO mice for the experiments and consistently found no significant change in tau level in these young mice.

**2. Does TBI induce neuronal injury? There is no data regarding this phenomenon. If it is true, TBI-induce DAPK1 KO model should be nod neuronal damage. This result should be added in the result section.**
Thanks for raising this question and kindly providing some suggestions. Indeed, TBI induces neuronal injury examined by neuronal damage marker, microtubule-associated protein 2 (MAP2). As showed in Supplemental Fig. S4A and B, MAP2 was decreased significantly in WT mice subjected to TBI, but not in DAPK1 KO mice, indicating that DAPK1 KO may prevent neurons from injury in mice after TBI, as described in new lines 291-296.

**3. TBI-induced brain changes should be shown in whole brain section, not in restricted area. In this case, we can understand TBI can increase DAPKl and cis-pTau level changes in the brain. Now, it is hard to think that these molecular changes come from TBI induction.**
As suggested, we have now added immunostaining of *cis* P-tau (Fig. 1D), DAPK1 (Fig. 1G) and P-tau (T231) (Supplemental Fig. S1) in sagittal section of the whole brain. Rectangle indicates the injured area by weight drop.

**4. Previous report showed DAPKl binds to NMDA receptor (NR2B) and activate it to induce calcium influx, resulting in neuronal death. Also, another report showed that DAPK1 directly binds to tau to phosphorylate it in stroke model (Cereb Cortex. 2015, 25(11) : 4559-4571, A novel mechanism of spine damages in stroke via DAPK1 and tau). Therefore, authors should discuss several possible mechanisms such as other kinases activation including GSK-3beta to phosphorylate tau under TBI condition.**

As suggested, we have now added to "Excitatory toxicity caused by NMDA receptors affects acute neurological diseases, such as ischemic stroke, as well as chronic neurodegenerative disease including AD (Chen et al., 2020; Choi, 1988; Parsons and Raymond, 2014). DAPK1 directly binds to NR2B subunit of NMDA receptor and mediates brain injury following ischemic stroke (Tu et al., 2010). In addition, DAPK1 interacts with tau to phosphorylate it, specifically at Ser262, and is involved in neuronal cell loss in a stroke mouse model (Pei et al., 2015). Moreover, DAPK1 mediates phosphorylation of amyloid precursor protein (APP) at Thr668 through c-Jun N-terminal kinase 3 (JNK3) and glycogen synthase kinase-3 beta (GSK-3β) activation (Kim et al., 2016b). Therefore, these mechanisms have the potential as strong upstream signaling candidates for DAPK1 to induce neuronal damage in TBI.", as described in new lines 509-517 in discussion.

**5. Authors showed the role of pinl on conversion of cis-pTau to trans-pTau using TBI model through the phosphorylation on ser71 pinl. But, there is no data about total pinl level under TBI condition as well as DAPKl KO mouse model. Authors should show these data.**

As suggested, we have now added immunostaining of Pin1 48 hours and 2 months after TBI in **Supplemental Fig. S5**. Total Pin1 levels were not significantly changed after TBI in WT and DAPK1 KO. In addition, we have now modified to "Compared with that in the WT mice, the phosphorylation of Pin1 at Ser71 (pSer71-Pin1) was barely detectable in the DAPK1 KO mice at 48 h and 2 months after TBI (Fig. 2K-N, **Supplemental Fig. S5A-D**), indicating that the *cis* P-tau induction linked the phosphorylation of Pin1", as described in new lines 329-331.

**Reviewer #2:**

**1. The authors should add how the specificity of each antibody was determined to Table 1.**

As suggested, we have modified Table 1 to add more information regarding specificity. Most of the experiments were conducted using the proven antibodies used by *Albayram et al., Nature communications, 2017.*

**2. Although the authors state that male mice were used in the study, there is not mention of age at time of behaviorally test.**

Thanks so much for the comments. We have now included the information on age as much as possible from the time performing TBI experiments to doing behavioral test, as labeled in Fig. 1A, Fig. 5 and Supplemental Fig. S7, described in their figure legends and results. Below is some example:

"we conducted closed-head TBI experiments **on 10- to 11-week-old C57BL/6 mice**, where ssTBI was induced by a single drop of a weight of 54 g from a height of 60 inches, while rmTBI was induced by five daily drops of a weight of 54 g from a height of 32 inches on a point near the center of the mouse head (Fig. 1A).", as described in new lines 265-268.

"we used an elevated plus maze apparatus to evaluate anxiety/risk-taking behaviors in mice (Adhikari et al., 2011; Kondo et al., 2015). All the groups spent similar amounts of time in the decision arm or center point both at **2 months after ssTBI and 10 days after rmTBI** (Fig. 5A, B, Supplemental Fig. S7A, B).", as described in new lines 380-384.

"T maze tests were performed to measure spatial working memory at 3.5 months (n= 10-11) **and 5 months after TBI** (n= 8-11).", as described in Fig. 5 legend

"Moreover, the mice subjected to TBI showed impaired spontaneous alteration both **at 3.5 and 5 months after TBI**, examined by Y maze apparatus to test hippocampal-dependent spatial leaning and working memory (Fig. 5E, F, Supplemental Fig. S7D).", as described in new lines 395-397

**3. There is no mention of how many mice were used in each experiment.**
As suggested, we have now modified figure legend in each experiment. For the behavior test, the number of mice is mentioned in the figure legend for each experiment (Fig. 5, Supplemental Fig. S7).

**4. What method was used to replicate positioning of the animal's head prior to TBI?**
As mentioned in Materials and Methods, this TBI method is a closed-head injury.
"The mouse model of closed-head TBI was established by weight drop as previously described (Flierl et al., 2009). Briefly, the mice were anaesthetized for 25-50 s using 4% isoflurane in a 70:30 mixture of air:oxygen. **The anaesthetized mice were placed on a Kimwipe (Kimberly-Clark, Irving, TX, USA) and positioned so that the center of the head was placed directly below a hollow glass tube**. A metal weight of 54 grams was used to impact on the dorsal aspect of the skull, causing rotational acceleration of the head.

**5. Was the skull exposed prior to TBI?**
The skull was not open. As mentioned in the manuscript, this is a method of dropping the weight on the closed head, inducing TBI after only anesthesia without opening the head of the mouse.

**6. What was the room temperature and where were mice kept during recovery?**
We conducted TBI experiments in an animal facility. The temperature there is usually set at 24-26 ℃. We kept the mouse in the new cage until it recovered and then returned it to the original cage.

**7. Since the animal exhibit seizures and other abnormality, were these variables included in the analysis of the behavioral and biological changes post TBI.**
Yes. We included all mice injured due to TBI.

**8. What was the section thickness?**
The thickness of the paraffin section is 5 μm. We have now modified to "The paraffin sections (**5 μm thick**) of the brain were deparaffinized with histoclear (National Diagnostics, Atlanta, GA, USA) and rehydrated with descending grades of ethanol", as described in new lines 158-159.

**9. What form of antigen retrieval was used for each antibody?**

We used a commercial antigen retrieval solution for all antibodies. We have added to "The slides were briefly boiled in antigen unmasking solution (Vector, Burlingame, CA, USA), for antigen enhancement.", as described in new lines 160-162.

**10. Was the overnight antibody incubation done at room temperature on in the cold?**
As mentioned in the materials and methods, the antibodies were incubated overnight at 4 °C, which was performed in a cold room.

**11. It would be helpful to specify what the Fiji analysis includes.**
As suggested, we have now modified to Fiji/ImageJ Coloc 2.

**12. Were the primary neurons derived from the same cortical region injured in the study and derived from the same strain obtained from Jackson Labs?**
We obtained pregnant mice from Jackson Lab for primary neuron culture. Under *in vitro* conditions, hypoxia was used to stress neurons mimic to TBI injury.

**13. Add the concentration of the DAPKl inhibitor in the methods section.**
As suggested, we have now modified to "The SH-SY5Y cells and primary cortical neurons were pretreated with a DAPK1 inhibitor **(0.1-0.5 uM)** for 48 h, and cell stress was induced under normoxic or 0.5% oxygen conditions for 48 h at DIV 10-12." in Materials and Methods.

**14. Since the authors state that the mice were fixed and embedded, where the animals used for immunoblotting an additional group?**
We divided the mouse brain into sagittal sections, half of brain fixed and embedded, and the other half of brain used for immunoblotting.

**15. The authors should include what was used as the control protein for calculation purposes and how many runs were performed in the immunoblotting section?**
As suggested, we have modified and added the figure legend (Figures, Supplemental Figures).

**16. What do the authors mean by the ... "number of repeats was increased according to the sample conditions".**
We are sorry for the mistake. The correct wording should be ".... The number of repeats was increased according to effect size or sample variation."

**17. The authors should define ssTBI vs rmTBI the first time the abbreviations are used.**
We mentioned it in the introduction.

"Repeated mild traumatic brain injury (rmTBI) is a major risk factor for chronic traumatic encephalopathy (CTE), which is associated with psychiatric alterations, such as memory and mood disorders (Aungst et al., 2014; Hay et al., 2016; Mez et al., 2017; Washington et al., 2016). Similarly, single moderate to severe TBIs (ssTBIs)..."

**18. Images of the extent of the lesion and a demonstration that there was axonal damage related to the injury should be included.**
As suggested, we have now added immunostaining of *cis* P-tau (Fig. 1D), DAPK1 (Fig. 1G) and P-tau (T231) (Supplemental Fig. S1) in sagittal section of the whole brain. Rectangle indicates the injured area by weight drop. In addition, we have examined whether TBI induces neuronal injury using neuronal damage marker, microtubule-associated protein 2 (MAP2). As

showed in Supplemental Fig. S4A and B, MAP2 was decreased significantly in WT mice subjected to TBI, but not in DAPK1 KO mice, indicating that DAPK1 KO may prevent neurons from injury in mice after TBI, as described in new lines 291-296.

**19. It would be helpful to the reader if the antibody used to detect a specific protein mentioned in the resits were aded to the text.**

As suggested, we have added antibodies in the text, as described below. "Interestingly, the phosphorylation of tau at Thr231 **(P-tau (Thr231)),** representing a total phosphorylated tau without conformation specificity, was prominently increased 48 h after TBI in....", as described in new lines 298-300.

"the phosphorylation of Pin1 at Ser71 **(pSer71-Pin1),** like the pathological *cis* P-tau, was significantly induced at 48 h, and accumulated at 2 months in WT mice after TBI (Fig. 2K-N)", as described in new lines 331-332 .

"Moreover, the levels of cis P-tau and phosphorylated Pin1 at Ser71 (pSer71-Pin1) were also increased under cell stress conditions in both the SH-SY5Y cells and primary neurons", as described in new lines 416-417.

**20. The authors use descriptive term such as robustly increased in the text. Better to indicate whether the changes were significant or not.**

As suggested, we have added p value in the Results, as described below.

"The *cis* P-tau levels were robustly increased 48 h after ssTBI **($p = 1.517 \times 10^{-16}$)** and rmTBI **($p = 3.458 \times 10^{-5}$)** in the cortical regions", as described in new lines 272-274.

"In the cerebral cortex, DAPK1 showed a trend of increasing expression beginning at 24 h **($p = 0.485$)**, and its expression was robustly increased at 48 h **($p = 5.999 \times 10^{-5}$)**" , as described in new lines 280-282.

**21. Iba-1 staining shown in Figs. 3E, F do not have the morphology of micro glia.**

As suggested, we have now modified Fig. 3E, F, G, H.

**22. What does "chronic stage of TBI in DAPKl KO mice" refer too? Does this refer to the ssTBI or rmTBI at what time point?**

That means more than 5 months in both ssTBI and rmTBI. The paper by *Albayram et al., Nature communications*, 2017 was also described as chronic phage at that time.

**23. The labeling above the blots in S6 and 7 need to be done more professionally so that the information is readable.**

As suggested, we have now modified all blots labeling in Supplemental Figures.

## 2nd Decision Letter

Dear Dr. Lee,

Thank you for submitting your manuscript to Progress in Neurobiology. We have received comments from reviewers on your manuscript. Your paper should become acceptable for publication pending suitable minor revision and modification of the article in light of the appended reviewer comments.

When resubmitting your manuscript, please carefully consider all issues mentioned in the reviewers' comments, outline every change made point by point, and provide suitable rebuttals for any comments not addressed.

To submit your revised manuscript go to https://www.editorialmanager.com/proneu/ and log in as an

Author where you will see a menu item called 'Submission Needing Revision'.

Please resubmit your manuscript by Apr 08, 2021.

We look forward to receiving your revised manuscript.

Kind regards,

Jeannie Chin
Associate Editor
Progress in Neurobiology

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Comments from the Editors and Reviewers:

Reviewer #1: This revised version of manuscript contains most of reviewers' requests. As a result, this manuscript became clearer than original one.

If possible, to show the DAPK1-pin1-cis-pTau-hyperphosphorylated tau pathway precisely, IHC images at Fig1 and 2 should be replaced with co-staining instead of separate staining. For example, IHC images for cis-pTau and DAPK1 at Fig1 might be a one image with co-staining. Also, at Fig2, co-staining of cis-pTau and Pin1 might be helpful to clarify their hypothesis.

Reviewer #2: The authors have addressed virtually all of the comments from the prior review. However, Supplement figure 1 is not acceptable. The authors need to provide a more convincing image of the ptau staining. What are the blue stripes? Figure Supplement 10 is troublesome. Why is the actin blot performed on the same blot as the cortical and hippocampal staining? Was actin run on the same gel? Why are figures !0B and 10D not lined up with their respective regional blots?

## 2nd Author Response Letter

**Responses to the Reviewers' Comments**

We once again deeply appreciate to both reviewers for their careful analysis and expert advice on improving the manuscript. We fully agree with the outstanding comments of the reviewers and accordingly have modified the manuscript as described below.

**Reviewer #1:**

**This revised version of manuscript contains most of reviewers' requests. As a result, this manuscript became clearer than original one.**

**If possible, to show the DAPK1-pin1-cis-pTau-hyperphosphorylated tau pathway precisely, IHC images at Fig1 and 2 should be replaced with co-staining instead of separate staining. For example, IHC images for cis-pTau and DAPK1 at Fig1 might be a one image with co-staining. Also, at Fig2, co-staining of cis-pTau and Pin1 might be helpful to clarify their hypothesis.**

Thanks for the suggestions. As suggested, we have now added co-immunostaining images of *cis* P-tau and DAPK1 on mouse brains (Fig. 1D). The previous separate staining images, Fig. 1D, E, F, G have been moved to Supplemental Fig. S1B, C.

In addition, we have now added co-immunostaining images of *cis* P-tau and Pin1 instead of separate staining (Fig. 2E, G).

**Reviewer #2:**

**The authors have addressed virtually all of the comments from the prior review. However, Supplement figure 1 is not acceptable. The authors need to provide a more convincing image of the ptau staining. What are the blue stripes?**

In this revision, we have now changed Supplementary Figure 1 to Supplementary Figure 1A. As suggested, we have now improved the quality of images for Supplement 1A using much higher resolution images to clearly show that there was a significant increase in P-tau in the cortex after ssTBI.

**Figure Supplement 10 is troublesome. Why is the actin blot performed on the same blot as the cortical and hippocampal staining? Was actin run on the same gel? Why are figures 10B and 10D not lined up with their respective regional blots?**

Thanks for the careful review. Since the molecular weights of β-actin (42 kDa) and *cis* P-tau (about 50-55 kDa) are quite close, we have performed western blots on different gels to detect *cis* P-tau and β-actin. We are sorry that we weren't so careful with Supplement 10 presentation. We have now aligned Supplement 10B with 10D for their respective regional blots.