nature portfolio

Peer Review File

S-Nitrosylation of p39 promotes its degradation and contributes to synaptic dysfunction induced by β-amyloid peptide

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this article authors have presented effect of S-Nitrosylation of p39 its effect on its degradation. They have demonstrated the effect this PTM on the regulation of CDK5 and effect on synaptic dysfunction associated with AD. There was increased expression of S-nitrosylated p39 in the brain of APP/PSEN1 mouse brain. In vitro AβO elevated levels of modified p39 and contributed to its degradation. S-nitrosylated p39 contributes to dendritic retraction and synaptic loss. The manuscript adds to the mechanism of AD and uncovers potential therapeutic target altering the synaptic modulation. The manuscript can be considered after addressing the following comments.

1. The authors showed the in vitro and in vivo nitrosylation. What is the possible way of nitrosylation. They state non-enzymatic route, but chemically there is no catalysts used. Authors need to explain possible mechanisms.
2. The relative quantification of nitrosylation in Figure 1 can be provided.
3. The study claims the proteasomal dependent degradation by indirectly blocking proteasomal pathway. It would be better if they show ubiquitination and markers of proteasomal degradation.
4. The S-nitrosylation happens site selectively at Cys265. How and where from this selectivity arise from? NO can react at any cysteine residue since it is not enzyme driven. Authors should address this aspect.
5. Authors can provide possible mechanisms of dendritic and synaptic effect by nitrosylation and how S-nitrosylation is regulated.
6. The typographical errors need to be corrected for example adivin as avidin etc.,
7. Related general references can be referred in the introduction.

Mol Neurobiol. 2015 Feb;51(1):268-80. doi: 10.1007/s12035-014-8672-2.
Methods Enzymol. 2019; 626: 499–538. doi: 10.1016/bs.mie.2019.08.004
ChemBiochem. 2020 Apr 17;21(8):1052-1079. doi: 10.1002/cbic.201900573.

Reviewer #2 (Remarks to the Author):

This study shows in models of Alzheimer’s disease (AD) that the Cdk5 activator p39 is S-nitrosylated at Cys265 (forming SNO-p39). This reaction promotes p39 degradation and contributes to dendrite retraction and spine loss. Concerning S-nitrosylation and Cdk5 pathways, several groups have previously demonstrated that NO-related species can S-

nitrosylate Cdk5 and another Cdk5 activator, p35. For example, this same group of Authors, reported that extremely high concentrations of NO inhibit Cdk5 activity; however, another group revealed that more pathophysiologically-relevant concentrations of NO stimulate SNO-Cdk5 formation in AD models, contributing to synaptic dysfunction. In addition, current group of Authors showed that S-nitrosylation of p35 promotes its degradation through the ubiquitin-proteasome system (UPS), but unlike S-nitrosylated p39, SNO-p35 increased dendritic spine density. Hence, the current study on SNO-p39 may provide additional insight into the effects of NO-related species on Cdk5 pathways and the pathogenesis of AD. However, although some of these new findings are interesting, there are major concerns about the study as currently formulated, as delineated below in order to help the Authors improve the work.

•One of the major concerns is the pathophysiological relevance of SNO-p39 formation compared to other S-nitrosylation events that are known to regulate the Cdk5 pathway. What is the mechanism for synaptic damage by the formation of SNO-p39 in this study? This is critical and the Authors do not address the pathway at all so there is no mechanistic information.

•Importantly, as stated above, published studies by the Lipton group demonstrated that NO-related species affect Cdk5 via S-nitrosylation and this leads to transnitrosylation and activation of Drp1, with consequent excessive mitochondrial fragmentation and damage resulting in energy compromise, thus contributing to synaptic loss in AD. Moreover, the cascade of transnitrosylation reactions involves SNO-Uch-L1 to Cdk5 to Drp1, and critically do NOT involve the canonical enzymatic effects of Uch-L1 or Cdk5—that is the kinase activity of Cdk5 does not play a role in the synaptic damage previously demonstrated (Cho et al. Science, 2009; Qu et al. PNAS, 2011; Nakamura et al. Science, 2021). But this cascade is not clearly discussed in the current manuscript. Perhaps even more critically, because S-nitrosylation directly effects Cdk5, S-nitrosylation of p39 (occurring upstream of SNO-Cdk5 in Cdk5 pathway) would have a very limited impact on Cdk5 activity. However, this concern is not addressed in the current study. Moreover, protein-protein transnitrosylation is a critical mechanism to transduce NO signaling pathways. Despite this well-accepted concept in the field, the current study does not examine if SNO-p39 affects S-nitrosylation of Cdk5 via transnitrosylation, thus coming up short of current expectations in the field for a publication of this type. Finally, the work is only performed in tissue culture where it shows dendritic spine/synaptic effects, so we do not know if this would occur in vivo in a mouse model of AD let alone in a human with AD.

Additional major concerns:

• The results in Fig. 1D, showing S-nitrosylation of p39 in mouse brains, are not convincing. The bands for SNO-p39 are very small and faint. This raises a concern that only a very small portion of p39 is S-nitrosylated. Can an unaltered blot please be shown prior to changes in contrast and brightness so that other bands can be judged by the Referees in this regard.
• Quantification and associated statistical analyses are missing from many figures (e.g., Fig 1A, 1B 1C, 2A, 2B, 2C, 3B, 4A, 4B, 5A, and 5B). The authors need to show results of quantification plus proper statistical analysis to confirm that experiments are repeated ≥3 times and that there are statistically significant differences.
• p35 is proteolytically cleaved to produce p29, becoming a more stable Cdk5 activator. The levels of p29, however, are not examined in this study. Additionally, the effect of SNO-p39 formation on Cdk5 activity is not monitored either. Thus, in this study, it is not clear if S-nitrosylated p39 indeed regulates Cdk5 pathway.
• The consensus in the field is that increased Cdk5 activity is a contributing factor to synaptic impairment in AD. However, in this study, amyloid beta-induced S-nitrosylation of the Cdk5 activator p39 promotes its degradation, leading to decreased expression of p39. Hence, the current findings are not in agreement or at least not explained in the context of the known roles of activated/S-nitrosylated Cdk5 in AD pathogenesis (for example, see Nakamura et al. Science 2021: 371(6526);eaaw0843, as cited by the Authors but not adequately discussed). It is critical for this manuscript to show the mechanism of how SNO-p39 triggers Cdk5 activation and contributes to the observed synaptic deficits.
• The authors demonstrate that p39 is specifically S-nitrosylated at Cys265. This Cys is also conserved in p35. However, the same group previously showed that this Cys is NOT a target of S-nitrosylation in p35. This may suggest that p39 is not an efficient target of S-nitrosylation compared to p35, and this should be discussed or further investigated.
• There are eight cysteine residues in p39; however, the Authors only mutated some of them to identify the S-nitrosylation target site. It is not clear why only certain cysteine residues (i.e., Cys195, 265, 297, 354) were selected for mutagenesis experiments and the rest (i.e., Cys135, 189, 198, 243) ignored. Please explain.
• The Authors claim that S-nitrosylation of p39 promotes its degradation via the ubiquitin-proteasome system (UPS); however, there is no evidence that p39 ubiquitination is indeed altered by S-nitrosylation. The authors need to show altered p39 ubiquitination upon S-nitrosylation. Along these lines, in the prior study, the same group previously showed that S-nitrosylation of p35 promotes its ubiquitination by the E3 ligase PJA2. Is the same mechanism employed for S-nitrosylation-mediated degradation of p39?
• In Figure 4, the authors show S-nitrosylation of p39 (and Cdk5) in a mouse model of AD. These are important findings, but the experiments lack an important negative control: omission of ascorbate from the biotin-switch assay. When examining S-nitrosylated proteins engendered by endogenous NO, it is critical to include an ascorbate negative

control in biotin-switch assay to verify that the bands are derived from S-nitrosylation rather than other redox modifications and also to prove that these bands are not background staining in the assay. We acknowledge that the Authors do show an ascorbate control in Figure 1D, which is good, but it is also needed in Figure 4.
• The Authors show that SNO-p39 levels found in the whole brain do not correlate with decreased expression of p39, but in the hippocampus, SNO-p39 levels correlate with decreased p39 expression. Why is this SNO-mediated degradation happening only in a certain brain region? The lack of mechanistic explanation for this region-specific effect lessens the significance of their finding.
• In the experiments of Fig. 5, does L-NAME or another NOS antagonist block the effect of amyloid-beta on spine retraction? If not, another oxidation reaction (e.g., ROS causing -SOH, SO2/3H derivatization) could be involved rather than S-nitrosylation, and this would also be prevented in by a non-nitrosylatable/non-oxidizable mutant.

Minor concerns:
• The first "N" in "S-nitrosylation" should be capitalized when used as the first word in a sentence.
• In the legends for Figure 1 and 4, it is stated that "the biotinylated proteins were immunoprecipitated (IP) with neutravidin-agarose". Neutravidin pull down is not an immunoprecipitation.
• In the Methods section, experimental procedures for the preparation of amyloid-beta oligomers are missing. The Authors should also describe how amyloid-beta oligomers were characterized before using them. Is the concentration of amyloid-beta listed in the figures and text the concentration of oligomers or the total concentration of amyloid-beta used?

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors present compelling evidence demonstrating the significant role of S-nitrosylation of p39 at Cys265 in the process of amyloid-β (Aβ) peptide-induced dendrite retraction and spine loss. The manuscript proposes a promising hypothesis that addresses an important aspect of Alzheimer's disease research.
Before publication, the authors may consider addressing the following points:

1. The introduction section could be streamlined to focus more directly on the novel aspects of the study, avoiding excessive coverage of well-established concepts related to the activation of iNOS and its signaling pathways etc.

2. While the discussion section effectively highlights the authors' findings, it could be enhanced by integrating additional relevant studies from the existing literature, thereby providing a more comprehensive context for the interpretation of the results.

Overall, this well-written manuscript has the potential to contribute significantly to the current understanding of Alzheimer’s disease pathology.

We would like to express our gratitude to the reviewers for the valuable feedback provided on our manuscript. We appreciate the thorough review process and the constructive comments provided by the reviewers. We have carefully considered all the suggestions and made the necessary revisions to address the concerns raised by the reviewers and we believe have significantly improved its quality and clarity.

Reviewers' comments:

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In this article authors have presented effect of S-Nitrosylation of p39 its effect on its degradation. They have demonstrated the effect this PTM on the regulation of CDK5 and effect on synaptic dysfunction associated with AD. There was increased expression of S-nitrosylated p39 in the brain of APP/PSEN1 mouse brain. In vitro AβO elevated levels of modified p39 and contributed to its degradation. S-nitrosylated p39 contributes to dendritic retraction and synaptic loss. The manuscript adds to the mechanism of AD and uncovers potential therapeutic target altering the synaptic modulation. The manuscript can be considered after addressing the following comments.

1. The authors showed the in vitro and in vivo nitrosylation. What is the possible way of nitrosylation. They state non-enzymatic route, but chemically there is no catalysts used. Authors need to explain possible mechanisms.

We appreciate the reviewer for highlighting this intriguing question, and we apologize for any lack of clarity in our original manuscript. In our study, we treated 293T cell lysates with the NO donor GSNO for 5 minutes and observed robust S-nitrosylation of p39. This finding indicates that NO can rapidly and spontaneously react with p39 to form SNO-p39. We have revised our manuscript to correct and clarify this point.

2. The relative quantification of nitrosylation in Figure 1 can be provided.

We appreciate the reviewer's helpful suggestion. We have incorporated the quantification results into Figure 1 in the revised manuscript.

3. The study claims the proteasomal dependent degradation by indirectly blocking proteasomal pathway. It would be better if they show ubiquitination and markers of proteasomal degradation.

We thank the reviewer for this insightful suggestion. We have included the results for poly-ubiquitination of p39 and the enrichment of p39 in the proteasome in Figure 2D-F in the revised manuscript.

4. The S-nitrosylation happens site selectively at Cys265. How and where from this selectivity arise from? NO can react at any cysteine residue since it is not enzyme driven. Authors should address this aspect.

We thank the reviewer for raising this interesting question. We agree that, in theory, NO can react with any cysteine residue since it is not enzyme-driven. However, it has been reported that S-nitrosylation of target proteins at cysteine residues is often facilitated

by flanking acidic and basic residues[1]. The study of SNO site prediction has become a significant area of research in recent years. We attempted to predict the S-nitrosylation site(s) in p39 using several software tools such as GPS-SNO and CPR-SNO. Despite these efforts, we were unable to validate the predicted sites through subsequent mutagenesis and biotin switch assays. Consequently, we employed traditional biochemical methods, generating mutants where each of the eight cysteine residues in p39 was individually replaced with alanine. Our results showed that only the mutation of Cys265 abolished p39 S-nitrosylation, indicating that Cys265 is the major S-nitrosylation site. We believe that the specificity of S-nitrosylation sites within proteins is conferred by structural motifs, allosteric regulators, and the interactions between NO synthases and target proteins[2].

5. Authors can provide possible mechanisms of dendritic and synaptic effect by nitrosylation and how S-nitrosylation is regulated.
We thank the reviewer for raising this interesting question. It has been reported that p39 is indispensable in postnatal neuronal differentiation and the formation of neuronal networks[3,4]. Additionally, several pathological factors, such as the production of Aβ, neuronal hyper-excitability, and neuro-inflammation, can trigger NO production and result in protein S-nitrosylation. Consistently, we found that treatment of cortical neurons with Aβ and NMDA resulted in p39 S-nitrosylation and its degradation (see Supplementary Fig. 3E-J in the revised manuscript). Therefore, we propose that the overproduction of NO, induced by Aβ production, neuronal hyper-excitability, or neuro-inflammation under pathological conditions, could result in dendritic and synaptic defects by promoting p39 S-nitrosylation and its degradation.

6. The typographical errors need to be corrected for example adivin as avidin etc.,
We apologize for the errors and appreciate the reviewer for pointing them out. We have corrected these errors in Figure 1 of the revised manuscript.

7. Related general references can be referred in the introduction.
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This study shows in models of Alzheimer's disease (AD) that the Cdk5 activator p39 is S-nitrosylated at Cys265 (forming SNO-p39). This reaction promotes p39 degradation and contributes to dendrite retraction and spine loss. Concerning S-nitrosylation and Cdk5 pathways, several groups have previously demonstrated that NO-related species can S-nitrosylate Cdk5 and another Cdk5 activator, p35. For example, this same group of Authors, reported that extremely high concentrations of NO inhibit Cdk5 activity;

however, another group revealed that more pathophysiologically-relevant concentrations of NO stimulate SNO-Cdk5 formation in AD models, contributing to synaptic dysfunction. In addition, current group of Authors showed that S-nitrosylation of p35 promotes its degradation through the ubiquitin-proteasome system (UPS), but unlike S-nitrosylated p39, SNO-p35 increased dendritic spine density. Hence, the current study on SNO-p39 may provide additional insight into the effects of NO-related species on Cdk5 pathways and the pathogenesis of AD. However, although some of these new findings are interesting, there are major concerns about the study as currently formulated, as delineated below in order to help the Authors improve the work.

•One of the major concerns is the pathophysiological relevance of SNO-p39 formation compared to other S-nitrosylation events that are known to regulate the Cdk5 pathway. What is the mechanism for synaptic damage by the formation of SNO-p39 in this study? This is critical and the Authors do not address the pathway at all so there is no mechanistic information.

We thank the reviewer for raising this interesting question and apologize for any lack of clarity in our initial explanation. As previous studies have shown, the level of p39 and its associated Cdk5 activity play crucial roles in synaptic formation and integrity[3,4]. Additionally, it has been reported that several pathological factors, such as Aβ production and hyperactivation of NMDA receptors by NMDA, can lead to overproduction of NO. In line with these findings, our study demonstrated that treating cortical neurons with Aβ and NMDA resulted in p39 S-nitrosylation and its subsequent degradation (See Supplementary Fig. 3E-J in the revised manuscript). Therefore, we propose that the overproduction of NO can lead to dendritic and synaptic defects by promoting p39 S-nitrosylation and degradation.

•Importantly, as stated above, published studies by the Lipton group demonstrated that NO-related species affect Cdk5 via S-nitrosylation and this leads to transnitrosylation and activation of Drp1, with consequent excessive mitochondrial fragmentation and damage resulting in energy compromise, thus contributing to synaptic loss in AD. Moreover, the cascade of transnitrosylation reactions involves SNO-Uch-L1 to Cdk5 to Drp1, and critically do NOT involve the canonical enzymatic effects of Uch-L1 or Cdk5—that is the kinase activity of Cdk5 does not play a role in the synaptic damage previously demonstrated (Cho et al. Science, 2009; Qu et al. PNAS, 2011; Nakamura et al. Science, 2021). But this cascade is not clearly discussed in the current manuscript. Perhaps even more critically, because S-nitrosylation directly effects Cdk5, S-nitrosylation of p39 (occurring upstream of SNO-Cdk5 in Cdk5 pathway) would have a very limited impact on Cdk5 activity. However, this concern is not addressed in the current study. Moreover, protein-protein transnitrosylation is a critical mechanism to transduce NO signaling pathways. Despite this well-accepted concept in the field, the current study does not examine if SNO-p39 affects S-nitrosylation of Cdk5 via transnitrosylation, thus coming up short of current expectations in the field for a publication of this type. Finally, the work is only performed in tissue culture where it shows dendritic spine/synaptic effects, so we do not know if this would occur in vivo

in a mouse model of AD let alone in a human with AD.

We thank the reviewer for raising this interesting question. Previous work by Lipton's group showed that S-nitrosylation activated Cdk5 activity and contributed to Aβ-induced spine loss[5]. However, in our study, we found that NO negatively regulates Cdk5 kinase activity by directly S-nitrosylating Cdk5 itself or by S-nitrosylating its activators p35 or p39. We acknowledge the discrepancy between our findings and those reported by Lipton's group, and we also found these results to be conflicting. To address this, we repeated the experiments following the protocol from Lipton's paper[5]. Our results showed that treatment of recombinant Cdk5/p35 with different concentrations of NO donors consistently resulted in Cdk5 inhibition (See Fig. 1 below).

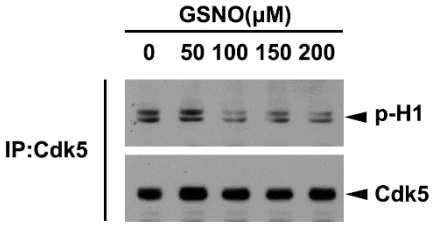

Figure 1. NO negatively regulates Cdk5 kinase activity. Recombinant Cdk5/p35 were treated with GSNO with the indicated concentrations for 30 mins and then subjected to in vitro kinase assay.

Moreover, we totally agree with the reviewer that protein-protein transnitrosylation is a critical mechanism for transducing NO signaling pathways. It is of great interest to determine whether SNO-p39 affects the S-nitrosylation of Cdk5, or if SNO-Cdk5 affects the S-nitrosylation of p39 via transnitrosylation. However, our experiments did not reveal any significant transnitrosylation between Cdk5 and p39. We have included this observation in the discussion section of the revised manuscript.

In addition, we concur with the reviewer that relying solely on tissue culture studies is insufficient to determine if these processes occur in vivo, particularly in a mouse model of AD or in humans with AD. Therefore, as per the reviewer's suggestion, we examined dendritic spine defects in vivo using APP/PS1 mice. Consistent with our cell culture data, we observed a decreased dendritic spine density in APP/PS1 mice. More importantly, expressing the p39-C265A mutant in the hippocampal region of APP/PS1 mice rescued these deficits, suggesting that p39 S-nitrosylation and its subsequent degradation play a crucial role in dendritic spine loss in the AD mouse model (See Fig. 6A-C in the revised manuscript).

Additional major concerns:

• The results in Fig. 1D, showing S-nitrosylation of p39 in mouse brains, are not convincing. The bands for SNO-p39 are very small and faint. This raises a concern that only a very small portion of p39 is S-nitrosylated. Can an unaltered blot please be shown prior to changes in contrast and brightness so that other bands can be judged by the Referees in this regard.

We apologize for the lack of clarity regarding this result and appreciate the reviewer's insightful suggestion. The faint bands observed for SNO-p39 are attributed to the low expression of p39 in the P1 brain samples. To address this, we redesigned our experiments and conducted biotin switch assays in adult WT and nNOS-KO mouse brains. We have incorporated the new data into revised Figure 1D. Consistently, our findings demonstrate that p39 can indeed undergo S-nitrosylation in WT mouse brains in an ascorbate-dependent manner, whereas this modification is absent in nNOS-KO mouse brains.

• Quantification and associated statistical analyses are missing from many figures (e.g., Fig 1A, 1B 1C, 2A, 2B, 2C, 3B, 4A, 4B, 5A, and 5B). The authors need to show results of quantification plus proper statistical analysis to confirm that experiments are repeated ≥3 times and that there are statistically significant differences.
Thank you for the valuable suggestion. We have now included the quantification results along with the associated statistical analyses in the revised manuscript.

• p39 is proteolytically cleaved to produce p29, becoming a more stable Cdk5 activator. The levels of p29, however, are not examined in this study. Additionally, the effect of SNO-p39 formation on Cdk5 activity is not monitored either. Thus, in this study, it is not clear if S-nitrosylated p39 indeed regulates Cdk5 pathway.
Thank you for bringing this up. We have considered the possibility of SNO-p39 or SNO-p35 affecting their cleavage or proteasome degradation. However, in our experiments, we did not observe significant production of p29 or p25 after GSNO treatment. Therefore, we believe that NO specifically affects the degradation of p35/p39, rather than their cleavage. Regarding the effect of SNO-p39 on Cdk5 activity, we examined the phosphorylation of Cdk5 substrates (Dcx and CRMP2) and found that NO negatively regulates Cdk5 activity (See Figure 2 below). This finding is consistent with our previous reports [6,7].

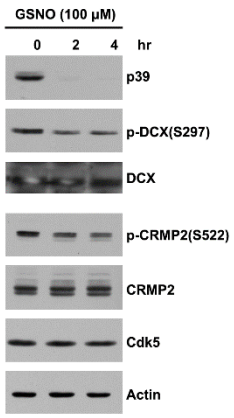

Figure 2. NO negatively regulates p39 level and Cdk5 kinase activity. Cultured cortical neurons were treated with GSNO for the indicated time points and then subjected to western blot analysis with indicated antibodies.

• The consensus in the field is that increased Cdk5 activity is a contributing factor to

synaptic impairment in AD. However, in this study, amyloid beta-induced S-nitrosylation of the Cdk5 activator p39 promotes its degradation, leading to decreased expression of p39. Hence, the current findings are not in agreement or at least not explained in the context of the known roles of activated/S-nitrosylated Cdk5 in AD pathogenesis (for example, see Nakamura et al. Science 2021: 371(6526);eaaw0843, as cited by the Authors but not adequately discussed). It is critical for this manuscript to show the mechanism of how SNO-p39 triggers Cdk5 activation and contributes to the observed synaptic deficits.

Thank you for your insightful comment. Indeed, the role of Cdk5 in synaptic impairment in Alzheimer's disease (AD) is complex and still not fully understood. While increased Cdk5 activity has been widely implicated in synaptic dysfunction, recent proteomics analyses have suggested a downregulation of Cdk5 levels in AD patients[8]. This apparent contradiction underscores the need for further investigation into the precise role of Cdk5 dysregulation in AD pathology.

Additionally, we acknowledge the discrepancy between our findings and those reported by Lipton's group regarding the effect of NO on Cdk5 activity[5,9]. In our study, we found that NO negatively regulates Cdk5 kinase activity by directly S-nitrosylating Cdk5 itself[6] or by S-nitrosylating its activators p35[7] or p39 in this manuscript. The discrepancy between our findings and those reported by Lipton's group regarding the effect of NO on Cdk5 activity prompted us to repeat their experiments using their protocol[5]. Our results confirmed that treatment of cells with different concentrations of NO resulted in Cdk5 inhibition (See Figure 1 above). One plausible explanation for these differences could be that the S-nitrosylation and subsequent loss of p39 attenuate overall Cdk5 activity in neurons. This attenuation may preferentially affect the phosphorylation of specific Cdk5 targets, ultimately leading to impaired dendritic spine and synapse formation. These insights align with findings from previous studies and shed light on the intricate mechanisms underlying synaptic impairment in Alzheimer's disease[3,4]. We have included this discussion in the revised manuscript to address the discrepancy and provide a comprehensive interpretation of our results. Thank you for your valuable input.

• The authors demonstrate that p39 is specifically S-nitrosylated at Cys265. This Cys is also conserved in p35. However, the same group previously showed that this Cys is NOT a target of S-nitrosylation in p35. This may suggest that p39 is not an efficient target of S-nitrosylation compared to p35, and this should be discussed or further investigated.

Thank you for your insightful observation. While Cys265 is conserved in both p35 and p39, our findings indicate that this site is specifically S-nitrosylated in p39, but not in p35. One plausible explanation for this difference could be attributed to the distinct expression patterns and subcellular localization of p35 and p39. It is well-documented that p39 is predominantly localized to the cell surface due to its myristoylation motif[10]. This localization likely brings p39 into close spatial proximity with neuronal nitric oxide synthase (nNOS), facilitating the endogenous production of nitric oxide (NO)

and its subsequent modification of p39. In contrast, the subcellular localization of p35 may limit its accessibility to NO, thereby preventing S-nitrosylation at Cys265. This differential regulation of S-nitrosylation between p35 and p39 underscores the complexity of NO signaling pathways and highlights the importance of considering cellular context in understanding protein modifications. We have incorporated this discussion into the revised manuscript to provide further insights into our findings. Thank you for bringing up this point.

• There are eight cysteine residues in p39; however, the Authors only mutated some of them to identify the S-nitrosylation target site. It is not clear why only certain cysteine residues (i.e., Cys195, 265, 297, 354) were selected for mutagenesis experiments and the rest (i.e., Cys135, 189, 198, 243) ignored. Please explain.
Thank you for bringing up this important point. Indeed, we conducted mutagenesis on all eight cysteine residues to identify potential S-nitrosylation sites. We apologize for the oversight in not including data for all eight mutants in the original manuscript due to space constraints. However, we have now rectified this in the revised manuscript, where we present comprehensive data for all mutants in Figure 3A-B and Supplementary Figure 1A-C. We appreciate your understanding and have made every effort to ensure the completeness and transparency of our findings in the revised version of the manuscript.

• The Authors claim that S-nitrosylation of p39 promotes its degradation via the ubiquitin-proteasome system (UPS); however, there is no evidence that p39 ubiquitination is indeed altered by S-nitrosylation. The authors need to show altered p39 ubiquitination upon S-nitrosylation. Along these lines, in the prior study, the same group previously showed that S-nitrosylation of p35 promotes its ubiquitination by the E3 ligase PJA2. Is the same mechanism employed for S-nitrosylation-mediated degradation of p39?
We appreciate the reviewer's insightful question. In response, we have incorporated the ubiquitination and proteasome data into Figure 2E-F in the revised manuscript to provide a comprehensive view of p39 degradation. Regarding the investigation of the E3 ligase, we attempted to knockdown PJA2 but did not observe a significant change in p39 protein levels. This suggests the involvement of another E3 ligase in p39 degradation. To explore this further, we are currently conducting co-immunoprecipitation (CO-IP) and proteomics studies. We anticipate that these additional experiments will shed light on the alternative E3 ligase involved in p39 degradation, and we look forward to sharing these findings in our future work.

• In Figure 4, the authors show S-nitrosylation of p39 (and Cdk5) in a mouse model of AD. These are important findings, but the experiments lack an important negative control: omission of ascorbate from the biotin-switch assay. When examining S-nitrosylated proteins engendered by endogenous NO, it is critical to include an ascorbate negative control in biotin-switch assay to verify that the bands are derived from S-nitrosylation rather than other redox modifications and also to prove that these

bands are not background staining in the assay. We acknowledge that the Authors do show an ascorbate control in Figure 1D, which is good, but it is also needed in Figure 4.

We appreciate the insightful suggestion from the reviewer. Indeed, we routinely perform the biotin switch assay both in the presence and absence of ascorbate to ensure the specificity of S-nitrosylation detection. Regrettably, due to space constraints, we did not include this control data previously. However, we have now rectified this omission by incorporating the negative control in supplementary Figure 2A of the revised manuscript. Thank you for bringing this to our attention, and we trust that this addition strengthens the robustness of our experimental approach.

• The Authors show that SNO-p39 levels found in the whole brain do not correlate with decreased expression of p39, but in the hippocampus, SNO-p39 levels correlate with decreased p39 expression. Why is this SNO-mediated degradation happening only in a certain brain region? The lack of mechanistic explanation for this region-specific effect lessens the significance of their finding.

We appreciate the reviewer's insightful question. Our approach initially involved conducting the biotin switch assay on whole brain samples to confirm the S-nitrosylation of p39 in vivo. Subsequently, we examined p39 protein levels in different brain regions. We observed a correlation between SNO-p39 and p39 protein levels in specific brain regions, likely influenced by the varying expression levels of nNOS across different brain regions. For instance, our data revealed high nNOS expression in the hippocampal region but not in the cortical region of the adult mouse brain (Figure 3 below). This observation suggests that the overproduction of NO might lead to significant p39 S-nitrosylation and subsequent degradation specifically in regions like the hippocampus. We have incorporated this explanation into the discussion section of the revised manuscript. Thank you for highlighting this aspect.

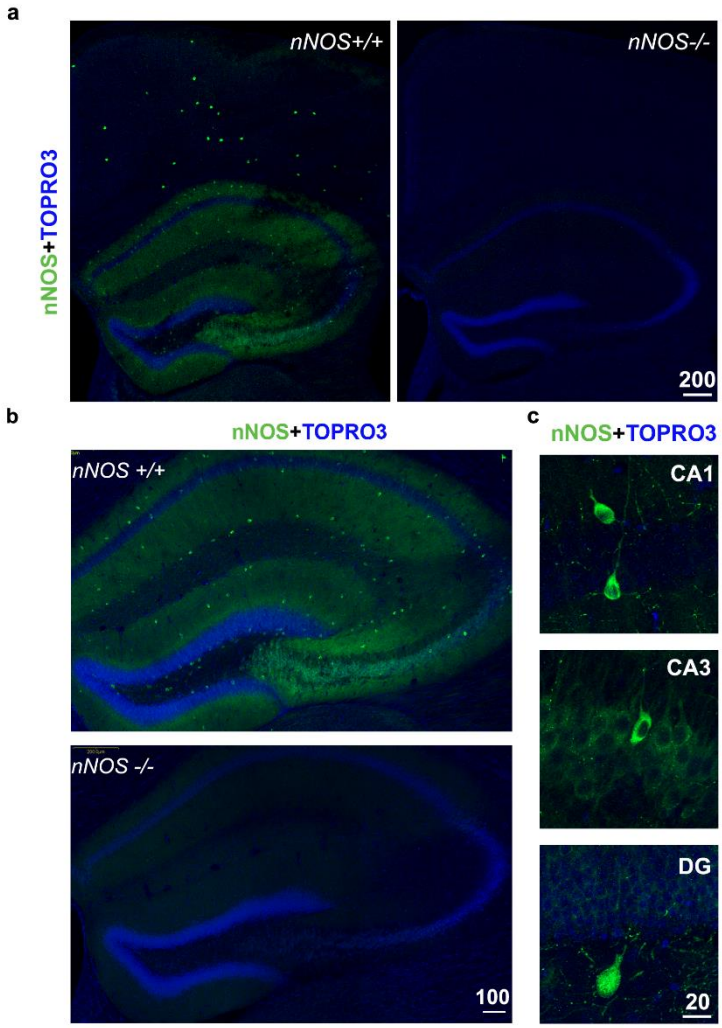

Figure 3. nNOS is widely expressed in the adult mouse hippocampus. (a) Representative micrographs of double-immunolabeling of TO-PRO-3 (blue, nuclear marker) and nNOS (green) in the coronal brain sections from WT and nNOS-KO mice. Scale bar: 200 μm. (b) Representative micrographs of double-immuno labeling with TO-PRO-3 (blue, nuclear marker) and nNOS (green) in the hippocampus of WT and nNOS-KO mice. Scale bar: 100 μm. (c) Representative micrographs of double-immunolabeling with TO-PRO-3 (blue, nuclear marker) and nNOS (green) in the CA1, CA3, and dentate gyrus in WT mice. Scale bar: 20 μm.

• In the experiments of Fig. 5, does L-NAME or another NOS antagonist block the effect of amyloid-beta on spine retraction? If not, another oxidation reaction (e.g., ROS causing -SOH, SO2/3H derivatization) could be involved rather than S-nitrosylation, and this would also be prevented in by a non-nitrosylatable/non-oxidizable mutant.

We appreciate the reviewer's insightful suggestion regarding L-NAME treatment experiments. Following this suggestion, we conducted experiments with L-NAME treatment to examine its effect on amyloid-beta-induced spine retraction. Encouragingly, our results demonstrated that L-NAME treatment effectively blocked the detrimental impact of amyloid-beta on spine retraction. We have included these

additional findings in supplementary Figure 4C-D of the revised manuscript. Thank you for this valuable input.

Minor concerns:
• The first "N" in "S-nitrosylation" should be capitalized when used as the first word in a sentence.
We apologize for the oversight and appreciate the reviewer for bringing these errors to our attention. We have rectified these mistakes in the revised manuscript. Thank you for highlighting these issues.

• In the legends for Figure 1 and 4, it is stated that "the biotinylated proteins were immunoprecipitated (IP) with neutravidin-agarose". Neutravidin pull down is not an immunoprecipitation.
We apologize for any confusion caused by the errors and appreciate the reviewer for bringing them to our attention. The corrections have been made in the legend of Figures 1 and 4 in the revised manuscript. Thank you for your attention to detail.

• In the Methods section, experimental procedures for the preparation of amyloid-beta oligomers are missing. The Authors should also describe how amyloid-beta oligomers were characterized before using them. Is the concentration of amyloid-beta listed in the figures and text the concentration of oligomers or the total concentration of amyloid-beta used?
Thank you for bringing this to our attention. We have now included the experimental procedures for the preparation of amyloid-beta oligomers in the methods section of the revised manuscript. We appreciate your thorough review and assistance in improving the clarity of our manuscript.

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors present compelling evidence demonstrating the significant role of S-nitrosylation of p39 at Cys265 in the process of amyloid-β (Aβ) peptide-induced dendrite retraction and spine loss. The manuscript proposes a promising hypothesis that addresses an important aspect of Alzheimer's disease research.
Before publication, the authors may consider addressing the following points:

1. The introduction section could be streamlined to focus more directly on the novel aspects of the study, avoiding excessive coverage of well-established concepts related to the activation of iNOS and its signaling pathways etc.
Thank you for your guidance. We have revised the introduction section in the manuscript to better highlight the novel aspects of our study. We appreciate your input and believe these changes will enhance the clarity and focus of our research.

2. While the discussion section effectively highlights the authors' findings, it could be

enhanced by integrating additional relevant studies from the existing literature, thereby providing a more comprehensive context for the interpretation of the results.

Thank you for your feedback. We have revised the discussion section in the manuscript to incorporate additional relevant studies, providing a more comprehensive interpretation of our results. We appreciate your valuable input and believe these changes will strengthen the discussion of our findings.

Overall, this well-written manuscript has the potential to contribute significantly to the current understanding of Alzheimer's disease pathology.

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1. Stamler, J.S., Toone, E.J., Lipton, S.A. & Sucher, N.J. (S)NO signals: Translocation, regulation, and a consensus motif. *Neuron* **18**, 691-696 (1997).
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4. Li, W.Q.*, et al.* p39 Is Responsible for Increasing Cdk5 Activity during Postnatal Neuron Differentiation and Governs Neuronal Network Formation and Epileptic Responses. *Journal of Neuroscience* **36**, 11283-11294 (2016).
5. Qu, J.*, et al.* S-Nitrosylation activates Cdk5 and contributes to synaptic spine loss induced by β-amyloid peptide. *P Natl Acad Sci USA* **108**, 14330-14335 (2011).
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8. Montero-Calle, A.*, et al.* Proteomics analysis of prefrontal cortex of Alzheimer's disease patients revealed dysregulated proteins in the disease and novel proteins associated with amyloid-β pathology. *Cell Mol Life Sci* **80**(2023).
9. Nakamura, T.*, et al.* Noncanonical transnitrosylation network contributes to synapse loss in Alzheimer's disease. *Science* **371**, 253-+ (2021).
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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have revised the manuscript considering all the reviewers' comments. The quality improved significantly and I recommend publication in its current form.

Reviewer #2 (Remarks to the Author):

Concerning Comments of Referee #1.
• Since cell lysates were used in many experiments, the Authors do NOT know if their mechanism of S-nitrosylation was enzymatic or non-enzymatic, and they should acknowledge this fact explicitly.

Concerning Comments of Referee #2
The revised manuscript submitted by Cheng et al. has addressed some of concerns from the original review; however, several critical issues persist as outlined below.

• Concerning Comment #1 of Referee #2, as well as the fourth major concern in the additional comments of the original review, regarding the mechanism for synaptic damage by the formation of SNO-p39 have not been sufficiently addressed. The authors failed to provide a mechanism explaining how decreased Cdk5 activity (according to their report, due to S-nitrosylation-triggered degradation of p39) contributes to dendritic spine loss. In contrast to the findings of the current study, it is widely accepted that increased Cdk5 activity mediates synaptic defects; hence, it is crucial to provide mechanistic insights into how SNO-p39 leads to dendritic spine loss.

Further to this point, the Authors claim that S-nitrosylation of Cdk5 and p39 inhibited its kinase activity but INCREASED kinase activity of Cdk5 in AD has been demonstrated by the team of LH Tsai (reviewed in https://doi.org/10.1016/j.molmed.2004.07.001). Hence, the Authors appear to be arguing that their findings are NOT pathologically relevant. This greatly detracts from the importance of the paper and has emerged as a real problem in publishing this manuscript.
Methodological issue – was pH tightly controlled in the Ck5 in vitro kinase activity assay, as pH is known to plummet with increasing concentrations of NO donors, and this can affect the activity assay as well.
How do the Authors know that S-nitrosylation specifically of p39 leads to its ubiquitination

and degradation – couldn't another protein also be S-nitrosylated in this experiment that affects p39 stability? How do the Authors know that their relatively large additions of GNSO are producing physiological NO levels and thus relevant results in their model systems? Along these lines, does inhibition of endogenous NOS protect p39 from degradation?
• Concerning comment #1 of Referee #2 on the in vivo analysis on dendritic spines, the new Figure 6 provided by the authors lacks a critical control (i.e., WT p39). Without a comparison of WT p39 and p39-C265A, it is not possible to assess the effects of SNO-p39 on dendritic spines. Thus, the new figure does not sufficiently address the concerns raised by this reviewer.

• Concerning comment #3 of Referee #2 of the additional major concerns on proteolytic cleavage of p39 to p29, the authors should present the results/data in the manuscript to demonstrate that GSNO exposure did not lead to p29 production (as they claim in their Response Letter) to support their premise that S-nitrosylation affects p39 degradation rather than its proteolytic cleavage.

• Regarding comment #7 of Referee #2 of the additional major concerns on p39 ubiquitination, the increase in ubiquitination of p39 in response to GSNO shown in the new figure is unclear or very subtle; hence, the new results do not sufficiently support their claim that NO decreases p39 levels via the ubiquitin-proteasome system.

SUMMARY
We have remaining concerns, especially concerning the association of decreased Cdk5 activity and synaptic impairment. The Authors claim this without providing any mechanistic explanation, and the finding flies in face of multiple other publications from the laboratories of Tsai, Lipton, and others who demonstrated the disease relevance of increased Cdk5 activity. There are also critical problems in the Authors' ubiquitination and in vivo experiments as well.

Reviewer #3 (Remarks to the Author):

Authors have replied to the comments of this reviewer properly and revised the manuscript adequately.

Reviewers' comments:

Reviewer #2 (Remarks to the Author):

Concerning Comments of Referee #1.

- Since cell lysates were used in many experiments, the Authors do NOT know if their mechanism of S-nitrosylation was enzymatic or non-enzymatic, and they should acknowledge this fact explicitly.

We appreciate the reviewer's helpful suggestion. We are also eager to understand the mechanism of S-nitrosylation and determine whether it is enzymatic or non-enzymatic. As suggested by the reviewer, we have included this discussion in the revised manuscript.

Concerning Comments of Referee #2

The revised manuscript submitted by Cheng et al. has addressed some of concerns from the original review; however, several critical issues persist as outlined below.

- Concerning Comment #1 of Referee #2, as well as the fourth major concern in the additional comments of the original review, regarding the mechanism for synaptic damage by the formation of SNO-p39 have not been sufficiently addressed. The authors failed to provide a mechanism explaining how decreased Cdk5 activity (according to their report, due to S-nitrosylation-triggered degradation of p39) contributes to dendritic spine loss. In contrast to the findings of the current study, it is widely accepted that increased Cdk5 activity mediates synaptic defects; hence, it is crucial to provide mechanistic insights into how SNO-p39 leads to dendritic spine loss.

We appreciate the reviewer's helpful suggestion. It has been reported that p39 is indispensable in postnatal neuronal differentiation and the formation of neuronal networks [1,2]. Additionally, various pathological factors such as Aβ production, neuronal hyper-excitability, and neuro-inflammation can trigger NO production, leading to protein S-nitrosylation. Consistently, we observed that treatment of cortical neurons with Aβ and NMDA resulted in p39 S-nitrosylation and subsequent degradation (see Supplementary Fig. 3E-J in the revised manuscript). Therefore, we propose that the overproduction of NO induced by these pathological conditions could contribute to dendritic and synaptic defects by promoting p39 S-nitrosylation and degradation. Furthermore, investigating downstream substrates of p39 S-nitrosylation involved in the regulation of dendritic spine loss in AD will be of significant interest. We have performed Mass Spectrometry and identified some promising candidates; we look forward to presenting these new findings in our next manuscript.

Further to this point, the Authors claim that S-nitrosylation of Cdk5 and p39 inhibited its kinase activity but INCREASED kinase activity of Cdk5 in AD has been demonstrated by the team of LH Tsai (reviewed in https://doi.org/10.1016/j.molmed.2004.07.001). Hence, the Authors appear to be arguing that their findings are NOT pathologically relevant. This greatly detracts from the importance of the paper and has emerged as a real problem in publishing this manuscript.

We thank the reviewer for raising this interesting question. Indeed, the role of Cdk5 in synaptic regulation is complex and still not fully understood. While increased Cdk5

activity has been widely implicated in synaptic dysfunction, recent proteomics analyses have suggested a downregulation of Cdk5 levels in AD patients[3]. Actually, Cdk5 can play either a positive or negative role in synapse functions; this is highly dependent on the phosphorylation of specific substrates. For example, the Cdk5-dependent phosphorylation of tropomyosin receptor kinase B (TrkB) in response to brain-derived neurotrophic factor is important for promoting the formation of dendritic spines in hippocampal neurons[4]. Regarding the inhibitory role of Cdk5 at synapses, Cdk5-dependent phosphorylation of the Rho guanine nucleotide exchange factor ephexin1 or the Wiskott–Aldrich syndrome protein family verprolin homologous protein 1 (WAVE1) reduces the number of dendritic spines through the modulation of actin dynamics[5].

We thank you for highlighting the study by LH Tsai's team (reviewed in https://doi.org/10.1016/j.molmed.2004.07.001), which demonstrates increased kinase activity of Cdk5 in Alzheimer's disease (AD). We acknowledge the importance of their findings in showing a context-specific increase in Cdk5 activity in AD pathology. It is indeed true that most studies have emphasized the role of Cdk5 in association with its cofactors p35 or p25, which influence its substrate specificity and activity. In our study, we have expanded upon this understanding by investigating the regulatory role of S-nitrosylation on p39, another crucial cofactor of Cdk5. Our findings suggest that S-nitrosylation-mediated degradation of p39 could contribute to synaptic spine loss under conditions of elevated β-amyloid peptide and nitric oxide levels. We believe our manuscript adds significant value by providing an additional layer of understanding to the complex role of Cdk5 in AD pathogenesis. By elucidating the regulatory mechanisms involving p39 and S-nitrosylation, we highlight a novel aspect of Cdk5 activity that complements existing research on p35/p25-associated functions. We have revised the manuscript to emphasize these points and discuss their implications in relation to the broader literature on Cdk5 in AD. We hope that our study will contribute to advancing the field's understanding of synaptic pathology mechanisms in AD. Thank you once again for your insightful comments and suggestions.

Methodological issue – was pH tightly controlled in the Ck5 in vitro kinase activity assay, as pH is known to plummet with increasing concentrations of NO donors, and this can affect the activity assay as well.

We thank the reviewer for raising this interesting question. We followed the protocol outlined in Lipton's paper and meticulously controlled pH during the *in vitro* kinase assay. Additionally, we included GSH as a control in the Cdk5 *in vitro* kinase assay, and our results demonstrate that GSH did not affect the assay outcomes.

How do the Authors know that S-nitrosylation specifically of p39 leads to its ubiquitination and degradation – couldn't another protein also be S-nitrosylated in this experiment that affects p39 stability? How do the Authors know that their relatively large additions of GNSO are producing physiological NO levels and thus relevant results in their model systems? Along these lines, does inhibition of endogenous NOS protect p39 from degradation?

We thank the reviewers for these insightful suggestions. We acknowledge the possibility that other S-nitrosylated proteins could potentially influence p39 stability. However, in our new Fig. 3C-F, we showed that GSNO treatment significantly accelerated the poly-ubiquitination and turnover of p39-WT, but not the S-nitrosylation-deficient mutant p39-C265A, suggesting that S-nitrosylation of p39 at Cys265

specifically leads to its ubiquitination and degradation.

Regarding the concentration of GSNO/SNOC used in our experiments, we treated cells with GSNO/SNOC at 100 μM, a concentration commonly used to mimic physiological and pathological conditions as reported in other studies[6,7].

Additionally, we appreciate the reviewer's interest in the inhibition of endogenous NOS experiments. Actually, we found that genetic deletion or pharmacological inhibition of endogenous NOS protects p39 from degradation. We have included these data in the new Fig. 2E-F in the revised manuscript.

- Concerning comment #1 of Referee #2 on the in vivo analysis on dendritic spines, the new Figure 6 provided by the authors lacks a critical control (i.e., WT p39). Without a comparison of WT p39 and p39-C265A, it is not possible to assess the effects of SNO-p39 on dendritic spines. Thus, the new figure does not sufficiently address the concerns raised by this reviewer.

We appreciate the reviewer's helpful suggestion, and we regret not presenting the WT-p39 data initially due to space constraints. However, we have previously conducted these experiments, and we have now included all relevant data in Fig. 6B-F of the revised manuscript.

- Concerning comment #3 of Referee #2 of the additional major concerns on proteolytic cleavage of p39 to p29, the authors should present the results/data in the manuscript to demonstrate that GSNO exposure did not lead to p29 production (as they claim in their Response Letter) to support their premise that S-nitrosylation affects p39 degradation rather than its proteolytic cleavage.

We appreciate the reviewer's valuable suggestion. Following this recommendation, we have included the new data in Fig. 2A-B and Fig. 2E-F of the revised manuscript.

- Regarding comment #7 of Referee #2 of the additional major concerns on p39 ubiquitination, the increase in ubiquitination of p39 in response to GSNO shown in the new figure is unclear or very subtle; hence, the new results do not sufficiently support their claim that NO decreases p39 levels via the ubiquitin-proteasome system.

We appreciate the reviewer's valuable suggestion. As recommended, we have included the new data in Fig. 3E of the revised manuscript.

SUMMARY

We have remaining concerns, especially concerning the association of decreased Cdk5 activity and synaptic impairment. The Authors claim this without providing any mechanistic explanation, and the finding flies in face of multiple other publications from the laboratories of Tsai, Lipton, and others who demonstrated the disease relevance of increased Cdk5 activity. There are also critical problems in the Authors' ubiquitination and in vivo experiments as well.

We would like to express our gratitude to the reviewers for their valuable feedback on our manuscript. We appreciate the thorough review process and the constructive comments provided by reviewer 2. However, we respectfully disagree with the reviewer's assessment that our findings conflict with those of multiple other publications from laboratories such as Tsai, Lipton, and others, which have

demonstrated the disease relevance of increased Cdk5 activity. As previously discussed, the role of Cdk5 in synaptic regulation is complex, and its impact can vary, potentially exerting both positive and negative effects on synapse function. This complexity is influenced by the phosphorylation of specific substrates, a process that may be modulated by the Cdk5/p35 or Cdk5/p39 complex.

Furthermore, we have carefully considered all suggestions regarding ubiquitination and in vivo experiments. We have made the necessary revisions to address the concerns raised by the reviewers, which we believe have significantly improved the quality and clarity of our manuscript.

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2. Li, W.Q., *et al.* p39 Is Responsible for Increasing Cdk5 Activity during Postnatal Neuron Differentiation and Governs Neuronal Network Formation and Epileptic Responses. *Journal of Neuroscience* **36**, 11283-11294 (2016).
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4. Lai, K.O., *et al.* TrkB phosphorylation by Cdk5 is required for activity-dependent structural plasticity and spatial memory. *Nature Neuroscience* **15**, 1506-1515 (2012).
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6. Qu, J., *et al.* S-Nitrosylation activates Cdk5 and contributes to synaptic spine loss induced by β-amyloid peptide. *P Natl Acad Sci USA* **108**, 14330-14335 (2011).
7. Nakamura, T., *et al.* Noncanonical transnitrosylation network contributes to synapse loss in Alzheimer's disease. *Science* **371**, 253-+ (2021).

Reviewers' comments:

Reviewer #2 (Remarks to the Author):

Cheng et al. (the “Authors”) have submitted a further revised manuscript (revision #2). The Authors have successfully presented essential control datasets for in vivo dendritic spine analysis and provided improved images for their ubiquitination assay. Additionally, the Authors have now included new data showing that inhibition of endogenous NOS prevents p39 degradation. These new results significantly improve the quality of the manuscript. However, the rationale for studying decreased Cdk5 activity (resulting from SNO-p39) in synaptic dysfunction in an (Alzheimer’s disease) AD context is still weak, in contrast to the well-accepted roles of increased CDk5 activity in AD (the opposite of what the Authors claim to have found here). The manuscript also lacks mechanistic insight into how SNO-p39 contributes to synaptic dysfunction. Concerning these issues, the Authors discuss the fact that Cdk5 can exert both positive and negative effects on synaptic function depending on the phosphorylation state of its substrate proteins. However, as far as we are aware, these properties are observed under basal physiological conditions, and there is little evidence supporting the premise that decreased Cdk5 activity contributes to loss of dendritic spines under pathological conditions such as in AD. Hence, the pathophysiological relevance of their findings remains questionable. If there is solid published evidence clearly indicating that decreased Cdk5 activity contributes to synaptic impairment in AD, then such studies should be explicitly discussed and referenced in this manuscript. Otherwise, the Reviewers are concerned that the findings may still represent a possible tissue culture artifact not relevant to the disease process.

Minor comment
Regarding the description of new Fig. 2F, it is stated that “we treated cultured cortical neurons with the nNOS inhibitor, L-NAME”; however, L-NAME is a non-selective inhibitor of NOSs and not specific to nNOS. Hence, this sentence is misleading and merits correction.

We would like to express our gratitude to the reviewers for their valuable feedback on our manuscript. We appreciate the thorough review process and the constructive comments provided by reviewer 2. We have carefully considered all suggestions and made the necessary revisions to address the concerns raised by the reviewers, which we believe have significantly improved the quality and clarity of our manuscript.

Reviewers' comments:

Reviewer #2 (Remarks to the Author):

Cheng et al. (the "Authors") have submitted a further revised manuscript (revision #2). The Authors have successfully presented essential control datasets for in vivo dendritic spine analysis and provided improved images for their ubiquitination assay. Additionally, the Authors have now included new data showing that inhibition of endogenous NOS prevents p39 degradation. These new results significantly improve the quality of the manuscript.
However, the rationale for studying decreased Cdk5 activity (resulting from SNO-p39) in synaptic dysfunction in an (Alzheimer's disease) AD context is still weak, in contrast to the well-accepted roles of increased CDk5 activity in AD (the opposite of what the Authors claim to have found here). The manuscript also lacks mechanistic insight into how SNO-p39 contributes to synaptic dysfunction. Concerning these issues, the Authors discuss the fact that Cdk5 can exert both positive and negative effects on synaptic function depending on the phosphorylation state of its substrate proteins. However, as far as we are aware, these properties are observed under basal physiological conditions, and there is little evidence supporting the premise that decreased Cdk5 activity contributes to loss of dendritic spines under pathological conditions such as in AD. Hence, the pathophysiological relevance of their findings remains questionable. If there is solid published evidence clearly indicating that decreased Cdk5 activity contributes to synaptic impairment in AD, then such studies should be explicitly discussed and referenced in this manuscript. Otherwise, the Reviewers are concerned that the findings may still represent a possible tissue culture artifact not relevant to the disease process.

We thank the reviewer for their helpful suggestion. In response, we have included the published evidence clearly indicating decreased Cdk5 activity in AD pathogenesis in the revised discussion section of our manuscript.

Minor comment
Regarding the description of new Fig. 2F, it is stated that "we treated cultured cortical neurons with the nNOS inhibitor, L-NAME"; however, L-NAME is a non-selective inhibitor of NOSs and not specific to nNOS. Hence, this sentence is misleading and merits correction.

We thank the reviewer for pointing this out. In response, we have revised the sentence

in the updated manuscript as suggested.