

Nonapoptotic caspase-3 guides C1q-dependent synaptic phagocytosis by microglia

Corresponding Author: Professor Ryuta Koyama

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)

In the presented manuscript Andoh and co-authors report their study of the neuronal-activity-dependent microglial synaptic phagocytosis. Using co-culture system, the authors can observe ramified-microglial phagocytosis without axonal shearing. The study reveals that intense increases in neuronal activity trigger presynaptic caspase-3 activation, subsequently promoting C1q tagging and microglial phagocytosis. Furthermore, employing a febrile seizure model, the authors demonstrate that microglial engulfment of inhibitory synapses in the hippocampus in vivo occurs through a molecular mechanism dependent on caspase-3 activation. The study contributes novel insights into the molecular connections between changes in neuronal activity and microglial phagocytosis, thereby deepening our understanding of a crucial issue in neuroscience: the molecular mechanisms of neural circuit coordination by microglia. The results and experimental system presented are deemed valuable to the field of neuronal and glial biology and are anticipated to guide future experiments. I included suggestions below that I believe will strengthen this study.

1. Given the intense nature of the stimuli inducing microglial phagocytosis in both the culture and in vivo models, it remains uncertain whether they accurately mirror microglial phagocytosis under more near-normal conditions. Furthermore, while responses to very strong stimuli were similar, the extent to which the characteristics of cultured microglia faithfully represent those of microglia in vivo remains unclear. In the co-culture system, it is essential to assess not only cell morphology but also other aspects, such as gene expression profiles, to facilitate meaningful comparisons with in vivo studies.
2. CNO treatment appears to change synaptic structure and distribution (Fig.1j, S2b). It is needed to investigate whether CNO treatment disrupts synaptic density and molecular accumulation, providing a comprehensive understanding of the potential effects of this treatment on cellular states.
3. Fig. S1h,j suggests that phagocytosis may occur due to increased neuronal activity in the absence of intense stimulation. Does decreased activity with Gi-DREADD decrease cleaved-caspase-3 staining intensity?
4. Considering the proposed spatial relationship between the accumulation of mitochondria in synapses and caspase-3 activation due to CNO treatment, it would be beneficial to assess the correlation between the presence or absence of cleaved caspase-3 staining signals and the proximity of mitochondria to presynapses after CNO treatment. This analysis will help evaluate the model suggesting that the region where caspase-3 activation occurs is the synaptic region.
5. In the culture system, the phagocytosis of the presynaptic structure was observed with CNO + C1qA. It is essential to discern whether this involves the incorporation of synaptophysin clusters that do not form synapses or if it encompasses both pre and postsynaptic structures. To address this, staining for postsynaptic markers will be conducted to assess the likelihood of either scenario.
6. In the culture system, the addition of C1qA is necessary. Is this requirement attributed to reduced production or secretion of C1qA in cultured microglia?
7. Fig. 1a-d. At what time point after CNO treatment were the cells fixed?
8. Line 75. Cite the research articles which report the activity-dependent accumulation of mitochondria at the presynapse,

not postsynapse (ref14).

9. Fig2e. There seems to be a labeling error.

10. Fig2f-i. At what time point after CNO treatment did the imaging begin?

11. Fig 4e and 5e. In Fig. 4e, it is observed that cleaved caspase-3 puncta are located at a portion of VGAT clusters. In Fig. 5e, the majority of VGAT clusters colocalize with cleaved caspase-3 signals. In addition, there are also many instances of non-colocalized caspase-3 signals. Could you clarify the differences between these two experiments?

Reviewer #2

(Remarks to the Author)

Short Summary

The present work by Andoh and others aimed to elucidate the role of presynaptic caspase-3 in microglial-induced phagocytosis of synapses. The authors developed and used an in vitro live imaging system and combined it with molecular and pharmacological tools. They tested the hypothesis that local neuronal activity mediates non-apoptotic caspase signaling that leads to selective microglial phagocytosis of synapses. Lastly, they used a mouse model of febrile seizures to validate the relevancy of this novel pathway. Mechanistically, the authors found that presynaptic caspase activity correlated with recombinant C1qa complement deposition and subsequent phagocytosis of the labeled compartment. Furthermore, this study demonstrated that excessive neuronal activity either by DREADDs (hM3Dq) or febrile seizure induction is sufficient to increase presynaptic caspase activity and subsequent complement labeling and phagocytosis by microglia. The results presented in this study reveal a novel non-apoptotic role of caspases in presynaptic microglial phagocytosis.

Overall Assessment

This study pursues a very relevant topic for the field: how does neuronal activity lead to the loss of only a subset of synapses? While the mechanistic work presented hereby is exciting, the authors should conduct several experiments to convincingly validate the suggested mechanism of action and address the major concerns listed below.

Major concerns:

1. The authors find that hM3Dq mediated over activation of neurons in culture is sufficient to activate caspase activity and lead to complement labeling and subsequent engulfment of specific synaptic compartments. However, this finding is counter to previous findings in the field. As the authors themselves cite, previous work in the dLGN found that lower neuronal activity correlated with a higher likelihood of synaptic engulfment by microglia. In light of these opposing findings and the availability of inhibitory DREADDs (hM4Di), this reviewer recommends conducting the inhibitory DREADDs experiment as a means to reconcile these opposing findings. Moreover, the authors seem to have the ideal system to quickly screen for synapse engulfment following DREADD mediated inhibition of neuronal activity.
2. Work in the field has implicated phosphatidyl-serine exposure in synaptic membranes as a potential mechanism to restrict complement labeling to specific synapses. While the authors mention this hypothesis in their discussion, this is not addressed in their experiments. There are many widely available tools to label PS both in vitro and in vivo which the authors could make use to address whether PS exposure is also being regulated by caspase activity.
3. A more specific and thorough validation of mSCAT3 is needed. The authors use DEVD as a caspase-3 substrate and activator. However, several studies have shown that this sequence is not specific to caspase-3 (see PMID: 17975551, PMID: 17117159, PMID: 18976637) as it can also cleave caspase-7. The authors should perform immunostaining and western blot against full and cleaved-caspase-7 to prove the specificity of mSCAT3.
4. The specificity regarding the role of caspase-3 in promoting c1q deposition is questionable. Most of the in vitro assessment of caspase-3 use Z-DEVD-FMK as a caspase-3 inhibitor. However, this inhibitor is known to be a non-specific, "pan-caspase-inhibitor" that also targets caspase-7 (see PMID: 17117159, PMID: 18976637). Since the authors then used Bax channel blocker and show that activation of caspase-9 is upstream of caspase-3, we suggest using a specific caspase-9 inhibitor such as XBIR-3. Another possibility would be to show that the expression of other caspases (particularly caspase-7) is not affected by the used inhibitor. Linked to this, the authors should blot against the cleaved-caspase-3 fraction to validate that caspase-3 is activated.
5. To strengthen the claim that non-apoptotic caspase-3 activity in hyperactive neurons is necessary to induce microglial synaptic phagocytosis, the authors should consider performing a more specific in vivo experiment. This reviewer recommends using either an inhibitory neuronal Cre line or an inhibitory neuron targeting Cre-virus combined with a caspase9 flx/flx mouse line and employ the febrile seizure model.

Minor Concerns:

1. The authors tie activation of caspase-3 to having non-apoptotic roles based on qualitative assessment of the neuronal nuclear morphology, however they should consider performing a more thorough assessment of this such as a TUNEL staining. Changes in nuclear morphology can occur at later stages in the apoptotic process.
2. The authors are testing the role of caspase-3 "nonlethal" activity, however, since caspase-3 seems to be activated by caspase-9 (initiator of the apoptotic death pathway), they should consider referring to this as a non-apoptotic caspase-3 function as nonlethal can be misleading and further assessments for other types of cell death should be tested to make this

claim.

3. The statistical N represented by p-values in several figures is not fully clear. Please include a more detailed description in the figure legends of statistical analysis. For, Figure 1 panel b, Figure 2 panel b, Figure 3 panel b, Supplementary Figure 2 e, Supplementary Figure 3 d, and Supplementary Figure 5 b and c, it is unclear whether these images/cells were collected across multiple experiments or are all from a single experiment.

4. All graphs with quantifications should have visible error bars.

5. Authors should consider quantifying if the number of phagocytosed synapses in Fig 2i changes with the administration of DEVD inhibitor.

6. Fig 3a diagram should also include that caspase-9 cleaves caspase-7. Figure 3b should also include cleaved caspase 9 staining. Figure 3 studies should include a specific inhibitor of caspase-9.

7. Figure 4C needs clarification on the statistical comparisons. Having representative images of VAGT and VGLUT1 in the same colors is confusing, consider changing to different colors.

8. Figure 5. To further establish that c1q is a potential cleaved-caspase-3 substrate, the authors should consider performing triple colocalization of VGAT, c 1q, and cl-caspase-3. Another alternative would be to perform an IP.

9. Representative images of synapse staining in Figure 6 g and h could benefit from separating the channels. Right now the images for Figure 6 g are too dense to clearly see the pre- and post-synaptic puncta.

Reviewer #3

(Remarks to the Author)

This work from Ando et al. applies multicolor time-lapse imaging with new optical tools to clarify the upstream events that lead to phagocytosis of presynaptic components in vitro. They also provided in vivo evidence of similar presynapse-specific elimination in the hippocampal dentate gyrus triggered by febrile seizures. The main findings in vitro include local presynaptic cleavage of caspase-3 after neuronal activity increase by DREADD, enhanced phagocytosis of presynaptic components by the combination of neuronal activity increase and exogenous complement component, C1q, and increase in exogenous C1q binding and microglial C3 receptor accumulation after local activation of caspase-3 by optogenetics. These results collectively indicate the presence of activity-dependent mechanisms of synapse phagocytosis by microglia mediated by local caspase-3 activation and complement-dependent recognition of target synapses. In the second half of the manuscript, Ando et al. extended the in vitro finding by setting up the model of synapse elimination in vivo triggered by febrile seizures. The main findings in this part show the tagging of inhibitory synapses by C1q in the dentate gyrus after seizure induction, followed by inhibitory synapse loss, without affecting the density of excitatory synaptic components. These in vivo experiments support the idea that activity-induced synaptic tagging selectively induces the reduction of specific presynaptic components, which will impact the overall function of the local circuits.

Activity-dependent remodeling of neuronal circuits has long been thought to be regulated by the signaling mechanisms confined to pre- and postsynaptic components. Recently, active roles of glial cells and related signaling pathways have been proposed, but the experimental evidence of the causal relationship between the neuronal signaling triggered by circuit activity and glial response is far from satisfactory. Therefore, this manuscript provides important new information for the field and has broad implications for understanding the complex spatiotemporal interactions between synapses and microglia in neural circuit remodeling. This manuscript presents two novel and significant findings (first, microglial synapse elimination triggered by neuronal activity and local caspase-3 activation; second, in vivo C1q-dependent inhibitory synapse elimination triggered by febrile seizures) as well as the introduction of new optical tools for monitoring and manipulating caspase-3 activity. As such, this manuscript has a potentially high impact after addressing the following points.

Major points

1. The most important remaining question is the link between caspase-3 activation and the binding of C1q on the surface of the presynaptic membrane. In the discussion, the authors proposed the involvement of phosphatidylserine externalization by caspase-3. Along this line, it is essential to know if surface phosphatidylserine increases after CNO treatment or photoactivation of CRY2-casp9. This can be tested using fluorescent probes detecting surface phosphatidylserine, such as AnnexinV or PSVue, in combination with the techniques of caspase-3 activation.

2. The authors reported the synaptic events triggered by the photoactivation of CRY2-casp9. These include accumulation of C1qA and an increase in microglial CR3 near the site of phagocytosis. It is likely that local photoactivation of CRY2-casp9 induces an increase in phagocytotic events per se. This data should be presented.

3. In the analysis of synapse elimination in the dentate gyrus after febrile seizures, the provided evidence supports the idea that complement tagging is involved in inhibitory synapse elimination. However, it is theoretically possible that, instead of microglia, astrocytes are the major players in complement-dependent synapse elimination. To clarify this point, the elimination of microglia by CSF1 inhibitor or related technologies in combination with seizure induction may be informative. Alternatively, the authors can measure the number of phagocytosed synaptic components within astrocytes using high-resolution tissue imaging.

4. Synaptophysin-mSCAT3 is useful in monitoring caspase-3 activity in axons. However, the design of this probe should be more clearly explained in the manuscript. Is mSCAT3 attached to the C-terminal cytoplasmic tail of synaptophysin? If so, after caspase-3-dependent cleavage of mSCAT3, ECFP remains on the synaptic vesicles while Venus is released. Do the authors observe this escape of Venus from synapses after caspase-3 activation? This may suggest some problems in the interpretation of FRET measurement, as the concentration ratio of ECFP and Venus in the presynaptic compartment is altered. In other words, it is possible that non-FRET probes attaching two fluorescent proteins via DEVD sequence show the

fluorescence ratio change similar to synaptophysin-mSCAT3. This point should be clarified.

Minor points

1. In this manuscript, “presynapse” refers to the axonal structure positive with fluorescent protein-tagged synaptophysin. It is well known that axons without any postsynaptic structures form many synaptic vesicle clusters in culture preparations. It is safe to use the word “axonal boutons” instead of “presynapses” when referring to the puncta positive with synaptophysin. Otherwise, the presence of postsynaptic markers (PSD-95) or active zone markers (bassoon) should be presented in several key experiments.
2. Figure 2g shows the important data supporting synapse elimination without axonal shearing. However, the images are either multicolor or after surface rendering, and it is difficult to identify the objects of interest. Side-by-side presentation of single-color raw images of mCherry and iRFP with high magnification will be more convincing.
3. The density of C1q puncta 6 h after seizure is 200,000 per square mm in Figure 5b. On the other hand, the density of C1q positive VGAT puncta is 1,000 per square mm in Figure 5i. A simple calculation suggests that only 0.5% of C1q puncta is associated with VGAT puncta. Is this true? If so, the remaining 99.5% of C1q puncta should be in contact with other components. This point should be discussed.
4. In Figure 1g, the inset shows the pixels with the highest and lowest values of the CFP/Venus ratio. It is not clear why this inset is necessary. Explanations should be provided.
5. In Figure 3h, the label “CX3CR1-tdTomato/Synaptophysin-mCherry” needs explanation. Why are the images of two fluorescent proteins combined?
6. Supplementary Figure 3d presents the ratiometric values without the values before CNO addition. If the design is the comparison between pre- and 6 h after treatment (as in the case shown in Figure 1i and k), a similar presentation is desirable.
7. In image analysis methods, many quantitative analyses are done after surface rendering. However, the surface rendering depends on the preceding binarization of the intensity data and should affect the subsequent analysis. The criteria for image binarization and surface rendering should be more precisely described.

Reviewer #4

(Remarks to the Author)

This is an elegant study with a logical succession of experiments to confirm the hypothesis that activity-dependent caspase-3 activation at presynapses guides complement-dependent microglial synaptic phagocytosis. Beautiful live imaging in cell cultures.

Globally the conclusions are supported by the data presented. The authors used live imaging in cultures, state-of-the-art technologies such as chemogenetics and optogenetics, and ended with some in vivo experiments.

What I do not understand regarding the viral vector strategy used for compiling figures 1 and 2, is that the authors do not use a control vector (i.e. AAV-synaptophysin-mCherry vector without GqDREADD construct; a vector used by the authors in their first validation experiments in supplement?) for the DREADD experiments? The most ideal way to compare the groups is a test group in which GqDREADD is expressed and CNO is used as the DREADD ligand, and a control group in which a viral vector without DREADD has been given but in which CNO is also administered. Now, the authors use GqDREADD-transduced neurons for both groups, with CNO in the test group and DMSO in the control group. In itself not a problem provided an additional control experiment is done with CNO alone to exclude off target effects of the DREADD agonist itself. Was DMSO also the solvent for CNO in the treatment group?

On page 11 and figure 4 the authors show that a caspase-3 inhibitor 15 minutes prior to febrile seizure induction reduced colocalization of inhibitory presynapses with C1q or C3 to baseline levels. Did the caspase-3 inhibitor also affected the seizures themselves? In other words, is the observed effect on presynaptic tagging by complement the effect of caspase-3 inhibition or the result of the lack of seizures?

Figure 6 panel 0: Were the experimenters blinded to the treatment groups? Especially in the behavioural scoring according to Racine's scale for mice receiving a single kainic acid injection. Which mouse behaviours were exactly scored? In my experience, the behavioural stages in mice following ip injection of kainic acid are not always easy to disentangle and there is also a lot of variation after systemic kainic injection. How robust was your model and read-out? The paper you refer to from Racine is about the scoring of electrical evoked seizures in amygdala or hippocampus in rats.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have responded to my queries either with additional experiments or by responding to the comments. I am fully satisfied with their responses.

Reviewer #2

(Remarks to the Author)

This reviewer thanks the authors for addressing all of our concerns thoroughly. Below are detailed responses to the authors regarding this reviewer's remaining concerns. Following minor edits, this reviewer recommends the work for publication.

Remaining concerns:

- This reviewer recommends adding representative images for supplementary figure 9 showing the reduced phagocytosis of synaptophysin-mCherry.
- The addition of representative images for the caspase-3 in response to inhibitory DREADDs would also strengthen supplementary figure 9.
- This reviewer recommends using ANOVA followed by a Sidak posthoc test for Figure 2E instead of the Mann-Whitney Rank Sum test. Mann-Whitney is unsuitable for this analysis because it does not account for multiple comparisons.
- Figure 8A legend states there should be arrowheads, but the arrowheads are missing in the image.
- This reviewer agrees that using caspase-9 flox/flox mice would not address the specificity of caspase-3's role in synaptic phagocytosis. However, this reviewer recommends emphasizing the non-apoptotic portion of caspase-3 in the discussion, i.e., expanding on the discussion point of how synaptic caspase-3 differs from apoptotic caspase-3 in the soma.

Reviewer #3

(Remarks to the Author)

The authors addressed my concerns by providing additional data and appropriately modifying the text in the revised manuscript. I support the publication of the manuscript after correcting the minor points listed below.

1. Figure 6g: Upper three images for Control and lower three images for FSs 24h, as in the case of Figure 6i? Labels should be added, together with scale bars.
2. Supplementary Fig. 7a: Labels for DMSO, CNO, and CNO-DEVD are missing.

Reviewer #4

(Remarks to the Author)

The authors have taken great effort to conduct additional experiments according to the suggestions of the various referees and incorporate them in the new version of the manuscript. The authors have also adequately answered my specific comments.

With regard to the additional data in Suppl Fig3d with CNO alone (no hM3Dq), these data points have a huge variation and are therefore not so convincing that CNO is completely inert in this experiment, but as the authors point out, the mean values in this CNO/no-hM3Dq group are not significantly different from those in the CNO/hM3Dq group, and so there is no effect.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors satisfactorily addressed my remaining minor concerns.

Reviewer #3

(Remarks to the Author)

The authors made appropriate corrections to the figures. I am satisfied with the current version of the manuscript.

Reviewer #4

(Remarks to the Author)

Dear editor, I have no further comments on the manuscript.

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Response to reviews

Comments and Answers

We are grateful to the editors and reviewers for their valuable comments and for this opportunity to improve our paper. Herein, we provide a point-by-point response to the reviewers' comments that we believe thoroughly address their concerns. The revised parts are shown in blue in the main text.

Reviewer #1 (Remarks to the Author):

General Comments:

In the presented manuscript Andoh and co-authors report their study of the neuronal-activity-dependent microglial synaptic phagocytosis. Using co-culture system, the authors can observe ramified-microglial phagocytosis without axonal shearing. The study reveals that intense increases in neuronal activity trigger presynaptic caspase-3 activation, subsequently promoting C1q tagging and microglial phagocytosis. Furthermore, employing a febrile seizure model, the authors demonstrate that microglial engulfment of inhibitory synapses in the hippocampus in vivo occurs through a molecular mechanism dependent on caspase-3 activation. The study contributes novel insights into the molecular connections between changes in neuronal activity and microglial phagocytosis, thereby deepening our understanding of a crucial issue in neuroscience: the molecular mechanisms of neural circuit coordination by microglia. The results and experimental system presented are deemed valuable to the field of neuronal and glial biology and are anticipated to guide future experiments. I included suggestions below that I believe will strengthen this study.

Answer: We thank the reviewer for the overall positive comments, and for the relevant and constructive suggestions.

27

28 **Comment 1:** Given the intense nature of the stimuli inducing microglial phagocytosis in both
29 the culture and in vivo models, it remains uncertain whether they accurately mirror microglial
30 phagocytosis under more near-normal conditions. Furthermore, while responses to very
31 strong stimuli were similar, the extent to which the characteristics of cultured microglia
32 faithfully represent those of microglia in vivo remains unclear. In the co-culture system, it is
33 essential to assess not only cell morphology but also other aspects, such as gene expression
34 profiles, to facilitate meaningful comparisons with in vivo studies.

35

36 **Answer 1:** We fully agree with the reviewer, and we have conducted RT-qPCR to assess the
37 gene expression profiles of microglia in the co-culture system. We confirmed that cultured
38 microglia express microglia-related genes, including homeostatic genes, but not monocyte-
39 related genes (lines 68–70, Supplementary Fig. 1c).

40

41 **Comment 2:** CNO treatment appears to change synaptic structure and distribution (Fig.1j,
42 S2b). It is needed to investigate whether CNO treatment disrupts synaptic density and
43 molecular accumulation, providing a comprehensive understanding of the potential effects of
44 this treatment on cellular states.

45

46 **Answer 2:** We thank the reviewer for raising this important point. We have assessed synaptic
47 densities and areas by immunocytochemistry and confirmed that CNO treatment does not
48 affect these parameters (lines 138–141, Supplementary Fig. 5).

49

50 **Comment 3:** Fig. S1h.j suggests that phagocytosis may occur due to increased neuronal
51 activity in the absence of intense stimulation. Does decreased activity with Gi-DREADD
52 decrease cleaved-caspase-3 staining intensity?

53

54 **Answer 3:** This is a very important point, and we have introduced hM4Di to decrease
55 neuronal activity. We have confirmed that decreased activity does not significantly decrease
56 synaptic caspase activity compared to the control group through real-time imaging of
57 synaptophysin-mSCAT3 (lines 201–203, Supplementary Fig. 9c).

58

59 **Comment 4:** Considering the proposed spatial relationship between the accumulation of
60 mitochondria in synapses and caspase-3 activation due to CNO treatment, it would be
61 beneficial to assess the correlation between the presence or absence of cleaved caspase-3
62 staining signals and the proximity of mitochondria to presynapses after CNO treatment. This
63 analysis will help evaluate the model suggesting that the region where caspase-3 activation
64 occurs is the synaptic region.

65

66 **Answer 4:** We agree with the reviewer and have assessed the correlation between
67 mitochondrial accumulation and caspase activity at presynapses by real-time imaging of mito-
68 DsRed and synaptophysin-mSCAT3. Our analysis indicates a positive correlation between
69 mitochondrial accumulation and caspase activity at presynapses (lines 132–135,
70 Supplementary Fig. 4).

71

72 **Comment 5:** In the culture system, the phagocytosis of the presynaptic structure was
73 observed with CNO + C1qA. It is essential to discern whether this involves the incorporation
74 of synaptophysin clusters that do not form synapses or if it encompasses both pre and
75 postsynaptic structures. To address this, staining for postsynaptic markers will be conducted
76 to assess the likelihood of either scenario.

77

78 **Answer 5:** We completely agree with the reviewer. However, it is challenging to investigate

whether phagocytosed synaptophysin-mCherry puncta formed synapses by immunostaining after live imaging due to the positional changes of neurites caused by astrocytic movement. Therefore, we examined the colocalization of postsynaptic markers with synaptophysin-mCherry that remained on the neuronal side after partial phagocytosis (trogocytosis) of synaptophysin-mCherry.

Our results show that 80% of the remaining synaptophysin-mCherry puncta are colocalized with excitatory or inhibitory postsynapses (lines 263–265, Supplementary Fig. 11a, b). This finding is consistent with additional data indicating that about 85% of all synaptophysin-mCherry in the field of view formed synapses (lines 265–267, Supplementary Fig. 11c, d). Furthermore, we confirmed that postsynaptic markers are not incorporated into microglia after real-time imaging of synaptophysin-mCherry phagocytosis (lines 267–269, Supplementary Fig. 11e, f).

These results suggest that almost all mCherry-labeled synaptophysin puncta were paired with postsynapses before phagocytosis and that microglia selectively trogocytose these presynaptic puncta from such synapses.

Comment 6: In the culture system, the addition of C1qA is necessary. Is this requirement attributed to reduced production or secretion of C1qA in cultured microglia?

Answer 6: We thank the reviewer for this insightful question. We have quantified C1q in microglia by immunocytochemistry. Our results indicate that the addition of C1qA does not increase the C1q area in microglia, suggesting that C1q production is not promoted (naïve: 20.683% of microglial area, C1qA: 20.615% of microglial area, median, n = 55, 57 cells, P > 0.05, Mann-Whitney Rank Sum Test). Based on these findings, we hypothesize that the requirement for exogenous C1qA may be due to reduced secretion of C1q in cultured microglia.

105

106 **Comment 7:** Fig. 1a-d. At what time point after CNO treatment were the cells fixed?

107

108 **Answer 7:** We thank the reviewer for pointing out this point. The cells were fixed 6 hours
109 after CNO treatment. We have included this information both in figure 1 panels and legends
110 (lines 575–588).

111

112 **Comment 8:** Line 75. Cite the research articles which report the activity-dependent
113 accumulation of mitochondria at the presynapse, not postsynapse (ref14).

114

115 **Answer 8:** We thank the reviewer for raising this important point. We have replaced ref14
116 with Vaccaro et al., EMBO reports, 2017 (lines 480–482).

117

118 **Comment 9:** Fig2e. There seems to be a labeling error.

119

120 **Answer 9:** We thank the reviewer for identifying this error, and we have corrected it in the
121 revised manuscript (Fig. 2e).

122

123 **Comment 10:** Fig2f-i. At what time point after CNO treatment did the imaging begin?

124

125 **Answer 10:** We thank the reviewer for raising this point. We started the 2-hour real-time
126 imaging 30 minutes after CNO treatment. We have added this information to the experimental
127 paradigm in Fig. 2h.

128

129 **Comment 11:** Fig 4e and 5e. In Fig. 4e, it is observed that cleaved caspase-3 puncta are
130 located at a portion of VGAT clusters. In Fig. 5e, the majority of VGAT clusters colocalize with

cleaved caspase-3 signals. In addition, there are also many instances of non-colocalized caspase-3 signals. Could you clarify the differences between these two experiments?

Answer 11: We appreciate the reviewer's comment. In these experiments, there was a difference in experimental conditions between Fig. 4 and Fig. 5 that was not fully detailed in the initial submission. Specifically, PSVue was injected into the dentate hilus in the experiments corresponding to Fig. 5 (we have included PSVue signals in this revision, which had been prepared in the initial manuscript but not presented). We hypothesize that seizures increase nonspecific labeling by PSVue, which further induces nonspecific binding by the cleaved caspase-3 antibody. This difference in experimental conditions likely accounts for the observed variation in caspase-3 signal between Fig. 4 and Fig. 5.

Reviewer #2 (Remarks to the Author):

Short Summary:

The present work by Andoh and others aimed to elucidate the role of presynaptic caspase-3 in microglial-induced phagocytosis of synapses. The authors developed and used an in vitro live imaging system and combined it with molecular and pharmacological tools. They tested the hypothesis that local neuronal activity mediates non-apoptotic caspase signaling that leads to selective microglial phagocytosis of synapses. Lastly, they used a mouse model of febrile seizures to validate the relevancy of this novel pathway. Mechanistically, the authors found that presynaptic caspase activity correlated with recombinant C1qa complement deposition and subsequent phagocytosis of the labeled compartment. Furthermore, this study demonstrated that excessive neuronal activity either by DREADDs (hM3Dq) or febrile seizure induction is sufficient to increase presynaptic caspase activity and subsequent complement labeling and phagocytosis by microglia. The results presented in this study reveal a novel non-apoptotic role of caspases in presynaptic microglial phagocytosis.

Answer: We thank the reviewer for the overall positive feedback and for the relevant comments and suggestions.

Overall Assessment:

This study pursues a very relevant topic for the field: how does neuronal activity lead to the loss of only a subset of synapses? While the mechanistic work presented hereby is exciting, the authors should conduct several experiments to convincingly validate the suggested mechanism of action and address the major concerns listed below.

Answer: We thank the reviewer for the constructive suggestions.

173 **Major concerns:**

174 **Comment 1:** The authors find that hM3Dq mediated over activation of neurons in culture is
175 sufficient to activate caspase activity and lead to complement labeling and subsequent
176 engulfment of specific synaptic compartments. However, this finding is counter to previous
177 findings in the field. As the authors themselves cite, previous work in the dLGN found that
178 lower neuronal activity correlated with a higher likelihood of synaptic engulfment by microglia.
179 In light of these opposing findings and the availability of inhibitory DREADDs (hM4Di), this
180 reviewer recommends conducting the inhibitory DREADDs experiment as a means to
181 reconcile these opposing findings. Moreover, the authors seem to have the ideal system to
182 quickly screen for synapse engulfment following DREADD mediated inhibition of neuronal
183 activity.

184

185 **Answer 1:** This is a very important point, and we have introduced hM4Di to decrease
186 neuronal activity. We have confirmed that decreased activity does not significantly decrease
187 synaptic caspase activity compared to the control group by real-time imaging of
188 synaptophysin-mSCAT3 (lines 201–203, Supplementary Fig. 9c). We have also assessed
189 synaptic phagocytosis by microglia and confirmed that it is decreased by inhibited neuronal
190 activity (lines 203–205, Supplementary Fig. 9d). From these results, we hypothesize that
191 caspase-dependent synaptic phagocytosis would be applied to highly active synapses in
192 balancing excitatory/inhibitory synapses during homeostatic circuit remodeling (lines 205–
193 207).

194

195 **Comment 2:** Work in the field has implicated phosphatidyl-serine exposure in synaptic
196 membranes as a potential mechanism to restrict complement labeling to specific synapses.
197 While the authors mention this hypothesis in their discussion, this is not addressed in their
198 experiments. There are many widely available tools to label PS both in vitro and in vivo which

the authors could make use to address whether PS exposure is also being regulated by caspase activity.

Answer 2: We thank the reviewer for raising this important point. We have examined PS exposure by using Annexin V for in vitro experiments (lines 162–168, Supplementary Fig. 7) and PSVue for in vivo experiments (lines, 301–305, Fig. 5e–j). We confirmed that PS exposure is promoted by increased neuronal activity and that this promotion is reversed to the control level when caspase activity is inhibited by Z-DEVD-FMK.

We would like to clarify that the PSVue data were already prepared at the time of the initial submission but were not included in the original manuscript. In response to the reviewer's suggestion, we have now incorporated these data into the revised manuscript.

Comment 3: A more specific and thorough validation of mSCAT3 is needed. The authors use DEVD as a caspase-3 substrate and activator. However, several studies have shown that this sequence is not specific to caspase-3 (see PMID: 17975551, PMID: 17117159, PMID: 18976637) as it can also cleave caspase-7. The authors should perform immunostaining and western blot against full and cleaved-caspase-7 to prove the specificity of mSCAT3.

Answer 3: As the reviewer has pointed out, the DEVD sequence is not specific to caspase-3 and can also be cleaved by caspase-7. Indeed, another caspase activity probe named VC3Ai, which utilizes the DEVD sequence to monitor DEVDase activity, has been shown to be cleaved by caspase-7 in MCF-7 cells that lack caspase-3 expression (PMID: 23857461). In our HeLa cell experiments, we demonstrated that both caspase-3 and caspase-7 are expressed and activated during apoptosis induced by TNF α /CHX, as shown by western blot analysis (Please see the following Fig. A). Therefore, mSCAT3 does not specifically detect

224 caspase-3 but rather monitors caspase-3-like activities, including those of caspase-3 and
 225 caspase-7.

226

227 **Comment 4:** The specificity regarding the role of caspase-3 in promoting c1q deposition is

Figure. A

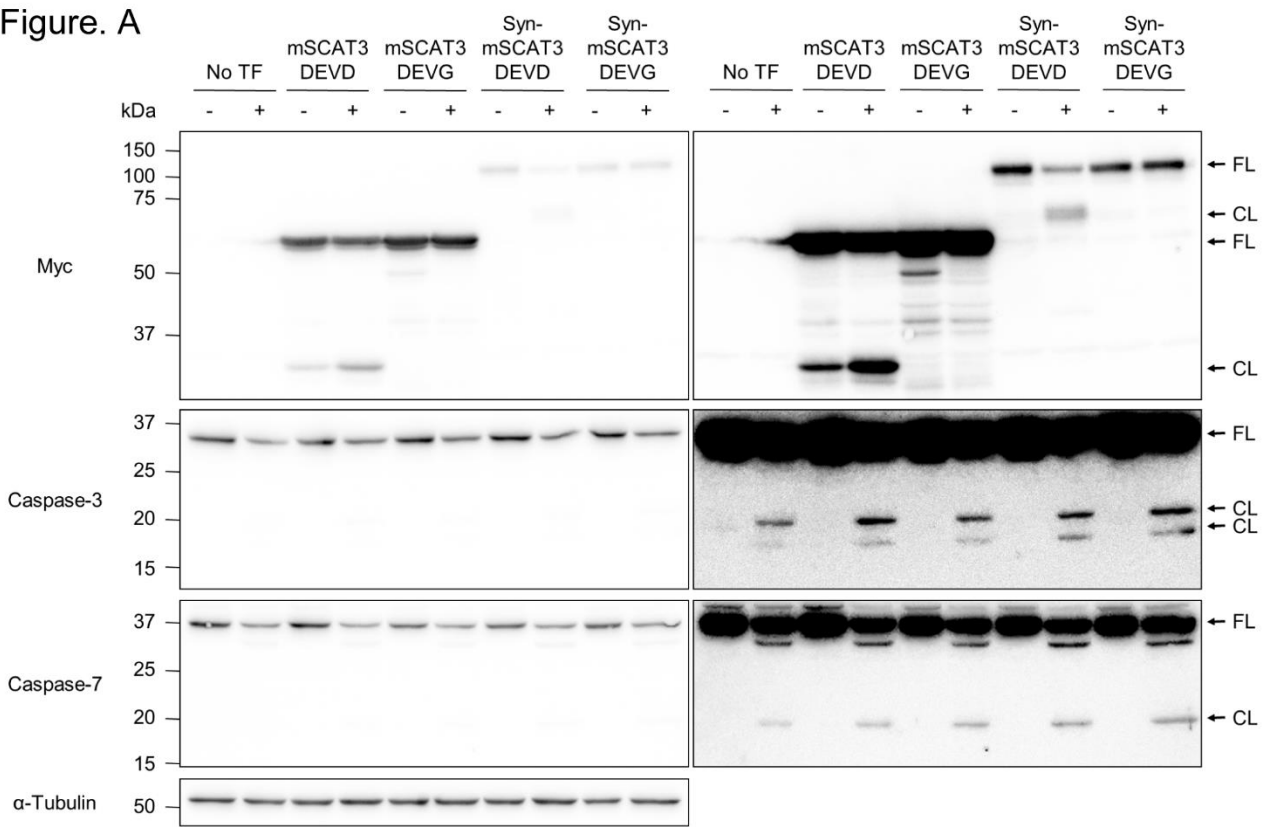


Figure A. Western blot images showing full-length (FL) and cleaved (CL) caspase-3 and caspase-7, as well as the full-length (FL) and cleaved (CL) forms of mSCAT3DEVG/DEVD and Syn-mSCAT3DEVG/DEVD in HeLa cells with (+) or without (-) apoptosis induction. Long exposure images are shown on the right.

228 questionable. Most of the in vitro assessment of caspase-3 use Z-DEVD-FMK as a caspase-
 229 3 inhibitor. However, this inhibitor is known to be a non-specific, “pan-caspase-inhibitor” that
 230 also targets caspase-7 (see PMID: 17117159, PMID: 18976637). Since the authors then used
 231 Bax channel blocker and show that activation of caspase-9 is upstream of caspase-3, we
 232 suggest using a specific caspase-9 inhibitor such as XBIR-3. Another possibility would be to

233 show that the expression of other caspases (particularly caspase-7) is not affected by the
234 used inhibitor. Linked to this, the authors should blot against the cleaved-caspase-3 fraction
235 to validate that caspase-3 is activated.

236

237 **Answer 4:** We thank the reviewer for raising this important point. Firstly, we do not intend to
238 deny the involvement of caspase-7 in the phagocytosis of highly active synapses. Additionally,
239 since cleaved caspase-9 activates both caspase-3 and caspase-7, using a specific caspase-
240 9 inhibitor would not clarify the specificity of caspase-3's involvement. Therefore, we
241 examined the expression of both caspase-3 and caspase-7 in neurons using western blot
242 analysis. Our results show that neurons express both caspase-3 and caspase-7, with
243 caspase-7 expression being comparatively weaker than in HeLa cells. This suggests that
244 caspase-3 is primarily responsible for the observed activity.

245 Moreover, while cleaved caspase-3 and caspase-7 are present in apoptotic
246 HeLa cells, neither cleaved caspase is detected in neurons, likely due to a less pronounced
247 caspase activation during neuronal activation compared to apoptosis (Please see **the**
248 **following Fig. B**). Immunohistochemistry and synaptophysin-mSCAT3 enable visualization
249 of localized caspase activation, which may be obscured in whole cell lysates.

250 To further clarify, we investigated the involvement of caspase-7 by
251 immunocytochemistry for cleaved caspase-7 following CRY2-casp9-induced caspase-9
252 activation. We did not detect cleaved caspase-7 signal at the photo-stimulated presynapse
253 and neighboring ones (**lines 224–228, Supplementary Fig. 10a**). This result is consistent
254 with previous reports demonstrating that local apoptosis at spines and dendrites is mainly
255 attributed to caspase-3. These findings suggest that synaptophysin-mSCAT3 mainly reflects
256 the activation of caspase-3 after increased neuronal activity. However, we acknowledge the
257 possibility that caspase-7 might also contribute to the cleavage of synaptophysin-mSCAT3

258 under certain conditions and have mentioned this point in the discussion (lines 400–413).

259

260 **Comment 5:** To strengthen the claim that non-apoptotic caspase-3 activity in hyperactive
261 neurons is necessary to induce microglial synaptic phagocytosis, the authors should consider

Figure. B

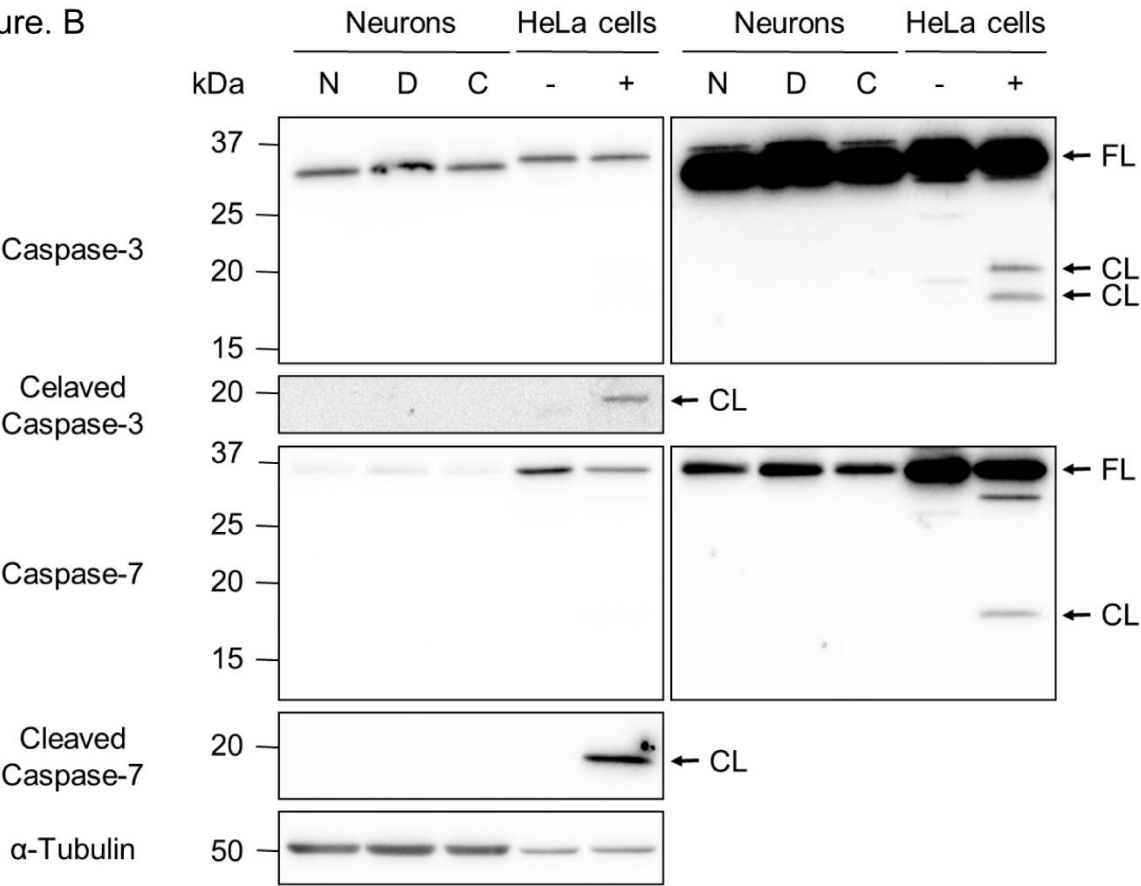


Figure B. Western blot images showing full-length (FL) and cleaved (CL) caspase-3 and caspase-7 in naïve (N), DMSO-treated (D), and CNO-treated (C) neurons, and in HeLa cells with (+) or without (-) apoptosis induction. Long exposure images are shown on the right.

262 performing a more specific in vivo experiment. This reviewer recommends using either an
263 inhibitory neuronal Cre line or an inhibitory neuron targeting Cre-virus combined with a
264 caspase9 flx/flx mouse line and employ the febrile seizure model.

265

266 **Answer 5:** We thank the reviewer for raising this important point. Unfortunately, caspase-9

267 flx/flx mice are not available to us at this time, and we were unable to perform the suggested
268 experiment. Nevertheless, we believe that our current findings, which demonstrate the
269 necessity of non-apoptotic caspase-3 activity in hyperactive neurons for inducing microglial
270 synaptic phagocytosis through a combination of in vitro and in vivo approaches, sufficiently
271 support our main claim.

272 Additionally, while we appreciate the reviewer's suggestion for further
273 strengthening the evidence for non-apoptotic caspase-3 activity, we believe that the proposed
274 experiment using caspase-9 flx/flx mice may not directly address this aspect because it would
275 primarily demonstrate the involvement of caspase-9 in the pathway, rather than distinguishing
276 between apoptotic and non-apoptotic roles of caspase-3. To further support our claim, we
277 have examined the density of GAD67-positive inhibitory neurons in the dentate gyrus after
278 febrile seizures and found no significant decrease in their density (**Supplementary Fig. 14**).
279 This finding indicates that inhibitory neurons are not undergoing apoptosis after febrile
280 seizures, reinforcing the notion that the observed caspase-3 activity in our study is indeed
281 non-apoptotic.

282

283

284 **Minor Concerns:**

285 **Comment 1:** The authors tie activation of caspase-3 to having non-apoptotic roles based on
286 qualitative assesment of the neuronal nuclear morphology, however they should consider
287 performing a more thorough assesment of this such as a TUNEL staining. Changes in nuclear
288 morphology can occur at later stages in the apoptotic process.

289

290 **Answer 1:** We agree with the reviewer and have performed TUNEL staining to more
291 thoroughly assess neuronal apoptosis. In both the control and CNO-treated groups, we
292 detected no TUNEL-positive neurons 6 hours after drug treatment (0%, 10 regions from 2

293 independent experiments) (lines 194–196). This confirms that hM3Dq-induced increased
294 activity does not induce neuronal death.

295

296 **Comment 2:** The authors are testing the role of caspase-3 “nonlethal” activity, however, since
297 caspase-3 seems to be activated by caspase-9 (initiator of the apoptotic death pathway), they
298 should consider referring to this as a non-apoptotic caspase-3 function as nonlethal can be
299 misleading and further assessments for other types of cell death should be tested to make
300 this claim.

301

302 **Answer 2:** This is a very important point, and we have replaced “nonlethal” with “nonapoptotic”
303 throughout the manuscript to avoid any potential misinterpretation. Additionally, we have
304 updated the title to reflect this change.

305

306 **Comment 3:** The statistical N represented by p-values in several figures is not fully clear.
307 Please include a more detailed description in the figure legends of statistical analysis. For,
308 Figure 1 panel b, Figure 2 panel b, Figure 3 panel b, Supplementary Figure 2 e,
309 Supplementary Figure 3 d, and Supplementary Figure 5 b and c, it is unclear whether these
310 images/cells were collected across multiple experiments or are all from a single experiment.

311

312 **Answer 3:** We thank the reviewer for the comment. We have now included detailed
313 information about the statistical analysis in each figure legend, clarifying whether the data
314 were collected from multiple experiments or a single experiment.

315

316 **Comment 4:** All graphs with quantifications should have visible error bars.

317

318 **Answer 4:** We thank the reviewer for this suggestion. In response, we have ensured that

319 error bars are included in all relevant graphs to reflect the variability in our data. Additionally,
320 where appropriate, we have used box-and-whisker plots and line graphs to more accurately
321 represent the data. We believe these changes enhance the clarity and precision of our data
322 presentation.

323
324 **Comment 5:** Authors should consider quantifying if the number of phagocytosed synapses
325 in Fig 2i changes with the administration of DEVD inhibitor.

326
327 **Answer 5:** We thank the reviewer for this comment. We have quantified the volume of
328 phagocytosed synapses in **Fig. 2e** in the presence of Z-DEVD-FMK, which provides relevant
329 insights into the effect of the DEVD inhibitor. To minimize the use of animals and adhere to
330 ethical guidelines, we have not conducted additional experiments on phagocytosed synapse
331 numbers in **Fig. 2j**.

332
333 **Comment 6:** Fig 3a diagram should also include that caspase-9 cleaves caspase-7. Figure
334 3b should also include cleaved caspase 9 staining. Figure 3 studies should include a specific
335 inhibitor of caspase-9.

336
337 **Answer 6:** We agree with the reviewer and have performed the immunostaining of cleaved
338 caspase-9 and cleaved caspase-7 after photoactivation of CRY2-casp9. As a result, we have
339 detected cleaved caspase-9 but not cleaved caspase-7 (**lines 221–228, Supplementary Fig.**
340 **10a**). Based on these findings, we did not include caspase-7 in Fig. 3a. We have also
341 confirmed that inhibition of caspase-9 activation by Z-LEHD-FMK TFA decreases synaptic
342 tagging by complements (**lines 232–235, Fig. 3d, e**). However, we have not examined
343 whether Z-LEHD-FMK TFA inhibits synaptic phagocytosis by microglia, as the primary
344 purpose of the experiments in Fig. 3g–j is to demonstrate that CRY2-casp9-induced synaptic

345 phagocytosis is complement-dependent.

346

347 **Comment 7:** Figure 4C needs clarification on the statistical comparisons. Having
348 representative images of VAGT and VGLUT1 in the same colors is confusing, consider
349 changing to different colors.

350

351 **Answer 7:** We thank the reviewer for pointing this out. We have added the statistical
352 information in the Fig. 4c legend and changed the VGLUT1 color to blue (**Fig. 4d, e**).

353

354 **Comment 8:** Figure 5. To further establish that c1q is a potential cleaved-caspase-3 substrate,
355 the authors should consider performing triple colocalization of VGAT, c 1q, and cl-caspase-3.
356 Another alternative would be to perform an IP.

357

358 **Answer 8:** We thank the reviewer for this valuable suggestion. We understand that the
359 reviewer's comment is aimed at demonstrating the relationship between cleaved caspase-3
360 and C1q. We attempted to perform the triple colocalization of VGAT, C1q, and cleaved
361 caspase-3. However, we found that the antibodies for both C1q and cleaved caspase-3 that
362 are suitable for immunostaining were anti-rabbit. We also tried using an anti-mouse antibody
363 against cleaved caspase-3 for these experiments, but unfortunately, this antibody did not
364 provide a detectable signal for cleaved caspase-3. Thus, we were unable to complete the
365 suggested triple colocalization.

366 As answered to Major Comment 2 of this reviewer, to address the association
367 between caspase-3 activation and the binding of C1q on the surface of the presynaptic
368 membrane, we examined PS exposure by using Annexin V and PSVue for in vitro and in vivo
369 experiments, respectively. We confirmed that PS exposure is promoted by increased
370 neuronal activity, which is reversed to the control level when caspase activity is inhibited by

371 Z-DEVD-FMK (lines 162–168, 299–310, Supplementary Fig. 7, Fig. 5e–j).

372 Regarding the suggestion to perform an immunoprecipitation (IP) experiment,
373 we found it challenging to isolate the inner molecular layer accurately and obtain a sufficient
374 amount of protein for IP due to the small size of the brain region. Therefore, we did not pursue
375 this approach.

376

377 **Comment 9:** Representative images of synapse staining in Figure 6 g and h could benefit
378 from separating the channels. Right now the images for Figure 6 g are too dense to clearly
379 see the pre- and post-synaptic puncta.

380

381 **Answer 9:** We thank the reviewer for the comment. We have separated the channels of
382 presynapses and postsynapses in the revised manuscript to improve clarity (Fig. 6g, i).

383

384

385

Reviewer #3 (Remarks to the Author):

General comments:

This work from Ando et al. applies multicolor time-lapse imaging with new optical tools to clarify the upstream events that lead to phagocytosis of presynaptic components in vitro. They also provided in vivo evidence of similar presynapse-specific elimination in the hippocampal dentate gyrus triggered by febrile seizures. The main findings in vitro include local presynaptic cleavage of caspase-3 after neuronal activity increase by DREADD, enhanced phagocytosis of presynaptic components by the combination of neuronal activity increase and exogenous complement component, C1q, and increase in exogenous C1q binding and microglial C3 receptor accumulation after local activation of caspase-3 by optogenetics. These results collectively indicate the presence of activity-dependent mechanisms of synapse phagocytosis by microglia mediated by local caspase-3 activation and complement-dependent recognition of target synapses. In the second half of the manuscript, Ando et al. extended the in vitro finding by setting up the model of synapse elimination in vivo triggered by febrile seizures. The main findings in this part show the tagging of inhibitory synapses by C1q in the dentate gyrus after seizure induction, followed by inhibitory synapse loss, without affecting the density of excitatory synaptic components. These in vivo experiments support the idea that activity-induced synaptic tagging selectively induces the reduction of specific presynaptic components, which will impact the overall function of the local circuits.

Activity-dependent remodeling of neuronal circuits has long been thought to be regulated by the signaling mechanisms confined to pre- and postsynaptic components. Recently, active roles of glial cells and related signaling pathways have been proposed, but the experimental evidence of the causal relationship between the neuronal signaling triggered by circuit activity and glial response is far from satisfactory. Therefore, this manuscript provides important new information for the field and has broad implications for understanding

the complex spatiotemporal interactions between synapses and microglia in neural circuit remodeling. This manuscript presents two novel and significant findings (first, microglial synapse elimination triggered by neuronal activity and local caspase-3 activation; second, in vivo C1q-dependent inhibitory synapse elimination triggered by febrile seizures) as well as the introduction of new optical tools for monitoring and manipulating caspase-3 activity. As such, this manuscript has a potentially high impact after addressing the following points.

Answer: We thank the reviewer for the overall positive comments and for the constructive suggestions.

Major points:

Comment 1: The most important remaining question is the link between caspase-3 activation and the binding of C1q on the surface of the presynaptic membrane. In the discussion, the authors proposed the involvement of phosphatidylserine externalization by caspase-3. Along this line, it is essential to know if surface phosphatidylserine increases after CNO treatment or photoactivation of CRY2-casp9. This can be tested using fluorescent probes detecting surface phosphatidylserine, such as AnnexinV or PSVue, in combination with the techniques of caspase-3 activation.

Answer 1: We agree with the reviewer and have examined PS exposure by using Annexin V and PSVue for in vitro and in vivo experiments, respectively. We confirmed that PS exposure is promoted by increased neuronal activity, which is reversed to the control level when caspase activity is inhibited by Z-DEVD-FMK (lines 162–168, 299–310, Supplementary Fig. 7, Fig. 5e–j).

We would also like to clarify that the PSVue data were already prepared at the time of the initial submission but were not included in the original manuscript. In response to

the reviewer's suggestion, we have now incorporated these data into the revised manuscript.

Comment 2: The authors reported the synaptic events triggered by the photoactivation of CRY2-casp9. These include accumulation of C1qA and an increase in microglial CR3 near the site of phagocytosis. It is likely that local photoactivation of CRY2-casp9 induces an increase in phagocytotic events per se. This data should be presented.

Answer 2: We thank the reviewer for raising this important point. We have performed real-time imaging of synaptic phagocytosis by microglia following photoactivation of CRY2-casp9 with or without C1qA addition. Our results show that C1qA addition significantly decreases the latency to CRY2-casp9-induced synaptic phagocytosis (lines 249–255, Supplementary Fig. 10b, c). This indicates that photoactivation of CRY2-casp9 alone induces synaptic phagocytosis, which is further accelerated by synaptic accumulation of C1q.

Comment 3: In the analysis of synapse elimination in the dentate gyrus after febrile seizures, the provided evidence supports the idea that complement tagging is involved in inhibitory synapse elimination. However, it is theoretically possible that, instead of microglia, astrocytes are the major players in complement-dependent synapse elimination. To clarify this point, the elimination of microglia by CSF1 inhibitor or related technologies in combination with seizure induction may be informative. Alternatively, the authors can measure the number of phagocytosed synaptic components within astrocytes using high-resolution tissue imaging.

Answer 3: We appreciate the reviewer for this insightful comment. We chose to investigate the role of astrocytes in synaptic phagocytosis due to practical considerations and the availability of tools in our lab. Additionally, in this study, administering a CSF1 inhibitor to postnatal mice while inducing febrile seizures caused significant damage to the animals,

resulting in high mortality rates. Therefore, we performed a synaptic engulfment assay focused on astrocytes. Our results indicate that febrile seizures do not enhance the phagocytosis of inhibitory presynapses by astrocytes in the dentate gyrus (lines 327–329, Supplementary Fig. 13). This finding supports our conclusion that microglia, rather than astrocytes, are the primary mediators of complement-dependent synapse elimination in this context.

Comment 4: Synaptophysin-mSCAT3 is useful in monitoring caspase-3 activity in axons. However, the design of this probe should be more clearly explained in the manuscript. Is mSCAT3 attached to the C-terminal cytoplasmic tail of synaptophysin? If so, after caspase-3-dependent cleavage of mSCAT3, ECFP remains on the synaptic vesicles while Venus is released. Do the authors observe this escape of Venus from synapses after caspase-3 activation? This may suggest some problems in the interpretation of FRET measurement, as the concentration ratio of ECFP and Venus in the presynaptic compartment is altered. In other words, it is possible that non-FRET probes attaching two fluorescent proteins via DEVD sequence show the fluorescence ratio change similar to synaptophysin-mSCAT3. This point should be clarified.

Answer 4: We thank the reviewer for raising this important point. mSCAT3 is tethered to the C-terminal cytoplasmic tail of synaptophysin, which implies that VENUS should be liberated from the vesicles. However, we did not observe VENUS release, possibly due to its low concentration relative to that of the synaptic vesicles. This phenomenon does not interfere with FRET measurements but may actually enhance them, as only ECFP, the donor, remains in the presynaptic compartment, minimizing the potential intermolecular FRET between the cleaved donor and acceptor.

We agree that non-FRET probes attaching two fluorescent proteins via DEVD

sequence can potentially show a ratio change. Indeed, another caspase activity probe called Apoliner, which uses membrane-tethered mRFP and NLS-EGFP linked by the caspase cleavage site, is available. In Apoliner, caspase activity is measured not by mRFP/EGFP ratio but rather by the translocation of NLS-EGFP (PMID: 18779587). However, using the Apoliner-type probe in this study would be challenging, as no obvious release of VENUS from synapses after neuronal stimulation was observed, as mentioned above.

Additionally, mSCAT3 has the advantage of requiring only one excitation laser for dual fluorescent imaging, simplifying the microscopy setup compared to non-FRET probes that need two lasers. A single excitation laser also permits concurrent imaging of other fluorophores like mRFP, facilitating faster and more complex imaging suitable for advanced studies. Another non-FRET probe is the VC3Ai probe, which fluoresces after cleavage of the DEVD sequence (PMID: 23857461). However, our in vivo use of VC3Ai in *Drosophila* epithelia suggests that this probe detects caspase activation in apoptotic cells well but is less sensitive than SCAT3 (PMID: 32791328).

Minor points:

Comment 1: In this manuscript, “presynapse” refers to the axonal structure positive with fluorescent protein-tagged synaptophysin. It is well known that axons without any postsynaptic structures form many synaptic vesicle clusters in culture preparations. It is safe to use the word “axonal boutons” instead of “presynapses” when referring to the puncta positive with synaptophysin. Otherwise, the presence of postsynaptic markers (PSD-95) or active zone markers (bassoon) should be presented in several key experiments.

Answer 1: We agree with the reviewer, and we have examined how many synaptophysin-mCherry puncta are associated with postsynaptic structures by immunostaining for synaptophysin, excitatory, and inhibitory postsynaptic markers. We first examined the

colocalization of postsynaptic markers with synaptophysin-mCherry that remained on the neuronal side after partial phagocytosis (trogocytosis) of synaptophysin-mCherry. As a result, 80% of the remaining synaptophysin-mCherry puncta were colocalized with excitatory or inhibitory postsynapses (lines 263–265, Supplementary Fig. 11a, b). Additionally, we confirmed that at the time of observation, about 85% of synaptophysin-mCherry puncta are associated with postsynapses (lines 273–275, Supplementary Fig. 11c, d).

Based on these results, although we acknowledge that approximately 20% of synaptophysin-mCherry puncta may not correspond to true presynapses, we have decided to use the term "presynapses" for simplicity. This terminology is justified by the high percentage of synaptophysin-mCherry puncta associated with postsynapses, providing a reasonable level of accuracy.

Comment 2: Figure 2g shows the important data supporting synapse elimination without axonal shearing. However, the images are either multicolor or after surface rendering, and it is difficult to identify the objects of interest. Side-by-side presentation of single-color raw images of mCherry and iRFP with high magnification will be more convincing.

Answer 2: We thank the reviewer for this valuable suggestion. We agree that side-by-side presentation of single-color raw images of mCherry and iRFP with higher magnification would be more convincing. Therefore, we have prepared and included these single-color images in the revised manuscript (Fig. 2i).

Comment 3: The density of C1q puncta 6 h after seizure is 200,000 per square mm in Figure 5b. On the other hand, the density of C1q positive VGAT puncta is 1,000 per square mm in Figure 5i. A simple calculation suggests that only 0.5% of C1q puncta is associated with VGAT puncta. Is this true? If so, the remaining 99.5% of C1q puncta should be in contact with other

542 components. This point should be discussed.

543

544 **Answer 3:** We thank the reviewer for raising this important point. We have included the
545 following sentences in the discussion (**lines 422–431**): “The current study suggests that
546 stronger and longer binding of C1q is crucial for the activation of the complement pathway
547 triggered by C1q, which is facilitated by increased neuronal activity, subsequent caspase
548 activation, and phosphatidylserine presentation. This might explain the presence of C1q
549 puncta without inducing microglial phagocytosis in the brain (**Fig. 5i**).

550 We hypothesize that C1q not colocalized with VGAT may be transiently
551 attaching and detaching from other neuronal compartments or being incorporated into glial
552 cells. Therefore, only those C1q puncta that are strongly and irreversibly tagged, particularly
553 when phosphatidylserine is present, are likely to be detected by microglia and targeted for
554 phagocytosis.”

555

556 **Comment 4:** In Figure 1g, the inset shows the pixels with the highest and lowest values of
557 the CFP/Venus ratio. It is not clear why this inset is necessary. Explanations should be
558 provided.

559

560 **Answer 4:** We thank the reviewer for the comment. The inset in Figure 1g was included to
561 highlight the regions with the highest and lowest mECFP/mVenus ratios within a single
562 presynapse. This detail is important because the ratio is not uniform across the presynapse,
563 and we wanted to clarify which parts of the presynapse were used for quantification. We have
564 now included a more detailed explanation in the legend of Figure 1g (**lines 594–596**).

565

566 **Comment 5:** In Figure 3h, the label “CX3CR1-tdTomato/Synaptophysin-mCherry” needs
567 explanation. Why are the images of two fluorescent proteins combined?.

568

569 **Answer 5:** We appreciate the reviewer's comment. To avoid activation of CRY2-casp9 by the
570 488 nm laser before real-time imaging, both microglia and presynapses were labeled with
571 red fluorescence, and the 561 nm laser was used when determining the field of view. We
572 have now included this explanation in the methods section (**lines 1183–1185**).

573

574 **Comment 6:** Supplementary Figure 3d presents the ratiometric values without the values
575 before CNO addition. If the design is the comparison between pre- and 6 h after treatment
576 (as in the case shown in Figure 1i and k), a similar presentation is desirable.

577

578 **Answer 6:** We thank the reviewer for raising this important point. In Fig. S3d, we aimed to
579 compare the degree of caspase activation 6 hours after treatment between groups.
580 Considering the values before each treatment, we have now calculated the differences
581 between the pre-treatment and 6 hours post-treatment values and compared them between
582 groups (**Supplementary Fig. 3d**).

583

584 **Comment 7:** In image analysis methods, many quantitative analyses are done after surface
585 rendering. However, the surface rendering depends on the preceding binarization of the
586 intensity data and should affect the subsequent analysis. The criteria for image binarization
587 and surface rendering should be more precisely described.

588

589 **Answer 7:** We agree with the reviewer for the comment. We have now included the following
590 sentences in the methods (**lines 1113–1115**): "For surface rendering, the brightness of
591 fluorescence images was adjusted to eliminate noise and halation. The thresholds for
592 binarization and surface rendering were then manually determined based on the signal
593 intensity."

594

595

596

597

598 **Reviewer #4 (Remarks to the Author):**

599 **General comments:**

600 This is an elegant study with a logical succession of experiments to confirm the hypothesis
601 that activity-dependent caspase-3 activation at presynapses guides complement-dependent
602 microglial synaptic phagocytosis. Beautiful live imaging in cell cultures.

603 Globally the conclusions are supported by the data presented. The authors used live imaging
604 in cultures, state-of-the-art technologies such as chemogenetics and optogenetics, and
605 ended with some in vivo experiments.

606

607 **Answer:** We thank the reviewer for encouraging comments and following suggestions.

608

609 **Comment 1:** What I do not understand regarding the viral vector strategy used for compiling
610 figures 1 and 2, is that the authors do not use a control vector (i.e. AAV-synaptophysin-
611 mCherry vector without GqDREADD construct; a vector used by the authors in their first
612 validation experiments in supplement?) for the DREADD experiments? The most ideal way
613 to compare the groups is a test group in which GqDREADD is expressed and CNO is used
614 as the DREADD ligand, and a control group in which a viral vector without DREADD has
615 been given but in which CNO is also administered. Now, the authors use GqDREADD-
616 transduced neurons for both groups, with CNO in the test group and DMSO in the control
617 group. In itself not a problem provided an additional control experiment is done with CNO
618 alone to exclude off target effects of the DREADD agonist itself. Was DMSO also the solvent
619 for CNO in the treatment group?

620

621 **Answer 1:** We thank the reviewer for this comment. First, we used two different AAVs to
622 infect cultured neurons to induce the expression of synaptophysin-mCherry and GqDREADD.
623 Since gene transduction by AAV itself could affect neuronal activity, we transduced

624 GqDREADD in all groups and used DMSO as a control for CNO. CNO was dissolved in
625 DMSO, and the final concentration of DMSO was set not to exceed 0.1%. However, we
626 understand the reviewer's concern and performed an additional control experiment to
627 investigate off-target effects of CNO on synaptic caspase activation. As a result, CNO
628 application did not increase synaptic caspase activation in neurons that do not express
629 DREADDs (lines 138-141, Supplementary Fig. 3d).

630

631 **Comment 2:** On page 11 and figure 4 the authors show that a caspase-3 inhibitor 15 minutes
632 prior to febrile seizure induction reduced colocalization of inhibitory presynapses with C1q or
633 C3 to baseline levels. Did the caspase-3 inhibitor also affected the seizures themselves? In other
634 words, is the observed effect on presynaptic tagging by complement the effect of caspase-3
635 inhibition or the result of the lack of seizures?

636

637 **Answer 2:** We thank the reviewer for raising this important point. We have confirmed that the
638 latency of FSs (time to induce clonic seizures after applying hyperthermia) is not significantly
639 different between the FSs and DEVD + FSs groups (FSs: 511.4 ± 49.8 seconds; DEVD +
640 FSs: 502.2 ± 41.7 seconds, mean $\pm$ SD, $n = 5$ mice, $P > 0.05$, Student's t test) (lines 305–
641 308).

642

643 **Comment 3:** Figure 6 panel 0: Were the experimenters blinded to the treatment groups?
644 Especially in the behavioural scoring according to Racine's scale for mice receiving a single
645 kainic acid injection. Which mouse behaviours were exactly scored? In my experience, the
646 behavioural stages in mice following ip injection of kainic acid are not always easy to
647 disentangle and there is also a lot of variation after systemic kainic injection. How robust was
648 your model and read-out? The paper you refer to from Racine is about the scoring of electrical
649 evoked seizures in amygdala or hippocampus in rats.

650

651 **Answer 3:** We thank the reviewer for the comment. This experiment was blinded by using
652 different experimenters for the induction of febrile seizures and kainic acid-induced seizures
653 (lines 1406-1407). As the reviewer points out, it is challenging to disentangle the behavioral
654 stages of kainic acid-induced seizures, especially in postnatal mice. We have developed our
655 own seizure scoring system based on careful observation of mouse behavior and our
656 previous study (Koyama et al., Nat Med, 2012). In the revised manuscript, we have included
657 the details of our seizure scale (lines 1392-1396).

658

Response to reviews

Comments and Answers

We are grateful to the editors and reviewers for their encouraging positive comments supporting the publication and for this opportunity to further improve our paper. Below, we provide a point-by-point response to the reviewers' comments. The revised sections are highlighted in blue in the main text.

Reviewer #1 (Remarks to the Author):

Comment 1: The authors have responded to my queries either with additional experiments or by responding to the comments. I am fully satisfied with their responses.

Answer 1: We sincerely appreciate this reviewer's thorough review and are grateful for the positive feedback. We're pleased that our additional experiments and responses have fully addressed the queries.

Reviewer #2 (Remarks to the Author):

Comment 1: This reviewer thanks the authors for addressing all of our concerns thoroughly. Below are detailed responses to the authors regarding this reviewer's remaining concerns. Following minor edits, this reviewer recommends the work for publication.

Answer 1: We sincerely appreciate this reviewer's thorough evaluation and positive comments. We are grateful for the constructive suggestions, and we have carefully addressed the remaining concerns in the following responses.

Remaining concerns:

Comment 2: This reviewer recommends adding representative images for supplementary figure 9 showing the reduced phagocytosis of synaptophysin-mCherry.

Answer 2: We agree with this reviewer's suggestion and have added representative images to Supplementary Figure 9 (Supplementary Fig. 9e).

Comment 3: The addition of representative images for the caspase-3 in response to inhibitory DREADDs would also strengthen supplementary figure 9.

Answer 3: We agree with this reviewer and have added the requested representative images (Supplementary Fig. 9c).

Comment 4: This reviewer recommends using ANOVA followed by a Sidak posthoc test for Figure 2E instead of the Mann-Whitney Rank Sum test. Mann-Whitney is unsuitable for this analysis because it does not account for multiple comparisons.

Answer 4: We thank the reviewer for raising this important point. In Figure 2e, our intention was to compare the synaptic volume phagocytosed by microglia before and after drug application within each experimental condition. As such, we applied the Mann-Whitney Rank Sum test since our focus was on within-group comparisons, not on multiple comparisons across different groups. However, we acknowledge that the original graph may have been misleading, so we have now separated the data into individual graphs for each experimental condition to enhance clarity (Figure 2e).

Comment 5: Figure 8A legend states there should be arrowheads, but the arrowheads are missing in the image.

Answer 5: We thank this reviewer for pointing out this oversight. We believe the comment refers to Supplementary Fig. 9a. We have now added the missing arrowheads in Supplementary Fig. 9a in the revised manuscript.

Comment 6: This reviewer agrees that using caspase-9 flox/flox mice would not address the specificity of caspase-3's role in synaptic phagocytosis. However, this reviewer recommends emphasizing the non-apoptotic portion of caspase-3 in the discussion, i.e., expanding on the discussion point of how synaptic caspase-3 differs from apoptotic caspase-3 in the soma.

Answer 6: We thank the reviewer for highlighting this important aspect. In response, we have expanded the discussion to emphasize the non-apoptotic role

of caspase-3 at presynapses and how it differs from its apoptotic role in the soma. Specifically, we now discuss two potential mechanisms that might explain this localized activation:

Mitochondrial Accumulation (lines 380–383): We hypothesize that the activation of caspase-3 being limited to the presynapse might be connected to the distribution of neuronal mitochondria, given that these energy hubs are transported from the soma to energy-demanding presynapses³⁵.

Involvement of IAPs (lines 394–398): One potential factor that enables this localized and transient activation is the inhibition of caspase-3 by inhibitor of apoptosis proteins (IAPs)^{1,36}. Following increased neuronal activity, the expression and activation of IAPs could occur in the neuronal soma, which is in consistent with case of local caspase activation observed during dendrite elimination⁶.

Reviewer #3 (Remarks to the Author):

Comment 1: The authors addressed my concerns by providing additional data and appropriately modifying the text in the revised manuscript. I support the publication of the manuscript after correcting the minor points listed below.

Answer 1: We sincerely appreciate this reviewer's positive feedback and the careful review of our manuscript. We are glad to have had the opportunity to address the points noted in the figures.

Comment 2: Figure 6g: Upper three images for Control and lower three images

for FSs 24h, as in the case of Figure 6i? Labels should be added, together with scale bars.

Answer 2: We thank this reviewer for pointing this out. We have added the labels and scale bars to Figure 6g.

Comment 3: Supplementary Fig. 7a: Labels for DMSO, CNO, and CNO-DEVD are missing.

Answer 3: We appreciate this reviewer's attention to detail. We have added the missing labels to Supplementary Figure 7a.

Reviewer #4 (Remarks to the Author):

Comment 1: The authors have taken great effort to conduct additional experiments according to the suggestions of the various referees and incorporate them in the new version of the manuscript. The authors have also adequately answered my specific comments.

Answer 1: We sincerely appreciate this reviewer's recognition of our efforts and the overall positive comments.

Comment 2: With regard to the additional data in Suppl Fig3d with CNO alone (no hM3Dq), these data points have a huge variation and are therefore not so

convincing that CNO is completely inert in this experiment, but as the authors point out, the mean values in this CNO/no-hM3Dq group are not significantly different from those in the CNO/hM3Dq group, and so there is no effect.

Answer 2: We thank the reviewer for this insightful comment. We acknowledge the observed variation in the CNO/no-hM3Dq group. This variability may partly arise from the metabolites of CNO. However, as shown in Supplementary Fig 3d, we compared the caspase activity between the Control (DMSO) group and each of the other groups. Since the mean values in the CNO/no-hM3Dq group are not significantly different from those in the DMSO group, we conclude that the caspase activation observed in the CNO/hM3Dq group is unlikely to be due to off-target effects of CNO.