nature portfolio

# Peer Review File

# Enhancing glymphatic fluid transport by pan-adrenergic inhibition suppresses epileptogenesis in male mice

Corresponding Author: Dr Fengfei Ding

**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 agreement with previous studies, Sun et al. show pan-inhibition of adrenergic receptors (PPA) reduces the severity and outcomes of KA-induced status epilepticus in mice. The authors extend these findings in a new direction by linking suppression of adrenergic systems to enhanced glymphatic fluid transport and hint at AQP4 as a mediator of this enhancement. The manuscript does a great job at correlating pan adrenergic suppression with increased glymphatic fluid influx and improved epileptogenesis outcomes. However, while the authors show strong correlations between improvements in a KA model for epileptogensis, PPA, and glymphatic fluid transport, many of the core conclusions are not fully supported by the data presented. Specifically, a demonstration that enhanced fluid transport itself suppresses epileptogenesis is missing in the present manuscript as many improvements observed could be explained by effects of PPA on neurons and other brain cell types without affecting glymphatic flow. In a revised manuscript, the title should be revised in addition to address a few specific comments below:

Major Concerns:
1. Evidence for enhanced glymphatic fluid transport and its direct role in suppressing epileptogenesis. The authors claim that a global increase in CSF-to-parenchyma transport/influx reflects enhanced glymphatic fluid transport and hint at the idea that this enhancement flushes out epilepsy-related damaging molecules like cytokines. Yet, the authors neither directly measure efflux (e.g., increased clearance of tracer injected in S1) nor capture the movement of cytokines from the brain to the periphery. The decrease in inflammatory signals weeks after PPA-treatment could be explained by direct effects of PPA on neurons and glia. PPA is expected to decrease neuronal activity which would increase microglia activity/function. If “boosting glympathic fluid transport with PPA restricted the inflammatory response by promoting the clearance of lactate, K+ and cytokines” is true, then the authors should show that 1) PPA enhances fluid efflux, 2) PPA changes the ratio of “cleared” molecules from the brain to the blood/periphery, and 3) a more specific increase in glymphatic fluid transport similarly improves epileptogenesis outcome. In addition, while PPA enhanced global influx (Fig. 2B), there did not appear to be a difference when parsed by region (Fig. 2C).

2. Incomplete assessment of AQP4 expression/polarization and it’s role in PPA-improvement of epileptogenesis. AQP4 MPI in Fig. 4A quantifies the whole field and suggests there is an increase in AQP4 expression across the whole FoV in KA-Vehicle which is reduced to vessels in PPA-treated brains. Presumably, this is due to a lack of AQP4 polarization and reflects its expression throughout astrocytes rather than at the endfeet. While Fig. 4B quantifies AQP4 polarization by normalizing to the background, if the background is not astrocyte specific then this polarization measure would be flawed. Evidence showing this background signal is in astrocytes (i.e. co-stain for astrocytes) would help alleviate this concern. Still, the functional implication of this polarization is unclear. AQP4 polarization is expected to increase efflux of water but it is not clear how this helps clearance of other inflammatory/damaging molecules. If increased glymphatic flow does help flush out these damaging molecules, where do they go?
3. Effects of PPA on neuronal activity vs. enhanced glymphatic fluid transport. The manuscript does a great job at correlating pan adrenergic suppression with increased glymphatic fluid influx and improved epileptogenesis outcomes. As stated earlier, however, the effects of PPA on neuronal activity itself can explain many of the observed effects. Indeed, suppression of neuronal activity via benzodiazepines is the go-to treatment for status epilepticus. How is glymphatic flow affected in current treatments, especially those that also suppress neuronal activity. The discussion could be improved with a comparison of PPA to current treatments.

Minor Concerns:
1. Methods:
a. How was somatosensory cortex defined and why was only LII/III analyzed? The example image in Fig. 4B shows the AQP4 background signal varies with cortical depth. How is this polarization index affected by cortical depth?
b. For analysis of tracer influx, it is unclear how many and which coronal sections were used.
2. Statistics.
a. The authors test for normality but always use the parametric Tukey's multiple comparisons test (when comparing more than one group). If a single group distribution is found non-parametric, authors should be conservative and use non-parametric tests to determine statistical significance. It's possible the authors found that Tukey's was appropriate for all multiple comparisons test. If so, please state so in the statistics section. If not, please run the appropriate non-parametric or parametric statistic.
b. It is unclear how variable the data in Fig. 6D is. While the sample size is listed, it is unclear if the statistic is being carried out animal as n versus population seizure numbers. Please clarify this. Also, given that this analysis is done on a by animal basis (as it should be), please add error bands to the cumulative distribution (error bands can be added via bootstrapping or by binning cumulative data). Alternatively, show data as a bar/scatter of animal means as shown everywhere else in the manuscript.
3. L227 reads "ovary-shaped cells" – did the authors mean oval-shaped cells?
4. L505-506 sentence missing words
5. Blood-brain-barrier dysfunction is a common hallmark of epileptogensis, yet the authors mention it once. Are there BBB perturbations at the observed timepoints? If so, does PPA treatment and enhanced glymphatic fluid transport help or degrade BBB integrity?

Reviewer #2

(Remarks to the Author)
Sun and colleagues presented a study in which they ameliorate the outcome of a chemical triggered epileptogenesis acting on the glymphatic influx via adrenergic receptor antagonist administration.
The manuscript is clear and all the important information is provided and figures are carefully crafted and very clear. The potential antiepileptic effect of the treatment has been evaluated in 2 sets of experiments, an early treatment in the three days after the KA injection and a delayed one once epilepsy fully developed. Several techniques have been used to support the findings: EEG, EMG, behavioral test and immunohistochemistry to name only some.
The study introduces a promising novel approach to improve epilepsy symptoms, in mice. However, the findings are oversold and/or not correctly interpreted.

Major points
- The main claim of the study is that "pan-adrenergic blockade suppresses epileptogenesis", however, it is supported by results. In fact, In the KA intra-hippocampal injection mouse model, epileptogenesis usually develops in 3-4 weeks. The experiments addressing an eventual suppression of epileptogenesis are the ones in which PPA was administered just after the status epilepticus, whose results in terms of epileptiform activity are reported in Fig. 3. Indeed, there is not a suppression of epileptogenesis, nor a clear anti-epileptic effect, because the significant decrease in seizure duration is not matched by a similar effect on seizure frequency and, still, seizure in treated mice last, in average, 40 sec. The decrease in inter-ictal spikes (IIS) cannot be considered as an antiepileptic effect of the treatment, since IIS have been hypothesized being a natural protective anti-epileptic mechanism to prevent seizures (see PJ Karoli et al., Brain, 2016). Having less IIS does not automatically mean anything about epilepsy. Of absolute interest, the improved behavioral performance of treated mice. The main message of this study must be changed accordingly to the results.
- The delayed pan-adrenergic blockade showed, indeed, an effective anti-epileptic effect - although it does not suppress epilepsy - decreasing both seizure frequency and duration, concomitant to a higher glymphatic influx. However, the effect was evaluated only for 24h, rising question about the long-term efficacy of the treatment. Based on the presented results, I would say that pan-adrenergic blockade has a short-term anti-epileptic effect. Note that I did not mention the differences of epileptiform activity observed during status epilepticus in treated vs untreated mice because it does not seem to significantly modify the triggered epileptogenesis (see comment above on Fig.3).
- The study lacks generalization because it presents results obtained in both extreme and narrow conditions. Extreme because the treatment was a cocktail targeting all the receptors of the adrenergic system (pan-adrenergic), that is a strong and nonspecific approach, unlikely to be used to develop novel anti-epileptic approaches. Narrow because a single model of induced epilepsy has been tested, a standard one, for sure, but also highly related to inflammation. It would be of interest to test other model of epilepsy, particularly the ones based on genetics or brain concussion. Narrow also for the already discussed evaluation of anti-epileptic effect of the delayed PPA blockade over a limited time.
- From the title, a direct cause-effect link between glymphatic system and epileptogenesis is clearly stated. In addition to the point about epileptogenesis already mentioned, the results of the paper highlight a direct correlation between anti-epileptic effect of PPA treatment and increased glymphatic influx at mice population level. However, because of nonspecificty of the treatment, targeting the entire adrenergic system, it is not possible to assess a direct causality between glymphatic influx and anti-epileptic effect. The hypothesis that this treatment both enhances the glymphatic influx and has an anti-epileptic action, independently, cannot be discarded. Surely a mouse-level evaluation of the glymphatic influx increase and the anti-epileptic effect exerted by PPA would make stronger the correlation between the two mechanisms, but a cleaner pharmacological approach, targeting specifically the glymphatic system is necessary to state the causality.

Minor points
- Please, avoid using 'suppress' instead of 'decreased'. It gives a false message to the reader. See the title, line 372 etc..

- Line 56: the Authors refer to epileptogenesis writing about AMPA receptors modifications. This definition correctly applies to long-term potentiation, but not to epileptogenesis. In fact, the cited references (10, 11) do not even mention epilepsy.
- Line 95: typo, status epilepticus.
- Line 106: mice were 'withdrawn from anesthesia' after KA injection: Was atipamezole used? Being it an alpha2-receptor antagonist, it could have hidden some potential effect of the PPA treatment.
- Line 108: I hope that mice were not injected i.p. with 1ml in a single shot. It is a very big amount of liquid. This quantity is over the limit of the ethical amount of i.p. injected solutions.
- Line 241: the statement about "Data and SW availability" is insufficient.
- Line 108, 315, 337, 352: typo, Kainate.
- Line 351: Bi-modal distributions are proposed for seizure frequency and open-field test. Although true for the latter, the seizure frequency has not an apparent bi-modal distribution.
- Line 386: please, do not use 'partially rescued' for non-significant data.

Reviewer #3

(Remarks to the Author)
In this manuscript, Sun et al. reports antiepileptogenic effects of pan-adrenergic inhibition by enhancing glymphatic fluid transport in the model of intrahippocampal kainic acid. Since the changes in seizures and glymphatic flux have been reported in response to modulating adrenergic receptors, the findings of the present study are not entirely novel; however, they confirm the earlier reports. Overall, the experiments are designed well but the results require better presentation. Please refer to the following comments for more details.

1) Fig 1F: Racine seizure scores for 3 mice in each group are not plotted. Did not all mice in each group develop SE? Did PPA treatment affect the number of mice developing KA-induced SE?
2) Fig 2B: Was there any difference in tracer distribution on the dorsal areas of the brain between the groups? Mention about the anatomical reference placed over the ventral image in the figure legend.
3) Fig 2C: Doublecheck brain slice displays showing the ratio of trace distribution areas. As per the legend, hotter the color, lesser the difference between the comparison groups (i.e., if the ratio=1, the trace distribution areas are equal for the comparison groups). If so, dorsal cortex should show the highest difference between KA-Veh and KA-PPA groups; however, this comparison is not statistically difference as per the graphs below. Clarify.
4) Fig 3: Did PPA reduce the number of mice with chronic seizures? Include seizure severity data.
5) N (no. of mice) are different for various graphs in Fig 3 panels. For example, why only 5 mice are shown for KA-Veh group in panel A (body weight), whereas 13 mice for KA-Veh group in panel D (spike freq)?
6) Given the bimodal distribution of mice in KA-PPA group for their seizure frequency and open-field activity, was there any correlation between seizure frequency and open-field activity?
7) Figs 4 and S2: AQP4 line polarization index around blood vessels in somatosensory cortex and CA1 area of hippocampus was found reduced in the KA-Veh group. However, the images and line analysis show AQP4 staining intensity in the perivascular region in the KA-Veh group either comparable to or even higher in the control group. The polarization index was found to be reduced because of overall increased expression of AQP4 in the KA-Veh group, which could be due to increased astrogliosis in the KA-Veh group. Please comment. How many blood vessels (small and large diameter) from each mouse were analyzed for this analysis?
8) Since glymphatic flow supports the optimal maintenance of the extracellular glutamate and K+, it would be best to measure the level of glutamate and K+ in extracellular dialysates collected, in addition to the cytokines data already provided.
9) Lines 105, 181, 188: Mention the reference point for mouse brain coordinates used for cannula implant.
10) Overall methods should provide more relevant details.
11) Line 315: kainite – typo error.
12) Line 356: Cite Fig 3F instead of 3E.
13) For trace image analysis, be consistent with the y-axis label. It should be “Glymphatic influx (%)”.
14) As per the results text, seizure duration graph should be part of panel D in Fig 3.

Reviewer #4

(Remarks to the Author)
This paper uses two or three different studies involving experimental manipulation of the adrenergic system (as an indirect methodological approach to modify glymphatic fluid transport), which is proposed to secondarily alter chronic epileptogenesis induced by status epilepticus via intrahippocampal KA injection in mice.
The first study used 72-hr video/EEG recording immediately following intrahippocampal KA injection to examine the effects of increased CSF influx (via adrenergic receptor agonists, PPA) on the latency and duration of seizures associated with the consequent status epilepticus (here divided into a first phase of recurrent seizures and a second phase of continuous spiking). PPA significantly delayed the onset of phase 1 convulsive seizures and prolonged seizure durations but did not affect the total number of seizures. PPA also shortened the duration of phase 2 spiking. These effects were thus on the status epilepticus, and not on epilepsy.
In a second study, PPA was administered for 3 days post-KA, and EEG recordings were performed for 2 hr at approximately 5 weeks post-KA. PPA had no effect on daily seizure frequency, but did shorten seizure durations from approximately 49 to 39 sec and reduced interictal spike frequencies from approximately 687 to 503 spikes/hr. These are minimal changes and

essentially inconsequential in regard to the claim of altering epileptogenesis.
Finally, in a separate set of mice, PPA was administered at 4 weeks post-KA and EEG recorded for 24 hours. Delayed PPA administration had the effect of reducing counts of spontaneous seizures for 12 hr and reducing interictal spike frequency by about 40% for 1-3 hr. These results provide no evidence for an effect on epileptogenesis; rather, they are simply short-term drug effects.
The observation that PPA can influence certain features of the intrahippocampal KA model (i.e., surrogate markers) may be somewhat interesting, but none of these experiments relate to epileptogenesis. The basic design and interpretations of these experiments do not support the proposed hypothesis that the glymphatic fluid transport has a role in epileptogenesis, and that the effects on glymphatic fluid transport can modify epileptogenesis.
The first major flaw is that most of the effects of PPA were on status epilepticus. As such, PPA appears to affect the nature and severity of the initial injury, and not epileptogenesis. This is an important and fundamental flaw of these studies, and on its own, negates the subsequent interpretations. Many researchers have already shown that experimental manipulation of the initial injury, as expected, can alter the subsequent epileptogenesis. In terms of providing evidence for an effect on the process of epileptogenesis, it is imperative that one apply the experimental manipulation after the insult, preferably well after the insult, so that one can be sure that the experimental manipulation is not merely blunting the injury.
The second major flaw in this study is that the authors have not studied the spontaneous recurrent seizures that are the definition of epilepsy. Not only do the electrical measures simply show an alteration in the characteristics of seizure activity of the status epilepticus, all of the later measures are indirect. The only measure that is relevant is the overall long-term frequency and severity of spontaneous seizures (i.e., “epileptic” or chronic seizures). To show a change in the actual epilepsy would require that the authors record spontaneous seizures for several months to determine if their frequency and severity are permanently altered. The changes reported here are actually quite small and simply not relevant to the question of epileptogenesis. For example, the effects on the duration and latency of seizures, as described by the authors, are unimportant, particularly since the total number of seizures was not affected. Similarly, late administration (4 weeks) of PPA produced some reduction in spontaneous seizures and interictal spikes for several hours, but again, this is not an effect on epileptogenesis – only a transient effect of the drugs. Chronic video/EEG recording for months post-KA would be required to assess whether any of the PPA applications examined here had an effect on the development and severity of epilepsy in this animal model. Unfortunately, these recordings were not performed, eliminating any the potential impact of this work.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have overall done a good job addressing reviewer comments. One points remains from the initial review (original concern 1, part 3) that was not fully addressed in the revision.

The authors propose that enhanced glymphatic flow alleviates pathophysiology in a mouse model for epilepsy by clearing toxic molecules from the brain. In the revised manuscript, the authors show regional differences in hypothalamus and ventral cortex, but based on the data, it seems that is no change in glymphatic flux or with Aqp4 polarization at the hippocampus. This is curious since hippocampal dysfunction underlies epileptogenesis. The authors make broad claims about PPA enhancing glymphatic flow that improves KA-induced outcomes, and so it seems that some of these changes would be anticipated to take place at the hippocampus. How do the authors reconcile these findings?

One minor point:

Mismatched scale bars in Fig. S3 C (Sensory Cortex)

Reviewer #2

(Remarks to the Author)
The paper that was revised and re-edited by Sun and colleagues improved substantially. I want to congratulate the Authors for adding new data and analysis, addressing all my concerns, particularly the PPA treatment far from KA injection, proving that it efficiently dampens the epileptogenesis process (fig.3). Also, the study conducted with AQP4-KO mice showing an aggravated epileptogenesis if compared to WT mice further clarifies the hypothesized mechanisms and support the paper's claims. I only have a single remark that I consider necessary to be fixed. In the abstract (line 50) and all over the manuscript, the Authors used the statement 'suppressed epileptogenesis' or 'suppressed the number of convulsive seizures' improperly. The right word for such statements should be 'decreased' or 'lowered'.
Once the mentioned sentences are corrected, I fully support the publication of this work in Nature Communications.

Minor comments:
- Page 6, line 237 dummy ??? (cannula).
- Page 9, line 395-396: please rephrase; the sentence does not make much sense.
- Page 11, line 473: 'the trend towards increased levels .. of cytokines' provides a misleading message since no significance has been found in the cited cytokines and even the mentioned trend is not evident in most of the plots of fig. S5.

Reviewer #3

(Remarks to the Author)
Authors have incorporated additional data in the revised manuscript that satisfactorily addresses the concerns raised by the reviewers on the original manuscript. The changes made have strengthened the manuscript. I have one major comment as follows:

Previous studies have shown increased seizures in AQP4-KO mice in response to various stimuli. Therefore, it is reassuring to find that intrahippocampal KA-induced seizures are worse in AQP4 KO mice. I think there should have been an additional group of AQP4-KO mice treated with PPA in this experiment. Since your hypothesis is that pan-adrenergic inhibition by PPA treatment suppresses epileptogenesis by augmenting glymphatic flux mediated through AQP4 expressed in astrocytic endfeet, treating AQP4-KO mice with PPA should not affect seizure parameters compared to vehicle-treated AQP4-KO mice. This experiment may also address a concern whether the antiseizure effects of PPA are mediated mainly through its augmenting effects on glymphatic flux or other mechanism(s) such as its actions on neuronal NE receptors.

Minor corrections to do:

1) Fig. 5G – Correct the timeline graphic. No PPA treatment at the time of KA injection.

2) Fig. S3D – Correct the error in statistical significance marks for HC.

3) Fig. S6 – BBB results are not reported or discussed in the text.

4) Line 626-628 (“The anti-epileptic drugs currently used in the clinic for acquired epilepsy include phenobarbital, phenytoin, carbamazepine, valproate, and magnesium134.”) – All these drugs are antiseizure drugs as opposed to antiepileptic drugs. Magnesium is not used for treating seizures.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors provide convincing evidence that PPA treatment improves outcomes in a model for epilepsy. However, their response fails to fully address concerns on the following points.

Comment 1

We appreciate the added paragraph to the discussion in Page 13-14. However, to our first and most important critique that “it seems that is no change in glymphatic flux or with Aqp4 polarization at the hippocampus”, the authors reply with an inaccurate statement. They state, “As showed in Fig 5C and 5I, we did find only a slight trend of increase in glymphatic influx in the hippocampus”. However, this is confusing since Figure 5C and 5I show that there is no difference in the average glymphatic influx at the hippocampus and therefore replying that there is “a slight trend of increase” seems to contradict their own data. In fact, this misuse of the word “trend” to suggest to the reader that there is a difference when the data shows otherwise now presents a major concern. All instances of “trend” when statistical significance was not found (Lines 403 , 430, and 496) should be removed from the manuscript altogether to prevent reader confusion.

Finally, while glymphatic influx at the hippocampus may be normally low and difficult to detect given anatomy, others have successfully measured changes in tracer flux using the same method of injection (PMID 38418877). Thus, it is just as likely that the lack of influx at the hippocampus is not due to methodology or imaging sensitivity but rather that there is simply no change following PPA treatment. Please update the discussion to reflect alternative explanations that include a different mechanism for the improvements following PPA treatment.

Comment 2

Please show both Ipsilateral and contralateral data
It is unclear if the new and significant differences found in hippocampus polarization shown in Fig. S3D are meaningful given that their means are barely noticeably higher in the KA-PPA vs KA-Veh group when compared to the Sham control.

Reviewer #3

(Remarks to the Author)
All the reviewers’ comments/concerns have been addressed very well by the authors. I have no further concerns and am in favour of publishing this revised manuscript in Nature Communications.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
We thank the authors for their thoughtful response and for revising their paper accordingly.

**point-by-point response to the reviewers' critiques**

## REVIEWER COMMENTS

**Reviewer #1** (Remarks to the Author): In agreement with previous studies, Sun et al. show pan-inhibition of adrenergic receptors (PPA) reduces the severity and outcomes of KA-induced status epilepticus in mice. The authors extend these findings in a new direction by linking suppression of adrenergic systems to enhanced glymphatic fluid transport and hint at AQP4 as a mediator of this enhancement. The manuscript does a great job at correlating pan adrenergic suppression with increased glymphatic fluid influx and improved epileptogenesis outcomes. However, while the authors show strong correlations between improvements in a KA model for epileptogensis, PPA, and glymphatic fluid transport, many of the core conclusions are not fully supported by the data presented. Specifically, a demonstration that enhanced fluid transport itself suppresses epileptogenesis is missing in the present manuscript as many improvements observed could be explained by effects of PPA on neurons and other brain cell types without affecting glymphatic flow. In a revised manuscript, the title should be revised in addition to address a few specific comments below:

**Response:** To the best of our knowledge, this is the first study to indicate that early pan-inhibition of adrenergic receptors attenuates epileptogenesis after acute kainic acid (KA)-induced status epilepticus (SE). In response to the first critique, we have repeated the experiments and recorded seizure activity on four consecutive days at three weeks as well as at two months post KA infusion (**Figure 2A-D, cited below**). There was a significant reduction in the number of seizures in the KA-PPA group as compared with the control (KA-Veh) group. PPA treatment also shortened the average seizure duration, which is consistent with prior findings from original submission. Scoring of the spontaneous seizures using the Racine Scale showed a reduction of severity in the KA-PPA group compared to the KA-Veh group. In response to the reviewer, we added a new set of experiments in which we administered PPA at 30 hours after KA infusion, after the cessation of the acute onset of SE. The new studies showed that early three-day PPA administration significantly reduced the number of seizures in the chronic stage monitored either at 3-4 weeks or two months post the initial onset of SE (**Figure 3, cited below**). In another newly conducted experiment, we tested our model in *Aqp4* knockout mice with chronically reduced glymphatic function[1,2]. As predicted, the genetic deletion of *Aqp4* aggravated KA-induced seizures with respect to acute SE and spontaneous seizure onsets in the chronic stage (**Figure 7, cited below**). Specifically, in acute SE, *Aqp4*-KO mice exhibited significantly shortened latency of first seizure onset, greater mean duration and severity of seizure onset in phase 1, and prolonged the duration and total epileptic spike number in phase 2 **(Figure 7B).** *Aqp4* knockout mice exhibited a significantly higher number of seizures, prolonged seizure duration, higher maximal Racine scale readouts and fewer seizure-free days in the chronic phase **(Figure 7D).**

New figures and Legends cited from the revised manuscript:

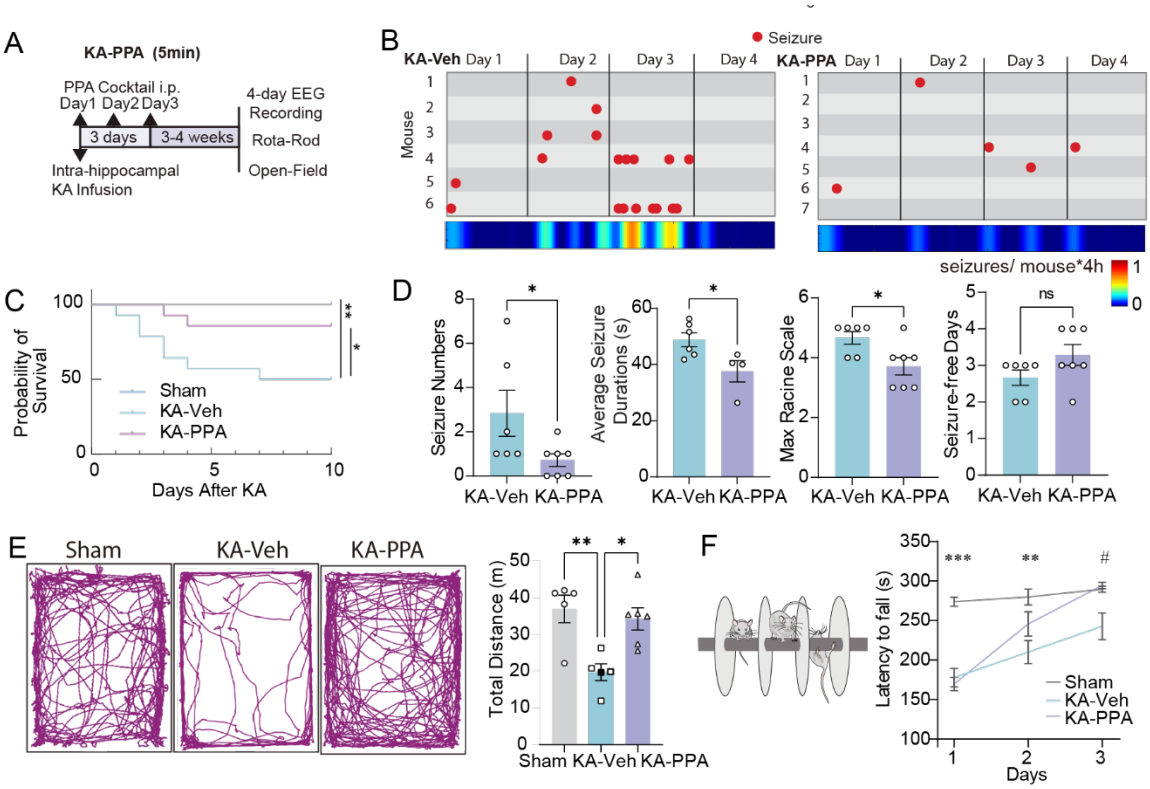



***Figure 2. Pan-adrenergic inhibition improves behavioral outcomes of KA-induced chronic epilepsy (A)*** *Timeline of the experimental design. Five minutes after intrahippocampal KA infusion, mice were randomly divided into two groups that received daily injections of either PPA or vehicle for three consecutive days. After 3-4 weeks, we implanted cortical EEG electrodes in a subgroup of mice and obtained EEG recordings obtained one week later. In parallel, we measured open-field and Rota-rod behavioral scores in a separate group of mice.* ***(B)*** *Seizure event plots show each convulsive seizure (red dot) onset time for individuals in 4-day recordings from the KA (left) and PPA-treated KA (right) groups. Grey bars (below) indicate the time distribution of seizure onsets for each entire group* ***(C)*** *Kaplan-Meier survival curve of Sham, KA-Veh and KA-PPA (5 min) groups after KA infusion (n = 12, Sham vs KA-Veh *P = 0.039, KA-Veh vs KA-PPA **P = 0.003, Log-rank test).* ***(D)*** *The numbers of seizures in 4-day recordings, duration of spontaneous seizures, maximal Racine scale in four days, and number of seizure-free days contrasted between KA-Veh and KA-PPA mice. n= 6-7 mice/group, seizure numbers, *P = 0.044 (Mann-Whitney test); seizure duration, *P = 0.031 (unpaired two-tailed t-test); maximal Racine scale, *P = 0.048 (Mann-Whitney test) and seizure-free days, P = 0.237 (unpaired two-tailed t-test).* ***(E)*** *The representative traces of motion trails in the open-field test among Sham, KA-Veh and KA-PPA groups (left). The comparisons of total distances travelled are presented on the right. (n = 5-6 mice/group, one-way ANOVA, Tukey's multiple comparisons test, Sham vs KA-Veh **P = 0.006, KA-Veh vs KA-PPA *P = 0.013).* ***(F)*** *Motor coordination and motor learning by the Rota-Rod test showing partial recovery with PPA treatment. Comparison of the latency to fall among Sham, KA-Veh and KA-PPA groups (two-way ANOVA, Tukey's multiple comparisons test, Sham vs KA-Veh, ***P < 0.001, **P < 0.01; KA-Veh vs KA-PPA #P < 0.05). Data are presented as mean ± SEM.*

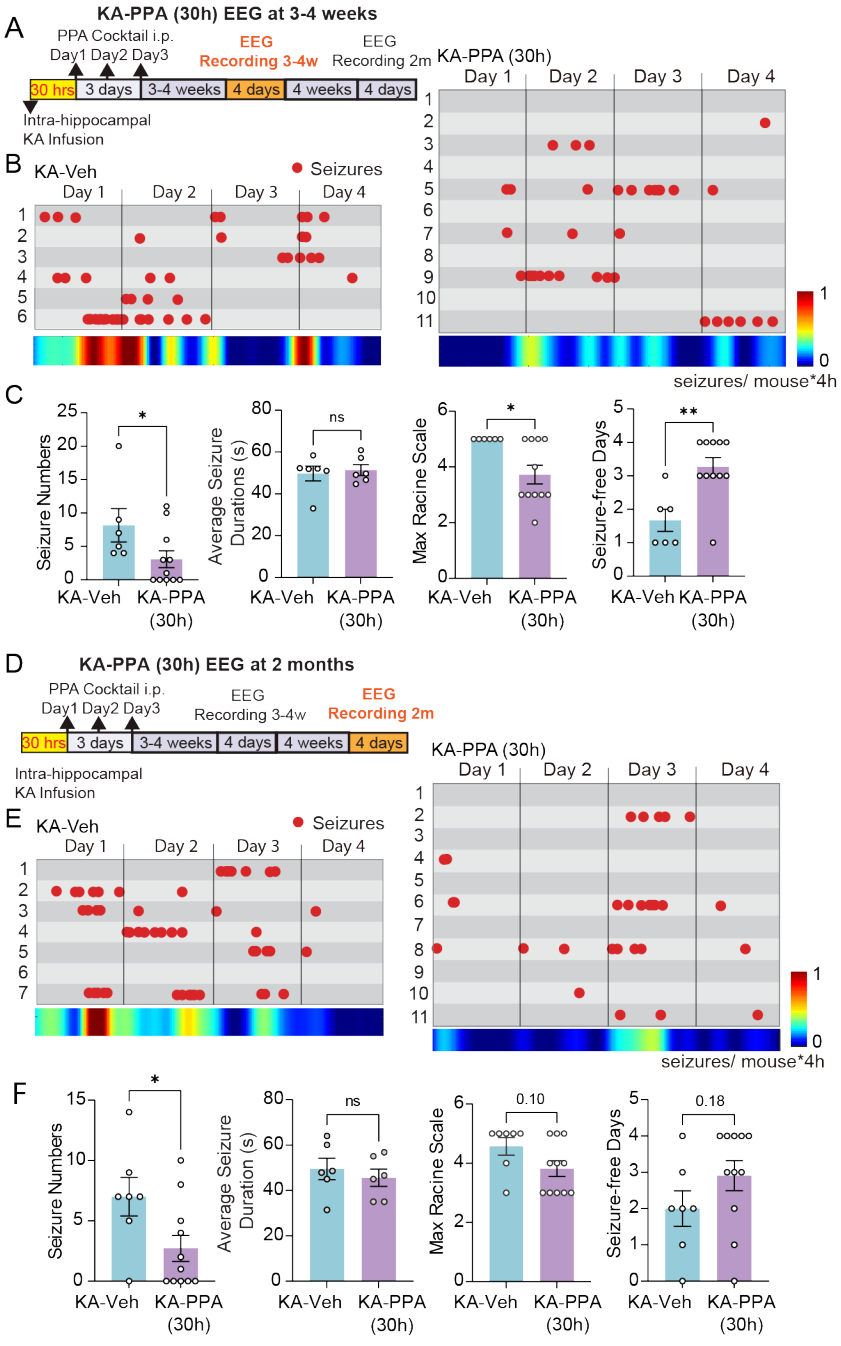



***Figure 3. 30-hour delayed pan-adrenergic inhibition reduces rate of spontaneous seizures in a chronic epilepsy model. (A)*** *Timeline of experimental design. 30 hours after intrahippocampal KA infusion, mice were randomly divided into two groups that received either a daily injection of PPA or vehicle for three*

*consecutive days. After 3-4 weeks, we implanted cortical EEG electrodes and obtained recordings one week later.* ***(B)*** *Seizure event plots show each convulsive seizure (red dot) onset time for individual rats in 4-day recordings from the KA-Veh (left) and KA-PPA (30 h) (right) groups. Heatmap (below) indicates the frequency of seizure onsets per mouse per 4-hour recording in each group.* ***(C)*** *The analysis of seizure numbers, duration of spontaneous seizures, maximal Racine scale in the 4-day recordings, and seizure-free days between the KA-Veh and KA-PPA (30 h) mouse groups at 3-4 weeks. n= 6-11 mice/group, maximal Racine scale, *P = 0.031 (Mann-Whitney test), seizure numbers, *P = 0.042 (Mann-Whitney test); average seizure duration, P = 0.719 (unpaired two-tailed t-test), and seizure-free days, **P = 0.004 (Mann-Whitney test).* ***(D)*** *Timeline of experimental design. At two months post-KA infusion, 4-day EEG recordings were obtained from the KA-Veh and KA-PPA (30 h) groups.* ***(E)*** *Seizure event plots show each convulsive seizure (red dot) onset time for individual rats in the 4-day recordings from the KA-Veh (left) and KA-PPA (30 h) (right) groups at two months. The heatmap (below) indicates the frequency of seizure onset per mouse during the 4-hour recordings in each mouse group.* ***(F)*** *The analysis of seizure numbers, duration of spontaneous seizures, maximal Racine scale in the 4-day recordings, and seizure-free days compared between the KA-Veh and KA-PPA (30 h) mouse groups at two months. n = 7-11 mice/group, maximal Racine scale, P = 0.100 (Mann-Whitney test), seizure numbers, *P = 0.035 (unpaired two-tailed t-test); average seizure duration, P = 0.535 (unpaired two-tailed t-test), and seizure-free days, P = 0.154 (Mann-Whitney test). Data are presented as mean ± SEM.*

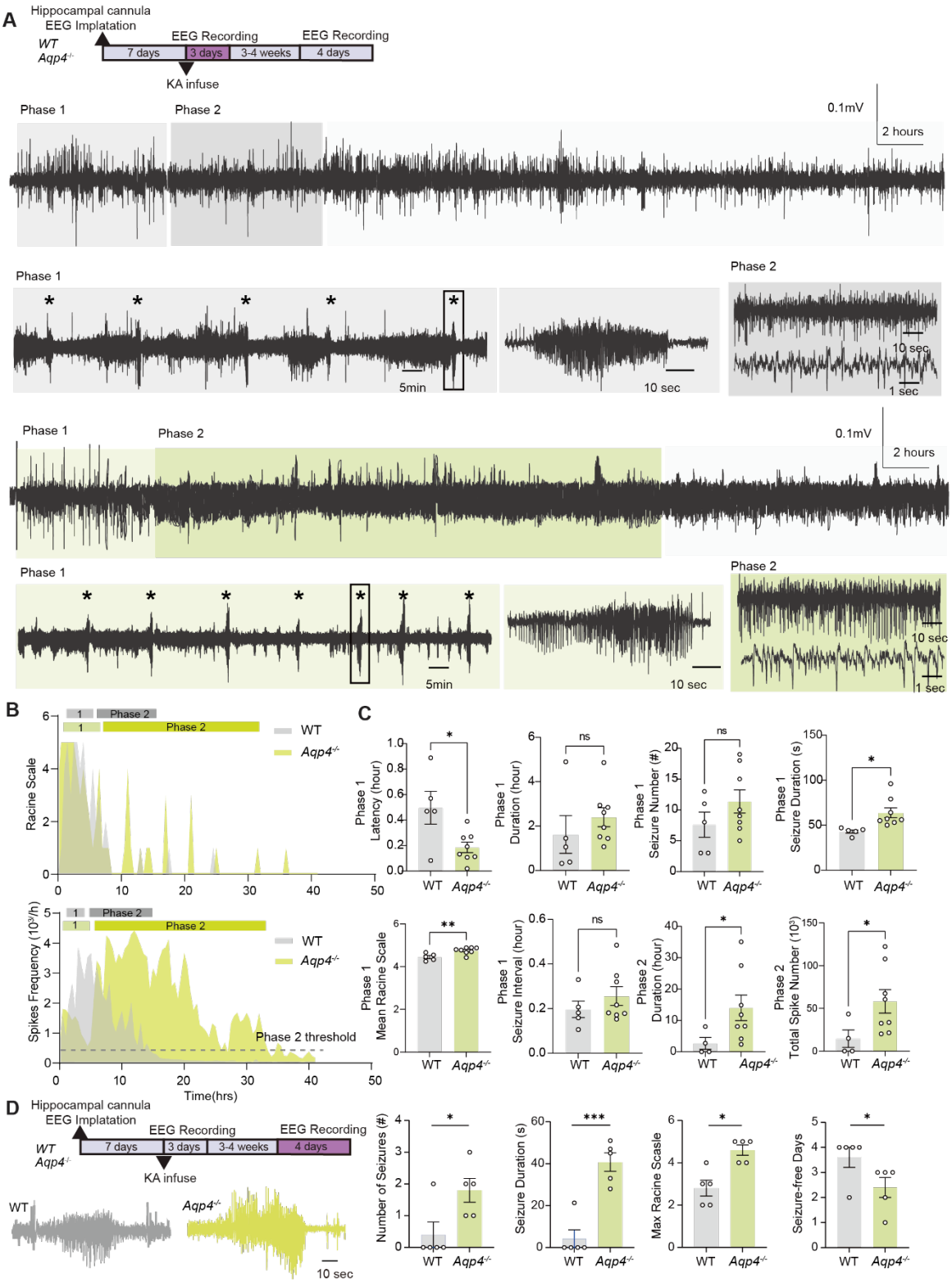

A
Hippocampal cannula
EEG Implatation
WT
Aqp4-/-
7 days
3 days
3-4 weeks
4 days
EEG Recording
EEG Recording
KA infuse
Phase 1
Phase 2
0.1mV
2 hours
5min
10 sec
1 sec
B
Racine Scale
Spikes Frequency (10²/h)
Phase 2 threshold
Time(hrs)
C
Phase 1 Latency (hour)
Phase 1 Duration (hour)
Phase 1 Seizure Number (#)
Phase 1 Seizure Duration (s)
Phase 1 Mean Racine Scale
Phase 1 Seizure Interval (hour)
Phase 2 Duration (hour)
Phase 2 Totlal Spike Number (10³)
D
Number of Seizures (#)
Seizure Duration (s)
Max Racine Scasle
Seizure-free Days

***Figure 7. Aqp4 deletion aggravates KA-induced seizures. (A)*** *Aqp4-knockout (KO) mice and C57 wildtype control mice received an intrahippocampal infusion of KA (20 mM, 50 nl, 10 nl/min, right CA1 region) followed by continuous 72-hour EEG recordings. After 3-4 weeks, the same groups of mice underwent 4-day EEG recordings. EEG trace shows a representative example of KA-induced status epilepticus (SE). As in Figure 1, the epileptiform activity was subdivided into two phases, with depiction of recurrent seizure onsets in Phase 1 (middle in light-colored box), and Phase 2 epileptiform discharges at higher temporal resolution (right in dark-colored box).* ***(B)*** *Time-course spike frequency analysis and hourly maximal Racine scale of the KA-induced status epilepticus from representative WT and Aqp4-KO mice after intrahippocampal KA infusion.* ***(C)*** *Histogram comparing the durations of the two phases of KA-induced SE in control and Aqp4-KO mice. Data are presented as mean ± SEM. Phase 1, latency of the first seizure onset post KA injection, n = 5-8 mice/group, *P = 0.019, unpaired two-tailed t-test; number of seizures, n = 5-8 mice/group, P = 0.302, Mann-Whitney's test; Phase 1 duration, n = 5-8 mice/group, P =0.378, unpaired two-tailed t-test; Seizure interval, n = 5-8 mice/group, P =0.343, unpaired two-tailed t test. Mean Racine Scale, n = 5-8 mice/group, P =0.007, unpaired two-tailed t test. Phase 2, duration, n = 4-8 mice/group, *P = 0.026, Mann-Whitney's test. Total spike number, n = 4-8 mice/group, *P = 0.047, Mann-Whitney's test.* ***(D)*** *The seizure numbers in 4-day recording, duration of spontaneous seizures, maximal Racine scale in the 4-day recordings, and seizure-free days compared between control and Aqp4-KO mice. n = 5 mice/group, seizure numbers, *P = 0.040 (Mann-Whitney's test); seizure duration, ***P < 0.001 (unpaired two-tailed t-test); Maximal Racine scale, *P = 0.024 (Mann-Whitney test) and seizure-free days, *P = 0.040 (Mann-Whitney's test). Data are presented as mean ± SEM.*

Major Concerns:1. Evidence for enhanced glymphatic fluid transport and its direct role in suppressing epileptogenesis. The authors claim that a global increase in CSF-to-parenchyma transport/influx reflects enhanced glymphatic fluid transport and hint at the idea that this enhancement flushes out epilepsy-related damaging molecules like cytokines. Yet, the authors neither directly measure efflux (e.g., increased clearance of tracer injected in S1) nor capture the movement of cytokines from the brain to the periphery. The decrease in inflammatory signals weeks after PPA-treatment could be explained by direct effects of PPA on neurons and glia. PPA is expected to decrease neuronal activity which would increase microglia activity/function. If "boosting glymphatic fluid transport with PPA restricted the inflammatory response by promoting the clearance of lactate, K+ and cytokines" is true, then the authors should show that 1) PPA enhances fluid efflux, 2) PPA changes the ratio of "cleared" molecules from the brain to the blood/periphery, and 3) a more specific increase in glymphatic fluid transport similarly improves epileptogenesis outcome. In addition, while PPA enhanced global influx (Fig. 2B), there did not appear to be a difference when parsed by region (Fig. 2C).

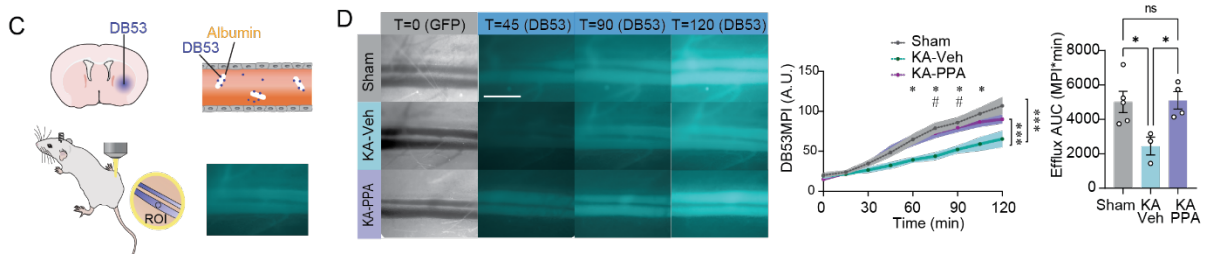
**Response:** Thank you for the insightful comments, which we address as follows:

**Point 1:** Yet, the authors neither directly measure efflux (e.g., increased clearance of tracer injected in S1) nor capture the movement of cytokines from the brain to the periphery. The decrease in inflammatory signals weeks after PPA-treatment could be explained by direct effects of PPA on neurons and glia. PPA is expected to decrease neuronal activity which would increase microglia activity/function.

**Answer:** In response to this comment, we have added *in vivo* glymphatic efflux measurements, which confirm that glymphatic efflux is significantly lower in KA-treated mice as compared with the sham group. PPA treatment effectively rescued the efflux deficit in KA-Veh mice. In **Figure 5J**, we present results showing that acute PPA treatment increased delta wave prevalence, which in a previous study

correlated positively with glymphatic transport[4]. We also showed that PPA administrated in the acute phase of SE reduced astrogliosis and partially reduced microgliosis in the chronic seizure stage **(Supplementary Figure 4**). It is not currently possible to measure the total cytokine efflux from the brain due to the presence of multiple source and efflux paths. For instance, analysis of cytokines in cerebrospinal fluid (CSF) collected from the cisterna magna will primarily reflect newly-produced CSF[5,6]. Additionally, measurements derived from blood are often confounded by the presence of cytokines arising from systemic inflammation [7,8]. Thus, these complexities impede efforts to capture the export of cytokines from the brain to the periphery.

*Data are presented in Figure 6C-D of the revised manuscript:*




*Cited from the revised manuscript, results section:*

*We also assessed glymphatic efflux with a real-time* in vivo *assay, following a recently published protocol[64]. In this experiment, we repeatedly imaged the femoral vein vascular compartments for up to two hours following intrastriatal infusion of the low molecular weight (0.96 kDa) fluorescent tracer DB53 (Direct Blue) in anesthetized mice* ***(Fig. 6C).*** *DB53 binds irreversibly to blood albumin, such that the blood signal therefore represents the total export of DB53 from striatum to blood[64333]. There was a significant reduction in DB53 fluorescence intensity over the femoral vein in chronic KA mice, which is indicative of suppressed glymphatic efflux. The mice with early PPA treatment exhibited efflux levels similar to the sham mice, suggesting a complete rescue of glymphatic efflux by PPA* ***(Fig. 6D)****.*

**Point 2:** If "boosting glympathic fluid transport with PPA restricted the inflammatory response by promoting the clearance of lactate, K+ and cytokines" is true, then the authors should show that 1) PPA enhances fluid efflux, 2) PPA changes the ratio of "cleared" molecules from the brain to the blood/periphery, and 3) a more specific increase in glymphatic fluid transport similarly improves epileptogenesis outcome.

**Answer:**

1) **PPA enhances fluid efflux:** New data shows that PPA rescued glymphatic efflux impairment in KA-Veh mice (as cited above).
2) **PPA changes the ratio of "cleared" molecules from the brain to the blood/periphery:** Unfortunately, there is no technique presently available that can address this point, as we state in the response to Point 1, above.
3) **A more specific increase in glymphatic fluid transport similarly improves epileptogenesis outcome:** We have not yet identified a simple (pharmacological) procedure for increasing glymphatic flow above baseline. However, we have reported that voluntary exercise enhances glymphatic flow[9] and others note that physical exercise during epileptogenesis can reduce the number of epileptic seizures and epilepsy comorbidities in the chronic stage [10,11]. In response to the reviewer's critique, we have undertaken new studies in *Aqp4*-KO mice, which have reduced glymphatic flow[1,2]. This new analysis showed that genetic

deletion of *Aqp4* accelerated KA-induced seizures and prolonged seizure duration in the acute phase and aggravated the spontaneous seizure onset in the chronic stage (**Figure 7**, shown above).

*Cited from the revised manuscript-result section:*

***Aqp4 knockout aggravated KA-induced SE and the severity of chronic epilepsy***

*We next tested whether a reduction in glymphatic flow in KA-induced epilepsy would worsen the outcome. Aqp4 knockout (Aqp4$^{-/-}$, Aqp4-KO) mice on a C57BL/6 background have previously been shown to exhibit reduced glymphatic flow [68,69] KA-Veh Aqp4-KO mice and their wildtype littermates exhibited similar neuronal discharges patterns as the FVB mice used in the prior experiments (**Fig. 1 and 7A**). As compared with wildtype littermate controls, Aqp4-KO mice exhibited significantly shortened latency of first seizure onset, greater mean duration and severity of seizure onset in phase 1, and prolonged the duration and total epileptic spike number in phase 2 (**Fig. 7B**). Aqp4-KO mice tended to have longer latency to phase 2 (**Fig. 7B-C**). There was a non-significant trend towards an increase in the number of seizures in AQP4-KO mice compared to controls (**Fig. 7C**). In several epilepsy models, C57BL/6 strain background mice develop less severe seizures than FVB mice[70,71]. Similarly, we here observed that four out of five wildtype mice did not exhibit spontaneous seizures during the four consecutive days of recording in the chronic phase (**Fig.7D**). In contrast, the Aqp4 knockout mice exhibited a significantly higher number of seizures, prolonged seizure duration, higher maximal Racine scale readouts and fewer seizure-free days (**Fig. 7D**) in the chronic phase as compared with wildtype littermates.*

**Point 3：** In addition, while PPA enhanced global influx (Fig. 2B), there did not appear to be a difference when parsed by region (Fig. 2C).

For better clarity on this point, we have prepared a new **Figure 5** presenting the influx data. PPA treatment significantly enhanced global influx and regional influx in the ventral cerebral cortex and hypothalamus regions in sham-Veh mice and in KA-Veh mice in the chronic stage (**Figure 5A-C, G-I**). In the mice with PPA administered concurrently with KA-infusion, there was a trend towards increased influx in the ventral cortex and hypothalamus (**Supplementary Figure S2**). In the latter analysis, we measured the glymphatic influx at five hours after the KA infusion, which was coincident with recurrent epileptic discharges (Figure 5.E). In response to this reviewer comment, we now present the brain regional data in a new **Supplementary Figure S2**.

*We revised the Methods section and the legends, as indicated below:*

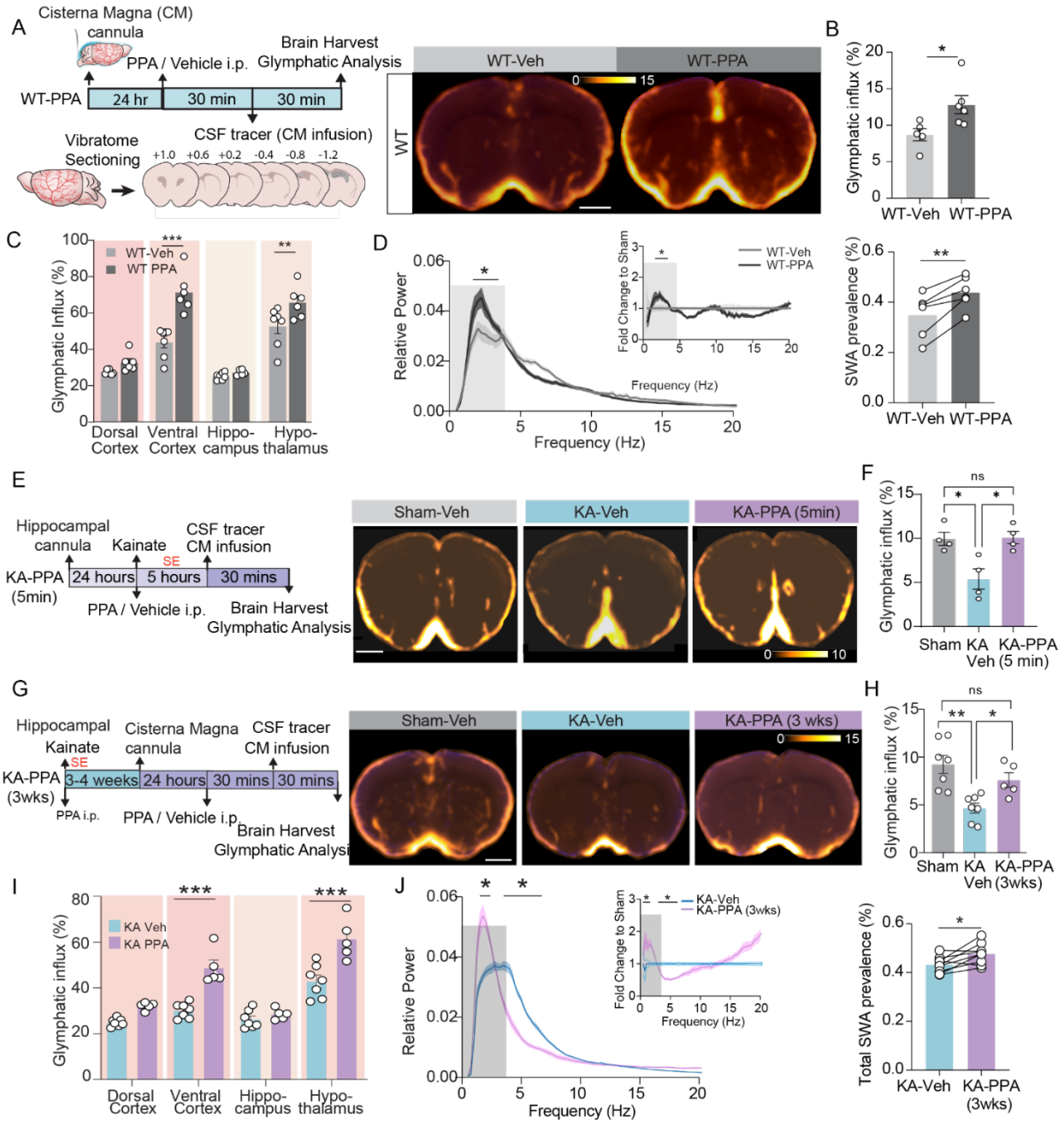



***Figure 5. PPA treatment enhances acute and chronic glymphatic tracer influx, while increasing EEG slow wave activity (A)*** *Glymphatic analysis in wildtype mice (PPA versus vehicle). A cisterna magna (CM) catheter was implanted 24 hours prior to the tracer infusion, and PPA was administered i.p. prior to infusion of the CSF tracer (bovine serum albumin, Alexa Fluor 647 conjugate, BSA-647, 65 kDa) in the CM. Population-based average images of CSF tracer distribution in PPA or saline-treated wildtype mice (right).* ***(B)*** *The influx area in a coronal brain slice (AP +0.6 mm) in PPA-treated mice compared the vehicle group (right, n = 5-6 mice/group; unpaired two-tailed t-test, *P = 0.028).* ***(C)*** *Analysis of the ratio of brain regional distribution of glymphatic influx between PPA-treated and vehicle-treated WT mice in*

*dorsal cortex (DC), hippocampus (HC), ventral cortex (VC), and hypothalamus (HT) in a coronal brain slice (AP -1.2 mm). Histograms summarizing the glymphatic influx area fraction. (mean ± SEM, n = 6-7 mice/group, Two-way ANOVA, Sidak's multiple comparisons test. VC, ***P < 0.001, HT, **P = 0.008).* ***(D)*** *EEG spectrum profile after PPA intraperitoneal injection in WT mice. Comparison of the power spectrum of 2-hour EEG recordings between PPA and vehicle treatment groups (left, *P < 0.05, two-way ANOVA, Tukey's multiple comparisons test). PPA treatment normalized the power spectrum the vehicle results, presented in the insert as fold change relative to vehicle-treated mice (upper right, *P < 0.05, two-way ANOVA, Tukey's multiple comparisons test) (insert). The total power of slow wave activity (SWA, 0.5-4 Hz) is compared between groups (right, n = 6, **P = 0.004, paired t-test) (right).* ***（E）*** *Glymphatic analysis in KA-treated mice (acute phase, 5 hours post KA infusion). Five minutes after KA treatment, mice received vehicle or PPA, and glymphatic function was evaluated 5 hours later by infusing CSF tracer (555-conjugated ovalbumin, OB-555, 45 kDa) in the cisterna magna (CM). Population-based images of brain coronal sections (Left, AP +0.6 mm, Scale bar = 1 mm) (right).* ***(F)*** *The influx areas of the brain sections (AP +0.6 mm) were compared among Sham-Veh, KA-Veh and KA-PPA groups, n = 4 mice/group, Sham-Veh vs KA-Veh, *P = 0.014, KA-Veh vs KA-PPA, *P = 0.012. One-way ANOVA, Tukey's post-hoc comparison).* ***(G)*** *Glymphatic analysis in KA-treated mice (chronic phase, 3-4 weeks post KA infusion). We administered intraperitoneal PPA 30 min prior to the CSF tracer (BSA-647, 65 kDa) CM infusion. Population-based images of brain coronal sections (Left, AP +0.6 mm, Scale bar = 1 mm) (right).* ***(H)*** *Quantitative analysis of CSF tracer influx in a coronal brain slice (AP +0.6 mm) (right, n = 5-6 mice/group, one-way ANOVA, Tukey's multiple comparisons test, Sham-Veh vs KA-Veh **P = 0.002., KA-Veh vs KA-PPA (3 weeks) *P = 0.045).* ***(I)*** *Regional influx analysis in a brain slice (AP -1.2 mm) after acute PPA administration in chronic epileptic mice. (n = 5-7 mice/group, VC, ***P < 0.001, HT, ***P < 0.001, two-way ANOVA, Sidak's multiple comparisons test).* ***(J)*** *EEG Power spectrum comparison between 2-hour PPA and baseline conditions. The power spectrum of 2-hour EEG recordings compared between KA-PPA (3 weeks) and KA-Veh treatment groups (Left, *P < 0.05, two-way ANOVA, Tukey's multiple comparisons test). The PPA treatment normalized the power spectrum to that in vehicle-treated chronic epilepsy mice (insert, upper right, *P < 0.05, two-way ANOVA, Tukey's multiple comparisons test). The total power of the Delta band (0.5-4 Hz) is presented on the right (n = 9, *P = 0.013, paired t-test). Data are presented as mean ± SEM.*

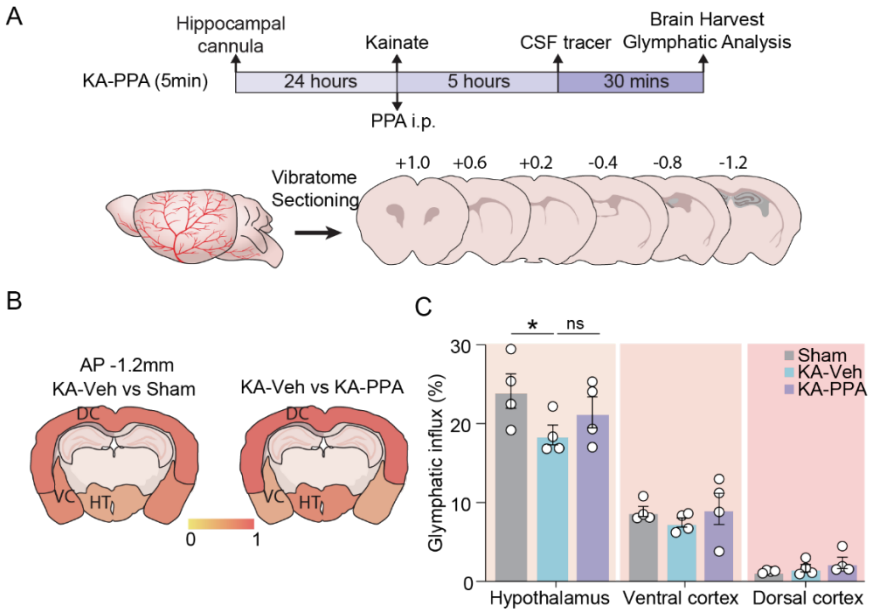



***Figure S2. Glymphatic influx after acute KA infusion treated by PPA. (A)*** *Glymphatic analysis of KA-induced status epilepticus (SE) and PPA treatment. We implanted a catheter in the cisterna magna (CM) 24 hours prior to the glymphatic evaluation. Five minutes after KA treatment, mice received vehicle or PPA, and their glymphatic function was evaluated five hours later by infusing CSF tracer (555-conjugated ovalbumin, OB-555, 45 kDa) in the CM. After 30 minutes of circulation, the brain was extracted and coronal brain sections (AP +1 ~ -1.2 mm) were cut using a Vibratome.* ***(B)*** *Regional analysis of CSF tracer distribution of coronal brain sections (Left, AP -1.2 mm). The color-coded brain regions display the ratio of tracer distribution areas between KA-Veh and Sham-Veh groups (upper left) and between KA-Veh vs KA-PPA groups (upper right).* ***(C)*** *The comparisons among Sham-Veh, KA-Veh and KA-PPA were presented below, including the hypothalamus, ventral cortex, and dorsal cortex regions. (n = 4-6 mice/group, Sham-Veh vs KA-Veh, *P = 0.016; KA-Veh vs KA PPA, P =0.290, Two-way ANOVA, Tukey's multiple comparison). Data are presented as mean ± SEM.*

2. Incomplete assessment of AQP4 expression/polarization and it's role in PPA-improvement of epileptogenesis. AQP4 MPI in Fig. 4A quantifies the whole field and suggests there is an increase in AQP4 expression across the whole FoV in KA-Vehicle which is reduced to vessels in PPA-treated brains. Presumably, this is due to a lack of AQP4 polarization and reflects its expression throughout astrocytes rather than at the endfeet. While Fig. 4B quantifies AQP4 polarization by normalizing to the background, if the background is not astrocyte specific then this polarization measure would be flawed. Evidence showing this background signal is in astrocytes (i.e. co-stain for astrocytes) would help alleviate this concern. Still, the functional implication of this polarization is unclear. AQP4 polarization is expected to increase efflux of water but it is not clear how this helps clearance of other inflammatory/damaging molecules. If increased glymphatic flow does help flush out these damaging molecules, where do they go?

**Response**：To avoid confusion in this matter, we now only present the data analyzed using the line polarization index in **Figure 6F** and **Figure S3D**. We have also revised the methods section to explain better the method for quantifying the AQP4 polarization. In a previous publication, we have reported a correlation between reduced glymphatic CSF tracer influx with loss of AQP4 polarization[12]. We emphasize in the revised manuscript that there are multiple glymphatic efflux

paths, including meningeal and cervical lymphatic vessels, arachnoid granulations, and the cranial and spinal nerves (reviewed[13]).

*Cite from the method section: For quantitation of AQP4 polarization, we followed the method published previously [12,14-16], as illustrated in **Fig. S3B.** In brief, we analyzed representative 70 μm segments centered around vessels using the line-plot tool in ImageJ. We then classified vessels into two groups based on their diameter (small vessel, < 10 μm, large vessel, 10-30 μm), and calculated the line polarization index (LPI) as the peak fluorescent intensity of the vascular end-feet divided by the average of the baseline. We undertook this analysis with images at 20X magnification.*

The polarized expression pattern of AQP4 plays an important role in facilitating glymphatic influx[1,2], and transport of CNS solutes[17] including amyloid-β[18], $K^+$ [19], and inflammatory cytokines[20,21]. Polarized AQP4 expression also mirrors the circadian rhythms of CSF amyloid-β levels [22]. There are reports of increased total AQP4 protein in traumatic brain injury [23], stroke [15,19], and Alzheimer's disease [24], which are linked to a reduction in glymphatic flow. The vascular polarization of AQP4 correlates with CSF tracer influx, as attested in numerous publications[2,12,25-27]. Perivascular polarized AQP4 expression ostensibly facilitates fluid and solutes transportation from the perivascular space of penetrating arteries into the interstitial space, which convey interstitial metabolic wastes along perivenous spaces, ultimately exiting the brain. The egress pathways for glymphatic flow follow along cranial nerves, cervical vessels, and lymphatic vessels enable fluid homeostasis in the brain. However, these efflux structures are not amenable for sampling of their fluid contents.

3. Effects of PPA on neuronal activity vs. enhanced glymphatic fluid transport. The manuscript does a great job at correlating pan adrenergic suppression with increased glymphatic fluid influx and improved epileptogenesis outcomes. As stated earlier**,** however, the effects of PPA on neuronal activity itself can explain many of the observed effects. Indeed, suppression of neuronal activity via benzodiazepines is the go-to treatment for status epilepticus. How is glymphatic flow affected in current treatments, especially those that also suppress neuronal activity.

**Response:** Currently there is limited experimental evidence demonstrating how benzodiazepines affect glymphatic function. According to one hypothesis, benzodiazepines might impair glymphatic flow due to their effect of reducing slow-wave sleep, which might underlie their contribution to an increased risk of dementia with long-term use[4,28,29]. Moreover, in preclinical studies, early benzodiazepine treatment clearly suppresses epileptic discharges during KA-induced SE[30-32], this standing in contrast to PPA, which primarily delayed the onset and prolonged seizure duration without affecting total seizure numbers during Phase 1 of SE (**Figure.1F** and **Table 1**). This discrepancy suggests that PPA and traditional antiepileptic drugs operate through distinctly different mechanisms. It is indeed plausible that the three types of adrenergic receptors that are targeted by PPA may exhibit differing effects on epileptiform discharges [33]. In *ex vivo* models of epilepsy induced by application of picrotoxin or 7.5 mM $[K^+]_e$ to CA3 pyramidal neurons, α1-agonist and α2-antagonist both slowed the discharges, while β-adrenergic receptor agonism increased the discharge rate[33]. In the present study, PPA did not reduce the number of seizures occurring in phase 1 of SE, but delays the phase 1 of SE, in addition, PPA significantly prolonged the duration of phase 1and slightly reduced the mean Racine scale score **(Figure 1F-G**). To circumvent interfering effects of PPA on acute SE, we have added new experiments with PPA administration at 30 hours after KA infusion, after the cessation of the acute onset of SE. This new analysis showed that administration of PPA at 30 hours after the induction of SE also significantly reduced the severity and onset of seizures in the chronic stage (**Figure 3, cited earlier in Page 3**).

Minor Concerns:
1. Methods:a. How was somatosensory cortex defined and why was only LII/III analyzed? The

example image in Fig. 4B shows the AQP4 background signal varies with cortical depth. How is this polarization index affected by cortical depth?

**Response:** In response to this critique, we undertook a new, blinded analysis of AQP4 immunostaining and quantified AQP4 vascular polarity in somatosensory cortex, hippocampus, hypothalamus, and ventral cortex. Comparison of AQP4 polarity in hippocampus, hypothalamus, ventral cortex and layer IV/V, layer II/III of somatosensory cortex. (**Figure S3**) recapitulated findings in the prior analysis.

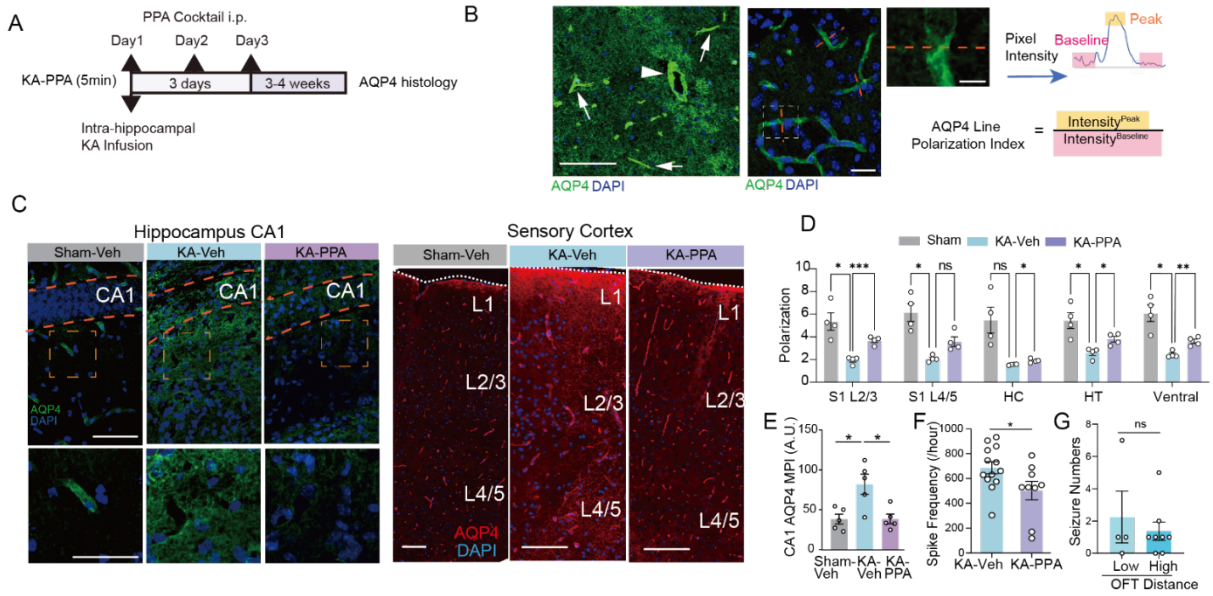



***Fig. S3 Quantification of AQP4 polarization in multiple brain regions.***

*(**A**) AQP4 immunostaining was analysis after 3-4 weeks after KA intrahippocampal infusion and PPA treatment. (**B**) Line-analysis of AQP4 Polarization Index (See Method for details). (**C**) Representative images of AQP4 expression in hippocampus CA1 region (Scale bar, 80 µm, insert, 50 µm) and sensory cortex (scale-bar :100 µm). (**D**) Regional analysis of small vessel AQP4 Polarization Index in Sham-Veh, KA-Veh and KA-PPA, including brain regions of sensory cortex Layer 2/3, Layer 4/5, hippocampus, hypothalamus and ventral cortex. (n = 4 mice/group, Two-way ANOVA, Tukey's multiple comparison. Sensory Cortex L2/3, Sham-Veh vs KA-Veh, *P = 0.039; KA-Veh vs KA-PPA, ***P <0.001; Sensory Cortex L4/5, Sham-Veh vs KA-Veh, *P = 0.026; KA-Veh vs KA-PPA, P =0.069; Hippocampus, Sham-Veh vs KA-Veh, P = 0.082; KA-Veh vs KA-PPA, *P =0.047; Hypothalamus, Sham-Veh vs KA-Veh, *P = 0.046; KA-Veh vs KA-PPA, P =0.031; Ventral Cortex, Sham-Veh vs KA-Veh, *P = 0.029; KA-Veh vs KA PPA, **P =0.009). (**E**) Mean pixel intensity of AQP4 in CA1 (n = 5 mice/group, one-way ANOVA, Tukey's multiple comparisons test, sham-Veh vs KA-Veh *P = 0.011, KA-Veh vs KA-PPA *P = 0.011). (**F**) The interictal spike frequency between KA-Veh and KA-PPA mice (n = 9-13 mice/group; *P = 0.041, unpaired t-test). (**G**) The seizure numbers in mice with less exploration (Total distance < 15 m) verse more exploration (Total distance > 15 m) in OFT (n = 4-8 mice/group; P = 0.532). Data are presented as mean ± SEM.*

b. For analysis of tracer influx, it is unclear how many and which coronal sections were used.

**Response:** We improved the depiction of the glymphatic influx changes in response to acute PPA administration, and reorganized the data in the revised **Figure 5**, including a presentation of the acute effect of PPA on influx in *wildtype* mice (**Figure 5A-D**), in sham and acute KA mice (**Figure 5E-F**), and in sham and chronic epilepsy model mice (**Figure 5G-J**). The representative coronal brain slices (AP +0.6 mm) consist of aligned and averaged images from all animals in each group, as described in the revised Methods sections. For quantitation of regional influx, we analyzed coronal slices (AP -1.2 mm) containing cortex, hippocampus, and hypothalamus regions of interest. In the revised manuscript, we expanded the figures depicting brain slices from representative mice to present the effect of PPA treatment on glymphatic influx during SE in **Figure S2**.

We revised the methods section as below. Please also refer the revised Figure 5 and legend in Page 9, and Figure S2 in Page 11.

***Methods:***

*The coronal brain sections (rostral to caudal: -1.2, -0.8, -0.4, +0.2, +0.6 and +1.0 mm from bregma) were then imaged using a 0.63 × lens with 2 X magnification on an MvX10 microscope (Olympus) equipped with a Lumencore 1600 (Prior) light source, using the Metamorph Basic (Olympus) software.*

2. Statistics.a. The authors test for normality but always use the parametric Tukey's multiple comparisons test (when comparing more than one group). If a single group distribution is found non-parametric, authors should be conservative and use non-parametric tests to determine statistical significance. It's possible the authors found that Tukey's was appropriate for all multiple comparisons test. If so, please state so in the statistics section. If not, please run the appropriate non-parametric or parametric statistic.

**Response:** We checked all the three-group comparisons with the Brown-Forsythe test or Bartlett's test for variance homogeneity. For any comparisons with positive results to these tests, we then applied Kruskal-Wallis test and Dunn's multiple comparisons tests. Several cytokine/chemokine measurements proved to have positive test results (**Figure S5**). Accordingly, we have updated the results, clarified the statistical methods for each cytokine/chemokine in the figure legend, and modified the relevant text statements in the revised manuscript.

b. It is unclear how variable the data in Fig. 6D is. While the sample size is listed, it is unclear if the statistic is being carried out animal as n versus population seizure numbers. Please clarify this. Also, given that this analysis is done on a by animal basis (as it should be), please add error bands to the cumulative distribution (error bands can be added via bootstrapping or by binning cumulative data). Alternatively, show data as a bar/scatter of animal means as shown everywhere else in the manuscript.

**Response:** We expanded the sample size of the PPA group to n = 10, thus matching the vehicle group (n=10), and quantified the seizure onsets during six hours post PPA administration (**Figure 4B-C**). The PPA groups showed a significant delay of seizure onset and decreased seizure numbers within the first six hours. Corresponding data during 12 hours post PPA administration are presented in **Figure S8**.

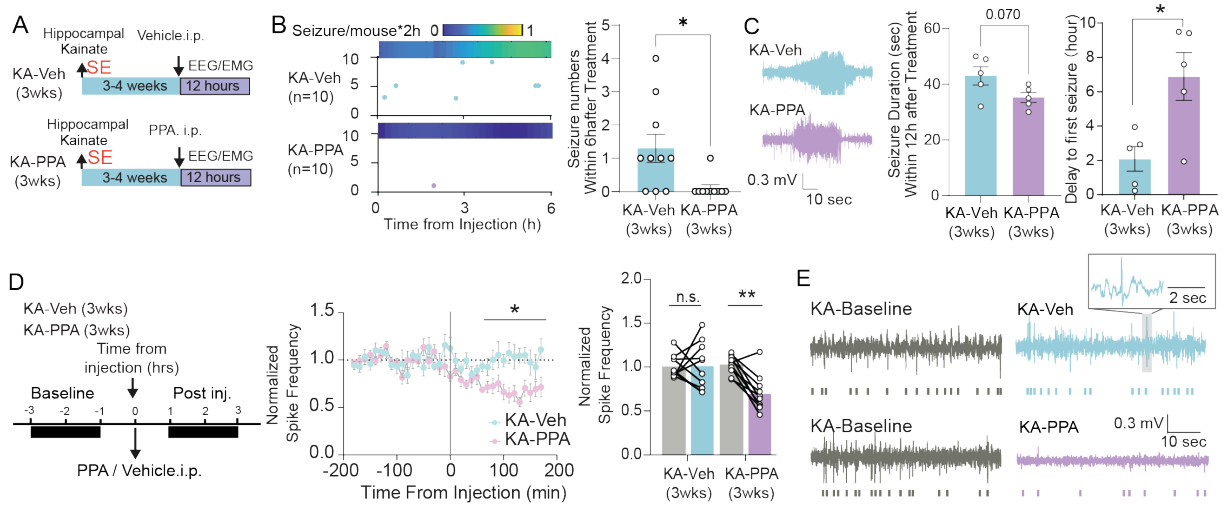



***Figure 4. Delayed administration of PPA at 3 weeks post-KA transiently reduces seizure burden***

***(A)*** *Timeline of experimental design of 3-week delayed PPA treatment. At 3-4 weeks post KA injection, mice received a single intraperitoneal dose of PPA or saline vehicle* ***(B)*** *Representative plot of seizures onset distributions injection during the first six hours in the PPA or vehicle treated epileptic mice. Each horizontal line represents a mouse and each dot indicates a seizure. The heatmap above indicates the seizure frequency of each group (left), the number of spontaneous convulsive seizures (middle). N = 10 mice/group, seizure numbers, *P = 0.070013, Mann-Whitney test.* ***(C)*** *For both KA-Veh and KA-PPA groups, five out of ten mice had convulsive seizures during 12-hour recording session. The average duration of convulsive seizures (left) and the delay to the first seizure (right) is summarized (n = 5 mice/group, *P = 0.013, unpaired two-tailed t-test).* ***(D)*** *Timeline (left) and normalized spike frequency is calculated based on the pre-PPA or -vehicle baseline (right). We quantified in the time window between two hours preceding and two hours after PPA or vehicle injection. (Left, n = 10 mice/group, 2-way ANOVA, Sidak's multiple comparisons test, *P < 0.05. Right, mean ± SEM, n = 10 mice/group, 2-way ANOVA, Sidak's multiple comparisons test. **P = 0.0021).* ***(E)*** *Representative traces (right) demonstrating interictal spikes before and after PPA or vehicle treatment in epileptic mice. Data are presented as mean ± SEM.*


***Fig. S8. Delayed PPA treatment in chronic epileptic mice did not reduce the number of seizures during the subsequent 12 hours****. Representative plot of seizures to show onset distributions of PPA- or vehicle-*

*treated epileptic mice during 12-hour recording sessions. Each horizontal line represents a mouse and each dot indicates a seizure. The heatmaps above indicates the seizure frequency of each group (left) and the number of spontaneous seizures (right). n = 10 mice/group, Seizure numbers, P = 0.135, Mann-Whitney test.). Data are presented as mean ± SEM.*

3. L227 reads “ovary-shaped cells” – did the authors mean oval-shaped cells?

**Response:** Corrected.

4. L505-506 sentence missing words

**Response**: Corrected.

5. Blood-brain-barrier dysfunction is a common hallmark of epileptogenesis, yet the authors mention it once. Are there BBB perturbations at the observed timepoints? If so, does PPA treatment and enhanced glymphatic fluid transport help or degrade BBB integrity?

**Response:**

In response to this critique, we have analyzed the integrity of the blood brain barrier (BBB) at 24 hours after KA infusion, following a recently published protocol involving intravenous cadaverine-conjugate administration[34]. The new analysis showed no significant breaching of the BBB in KA-Veh and KA-PPA mice compared with the sham group. As a positive control, we administered lipopolysaccharide (LPS,10 mg/kg, i.p.) in a separate group of *wildtype* mice, which resulted in a significant breaching of the BBB, thus confirming the fitness of the assay. We have revised the manuscript accordingly, and now present these new data as **Figure S6.**

Legend cited from the revised manuscript:

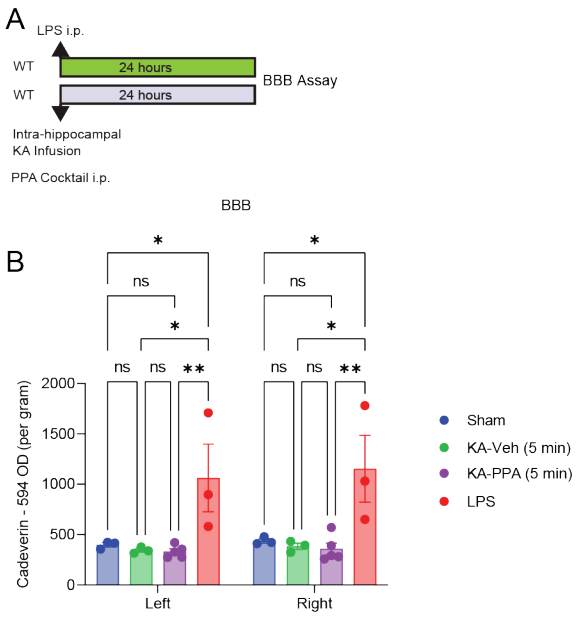



***Fig. S6. The blood brain barrier (BBB) is not compromised at 24 hours after KA-induced SE (A)*** *As a positive control, lipopolysaccharide (LPS; 10 mg/kg, i.p.) was applied 24 hours prior to assaying the BBB permeability according to cerebral uptake of i.v. cadaverine. Other groups of mice received vehicle, KA intrahippocampal infusion, or PPA i.p. 24 hours prior to the BBB test. For each hemisphere, the cadaverine OD values (594 nm) were normalized by tissue weight.* ***(B)*** *OD value comparison for each hemisphere. (N = 3-5. Left, Sham vs LPS *P = 0.038, KA vs LPS *P = 0.022, KA PPA vs LPS **P = 0.008. Right, Sham vs LPS *P = 0.022, KA vs LPS *P = 0.013, KA PPA vs LPS **P = 0.004. Two-way ANOVA, Tukey's multiple comparison) Data are presented as mean ± SEM.*

**Reviewer #2** (Remarks to the Author): Sun and colleagues presented a study in which they ameliorate the outcome of a chemical triggered epileptogenesis acting on the glymphatic influx via adrenergic receptor antagonist administration.The manuscript is clear and all the important information is provided and figures are carefully crafted and very clear. The potential antiepileptic effect of the treatment has been evaluated in 2 sets of experiments, an early treatment in the three days after the KA injection and a delayed one once epilepsy fully developed. Several techniques have been used to support the findings: EEG, EMG, behavioral test and immunohistochemistry to name only some.The study introduces a promising novel approach to improve epilepsy symptoms, in mice. However, the findings are oversold and/or not correctly interpreted.

Major points: The main claim of the study is that "pan-adrenergic blockade suppresses epileptogenesis", however, it is supported by results. In fact, In the KA intra-hippocampal injection mouse model, epileptogenesis usually develops in 3-4 weeks. The experiments addressing an eventual suppression of epileptogenesis are the ones in which PPA was administered just after the status epilepticus, whose results in terms of epileptiform activity are reported in Fig. 3. Indeed, there is not a suppression of epileptogenesis, nor a clear anti-epileptic effect, because the significant decrease in seizure duration is not matched by a similar effect on seizure frequency and, still, seizure

in treated mice last, in average, 40 sec. The decrease in inter-ictal spikes (IIS) cannot be considered as an antiepileptic effect of the treatment, since IIS have been hypothesized being a natural protective anti-epileptic mechanism to prevent seizures (see PJ Karoli et al., Brain, 2016). Having less IIS does not automatically mean anything about epilepsy. Of absolute interest, the improved behavioral performance of treated mice. The main message of this study must be changed accordingly to the results.

**Response:** We are grateful for these insightful comments. In response, we repeated the experiments with EEG recording over four consecutive days at three weeks after KA-induced SE. As shown in the revised **Figure 2B-D**, there were fewer seizures in the KA-PPA group as compared with the KA-Veh group. The PPA treatment also shortened the average seizure duration at late follow-up, which is consistent with our prior findings. Scoring of the clinical severity of the spontaneous seizures using the Racine Scale also showed lesser seizure severity in the KA-PPA than in the KA-Veh group. For the revised Figure and its legend, see page 2 above.

As also noted above in response to reviewer 1, we have added an additional experiment with PPA administration at 30 hours after KA infusion, thereby avoiding the possible interference with SE by acute PPA. The delayed PPA treated significantly reduced the severity and onset of seizures in the chronic stage monitored at 3-4 weeks and two months after KA injection, as depicted in the revised **Figure 3** and its caption, which are presented on page 3, above.

The delayed pan-adrenergic blockade showed, indeed, an effective anti-epileptic effect - although it does not suppress epilepsy - decreasing both seizure frequency and duration, concomitant to a higher glymphatic influx. However, the effect was evaluated only for 24h, rising question about the long-term efficacy of the treatment. Based on the presented results, I would say that pan-adrenergic blockade has a short-term anti-epileptic effect. Note that I did not mention the differences of epileptiform activity observed during status epilepticus in treated vs untreated mice because it does not seem to significantly modify the triggered epileptogenesis (see comment above on Fig.3).

**Response:** In this study, we focused on the potential long-term therapeutic effect of three consecutive days of PPA treatment on the acute phase of SE. It is correct that a single dose of PPA also reduced the frequency of seizures (**Figure 4B-E**)**.** As noted above in response to reviewer 1, regarding effect of PPA on epileptogenesis, we conducted a supplementary experiment in which PPA was administered 30 hours after KA infusion, thereby avoiding interference with the phase of established SE. The results of this new study show that PPA treatment initiated after SE significantly reduced the severity of epileptogenesis in the chronic stage (**Figure 3**).

The study lacks generalization because it presents results obtained in both extreme and narrow conditions. Extreme because the treatment was a cocktail targeting all the receptors of the adrenergic system (pan-adrenergic), that is a strong and nonspecific approach, unlikely to be used to develop novel anti-epileptic approaches. Narrow because a single model of induced epilepsy has been tested, a standard one, for sure, but also highly related to inflammation. It would be of interest to test other model of epilepsy, particularly the ones based on genetics or brain concussion. Narrow also for the already discussed evaluation of anti-epileptic effect of the delayed PPA blockade over a limited time.

**Response:** We agree with the reviewer that the PPA treatment has a broad effect; this is by intention. We utilized the pan-adrenergic inhibition (in emulation of the absent noradrenergic tonus during REM sleep) to enhance glymphatic function, as previous work showed this cocktail to normalize seizure-related pathophysiology such as excessive extracellular $K^+$ release in stroke[19 35], and cerebral edema in post-traumatic brain injury[36], which is a major cause of acquired epilepsy[37]. Those studies also documented less potent effects of individual adrenergic receptor antagonists on glymphatic clearance and prognosis after injury. In other epilepsy studies, these three types of adrenergic receptors exhibit

diverse effect on epileptiform discharges [33]. For example, in an *ex vivo* model of epilepsy in CA3 pyramidal neurons induced by picrotoxin or 7.5 mM $[K^+]_e$, α1-agonist and an α2-antagonist slowed the discharges, while β-adrenergic receptor agonist increased the discharge rate[33].

To expand the narrow time window of PPA application, we conducted a supplementary experiment at two months after the initial SE. In this experiment, we administered PPA at 30 hours after KA-infusion. At two months' follow-up, mice with this early three-day PPA treatment had significantly fewer seizures during the recording sessions as compared with sham mice (**Figure 3D-F**).

We agree with the reviewer that our approach should be generalized to investigations in other epilepsy models.

From the title, a direct cause-effect link between glymphatic system and epileptogenesis is clearly stated. In addition to the point about epileptogenesis already mentioned, the results of the paper highlight a direct correlation between anti-epileptic effect of PPA treatment and increased glymphatic influx at mice population level. However, because of nonspecificity of the treatment, targeting the entire adrenergic system, it is not possible to assess a direct causality between glymphatic influx and anti-epileptic effect. The hypothesis that this treatment both enhances the glymphatic influx and has an anti-epileptic action, independently, cannot be discarded. Surely a mouse-level evaluation of the glymphatic influx increase and the anti-epileptic effect exerted by PPA would make stronger the correlation between the two mechanisms, but a cleaner pharmacological approach, targeting specifically the glymphatic system is necessary to state the causality.

**Response:** As we noted above in response to reviewer 1, there is no simple pharmacological way to increase glymphatic flow. We have shown that voluntary exercise enhances glymphatic flow[9], and that our present pan-inhibition of adrenergic receptors enhances glymphatic function[4,35,36]. In new experiments, we employed *Aqp4*-KO mice, which have impaired glymphatic fluid transport[1,2]. This new analysis showed that genetic deletion of *Aqp4* accelerated KA-induced seizures, and prolonged seizure duration in the acute phase and aggravated the spontaneous seizure onset in the chronic stage (**Figure 7**).

These data are consistent with a prior study in which we induced seizures via electrical stimulation of the hippocampus; *Aqp4*-KO mice had much longer seizure duration, in spite of having an elevated seizure threshold as compared with controls [38]. There are also reports that *Aqp4*-KO has a proconvulsant effect in KA-induced SE and post-traumatic seizures [39-41]. In addition, *Aqp4*-KO mice have greater seizure susceptibility in response to application of the proconvulsant (GABA-A antagonist) pentylenetetrazol (PTZ). Thus, we utilized a validated model of reduced glymphatic flow, as distinct from endeavoring to enhance glymphatic flow with PPA.

For the revised Figure 7 and its caption, see page 5-6 above.

Minor points: Please, avoid using 'suppress' instead of 'decreased'. It gives a false message to the reader. See the title, line 372 etc.

**Response:** Corrected.

Line 56: the Authors refer to epileptogenesis writing about AMPA receptors modifications. This definition correctly applies to long-term potentiation, but not to epileptogenesis. In fact, the cited references (10, 11) do not even mention epilepsy.

**Response:** Thank you for the comment. We have updated the references accordingly.

Line 95: typo, status epilepticus.

**Response:** Corrected

Line 106: mice were 'withdrawn from anesthesia' after KA injection: Was atipamezole used? Being it an alpha2-receptor antagonist, it could have hidden some potential effect of the PPA treatment.

**Response:** To eliminate the confounding factors from anesthetic agents, we performed the additional experiments, as presented in **Figures 2 and 5** in the revised manuscript, in awake non-anesthetized mice pre-implanted with intrahippocampal cannula. The analysis showed that the effects of PPA treatment did not depend on the presence of anesthesia. In the previous dataset, we had anesthetized the mice with i.p. KX (ketamine, a NMDAR antagonist and xylazine, an α2-adrenerigic receptor agonist) for their fixation on a stereotaxic frame. This anesthesia mixture increases glymphatic influx [4,18]. Whereas the anesthetic effect of KX lasts 1-2 hours, the pharmacological effects of PPA last for 6-8 hours.

Line 108: I hope that mice were not injected i.p. with 1ml in a single shot. It is a very big amount of liquid. This quantity is over the limit of the ethical amount of i.p. injected solutions.

**Response:** We applied saline in the volume to avoid post-surgery dehydration. Our procedure is approved by the University of Rochester UCAR Protocol 2011-022 as follows: Experimental substance table, section 8.1.7.1, 0.9% saline, 0.1-1 ml. In fact, administration of 1 ml saline i.p. is commonly reported in the literature [42]. We have revised the manuscript with the following qualification: “To support fluid homeostasis during surgery, we intraperitoneally administered 1 mL of saline (0.9%) at a temperature of 36 °C shortly after induction of anesthesia.”

Line 241: the statement about "Data and SW availability" is insufficient.

Response: Corrected.

Line 108, 315, 337, 352: typo, Kainate.

Response: Corrected.

Line 351: Bi-modal distributions are proposed for seizure frequency and open-field test. Although true for the latter, the seizure frequency has not an apparent bi-modal distribution.

**Response:** Corrected.

Line 386: please, do not use 'partially rescued' for non-significant data.

**Response:** The neuronal loss caused by KA in sensory cortex (S1) and in the hippocampal CA1 region was significantly rescued by early PPA treatment (original **Supplementary Figure S3C**). We use the term ‘partially rescued’ to qualify that the neuronal counts in PPA-KA group were still significantly lower than those in sham group, albeit to a lesser extent than in the KA-alone group.

**Reviewer #3** (Remarks to the Author): In this manuscript, Sun et al. reports antiepileptogenic effects of pan-adrenergic inhibition by enhancing glymphatic fluid transport in the model of intrahippocampal kainic acid. Since the changes in seizures and glymphatic flux have been reported in response to modulating adrenergic receptors, the findings of the present study are not entirely novel; however,

they confirm the earlier reports. Overall, the experiments are designed well but the results require better presentation. Please refer to the following comments for more details.

**Response:** As noted also in our response to reviewer #2, we utilized the pan-adrenergic inhibition approach to enhance glymphatic function based on our earlier findings of its efficacy in normalizing seizure-related pathophysiology such as excessive extracellular $K^+$ release in ischemic stroke[19 35], and cerebral edema in post-traumatic injury[36], which is a major cause of acquired epilepsy[37]. Those studies also demonstrated lesser effect of individual adrenergic antagonists on glymphatic clearance and on clinical prognosis. In previous experimental epilepsy studies, the three types of adrenergic receptors exhibited differing effects on epileptiform discharges [33]. For example, in an *ex vivo* model of epilepsy induced by picrotoxin or 7.5 mM $[K^+]_e$ in CA3 pyramidal neurons, an α1-agonist and an α2-antagonist slowed down the discharges, while a β-adrenergic receptor agonist increased the discharge rate[33]. Therefore, we have proposed that pan-adrenergic inhibition should have a composite net effect favoring its efficacy in the context of epileptogenesis.

1) Fig 1F: Racine seizure scores for 3 mice in each group are not plotted. Did not all mice in each group develop SE? Did PPA treatment affect the number of mice developing KA-induced SE?

**Response**: In **Figure 1**, we present data from the subset of animals with video recording for quantitation of the Racine Scale. To avoid confusion, we therefore labeled the Racine Scale quantification with as a separate panel (**Figure 1G)**, with the legend reading, *"**(G)** In a subset of the mice, Racine scale was quantified based on video recordings obtained during the EEG recordings."* All the mice develop KA-induced SE in the acute phase. As shown in **Figure 1F**, PPA treatment did not reduce the number of seizures occurring in phase 1, but a delayed phase 1 of SE increased the onset interval, which slightly but significantly reduced the mean Racine scale score.

2) Fig 2B: Was there any difference in tracer distribution on the dorsal areas of the brain between the groups? Mention about the anatomical reference placed over the ventral image in the figure legend.

**Response:** We found the PPA treatment significantly increased the glymphatic influx in hypothalamus and ventral cortex in wildtype control mice and chronic epilepsy model mice, as compared to their respective controls (**Figure 5**). There was a non-significant trend towards increased glymphatic influx in dorsal cortex after PPA treatment (**Figure 5**). The ventral images in the original **Figure 2B** are representative images. As stated throughout the revised manuscript, we used coronal sections for quantitation of glymphatic influx. To avoid confusion on this point, we have removed the ventral images in the revised manuscript. Please find the revised Figure 5 and legend above in Page 9.

3) Fig 2C: Doublecheck brain slice displays showing the ratio of trace distribution areas. As per the legend, hotter the color, lesser the difference between the comparison groups (i.e., if the ratio=1, the trace distribution areas are equal for the comparison groups). If so, dorsal cortex should show the highest difference between KA-Veh and KA-PPA groups; however, this comparison is not statistically difference as per the graphs below. Clarify.

**Response**: Thank you for pointing out the mislabeled color. The correct ratio of KA-Veh over Sham in original **Figure 2C** is actually 0.812, and KA-Veh over KA-PPA is 0.842. We checked and updated the reported ratios of glymphatic influx between KA-vehicle and sham-vehicle groups, and between KA-vehicle and KA-PPA groups in **Figure S2B** of the revised manuscript. Please find details in the response to reviewer 1, Point 3 on Page 8.

4) Fig 3: Did PPA reduce the number of mice with chronic seizures? Include seizure severity data.

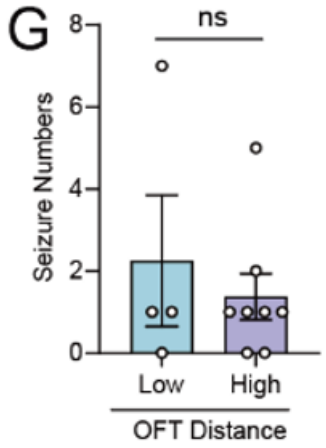
**Response:** We now present the survival curves of sham, KA-Veh and KA-PPA treatment groups in the revised **Figure 2C.** The seizure severity as evaluated in 3-4 weeks after the initial SE is presented in **Figure 2D**. We also evaluated the spontaneous seizure severity at two months after the initial SE, as presented in the revised **Figure 3D-F**. We present the revised **Figures 2 and 3** and their captions on pages 2-3, above.

5) N (no. of mice) are different for various graphs in Fig 3 panels. For example, why only 5 mice are shown for KA-Veh group in panel A (body weight), whereas 13 mice for KA-Veh group in panel D (spike freq)?

**Response**: We measured body weight in a separate batch of mice, as indicated in the legend of the revised figure 3. We regret the error in the sample size of the spike frequency data, which we have corrected in the revised manuscript. To avoid interference from anesthetics administrated prior to the intrahippocampal KA infusion, we undertook additional experiments in mice with pre-implanted cannula, and later conducted the KA infusion while the animals were awake. We also updated the behavioral data accordingly.

6) Given the bimodal distribution of mice in KA-PPA group for their seizure frequency and open-field activity, was there any correlation between seizure frequency and open-field activity?

**Response:** In the revised manuscript, we retrospectively compared the seizure numbers between subgroups of mice with less or more explorative behavior, as presented in the revised **Figure S3G**. There was no significant difference between the two groups.




*Legend cited from the revised manuscript:*

***(G)** The seizure numbers in mice with less exploration (Total distance < 15 m) verse more exploration (Total distance > 15 m) in the open field test (OFT; n = 4-8 mice/group; P = 0.532). Data are presented as mean ± SEM.*

7) Figs 4 and S2: AQP4 line polarization index around blood vessels in somatosensory cortex and CA1 area of hippocampus was found reduced in the KA-Veh group. However, the images and line analysis show AQP4 staining intensity in the perivascular region in the KA-Veh group either comparable to or even higher in the control group. The polarization index was found to be reduced because of overall increased expression of AQP4 in the KA-Veh group, which could be due to increased astrogliosis in the KA-Veh group. Please comment. How many blood vessels (small and large diameter) from each mouse were analyzed for this analysis?

**Response**: The total AQP4 protein expression was enhanced in the KA-Veh group, but was normalized in the KA-PPA group (**Figure 6E of the revised manuscript**). Total AQP4 protein overexpression, which could likely bear some relation with neuroinflammation and astrogliosis, was reported previously in traumatic brain injury [23], stroke [15,19], and Alzheimer's disease [24]. AQP4 water channels are expressed selectively in astrocytes, mainly in perivascular astrocytic processes plastered around the cerebral vasculature [43]. Therefore, we suppose that there was concurrent overexpression of AQP4 protein and loss of AQP4 perivascular polarity in the model mice.

We used a published method of measuring the line polarization index to quantify AQP4 polarization [15,44], which is described in some detail in the revised methods section. We included three small and large vessels from Layer II/III for each animal in **Figure 6F** and three small vessels for each brain region in **Figure S3D.** We present the revised **Figure S3** on page 13. For clarity, we updated the method section and cited below.

*Citation from the revised methods section:*

*For quantitation of AQP4 polarization, we followed the method published previously [12,14-16], as illustrated in **Fig. S3B.** In brief, we analyzed representative 70 μm segments centered around vessels using the line-plot tool in ImageJ. We then classified vessels into two groups based on their diameter (small vessel, < 10 μm, large vessel, 10-30 μm), and calculated the line polarization index (LPI) as the peak fluorescent intensity of the vascular end-feet divided by the average of the baseline. We undertook this analysis with images at 20X magnification.*

8) Since glymphatic flow supports the optimal maintenance of the extracellular glutamate and K+, it would be best to measure the level of glutamate and K+ in extracellular dialysates collected, in addition to the cytokines data already provided.

**Response:** We appreciate the reviewer's suggestion. Previous animal and human studies have shown that extracellular glutamate and potassium levels both increase during seizure[45-52]. However, the available interictal data are not conclusive in this regard. In studies of human seizure patients, there was no significant change in glutamate release to cerebral microdialysis at the seizure foci [53,54], nor was there any change in the glutamate signal to magnetic resonance spectroscopy (MRS)[55-57]. Other studies found elevated[58] or decreased[59] baseline glutamate levels ($[K^+]_e$) during the interictal state , Various studies show significant changes in the expression of proteins mediating potassium dynamics (Potassium inwardly-rectifying channel (Kir4,1), Na-K-ATPase, Connexin 43) in epilepsy[60-64]. However, it remains uncertain how the absolute $[K^+]_e$ may change in epilepsy[65,66]. Thus, we believe that an additional analysis of $[K^+]_e$ would likely not add critical new insights about seizure biology, but would rather reflect whether or not the mice had experienced a seizure during the collection of the micro-dialysis samples. Furthermore, we note that insertion of a microdialysis probe will certainly perturb glymphatic flow[67].

9) Lines 105, 181, 188: Mention the reference point for mouse brain coordinates used for cannula implant.

**Response**: Corrected

10) Overall methods should provide more relevant details.

**Response**: We have expanded the presentation of the methods section

11) Line 315: kainite – typo error.

**Response**: Corrected

12) Line 356: Cite Fig 3F instead of 3E.

**Response**: Corrected

13) For trace image analysis, be consistent with the y-axis label. It should be “Glymphatic influx (%)”.

**Response**: Corrected

14) As per the results text, seizure duration graph should be part of panel D in Fig 3.

**Response**: Corrected

**Reviewer #4** (Remarks to the Author): This paper uses two or three different studies involving experimental manipulation of the adrenergic system (as an indirect methodological approach to modify glymphatic fluid transport), which is proposed to secondarily alter chronic epileptogenesis induced by status epilepticus via intrahippocampal KA injection in mice. The first study used 72-hr video/EEG recording immediately following intrahippocampal KA injection to examine the effects of increased CSF influx (via adrenergic receptor agonists, PPA) on the latency and duration of seizures associated with the consequent status epilepticus (here divided into a first phase of recurrent seizures and a second phase of continuous spiking). PPA significantly delayed the onset of phase 1 convulsive seizures and prolonged seizure durations but did not affect the total number of seizures. PPA also shortened the duration of phase 2 spiking. These effects were thus on the status epilepticus, and not on epilepsy. In a second study, PPA was administered for 3 days post-KA, and EEG recordings were performed for 2 hr at approximately 5 weeks post-KA. PPA had no effect on daily seizure frequency, but did shorten seizure durations from approximately 49 to 39 sec and reduced interictal spike frequencies from approximately 687 to 503 spikes/hr. These are minimal changes and essentially inconsequential in regard to the claim of altering epileptogenesis. Finally, in a separate set of mice, PPA was administered at 4 weeks post-KA and EEG recorded for 24 hours. Delayed PPA administration had the effect of reducing counts of spontaneous seizures for 12 hr and reducing interictal spike frequency by about 40% for 1-3 hr. These results provide no evidence for an effect on epileptogenesis; rather, they are simply short-term drug effects. The observation that PPA can influence certain features of the intrahippocampal KA model (i.e., surrogate markers) may be somewhat interesting, but none of these experiments relate to epileptogenesis. The basic design and interpretations of these experiments do not support the proposed hypothesis that the glymphatic fluid transport has a role in epileptogenesis, and that the effects on glymphatic fluid transport can modify epileptogenesis.

The first major flaw is that most of the effects of PPA were on status epilepticus. As such, PPA appears to affect the nature and severity of the initial injury, and not epileptogenesis. This is an important and fundamental flaw of these studies, and on its own, negates the subsequent interpretations. Many researchers have already shown that experimental manipulation of the initial injury, as expected, can

alter the subsequent epileptogenesis. In terms of providing evidence for an effect on the process of epileptogenesis, it is imperative that one apply the experimental manipulation after the insult, preferably well after the insult, so that one can be sure that the experimental manipulation is not merely blunting the injury. The second major flaw in this study is that the authors have not studied the spontaneous recurrent seizures that are the definition of epilepsy. Not only do the electrical measures simply show an alteration in the characteristics of seizure activity of the status epilepticus, all of the later measures are indirect. The only measure that is relevant is the overall long-term frequency and severity of spontaneous seizures (i.e., "epileptic" or chronic seizures). To show a change in the actual epilepsy would require that the authors record spontaneous seizures for several months to determine if their frequency and severity are permanently altered. The changes reported here are actually quite small and simply not relevant to the question of epileptogenesis. For example, the effects on the duration and latency of seizures, as described by the authors, are unimportant, particularly since the total number of seizures was not affected. Similarly, late administration (4 weeks) of PPA produced some reduction in spontaneous seizures and interictal spikes for several hours, but again, this is not an effect on epileptogenesis – only a transient effect of the drugs. Chronic video/EEG recording for months post-KA would be required to assess whether any of the PPA applications examined here had an effect on the development and severity of epilepsy in this animal model. Unfortunately, these recordings were not performed, eliminating any the potential impact of this work.

**Response**: In response to the first critique, we conducted an additional experiment in which we first administered PPA at 30 hours after the kainic acid (KA) infusion, thereby avoiding interference with the SE phase. This delayed PPA administration significantly reduced the severity and onset of seizures in the chronic stage (**Figure 3**). Importantly, the alleviation of spontaneous seizures in the chronic phase was evaluated at both three weeks and two months after KA infusion. We present the revised **Figure 3** and its caption on page 3 above.

In addition to these considerations, we repeated the experiment with PPA treatment 5 min post KA-injection in conjunction with EEG/EMG recordings for four consecutive days at three weeks and two months post KA-induced SE. As shown in **Figure 2A-D** of the revised manuscript (see Page 2, above), we observed a suppression of the number of seizures in the KA-PPA group as compared with the KA-Veh group at three weeks post KA. The PPA treatment also shortened the average seizure duration, which is consistent with our prior findings from original submission. Scoring of the spontaneous seizures using the Racine Scale also showed lesser severity of seizures in the KA-PPA group compared to the KA-Veh group.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have overall done a good job addressing reviewer comments. One point remains from the initial review (original concern 1, part 3) that was not fully addressed in the revision.

The authors propose that enhanced glymphatic flow alleviates pathophysiology in a mouse model for epilepsy by clearing toxic molecules from the brain. In the revised manuscript, the authors show regional differences in hypothalamus and ventral cortex, but based on the data, **it seems that is no change in glymphatic flux or with Aqp4 polarization at the hippocampus.** This is curious since hippocampal dysfunction underlies epileptogenesis. The authors make broad claims about PPA enhancing glymphatic flow that improves KA-induced outcomes, and so it seems that some of these changes would be anticipated to take place at the hippocampus. How do the authors reconcile these findings?

**Comment 1-it seems that is no change in glymphatic flux at the hippocampus.**

**Response:** Thanks for the comments. As showed in **Fig 5C** and **5I**, we did find only a slight trend of increase in glymphatic influx in the hippocampus. However, these results might be biased by the low tracer intensity that represents glymphatic flux in the hippocampal regions. The relatively low glymphatic flux in hippocampus was also found in multiple previous rodent studies[1-6]. The underlying mechanisms were not fully understood. We speculate that it may be due to the differences in the routes for cerebrospinal fluid (CSF) entry and the structures of vasculatures between hippocampus and cortex.

In cortex, the penetrating arteries enter the brain parenchyma from the pial surface, allowing the perivascular glymphatic flow to reach the deeper cortical layers directly from subarachnoid space. Hippocampus is dependent on the blood supply from the collateral branches of the posterior cerebral artery (PCA)[7-10], which generates a distinctive glymphatic influx pathway from the cortex. On the other hand, the approaches of tracer delivery might also affect tracer distribution features in the brain. This is supported by a recent study indicating that the hippocampal tracer intensity could be potentially enhanced if the tracer was delivered intranasally[11]. Additionally, cortex was reported to have significantly higher artery and capillary density than hippocampus[12,13], which facilitate the CSF entry via the perivascular space.

In summary, the low basal intensity levels of fluorescence tracer in hippocampal regions, might explain the trending but not significant enhancement of tracer intensity in hippocampus as result of PPA treatment. .

**Comment 2- There is no changes in AQP4 polarization in the hippocampus.**

**Response:** We have in response to the reviewer's comments added the quantification of AQP4 polarization in the contralateral hippocampus. We think it is more accurate than the ipsilateral hippocampus quantification, since the kainic acid (KA) infusion site caused astrogliosis that could affect AQP4 polarization[15,16]. The data is included in **Fig. S3E**, and also presented below. It showed that AQP4 depolarization is significantly upregulated in KA-Veh mice versus Sham-Veh, which can be significantly rescued by PPA treatment.

In response to this comment, we added a paragraph to the discussion in Page 13-14.

*"A limitation of this study needs to be addressed. The unique vasculature of the hippocampus, primarily supplied by collateral branches of the posterior cerebral artery, poses a challenge for the evaluation of glymphatic influx. Our reliance on the conventional cisterna magna (CM) infusion technique, which tends to be more effective in cortical regions than in the hippocampus, may have influenced our observations[128–131]. This limitation highlights the need for alternative approaches, such as intranasal tracer delivery[132] or MRI imaging with specific tracers[33], to better assess glymphatic transport in the hippocampus."*

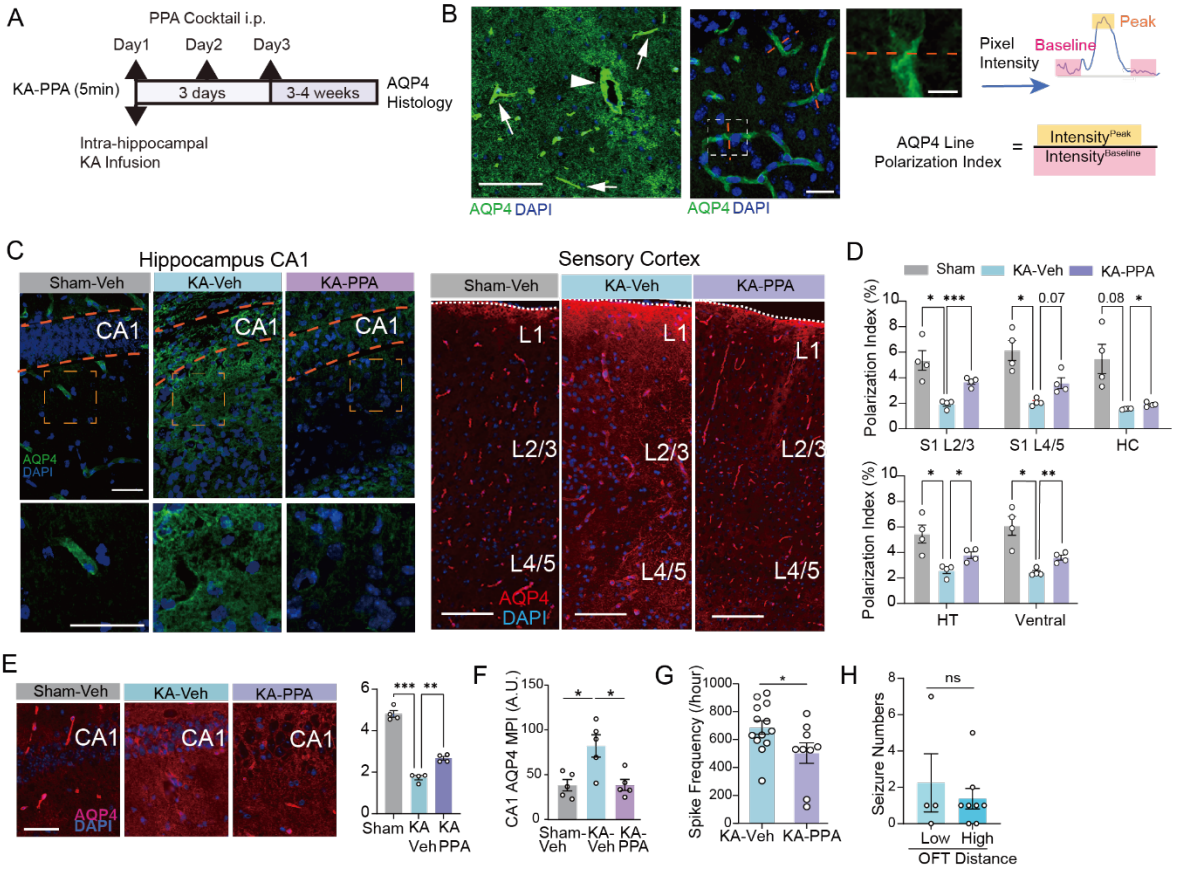



***Fig. S3 Quantitation of AQP4 polarization in various brain regions. (A)*** *Analysis of AQP4 immunostaining at 3-4 weeks after intrahippocampal KA infusion and PPA treatment.* ***(B)*** *Line-analysis of AQP4 polarization*

*index (PI, see Methods for details).* ***(C)*** *Representative images of AQP4 expression in CA1 hippocampal region (Scale bar, 50 µm) and sensory cortex (scale-bar :100 µm).* ***(D)*** *Regional analysis of small vessel AQP4 PI Index in Sham-Veh, KA-Veh and KA-PPA, including sensory cortex layer 2/3, and layer 4/5, hippocampus, hypothalamus and ventral cortex. (n = 4 mice/group, Two-way ANOVA, Tukey's multiple comparison. Sensory cortex layer 2/3, Sham-Veh vs KA-Veh, *P = 0.039; KA-Veh vs KA-PPA, ***P <0.001; Sensory cortex layer 4/5, Sham-Veh vs KA-Veh, *P = 0.026; KA-Veh vs KA-PPA, P =0.069; hippocampus, Sham-Veh vs KA-Veh, P = 0.082; KA-Veh vs KA-PPA, *P =0.047; hypothalamus, Sham-Veh vs KA-Veh, *P = 0.046; KA-Veh vs KA-PPA, *P =0.031; ventral cortex, Sham-Veh vs KA-Veh, *P = 0.029; KA-Veh vs KA PPA, **P =0.009).* ***(E)*** *Small vessel AQP4 PI Index of contralateral hippocampus (n = 4 mice/group, One-way ANOVA, Tukey's multiple comparison. Sham-Veh vs KA-Veh, ***P < 0.001; KA-Veh vs KA-PPA, **P =0.001).* ***(F)*** *Mean pixel intensity of AQP4 immunofluorescence in CA1 (n = 5 mice/group, one-way ANOVA, Tukey's multiple comparisons test, Sham-Veh vs KA-Veh *P = 0.011, KA-Veh vs KA-PPA *P = 0.011).* ***(G)*** *The interictal spike frequency between KA-Veh and KA-PPA mice (n = 9-13 mice/group; *P = 0.041, unpaired t-test).* ***(H)*** *The seizure numbers in mice with less exploration (Total distance < 15 m) verse more exploration (Total distance > 15 m) in open field test (n = 4-8 mice/group; P = 0.532). Data are presented as mean ± SEM.*

On the other hand, although we induced the initial status epilepticus (SE) by KA intrahippocampal infusion, it is reported that the global inflammation and hyperexcitability also contribute to epileptogenesis[17,18]. In alignment with prior research[19-21], we found neuronal loss and glial activation, as well as the AQP4 depolarization in regions other than hippocampus, such as dorsal cortex, ventral cortex and hypothalamus(Fig. S3 and S4).

One minor point:

Mismatched scale bars in Fig. S3 C (Sensory Cortex)

Thanks. We have corrected that as cited above.

Reviewer #2 (Remarks to the Author):

The paper that was revised and re-edited by Sun and colleagues improved substantially. I want to congratulate the Authors for adding new data and analysis, addressing all my concerns, particularly the PPA treatment far from KA injection, proving that it efficiently dampens the epileptogenesis process (fig.3). Also, the study conducted with AQP4-KO mice showing an aggravated epileptogenesis if compared to WT mice further clarifies the hypothesized mechanisms and support the paper's claims. I only have a single remark that I consider necessary to be fixed. In the abstract (line 50) and all over the manuscript, the Authors used the statement 'suppressed epileptogenesis' or 'suppressed the number of convulsive seizures' improperly.

The right word for such statements should be 'decreased' or 'lowered'.
Once the mentioned sentences are corrected, I fully support the publication of this work in Nature Communications.

Minor comments:
- Page 6, line 237 dummy ??? (cannula).
- Page 9, line 395-396: please rephrase; the sentence does not make much sense.
- Page 11, line 473: 'the trend towards increased levels .. of cytokines' provides a misleading message since no significance has been found in the cited cytokines and even the mentioned trend is not evident in most of the plots of fig. S5.

Response:

Thank you. We have corrected all the mistakes listed by the reviewer as cited below.

*'In brief, mice were implanted with a guide cannula (26G, C315G SPC, 4.5 mm below pedestal) sealed with a dummy cannula (33G, C315DC/SP, 4.5 mm projection) (PlasticsOne, Roanoke, VA) into the right striatum (Bregma: AP +0.6; ML -2.0, DV -3.3 mm).'*

*'To avoid direct pharmacological manipulation during SE, we first applied the initial dose of PPA at 30 hours post KA in a separate set of experiments* ***(Fig. 3A)****.'*

*'Concentrations of the pro-inflammatory cytokines, G-CSF, and the pro-inflammatory chemokine KC (CXCL1) were significantly higher in KA-Veh mice versus sham mice. PPA treatment significantly lowered the levels of G-CSF as compared with KA-Veh mice (****Fig. S5B-D****).* ~~*There was a trend towards increased levels in KA-Veh mice of several pro-inflammatory cytokines, including IL-1a, IL-2, IL-1β, IL-6, IL-9 IL-13, CXCL2, CCL2, CCL3, and CCL4, with a non-significant trend towards a reduction in KA-PPA mice (****Fig. S5B-D****).*~~*'*

Reviewer #3 (Remarks to the Author):

Authors have incorporated additional data in the revised manuscript that satisfactorily addresses the concerns raised by the reviewers on the original manuscript. The changes made have strengthened the manuscript. I have one major comment as follows:

Previous studies have shown increased seizures in AQP4-KO mice in response to various stimuli. Therefore, it is reassuring to find that intrahippocampal KA-induced seizures are worse in AQP4 KO mice. I think there should have been an additional group of AQP4-KO mice treated with PPA in this experiment. Since your hypothesis is that pan-adrenergic inhibition by PPA treatment suppresses epileptogenesis by augmenting glymphatic flux mediated through AQP4 expressed in astrocytic endfeet, treating AQP4-KO mice with PPA should not affect seizure parameters compared to vehicle-treated AQP4-KO mice. This

experiment may also address a concern whether the antiseizure effects of PPA are mediated mainly through its augmenting effects on glymphatic flux or other mechanism(s) such as its actions on neuronal NE receptors.

**Response:**

We appreciate the importance of the reviewer's request for dissecting the role of adrenergic signaling in AQP4 KO mice in epileptogenesis. However, we have worked with the AQP4 KO mice for decades and have shown that these mice have developed several adaptations to the deletion of AQP4 and that these involve signaling mechanisms. For example, in the AQP4 KO mice,1) the extracellular space volume and MRI diffusion parameters are significantly altered[3,22], 2)Calcium signaling in astrocytes are impaired[23], 3) Immune responses are dampened[24], and 4) the sleep-wake pattern is altered (unpublished). We have therefore refrained from utilizing AQP4 mice in elaborate mechanistic analysis in the past. We are concerned that adrenergic signaling is altered in AQP4 KO mice and that treating AQP4 KO mice with PPA after KA infusion will result in data that not clearly can be used in the study. For example, a downregulation of adrenergic receptors in AQP4 KO mice will lead to a smaller amplitude of effect of PPA, but we will not be able to ascribe the reduced effect of PPA without a 1-2 year additional studies comparing adrenergic receptor expression as well as cellular effect the alteration in GPCR intracellular signaling in AQP4 KO and wildtype mice. We prefer not to add studies that clearly will advance the main topic of the study and we have in response to the reviewer added the following discussion in the limitation section of the discussion (Page 14):

*"It should be noted that aqp4 knockout mice display several adaptive changes, including an expansion of the extracellular space volume, altered diffusion parameters and changes in astrocytic volume regulation and $Ca^{2+}$ signaling[134-136]. In addition to the low glymphatic activity, these changes may contribute worsening of the KA-induced seizure in aqp4 knockout mice."*

Minor corrections to do:

1) Fig. 5G - Correct the timeline graphic. No PPA treatment at the time of KA injection.

2) Fig. S3D - Correct the error in statistical significance marks for HC.

3) Fig. S6 - BBB results are not reported or discussed in the text.

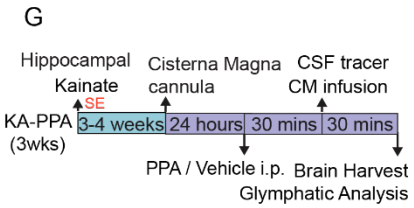
4) Line 626-628 ("The anti-epileptic drugs currently used in the clinic for acquired epilepsy include phenobarbital, phenytoin, carbamazepine, valproate, and magnesium134.")

- All these drugs are antiseizure drugs as opposed to antiepileptic drugs. Magnesium is not used for treating seizures.

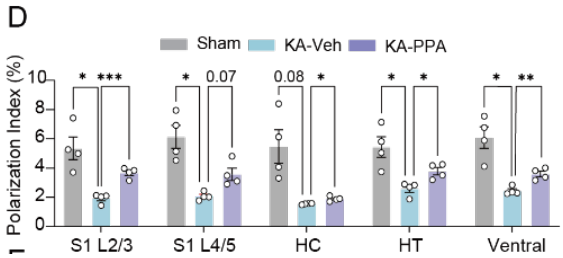
**Response:**

Thank you for the comments. We have corrected all the mistakes listed by the reviewer.

1) The 'PPA treatment' has been removed.




2) The statistical analysis is correct in Fig. S3D. Although the Mean of Sham hippocampal AQP4 polarization is higher than that in KA mice, the turkey post-hoc test did show non-significance ($P$ = 0.082). It is likely due to the large variance of Sham group. We also added the p values to the bar chart.




3) We updated the discussion to include Fig. S6 in Page 13 as cited below.

*'Additionally, our assessment of BBB permeability indicated that PPA did not significantly impact BBB integrity at the late phase of SE (24 hours after SE,* ***Fig. S6****). We propose that boosting glymphatic fluid transport with PPA restricted the inflammatory response by promoting the clearance of lactate, $K^+$, cytokines and other agents promoting excitability [92,119] independent of changes in BBB permeability, with additional benefits obtained via adrenergic reguation of inflammatory cells.'*

4) The 'magnesium' has been removed.

*'The anti-epileptic drugs currently used in the clinic for acquired epilepsy include phenobarbital, phenytoin, carbamazepine and valproate~~, and magnesium~~[141].'*

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Reviewer #1 (Remarks to the Author):

The authors provide convincing evidence that PPA treatment improves outcomes in a model for epilepsy. However, their response fails to fully address concerns on the following points.

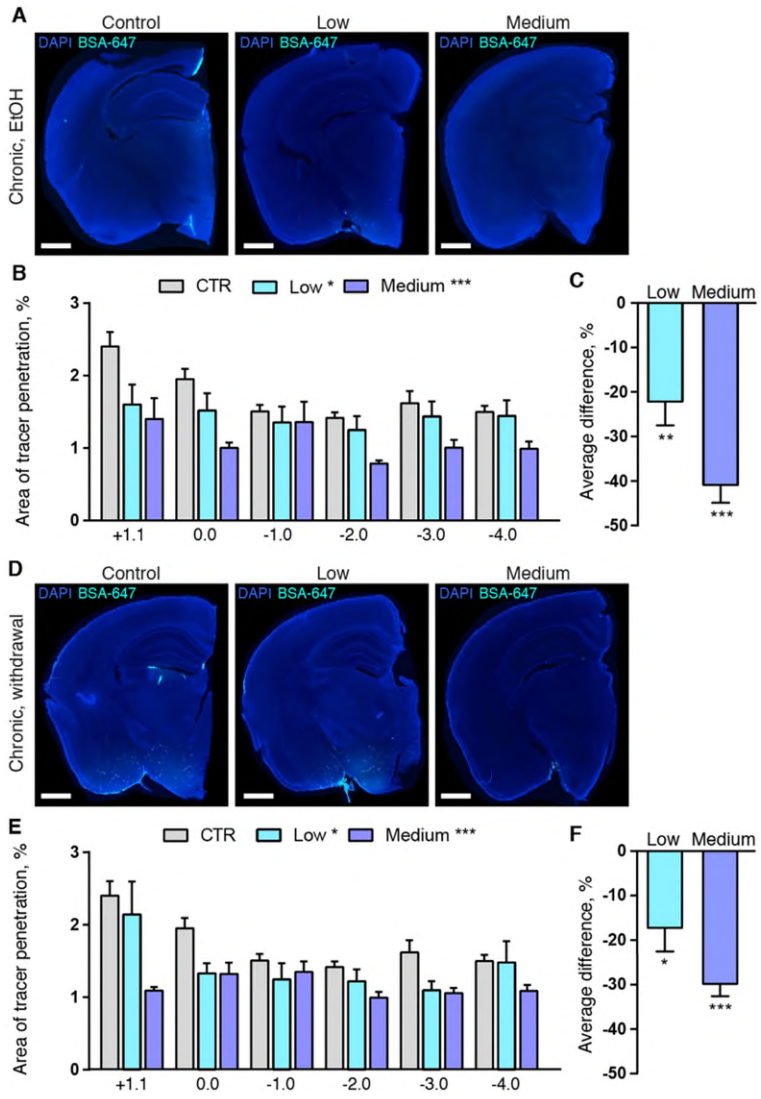
Comment 1

We appreciate the added paragraph to the discussion in Page 13-14. However, to our first and most important critique that "it seems that is no change in glymphatic flux or with Aqp4 polarization at the hippocampus", the authors reply with an inaccurate statement. They state, "As showed in Fig 5C and 5I, we did find only a slight trend of increase in glymphatic influx in the hippocampus". However, this is confusing since Figure 5C and 5I show that there is no difference in the average glymphatic influx at the hippocampus and therefore replying that there is "a slight trend of increase" seems to contradict their own data. In fact, this misuse of the word "trend" to suggest to the reader that there is a difference when the data shows otherwise now presents a major concern. All instances of "trend" when statistical significance was not found (Lines 403 , 430, and 496) should be removed from the manuscript altogether to prevent reader confusion.

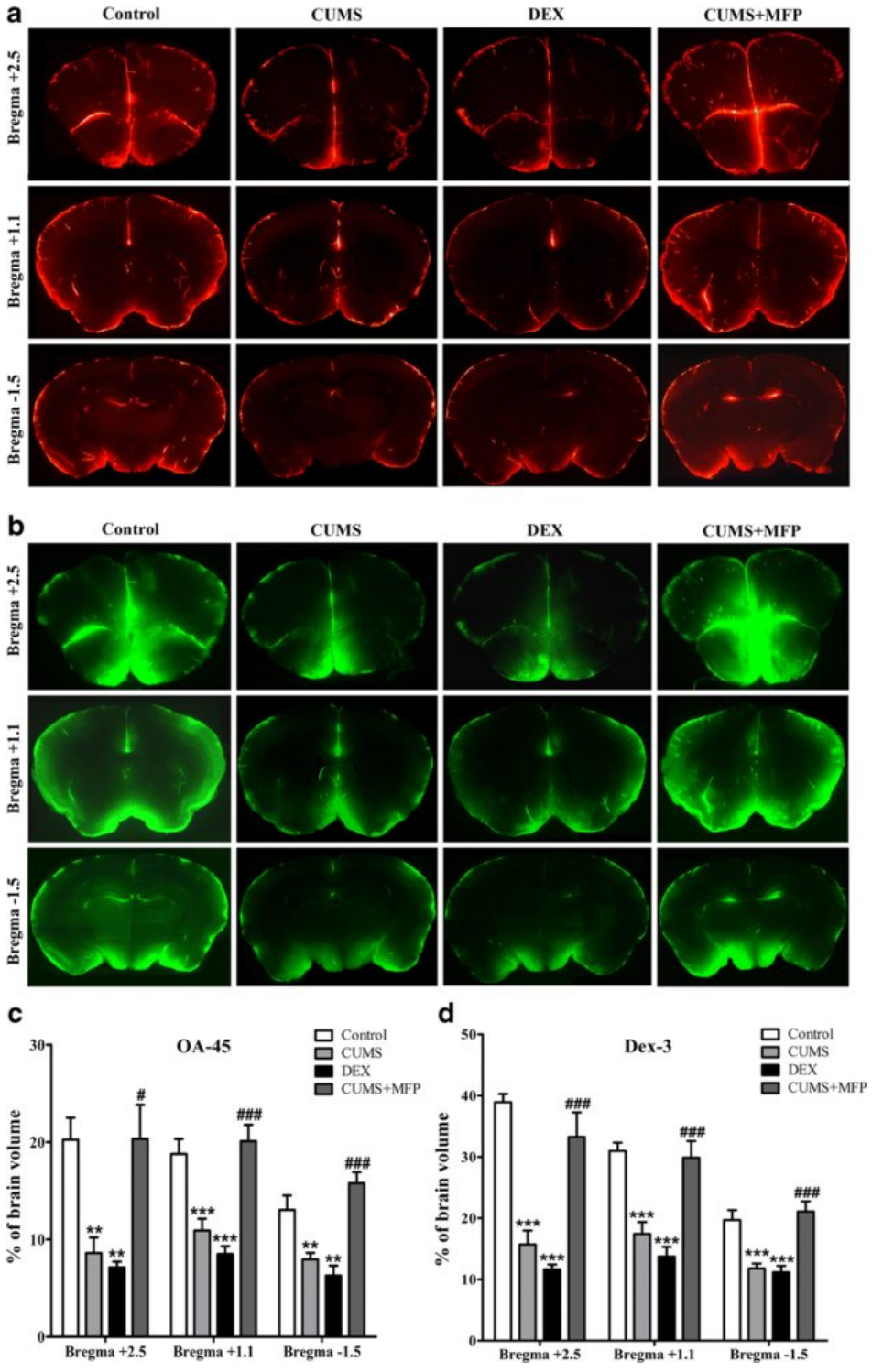
Finally, while glymphatic influx at the hippocampus may be normally low and difficult to detect given anatomy, others have successfully measured changes in tracer flux using the same method of injection (PMID 38418877). Thus, it is just as likely that the lack of influx at the hippocampus is not due to methodology or imaging sensitivity but rather that there is simply no change following PPA treatment. Please update the discussion to reflect alternative explanations that include a different mechanism for the improvements following PPA treatment.

**Response:**

In response to the reviewer's comments eliminated all statements regarding trends in data throughout the manuscript. It is correct that we did not find a significant increase in glymphatic influx following PPA treatment in the hippocampus regions of kainate acid (KA)-treated mice versus vehicle controls. This observation may reflect the severe astrogliosis as a result of KA local infusion[1-5]. Using he tracer molecules (Albumin from Bovine Serum, Alexa Fluor 647 Conjugate, 66kDa (Invitrogen A34785) or Ovalbumin, Alexa Fluor 555 Conjugate, 45kDa (Invitrogen O34782)) and a circulation time of 30 minutes, we observed lower fluorescent intensity in hippocampus as compared to the cortex and hypothalamus, which is consistent with other studies (Figures cited below) [6,7]. The mechanism is not fully understood; but may relate to the vasculature supply[8,9] or the anatomy of hippocampus[10-13].

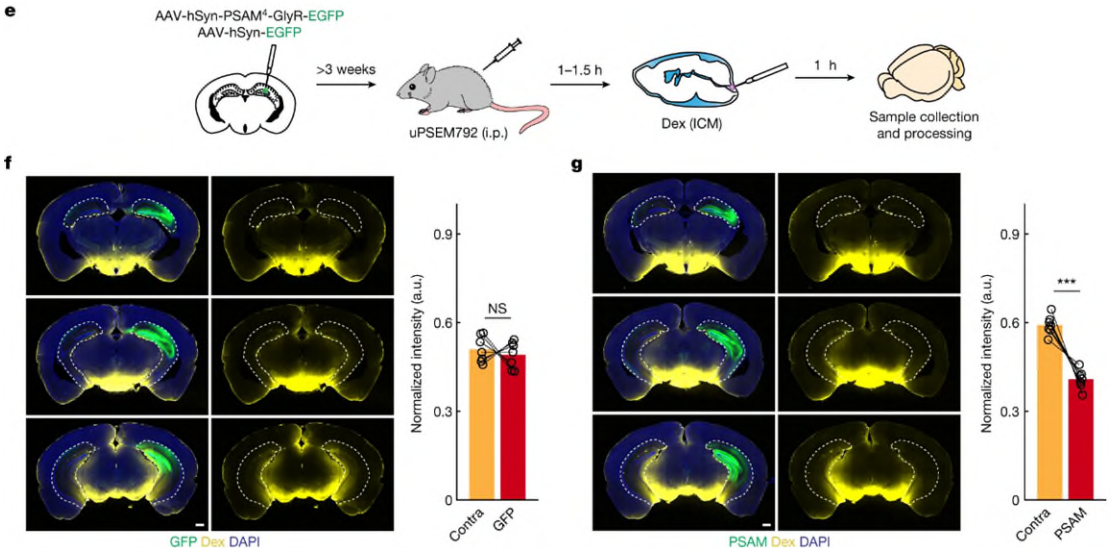



*Lundgaard, et al. 2018. BSA-66kDa 30min circulation*




*Wang, et al. 2019, OA-45kDa and Dex-3kDa, 30 min circulation.*

The tracer influx visualization is highly dependent on the molecule weights of tracer and circulation time[14,15]. In the reference (PMID 38418877)[16] referred by the reviewer, the authors used smaller tracer molecule (Dextran-Texas Red, 3 kDa (Invitrogen, D3328), or dextran–fluorescein, 3 kDa (Invitrogen, D3306)) and a longer circulation time of 60 minutes. In addition, strong and sustained chemogenetic inhibition of hippocampal neurons were induced during tracer circulation. Please see figure below. The differences in tracer accumulation is minimal albeit a side-to-side comparison in the same mouse was possible. We could not employ a similar strategi since the epileptic mice must be compared to littermate non-epileptic mice. Inter-animal variability will naturally add variability to the glymphatic analysis. Thus, we do not believe that our observations are surprising.




*Kipnis, et al. 2024. Figure 1. Dextran-3kDa 60 min circulation.*

Global neuroinflammation and related gliosis are deciphered to contribute to epileptogenesis in multiple aspects [17–22]. Because the initiation and propagation of temporal lobe seizure involve cortex and limbic system[23], we propose that PPA treatment probably benefits epileptogenesis via its anti-inflammatory effects and by promoting brain-wide clearance.

We updated the revised discussion section and cited below.

*"This also explains why, even though we did not find significant glymphatic enhancement in hippocampus (**Fig. 5C and 5I**), probably due to severe astrogliosis as result of kainite acid local infusion[120,121], PPA treatment beneficial effects on epileptogenesis may reflect an improvement of brain-wide clearance[122]."*

*"A limitation of this study is that the intracisternal magna infusion protocol utilized (30 minutes circulation time and Bovine Serum Albumin/Ovalbumin fluorescent tracer, 66kDa/45KDa) was not optimized for demonstrating hippocampal glymphatic influx. The influx of large CSF tracers into cortical regions are always higher than in the hippocampus[134–137]. Alternative approaches are needed to better assessed glymphatic transport in the hippocampus, such as extending CM*

*infusion with longer circulation time (60 minutes) and using a smaller fluorescent tracer (Dextran, 3kDa)[138] or in vivo MR imaging of contrast agents[30].”*

Comment 2

Please show both Ipsilateral and contralateral data
It is unclear if the new and significant differences found in hippocampus polarization shown in Fig. S3D are meaningful given that their means are barely noticeably higher in the KA-PPA vs KA-Veh group when compared to the Sham control.

**Response:**

We have in response to the comments of the reviewer analyzed all the brain regions listed and updated **Figure S3** shown below.

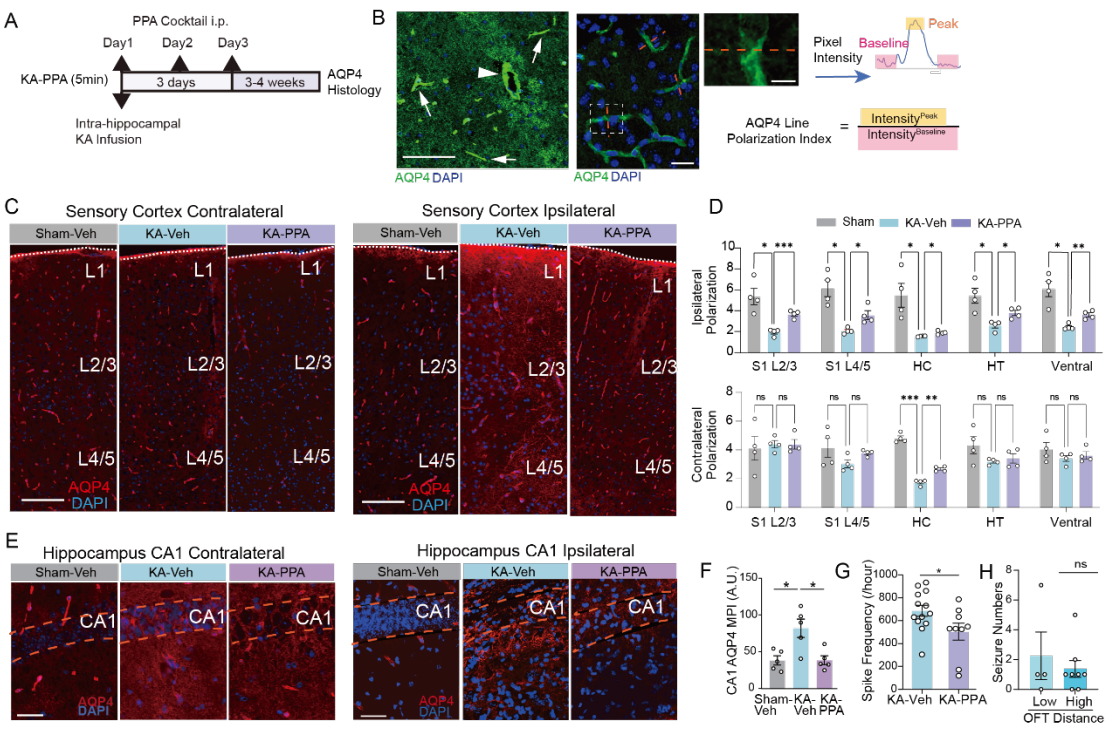



***Fig. S3 Quantitation of AQP4 polarization in various brain regions. (A)*** *Analysis of AQP4 immunostaining at 3-4 weeks after intrahippocampal KA infusion and PPA treatment.* ***(B)*** *Line-analysis of AQP4 polarization index (PI, see Methods for details).* ***(C)*** *Representative images of AQP4 expression in bilateral sensory cortex (scale-bar :100 µm).* ***(D)*** *Regional analysis of bilateral small vessel AQP4 PI Index in Sham-Veh, KA-Veh and KA-PPA, including sensory cortex layer 2/3, and layer 4/5, hippocampus, hypothalamus and ventral cortex. (n = 4 mice/group, Two-way ANOVA, Tukey's multiple comparison. Upper Panel, Ipsilateral, Sensory cortex layer 2/3, Sham-Veh vs KA-Veh, *P = 0.039; KA-Veh vs KA-PPA, ***P <0.001; Sensory cortex layer 4/5, Sham-Veh vs KA-*

*Veh, *P = 0.026; KA-Veh vs KA-PPA, P =0.069; hippocampus, Sham-Veh vs KA-Veh, P = 0.082; KA-Veh vs KA-PPA, *P =0.047; hypothalamus, Sham-Veh vs KA-Veh, *P = 0.046; KA-Veh vs KA-PPA, *P =0.031; ventral cortex, Sham-Veh vs KA-Veh, *P = 0.029; KA-Veh vs KA PPA, **P =0.009. Lower Panel, Contralateral, Sham-Veh vs KA-Veh, ***P < 0.001; KA-Veh vs KA-PPA, **P =0.001).* ***(E)*** *Representative images of AQP4 expression in bilateral CA1 hippocampal region (Scale bar, 50 µm)* ***(F)*** *Mean pixel intensity of AQP4 immunofluorescence in CA1 (n = 5 mice/group, one-way ANOVA, Tukey's multiple comparisons test, Sham-Veh vs KA-Veh *P = 0.011, KA-Veh vs KA-PPA *P = 0.011).* ***(G)*** *The interictal spike frequency between KA-Veh and KA-PPA mice (n = 9-13 mice/group; *P = 0.041, unpaired t-test).* ***(H)*** *The seizure numbers in mice with less exploration (Total distance < 15 m) verse more exploration (Total distance > 15 m) in open field test (n = 4-8 mice/group; P = 0.532). Data are presented as mean ± SEM.*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