nature biomedical engineering | PEER-REVIEW INFORMATION

Brain-wide Cas9-mediated cleavage of a disease-causing gene eases amyloid-related pathologies in mouse models of familial Alzheimer's disease

Corresponding author: Nancy Ip

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Mon 20 April 2020
Decision on Article nBME-20-0510

Dear Prof Ip,

Thank you again for submitting to *Nature Biomedical Engineering* your Article, "AAV-mediated brain-wide genome editing ameliorates amyloid-associated pathologies in familial Alzheimer's disease mouse models". The manuscript has been seen by three experts, whose reports you will find at the end of this message. You will see that although the reviewers have some good words for the work, they articulate concerns about the degree of support for the claims, and in this regard provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

* negative control treatments using the viral vectors with nonspecific gRNAs;

* behavioral/memory tests of treated animals to ascertain physiological improvements with treatment;

* a deeper assessment of the off-target editing activity, as suggested by reviewer #1.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 25 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*. Because of the COVID-19 pandemic, should you be unable to carry out experimental work in the near future we advise that you reply to this message with a revision plan in the form of a preliminary point-by-point rebuttal to the comments from all reviewers that also includes a response to any points highlighted in this decision. We should then be able to provide you with additional feedback.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

João

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Dr João Duarte
Senior Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

Comments to the Authors

Duan et al. present a study that investigates how genome editing targeting the human APPswe mutation in two different AD mouse models (5xFAD and APP/PS1) affects AD-associated pathologies in the hippocampus and other brain regions. In the core of the paper (Fig 2-5, 7), it is shown that AAV-mediated delivery of Cas9-SW1 decreases several AD pathologies in 5xFAD and APP/PS1 mice, including Abeta plaques, gliosis, and synaptic plasticity dysfunction (LTP). While these data are very clearly and beautifully presented, nevertheless, the manuscript lacks required studies to show that Cas9-SW1 prevent or restores memory impairments (Major Comment 1).

To target the APPswe mutation, they designed two sgRNA (SW1 and SW2) to specifically target the APPswe mutation. AAV9-mediated delivery of Cas9-SW1 to hippocampi from 5XFAD mice shows that this construct disrupts the mutant APPswe allele with an editing efficiency (indel) of 27% (Fig. 1, Suppl Fig. 1e); although the 27% editing efficiency might be an overestimation (see Major Comment 2). Whether 27% or little less, this is still a good percentage of editing efficiency and quite more than recent report, where similar CRISPR constructs targeting APPswe only displayed a 2% editing efficiency in the brain (Gyorgy et al., 2018 Mol Ther Nuc Acids).

Importantly, the authors also generated a novel viral vector, AAV-PHP.eB containing the WPRE construct (AAV-PHP.eB:SW1-Syn::Cas9-mWPRE) to systemically deliver Cas-SW1 to the entire brain and activate Cas9 (Fig 6), that moreover, led to a 30% editing efficiency of the APPswe allele (Suppl Fig 7). One problem, however, is that no experiment is performed to demonstrate if (or not) a control viral construct AAV-PHP.eB-mWPRE lacking Cas9-SW1 induces any adverse effects in the brain (Major Comment 3).
Regarding the specificity of the Cas9-SWI, the authors performed sequencing and showed that i) the number

of somatic mutations in virus-infected and uninfected regions in the same mouse brain was similar (~35,000) (Suppl Fig 1f), and 2) no mutations were detected in the top 10 predicted potential off-target sites in virus-infected regions (Suppl. Table 1). This section, however, is weak and should be improved (should be Major Comment 4).

Together, the data presented intriguingly suggest that AAV-mediated brain-wide genome editing of human mutant APP in two AD mouse models may be therapeutic strategy for Alzheimer's Disease by reducing AD-associated pathologies, although there are several issues regarding the specificity of Cas9-SW1 and its beneficial effect on cognition that are missing. Without addressing these major concerns (#1-4) this reviewer is not favorable to publish the data in Nature Biomedical Engineering.

Major comments:
1. Lack of behavioral studies impedes understanding the real potential of the Cas9-SW1 strategy used here. The authors should test if Ca9-SWI reduced (prevented or rescued; see point 6) typical memory deficits in any of the two AD mouse models studied here. Of particular interest would be determining the effects of regional versus systemic delivery of Ca9-SWI on memory deficits that are dependent only on the hippocampus (e.g. NOL) and on additional brain regions (e.g. NOR).

2. However, it seems that the 27% editing efficiency is an overestimation given that not only indels but also nucleotide changes are included in the calculations (Suppl Fig. 1e). Explain better in text.

3. In all the experiments shown, AD mouse models without any viral treatment are used as controls: this is not correct. For the more general AAV9 vector, the authors might simply refer to the literature in which intrahippocampal injection of empty AAV9 vector (alone and with capsid PHP.eB or PHP.B) have been used in AD mouse models without generating adverse effects. However, since this is the first study using systemic injection of AAV-PHP.eB-WHRE, some AD pathological and/or cognitive deficits should be tested in AD animals treated with AAV-PHP.eB:SW1-Syn::Cas9-mWPRE and with AAV-PHP.eB-WHRE lacking Cas9-SW1 as control.

4. The deep sequencing data showing that ~35,000 mutations were found in both virus-infected and uninfected regions in the same mouse brain is insufficient to conclude that no Cas9-SW1–induced off-target activity was detected in vivo. It is imperative that a detailed analysis on the existing deep sequencing data i) to determine the percentage of mutations shared not shared (simple graph), ii) to determine all the non-shared mutations in viral infected regions (show list of genes, mutations and potential consequences), and iii) discuss these genes in the context of brain function in the Discussion section.

5. It should be tested if Cas9-SW1 decreases phosphorylation of Tau, the other critical hallmark of AD.

6. Throughout the paper (including in the abstract) the terms "rescue and restore" are used. In my opinion, these terms are incorrectly used -specially for the 5xFAD mice- and should only be used if indeed the treatment was able to revert the presence of AD pathologies hallmarks; if not, terms such as prevents, reduced and ameliorates should be used. Only some data with APP/PS1 mice, where treatment was started after the onset of AD pathologies, would fall under the "rescue and restore" terminology. The text should be carefully revised for these terms.

7. Analyzing PSD-95 IR in tissue is not reliable as it gets easily trapped in the PSD. It has to be shown (here or referred to the literature) that the particular Ab used to detect PSD-95 does not show IR in neurons lacking PSD95. If not, this experiment should be performed in cultured hippocampal neurons where PSD-95-IR false positives do not occur.

Question/Suggestion:
1. Should the title not include "APP": for example "editing ….of mutant human APP"?

Minor comments:
1. From the text and data shown in HEK cells, it is not clear why SW1 was selected over SW2: Explain better. Also, was in any opportunity both constructs simultaneously used?

2. It is claimed that the reduced aggregation of lysosomes (detected with LAMP) in neurites surrounding amyloid plaques indicates that Cas9-SW1 editing reduces neurite dystrophy (note that figure 4f legends states “neuronal dystrophy”). It seems to me an overstatement of the data. Is it possible to show more directly changes in neurites (maybe in vitro)??

3. Figure 2A lacks additional protein as internal loading control.

4. Image of Figure 2e seems not well chosen in that Abeta IR is higher in areas enriched for HA-IR (and thus CRISPR-SW1) compared to areas with low HA-IR.

5. It would be clearer if the images on microgliosis and astrogliosis (Fig 3 for example) also includes HA-IR (and thus CRISPR-SW1).

6. It is stated that number of Iba1-labeled microglia is reduced by approximately 50%: this seems quite an overestimation on what is shown in graph of Fig. 3a,b.

7. Why AAV-PHP.eB-Cas9-SWI infects very limited the dentate gyrus (following Suppl Fig 7d) but able to form indel mutations as efficient as for example CA1 (following Suppl Fig 7b)?

8. Why are in Figure 7 amyloid plaques labeled with the dye X34 in stead of performing immunohistochemistry for Aβ as for the other figures?

9. It should be briefly discussed why the similar CRISPR Cas9-SWE construct by Gyorgy et al., 2018 (Mol Ther Nuc Acids) did only have a 2% editing efficiency in the brain.

10. In a recent study, epigenome editing of the critical synaptic protein PSD-95 was established as an effective therapeutic strategy to treat an AD mouse model (Bustos et al., Brain 2019). Discuss the advantages of epigenome editing vs genome editing strategies in AD.

11. In the M&M section it is indicated that mice were injected at a dose of 1x 10^13 vg/mice with AAV-PHP.eB:Cas9-SW1. That is 100 times the amount when “empty” AAV-PHP.eB viral vectors. Shortly discuss posible causes.

12. Indicate in the M&M more details on the viral titer concentrations generated (AAV9-Cas9-SW1 and AAV-PHP.eB:Cas9-SW1) and volumes used to perform intrahippocampal and systemic injections.

Reviewer #2 (Report for the authors (Required)):

The paper by Duan and colleagues describes the use and evaluation of CRISPR/Cas9 against the Swedish mutant of the APP gene. The authors have carefully tested this gene therapeutic strategy both intracerebrally and systemically on two different transgenic mouse models. Interestingly they found that both administration routes resulted in significantly less brain pathology as compared to control mice. This work represents a promising and clear step forward from the study by György et al and is an important contribution to the development of CRISPR/Cas-based treatment strategies for dominantly inherited forms of neurodegenerative disorders. The manuscript is fairly clearly written and structured. However, there are a number of unclarities that need to be adressed.

Major points:
- 1. The authors should somewhere state the degree of APP overexpression for the two mouse models and also reflect on how the editing efficacy in their systems might differ between these models and the physiological situation in patients.
- 2. The design and function of the EGxxFP system should be described in detail.
- 3. For the 5xFAD mice, the authors are targeting the Swedish site. But as also two other APP mutations are present, the authors should acknowledge the fact that they are simultaneously disrupting the effect of also these other mutations.
- 4. Figure 4 (and elsewhere): How have the authors made sure that their calculations were done on tissue

sections from representative areas?
- 5. Figure 4C: The control section seems to have been unevenly stained, which could result in a falsely positive treatment effect.
- 6. Figure 4F: The authors should explain and motivate why LAMP-1, a lysosomal marker, here is used as a marker for neuronal dystrophy.
- 7. Page 18 / Line 292 (and elsewhere): The authors describe that the treatment results in reduced pathology, but should use another terminology. What they in fact demonstrate is that the treated mice do not develop as much pathology as the control-treated mice.
- 8. For figure 2 and 3, it is not described in the legends what staining method that was used for Aβ visualization. For fig. 5 the 4G8 antibody was used and for fig. 6 the X34 dye, whereas IHC was used again for fig. 7 (but with no ab stated). Please provide the lacking details and explain why different methods were used for the different mice/CRISPR administrations.
- 9. Discussion: The authors should not use the term “AD transgenic models” but instead “transgenic models for Aβ pathology”, or similar. In the same way, they should not describe the pathologies as “AD pathologies” but instead “Aβ-related pathologies, similar to what can be seen in AD brain”, or similar.
- 10. Supplemental figure 1: Please explain in which genes these potential off target sequences are found.
- 11. Please make sure that all the figure legends state clearly if the images relate to locally or systemically treated animals.
- 12. For the statistical analyses, a separate section should be added to the Material and Methods, in which it is properly explained what methods that were used for which analyses.
- 13. As for T-testing, the authors have used both unpaired and paired tests. However, only the unpaired variant should have been used since the control mice belong to a different population than the treated mice. Paired T-tests should be reserved to situations where the same mice are investigated before and after treatment.
- 14. Please state more clearly how control mice were treated. Empty vector? Scrambled gRNA sequences?
- 15. Supplementary figure 7c: Please explain why there was much less HA staining in the control mice. That should not have been the case If they were injected with an AAV vector that did not include the target specific gRNA but that still expressed HA. And if the control vector did not express HA there should be no such staining at all.

Minor points:
- 1. Mention gender of animals used for the experiments
- 2. Page 4: “Tau” should be “tau”.
- 3. Page 18 / Line 282: Please point out that the injections were made intracerebrally.
- 4. Page 21-22 / Line 343-350: This part should instead be included in the Discussion.
- 5. Page 27 / Line 400: “is” should be “was”
- 6. Supplementary figure 5: Please state in the legend that the effects were seen only in CAS9-expressing areas (similar to supplementary figures 2 and 5)?

Reviewer #3 (Report for the authors (Required)):

Summary
In the manuscript provided by Duan et al, the authors report on their work to develop and deploy adeno-associated virus (AAV) vectors to deliver CRISPR genome editing activity for the targeted disruption of a disease-causing allele of amyloid precursor protein (APPswe) in a transgenic mouse model of Alzheimer’s Disease. They explain their rationale for choosing this particular allele, validate the specificity of their novel reagents in vitro, and demonstrate that disruption of APPswe reduces the severity of the disease phenotypes histologically and electrophysiologically. The use of two different mouse models allowed them to demonstrate that these vectors could be used as a therapy to improve outcomes after the onset of clinical symptoms. Finally, the application of the recently developed serotype “PHP.eB” facilitates the vectors’ delivery to large regions of the brain without invasive intracranial surgery. In conclusion, this type of approach has potential for
translation into a clinical therapy with several caveats. These include 1) this approach is not broadly applicable, in that it requires specific conditions for the target alleles, i.e. dominant alleles with base change in the seed, and 2) currently there is little/no evidence that PHP.eB serotype works to bypass the BBB in humans/NHP models (Matsuzaki et al. Neuroscience Letters, 2017; Liguore et al. Mol Therapy, 2019). These

caveats are disclosed in the main text.

* Your reasoned opinion on the degree of advance (fundamental, mechanistic, methodological, technological, therapeutic, translational and/or clinical) of the work with respect to the state of the art. If the results or conclusions are not original, please provide relevant references.

The individual components mostly represent established tools and techniques, however, it is the combined usage of these components that make this a significant advancement. Specifically, the work demonstrates a concise and well executed workflow of design choices and methods to non-invasively deliver gene therapy in the rodent brain.

* Your reasoned opinion on the broad implications of the findings.

The present work is encouraging for those who work in the field of gene therapy by demonstrating what is possible once certain tools (i.e. BBB-bypassing serotypes of AAV, “small” Cas9 orthologs, etc.) are available for clinical use. Even if the targeting constraints limit the field to a subset of inherited diseases, this approach can have a significant impact for those afflicted.

Major Technical
MAJOR: The effects of injecting AAVcas9 alone or AAVcas9+ nonspecific gRNA have not been included as a negative control. There are too many uncontrolled variables between the groups (uninjected vs injected with AAV-Cas9+Sw1). A control injection of a control gRNA would be more appropriate to control for surgery, inflammation, overexpression of CRISPR/Cas9, etc. If evidence supports that there is no difference from injected control virus to uninjected animals that data should be referenced.

No behavioral outcomes were examined. Although “improvements” in pathology were observed, were they enough to overcome cognitive impairment?

The use of the paired t-test are not appropriate as used and authors should consult statistician. The group sizes also appear to be small for the types of assays performed.

Minor Technical

Line 165 – There is no evidence specific to NHEJ function that is shown in figure 1. Statement should be removed or qualified to reflect the test performed.

Missing/unclear details

Line 162 Figure 1e – “Con” is displayed in the graphic, but is not defined anywhere until Sup Fig 1. Please modify legend in Figure 1 to include (Con) definition. What method was used to obtain the biopsies used for genomic DNA isolation? It is unclear whether these are dissected hippocampal biopsies or whole punch.

Line 276 Fig 4h – Please explain what the basis for the analysis of “volume” is in the y-axis. If this is based on images, then I would expect it to be “area”. In any case, the criteria for quantification of “Total dystrophy” should be explained in more detail.

Line 328 – Please elaborate on the provenance and/or divulge the actual sequence of the “truncated WPRE” that was appended to the vector genome. The cited reference (#36, Patricio et al) discusses the effect of WPRE on AAV expression/safety, but does not appear to contain details that would indicate characterization of a truncated variant. This presumably novel element has value to those who design vectors for gene therapy. How does it differ from the deletion mutants that are characterized in this reference: 10.1261/rna.061192.117?

Line 380 (As in Fig4) please explain what the basis for “volume” is in the y-axis. Criteria for quantification of “Total dystrophy” should be explained in more detail.

Missing citations
Line 328 – Include citation or information pertaining to the provenance of “truncated WPRE”.

Optional suggestions
Line 312 Fig 5g – As presented, the GFAP images in (g) are qualitatively different between samples, in that Con astrocytes show “thicker” processes than those in SW1. Does the quantification of (GFAP+ area (% area)), as in Fig 3e, reveal any quantifiable difference?

Stylistic suggestions
Line 271 Fig 4e – consider relabeling the y-axis with something that does not include a “/” symbol. “Syn/Psd95 contacts”) implies a ratio of two values.

Tue 19 Jan 2021
Decision on Article NBME-20-0510A

Dear Professor Ip,

Thank you for your revised manuscript, "AAV-mediated brain-wide genome editing ameliorates amyloid-associated pathologies in familial Alzheimer's disease mouse models", which has been seen by the original reviewers. In their reports, which you will find at the end of this message, you will see that the reviewers acknowledge the improvements to the work, and that reviewer #2 raises a few additional technical criticisms that we hope you will be able to address. In particular, we would expect that the next version of the manuscript provides a revision of the descriptions of data in the text regarding the therapeutic effect; an assessment of the degree of gene editing in both models; a discussion of the possibility of AAV spread through the brain following intracranial injection; and for the systemic administration, a more appropriate control group as injection with an empty vector or with a vector containing a scrambled gRNA.

As before, when you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

As a reminder, please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

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* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 12 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*. Because of the COVID-19 pandemic, should you be unable to carry out experimental work in the near future we advise that you reply to this message with a revision plan in the form of a preliminary point-by-point rebuttal to the comments from all reviewers that also includes a response to any points highlighted in this decision. We should then be able to provide you with additional feedback.

We look forward to receive a further revised version of the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

João

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Dr João Duarte
Senior Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

The authors are commended for an excellent response to the comments of reviewer 1, especially under the current covid-19 pandemic situation. The addition of the behavioral studies, the use of AAV-PHP.eB-WPRE with a scrambled sgRNA as control, and the new bioinformatic analyses have significantly strengthened the manuscript and the reported findings. Thus, all prior points of the main critiques have been adequately addressed and while some minor comments were not ideally answered, I have no further concerns.

Brigitte van Zundert

Reviewer #2 (Report for the authors (Required)):

The manuscript by Duan et al has been much improved compared to the original submission. The addition of behavioral assessments have strengthened and made the study even more interesting. However, there are still some questions and concerns that need to be addressed.

Major remarks:
Please reconsider how to express and interpret the data. In the results section it should be described that treated mice “displayed lower Abeta levels compared to control mice” etc. In the discussion section it can be stated that the treatment “ameliorated”, “alleviated”, “prevented” or “reduced” the different features, but please then carefully consider which such interpretations that the data support.

Whereas the authors provide some estimates on the degree of gene editing it would be important to have such assessments performed on both mouse models used. If possible, it would also be desirable to analyze tissues from individual mice with next generation sequencing in order to see whether mice that were more efficiently edited also displayed lesser pathology or less cognitive impairment than non-treated mice.

For the part of the study based on intracranial injections the authors used the contralateral side as control. However, AAVs have been described to spread to the contralateral side upon intracranial injection. Did they ascertain that this did not occur in their models?

For the part of the study based on peripheral administration the authors used non-injected mice as controls. A better control would have been to use mice injected with empty vector or vector expressing a scrambled gRNA sequence.

The authors used AAV with higher titers than what can generally be obtained. Please include a description on how the capsids could be generated with such impressive yields.

Please make more clear that dominantly inherited familial AD only represents less than 5% and that the current treatment paradigm can not be transferred to the vast majority of AD patients.

Please include more reflections to the discussion section regarding the treatment effects of the non-amyloid pathology parameters that were investigated.

Minor remarks

Title:
Shouldn’t the treatment effects on behavior also be reflected in the title?

Abstract:
Row 33: The abbreviation for the amyloid precursor protein (APP) should be introduced, as it is used further down the abstract.

Rows 33-34: It is unclear what is meant by “which affect multiple brain regions”. Maybe this part of the sentence could be left out?

Row 35: “brain-wide amelioration of Alzheimer’s disease phenotypes” should be changed to: “amelioration of Alzheimer’s disease-related phenotypes”.

Row 41: “AAV-Cas9 virus” should be changed to “AAV-Cas9”, as “virus” is already included in the AAV

abbreviation.

Rows 42-43: “amyloid-beta–associated pathologies throughout the brain and alleviates the cognitive dysfunctions” should be changed to: “amyloid-beta–associated brain pathology and alleviates cognitive dysfunction”.

Introduction:
General: Gene names should be written in italic.

Row 60: “Microbial” could be removed.

Row 82: “heterozygous disease mutation” should be changed to “heterozygous disease causing mutation”.

Rows 102-103: “other nervous system disorders caused by autosomal dominant mutations and affect multiple brain regions” should be: “other central nervous system disorders caused by autosomal dominant mutations and affecting multiple brain regions”.

Results:
General: Please express more clearly that three months old mice were used for the treatment intervention.

General: Please consider changing “virus infected” to “transduced” throughout the text.

General: Please make sure to use the correct abbreviations for proteins and genes, respectively. For example, PS1 = protein, PSEN1= gene.

Row 135: “into the hippocampus in 3-month-old” should be changed to “into the hippocampus of 3-month-old”.

Row 138: No protein expression data are shown to support that “the mutation abolishes the expression”.

Row 155-157: The authors should specify if they refer to their own or to already published data. Also, they should make clear if what they state is true for mouse neurons, human neurons or both.

Figure 1: Row 6: “Cas9” should be changed to “SaCas9”.
Row 179: “To examine whether and how the disruption of the APPswe mutation in 5XFAD mice regulates Aβ-associated pathologies” should be changed to: “To examine if the disruption of the APPswe mutation in 5XFAD mice can alleviate Aβ-associated pathologies”.

Row 181: “injection into the hippocampus in 3-month-old” should be changed to “injection into the hippocampus of 3-month-old”

Rows 192-193: The text suggests that comparisons were made in injected and non-injected mice, while the statistic test used is paired. Please specify whether the percentages and staining shown refer to the injected and non-injected areas of the same mice (paired t test) or if they refer to injected and non-injected mice (unpaired t test).

Row 224: “APPswe mutation” should be changed to “APPswe allele”.

Figure 3: “ameliorate” should be replaced with: “was associated with lower” (as the data do not reflect whether the effects were due to reduction or prevention).

Row 247: “ameliorate hippocampal” should be: “affect hippocampal”.

Fig. 4: Please clarify what microscopy method that was used to make these images and reconsider if you can claim that these images provide support that PSD95 and synaptophysin interact with each other.

Row 326: “deliver Cas9-SW1 specifically into the adult brain via noninvasive systemic administration.” should be changed to: “achieve a higher degree of Cas9-SW1 delivery into the adult brain via systemic administration.”

Row 347: “any adverse effects” should be changed to: ”any apparent adverse effects”.

Row 351: “globally” can be removed.

Row 375: “Aβ-deposition transgenic mice exhibit impaired” should be changed to: “APP transgenic mice exhibit impaired”
Figure 7: The inflammatory and cognitive measures should be divided into two separate figures. A statistic comparison of Con (WT) and 5xFAD (SW1) would also be good to include.

Row 421: “Aβ-deposition transgenic mice” should be changed to: ”APP transgenic mice”

Rows 451-452: Please consider changing “However, it might not be possible to translate germline editing to humans.” to: “However, it might not be possible to translate germline editing to a viable AD treatment approach.”

Methods:
Please explain why AAV9 was used for the intracerebral injections.

Reviewer #3 (Report for the authors (Required)):

The authors have adequately addressed concerns. I recommend the article for publication.

Tue 19 Jan 2021
Decision on Article NBME-20-0510A

Dear Prof Ip,

On behalf of my colleague João Duarte, thank you for your patience in waiting for the feedback on your revised manuscript, "AAV-mediated brain-wide genome editing ameliorates amyloid-associated pathologies in familial Alzheimer's disease mouse models". Having consulted with Reviewer #2 (whose comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please upload the files specified in the instructions file.

Also, please consider the remaining minor points from the reviewer.

For primary research originally submitted after December 1, 2019, we encourage authors to take up transparent peer review. If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. **If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'.** In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons. If any reviewers have signed their comments to authors, or if any reviewers explicitly agree to release their name, we will include the names in the peer-review supplementary file. More information on transparent peer review is available.

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

P.S. Nature Research journals encourage authors to share their step-by-step experimental protocols on a protocol-sharing platform of their choice. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can be found at www.nature.com/protocolexchange/about.

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Reviewer #2 (Report for the authors (Required)):

The manuscript has been further improved with the new adjustments. My only remaining recommendation for the authors would be to carefully go through the text and make sure that the nomenclature and the use of abbreviations is consistent. For example, they currently use at least five different ways of describing the Swedish APP mutation:

Row 40, 137: APP Swedish
Row 87-88: APP Swedish (APPswe) (KM670/671)
Row 107: (KM670/671NL)
Row 136: K670N/M671L [Swedish]
Row 143: APPswe mutation

Rebuttal 1

Response to reviewers (nBME-20-0510)

We thank the reviewers for their positive and constructive comments about our manuscript as well as their support for publishing our manuscript in the journal. We have taken all reviewers' comments into consideration, conducted additional analyses, and provided extensive additional data to strengthen the conclusions of our study. In addition, we have provided more details about our methods and analyses. We believe that the revised manuscript addresses most of the reviewers' concerns. Our point-by-point responses are presented below.

Reviewer #1 (Report for the authors (Required)):

Comments to the Authors

Duan et al. present a study that investigates how genome editing targeting the human APPswe mutation in two different AD mouse models (5xFAD and APP/PS1) affects AD-associated pathologies in the hippocampus and other brain regions. In the core of the paper (Fig 2-5, 7), it is shown that AAV-mediated delivery of Cas9-SW1 decreases several AD pathologies in 5xFAD and APP/PS1 mice, including Abeta plaques, gliosis, and synaptic plasticity dysfunction (LTP). While these data are very clearly and beautifully presented, nevertheless, the manuscript lacks required studies to show that Cas9-SW1 prevent or restores memory impairments (Major Comment 1).
To target the APPswe mutation, they designed two sgRNA (SW1 and SW2) to specifically target the APPswe mutation. AAV9-mediated delivery of Cas9-SW1 to hippocampi from 5XFAD mice shows that this construct disrupts the mutant APPswe allele with an editing efficiency (indel) of 27% (Fig. 1, Suppl Fig. 1e); although the 27% editing efficiency might be an overestimation (see Major Comment 2). Whether 27% or little less, this is still a good percentage of editing efficiency and quite more than recent report, where similar CRISPR constructs targeting APPswe only displayed a 2% editing efficiency in the brain (Gyorgy et al., 2018 Mol Ther Nuc Acids).
Importantly, the authors also generated a novel viral vector, AAV-PHP.eB containing the WPRE construct (AAV-PHP.eB:SW1-Syn::Cas9-mWPRE) to systemically deliver Cas-SW1 to the entire brain and activate Cas9 (Fig 6), that moreover, led to a 30% editing efficiency of the APPswe allele (Suppl Fig 7). One problem, however, is that no experiment is performed to demonstrate if (or not) a control viral construct AAV-PHP.eB-mWPRE lacking Cas9-SW1 induces any adverse effects in the brain (Major Comment 3).
Regarding the specificity of the Cas9-SWI, the authors performed sequencing and showed that i) the number of somatic mutations in virus-infected and uninfected regions in the same mouse brain was similar (~35,000) (Suppl Fig 1f), and 2) no mutations were detected in the top 10 predicted potential off-target sites in virus-infected regions (Suppl. Table 1). This section, however, is weak and should be improved (should be Major Comment 4).

Together, the data presented intriguingly suggest that AAV-mediated brain-wide genome editing of human mutant APP in two AD mouse models may be therapeutic strategy for Alzheimer's Disease by reducing AD-associated pathologies, although there are several issues regarding the specificity of Cas9-SW1 and its beneficial effect on cognition that are missing. Without addressing these major concerns (#1-4) this reviewer is not favorable to publish the data in Nature Biomedical Engineering.

We thank the reviewer for these insightful comments. We conducted additional experiments and analysis to address the 4 major concerns raised by the reviewer; our point-to-point responses are presented below.

Major comments:

1. Lack of behavioral studies impedes understanding the real potential of the Cas9-SW1 strategy used here. The authors should test if Ca9-SWI reduced (prevented or rescued; see point 6) typical memory deficits in any of the two AD mouse models studied here. Of particular interest would be determining the effects of regional versus systemic delivery of Ca9-SWI on memory deficits that are dependent only on the hippocampus (e.g. NOL) and on additional brain regions (e.g. NOR).

We thank the reviewer for this constructive comment. As suggested, we performed additional behavioral experiments to evaluate the cognitive functions and memory performance of 5XFAD mice after systemic administration of Cas9-SW1.

In previous studies, Aβ-deposition transgenic mice exhibited impaired habituation and working memory as well as increased risk-taking behavior (**Fu et al., *Proceedings of the National Academy of Sciences of the U S A*, 2016; Oakley et al., *Journal of Neuroscience*, 2006; Jawhar et al., *Neurobiology of Aging*, 2012; Tible et al., *Aging Cell*, 2019**). Concordant with the alleviation of amyloid-associated pathologies in 5XFAD mice, the present study provides new findings showing that systemic delivery of Cas9-SW1 improves the performance of the transgenic mice in different behavioral tests—specifically habituation ability, working memory, and risk-taking behavior in the exploratory open field test, Y-maze spontaneous alternation test, and elevated plus maze test, respectively. Accordingly, we have included descriptions of these tests in the *Methods* along with new findings in the revised manuscript (highlighted in text; **Pages 24 and 34–35**; **new Fig. 7e–g**).

While it is certainly of interest to investigate whether intrahippocampal and systemic delivery of Cas9-SW1 differentially affect behaviors that are dependent only on the hippocampus or additional brain regions, it is technically difficult to differentiate such subtle effects on the basis of behavioral tests. We hope that the reviewer agrees that this is out of the scope of the current study.

2. However, it seems that the 27% editing efficiency is an overestimation given that

not only indels but also nucleotide changes are included in the calculations (Suppl Fig. 1e). Explain better in text.

We thank the reviewer for raising this issue. The 27% editing efficiency was calculated on the basis of the intensity of enzyme-cut and enzyme-uncut bands in the T7 endonuclease assay according to a well-established formula shown below (**Guschin et al., *Methods in Molecular Biology*, 2010**). The single nucleotide changes in **Supplementary Fig. 1e** are not included in the calculations, because T7 endonuclease selectively detects indels and not single nucleotide mismatches (**Guan et al., *Biochemistry*, 2004**). Therefore, the 27% editing efficiency is not an overestimate.

$$\text{Editing efficiency } \% = 1 - \sqrt{1 - \frac{\text{cut bands}}{(\text{uncut band} + \text{cut bands})}} \times 100$$

We would like to clarify that the nucleotide changes shown in **Supplementary Fig. 1e** are unlikely to be Cas9-induced editing events because they are far from the Cas9 cleavage site; we have added this information in the revised manuscript (highlighted in text**; Pages 46**).

3. In all the experiments shown, AD mouse models without any viral treatment are used as controls: this is not correct. For the more general AAV9 vector, the authors might simply refer to the literature in which intrahippocampal injection of empty AAV9 vector (alone and with capsid PHP.eB or PHP.B) have been used in AD mouse models without generating adverse effects. However, since this is the first study using systemic injection of AAV-PHP.eB-WHRE, some AD pathological and/or cognitive deficits should be tested in AD animals treated with AAV-PHP.eB:SW1-Syn::Cas9-mWPRE and with AAV-PHP.eB-WHRE lacking Cas9-SW1 as control.

As suggested by the reviewer, we have included the literature in which intrahippocampal injection of empty AAV9 vector was widely used in mice without generating adverse effects (**Sun et al., *Nature communications*, 2019; Hudry et al., *Neuron*, 2019; Hocquemiller et al., *Human gene therapy*, 2016**) (highlighted in text; **Page 31**).

As our study is the first to examine the effects of systemic injection of AAV-PHP.eB:SW1-Syn::Cas9-mWPRE in familial AD mouse models, we conducted new experiments to include AAV-PHP.eB-WPRE with a scrambled sgRNA as a control to characterize the effects of AAV-PHP.eB:SW1-Syn::Cas9-mWPRE in Aβ-deposition transgenic mice. The new data show that the control virus does not affect Aβ-related phenotypes in 5XFAD mice, as amyloid plaque burden was

relatively unchanged upon injection of Cas9-Con virus (highlighted in text; **Page 21; new Supplementary Fig. 8**).

4. The deep sequencing data showing that ~35,000 mutations were found in both virus-infected and uninfected regions in the same mouse brain is insufficient to conclude that no Cas9-SW1–induced off-target activity was detected in vivo. It is imperative that a detailed analysis on the existing deep sequencing data i) to determine the percentage of mutations shared not shared (simple graph), ii) to determine all the non-shared mutations in viral infected regions (show list of genes, mutations and potential consequences), and iii) discuss these genes in the context of brain function in the Discussion section.

As suggested by the reviewer, we conducted a more detailed analysis of the existing deep-sequencing data of the virus-infected and uninfected regions in the same mouse brain ($n = 2$).

i. Among the ~35,000 mutations detected in the virus-infected or uninfected brain regions in 2 mice injected with the virus, ~23,400 were shared in both virus-infected and uninfected brain regions for each mouse, respectively. Meanwhile, there were 10,315 (30.6%) and 10,656 (30.9%) non-shared mutations in the virus-infected regions in mouse 1 and mouse 2, respectively (**new Supplementary Table 1**). Of note, among those mutations, we identified 1,345 mutations that shared by both mice. To retrieve the somatic mutations that may be generated by off-target events, we conducted filtering for those 1,345 mutations by removing those resided in the repetitive regions or overlapped with the germline mutations, which results in 257 somatic mutations (listed in **new Supplementary Dataset**). Among the 257 mutations, 126 resided in the gene body regions of 46 genes (**new Supplementary Table 2**).

ii. We found that these 46 genes are not enriched in any biological pathways according to Gene Ontology and pathway analyses. Meanwhile, among 46 genes, 17 genes were marked by 23 somatic mutations in exons or untranslated regions, which might exert biological functions by modifying gene expression and protein function (**new Supplementary Table 2**). Of note, cell-type specific transcriptome data suggested that three of these genes (*Sp110*, *Sp140*, and *Abcg2*) were expressed in the mouse brain (**Zhang et al., *Journal of Neuroscience,* 2014**).

iii. Particularly, for those 3 genes (*Sp110*, *Sp140*, and *Abcg2*), Sp110 and Sp140 belong to the nuclear body protein family and potentially regulate gene transcription (**Bloch et al., *Molecular and Cellular Biology,* 2000**), whereas Abcg2 belongs to the ATP-binding cassette (ABC) transporter superfamily, which serves as a xenobiotic exporter. (**Mo et al., *International Journal of***

***Biochemistry and Molecular Biology*, 2012**). Notably, *Sp110* and *Sp140* are only expressed in brain myeloid and endothelial cells, whereas *Abcg2* is mainly expressed in endothelial cells. Given that these genes were not prominently expressed in neurons (**Zhang et al., *Journal of Neuroscience*, 2014**), whereas the synapsin-driven Cas9 system can only target the neurons, the identified mutations are unlikely to affect the expression and hence functions of these genes in neurons. We have included the discussion in the revised manuscript (highlighted in text**; Page 29**).

5. It should be tested if Cas9-SW1 decreases phosphorylation of Tau, the other critical hallmark of AD.

We thank the reviewer for raising this issue. The 5XFAD and APP/PS1 Aβ-deposition transgenic mouse models do not develop neurofibrillary tangle-like hyperphosphorylated tau like that observed in human AD (**Jankowsky and Zheng, *Molecular Neurodegeneration*, 2017**). Therefore, to determine whether Cas9-SW1 decreases tau phosphorylation, we need to examine its effect in Aβ-deposition transgenic mouse models crossed with a novel humanized tau mouse model (**Saito et al., *Journal of Biological Chemistry*, 2019**). We hope that the reviewer agrees that this is out of the scope of the current study.

6. Throughout the paper (including in the abstract) the terms "rescue and restore" are used. In my opinion, these terms are incorrectly used -specially for the 5xFAD mice- and should only be used if indeed the treatment was able to revert the presence of AD pathologies hallmarks; if not, terms such as prevents, reduced and ameliorates should be used. Only some data with APP/PS1 mice, where treatment was started after the onset of AD pathologies, would fall under the "rescue and restore" terminology. The text should be carefully revised for these terms.

As suggested by the reviewer, in the revised manuscript, we revised "rescue"/"restore" to "prevent"/"ameliorate"/"alleviate" where appropriate when referring to Cas9-SW1–treated 5XFAD mice; these changes are highlighted in the text. Meanwhile, we kept the original terms when referring to APP/PS1 mice when treatment started after the onset of AD pathologies.

7. Analyzing PSD-95 IR in tissue is not reliable as it gets easily trapped in the PSD. It has to be shown (here or referred to the literature) that the particular Ab used to detect PSD-95 does not show IR in neurons lacking PSD95. If not, this experiment should be performed in cultured hippocampal neurons where PSD-95-IR false positives do not occur.

We thank the reviewer for raising this issue. The PSD-95 antibody we used in this study is both well characterized and widely used and to label PSD-95–expressing postsynaptic clusters in tissues (**Planagumà et al., *Annals of Neurology*, 2016; Alves**

et al., ***Molecular Neurodegeneration*****, 2016; Shao et al.,** ***Acta Neuropathologica*****, 2011; Yuan, Peng, and Grutzendler,** ***Journal of Neuroscience*****, 2016; Cai et al.,** ***Journal of Cell Biology*****, 2016; Han et al.,** ***Journal of Neuroinflammation*****, 2017**). Furthermore, we indicated the excitatory synapses in the mouse brain sections by co-labeling with PSD-95 and the presynaptic marker synaptophysin. The best approach to examine the beneficial effects of *APP* gene editing on synapse regulation in Aβ-deposition transgenic mouse models is to quantify the excitatory synapses in mouse brain sections by performing immunohistochemical analysis.

Question/Suggestion:
1. Should the title not include "APP": for example "editing ….of mutant human APP"?

We thank the reviewer for this suggestion. While we are happy to make this revision, it would not conform to the *nBME* journal guidelines, which limit the length of article titles to 130 characters including spaces.

Minor comments:
1. From the text and data shown in HEK cells, it is not clear why SW1 was selected over SW2: Explain better. Also, was in any opportunity both constructs simultaneously used?

We thank the reviewer for raising this issue. When we designed the sgRNAs for the *APP* Swedish mutation, SW1 and SW2 were the best candidates and they are closely located on the genomic sequence. While their genome-editing efficiency was similar, that of SW1 was slightly superior. In addition, the SW1 cleavage site is on the nucleotides that encode the β-secretase cleavage site—the disruption of which would abolish Aβ generation (**Fig. 1a**). We have included this justification in the revised manuscript (**Page 6**).

We did not use both SW1 and SW2 together because we were aiming for a "cleaner" effect generated by a single-guide RNA SW1.

2. It is claimed that the reduced aggregation of lysosomes (detected with LAMP) in neurites surrounding amyloid plaques indicates that Cas9-SW1 editing reduces neurite dystrophy (note that figure 4f legends states "neuronal dystrophy"). It seems to me an overstatement of the data. Is it possible to show more directly changes in neurites (maybe in vitro)??

We thank the reviewer for the helpful comments. The lysosomal protein LAMP1 is a widely used and well-characterized marker that can label the dystrophic neurites at amyloid plaque sites in transgenic mouse models of Aβ pathology (**Gowrishankar et al.,** ***Proceedings of the National Academy of Sciences of the U S A*****, 2015**). As suggested, we have revised the manuscript by changing "neuronal dystrophy" to

"LAMP1^{+} dystrophic neurites" in the legend of **Fig. 4f**. While we observed dystrophic neurites in adult Aβ-deposition transgenic mice, it would be difficult to examine similar neurite changes in an *in vitro* culture system. Given that the current well-accepted protocols for preparing cultured neurons involve embryonic mice, such a cultured neuronal system is very different from the *in vivo* system. We hope the reviewer agrees that examining the dystrophic neurites *in vitro* might be influenced by other factors, e.g. whether the cultured neurons subjected to different conditions might not have the same differentiation status, and the interplay among amyloid plaques, microglia, and neurons might be lost in the cultured condition.

3. Figure 2A lacks additional protein as internal loading control.

As suggested by the reviewer, we have included GAPDH as a loading control (**Fig. 2a**).

4. Image of Figure 2e seems not well chosen in that Abeta IR is higher in areas enriched for HA-IR (and thus CRISPR-SW1) compared to areas with low HA-IR.

We apologize for not making this clearer. The higher Aβ immunoreactivity in the HA-expressing subiculum region than that in the other areas with low HA expression is due to the following reasons. In 5XFAD mice, the subiculum exhibits high accumulation of amyloid plaques. Furthermore, because we injected CRISPR-SW1 mainly into the subiculum, HA expression was largely confined to neurons in the subiculum. Therefore, when examining the brain sections of a mouse that expresses Cas9-SW1, Aβ immunoreactivity appeared to be higher in HA-expressing regions than other regions (**Supplementary Fig. 2b**). Nonetheless, compared to that in the uninjected group, the plaque-positive area decreased in the subiculum upon SW1-mediated genome editing. Accordingly, in the revised manuscript, we have replaced the representative images for a better presentation (**Fig. 2e**).

5. It would be clearer if the images on microgliosis and astrogliosis (Fig 3 for example) also includes HA-IR (and thus CRISPR-SW1).

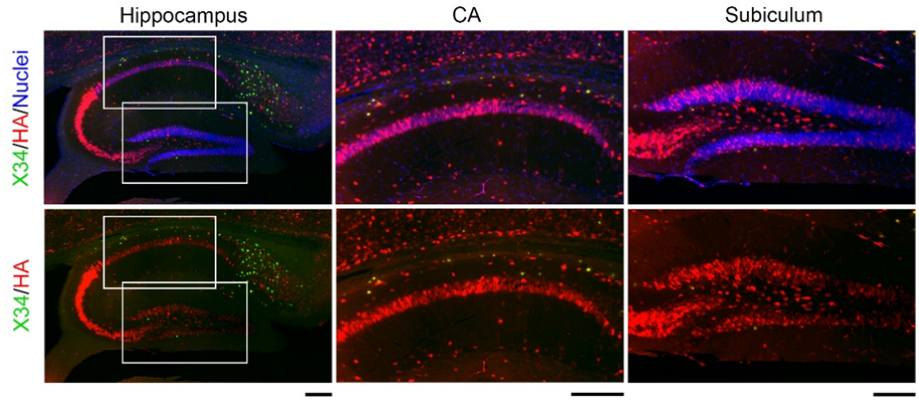
We fully agree with the reviewer's comment. However, both antibodies used in this study for HA and Iba1 staining have been raised from rabbits, making it technically difficult to co-stain both proteins on the same section. Nonetheless, we have validated HA expression (and thus Cas9-SW1) in separate brain sections of the same mouse brain (**Fig. 2e and Supplementary Fig. 2b**), in which gliosis was reduced upon Cas9-SW1 editing (**Fig. 3**).

6. It is stated that number of Iba1-labeled microglia is reduced by approximately 50%: this seems quite an overestimation on what is shown in graph of Fig. 3a,b.

We apologize for mislabeling the decrease in the percentage of $Iba1^+$ microglia. There was an average of 1,566 $Iba1^+$ microglia per mm^2 in the subiculum in 6-month-old 5XFAD mice, which decreased to 1,016 per mm^2 (64.9%) in the SW1-edited transgenic mice. We have revised the manuscript accordingly.

7. Why AAV-PHP.eB-Cas9-SWI infects very limited the dentate gyrus (following Suppl Fig 7d) but able to form indel mutations as efficient as for example CA1 (following Suppl Fig 7b)?

We thank reviewer for this comment. While AAV-PHP.eB-Cas9-SW1 infected the dentate gyrus to a slightly lesser extent than the CA1 region, Cas9-HA staining was obvious in the dentate gyrus. However, the HA signal was masked because of the high density of nuclear staining in the region. Accordingly, we removed the nuclear staining in the images for better visualization (see below; lower panels). We have removed the nuclear staining and added enlarged image of dentate gyrus in **Supplementary Fig. 7d** accordingly.



8. Why are in Figure 7 amyloid plaques labeled with the dye X34 in stead of performing immunohistochemistry for Aβ as for the other figures?

We thank reviewer for this comment. Systemic administration of Cas9-SW1 resulted in relatively low HA expression. When we used the same immunohistochemistry protocol to co-stain HA and Aβ with antigen retrieval, the HA staining signal was too weak for imaging. Therefore, we labeled the amyloid plaques using X34 dye together with HA staining for the Cas9-SW1 systemic administration experiment (**Fig. 6**). Nonetheless, we included the Aβ antibody immunohistochemistry analysis for amyloid plaques in **Fig. 6b–d** and confirmed that systemic administration of Cas9-SW1 resulted in decreased amyloid deposition.

9. It should be briefly discussed why the similar CRISPR Cas9-SWE construct by Gyorgy et al., 2018 (Mol Ther Nuc Acids) did only have a 2% editing efficiency in

the brain.

We thank the reviewer for this comment. Accordingly, we have included discussion on the possible discrepancy in the editing efficiency using the 2 approaches (**Page 27**). The CRISPR Cas9-SWE construct used by **Gyorgy et al.** (***Molecular Therapy - Nucleic Acids*, 2018**) was based on the Cas9 from *Streptococcus pyogenes* (SpCas9; 1,368 amino acids); we packaged SpCas9 and sgRNA into 2 separate AAV vectors owing to the size limit for AAV packaging. In contrast, we utilized a smaller Cas9 from *Staphylococcus aureus* (SaCas9; 1,053 amino acids), whose sgRNA can be packaged into an all-in-one AAV vector. Our results show that the all-in-one AAV strategy described herein substantially increases the Cas9 editing efficiency *in vivo*.

10. In a recent study, epigenome editing of the critical synaptic protein PSD-95 was established as an effective therapeutic strategy to treat an AD mouse model (Bustos et al., Brain 2019). Discuss the advantages of epigenome editing vs genome editing strategies in AD.

We thank the reviewer for this insightful comment. Epigenetic enhancement of PSD-95 expression in the hippocampus is a general approach for ameliorating synaptic and behavioral deficits irrespective of disease mutations. However, other AD pathologies such as amyloid plaque burden and gliosis would remain unaffected using this approach. Compared to epigenome editing, genome editing ameliorates multiple amyloid-associated pathologies including Aβ plaque burden, microgliosis, synaptic deficits, and cognitive impairment.

11. In the M&M section it is indicated that mice were injected at a dose of 1x 10^13 vg/mice with AAV-PHP.eB:Cas9-SW1. That is 100 times the amount when "empty" AAV-PHP.eB viral vectors. Shortly discuss possible causes.

We thank the reviewer for this comment. In our study, we injected AAV-PHP.eB:Cas9-SW1 at 1×10^{13} vg per mouse. In the previous paper (**Chan et al., *Nature Neuroscience*, 2017**), 1×10^{11} vg AAV-PHP.eB-CAG-NLS-GFP was injected into the mice for the brain-wide expression of GFP. The Cas9-encoding sequence (~3,200 bp) is much larger than the GFP-encoding sequence (~720 bp). Furthermore, the size of the Cas9 construct (~4,900 bp) is close to the packaging limit of AAV virus. These factors explain why AAV-PHP.eB:Cas9-SW1 was barely expressed at a dose of 1×10^{11} vg per mouse. After testing different doses of the virus, we found that Cas9 was well expressed and induced efficient brain-wide editing upon injection at a dose of 1×10^{13} vg per mouse.

12. Indicate in the M&M more details on the viral titer concentrations generated (AAV9-Cas9-SW1 and AAV-PHP.eB:Cas9-SW1) and volumes used to perform intrahippocampal and systemic injections.

We thank the reviewer for this comment. Accordingly, we have included the concentration and volumes in the *Methods* section (**Page 31**). The viral titer concentration for AAV9-Cas9-SW1 was 3×10^{13} vg/mL; we used 2 μL for each hippocampal injection. The viral titer concentration for AAV-PHP.eB:Cas9-SW1 was 1×10^{14} vg/mL; we used 100 μL for systemic injection.

Reviewer #2 (Report for the authors (Required)):

The paper by Duan and colleagues describes the use and evaluation of CRISPR/Cas9 against the Swedish mutant of the APP gene. The authors have carefully tested this gene therapeutic strategy both intracerebrally and systemically on two different transgenic mouse models. Interestingly they found that both administration routes resulted in significantly less brain pathology as compared to control mice. This work represents a promising and clear step forward from the study by György et al and is an important contribution to the development of CRISPR/Cas-based treatment strategies for dominantly inherited forms of neurodegenerative disorders. The manuscript is fairly clearly written and structured. However, there are a number of unclarities that need to be adressed.

Major points:
- 1. The authors should somewhere state the degree of APP overexpression for the two mouse models and also reflect on how the editing efficacy in their systems might differ between these models and the physiological situation in patients.

We thank the reviewer for this comment. While there are no reports on the exact copy numbers of *APP* in 5XFAD or APP/PS1 mice, we observed similar editing efficiency in these mice. Given that CRISPR/Cas9-mediated gene disruption is reported to be insensitive to gene copy number (**Yuen et al.,** ***Nucleic Acids Research,*** **2017**), we believe that our genome-editing method can achieve similar editing efficiency in humans.

- 2. The design and function of the EgxxFP system should be described in detail.

We thank the reviewer for this comment. We used the EGxxFP system (**Mashiko et al.,** ***Scientific Reports,*** **2013**) to evaluate the specificity of sgRNAs SW1 and SW2. We used this EGxxFP system to confirm that sgRNAs SW1 and SW2 only target the *APP* allele that carries the Swedish mutation and not the WT allele. The EGxxFP reporter contains the 5′ and 3′ fragments of GFP that share a ~500-bp overlapping region. We inserted the APP_{WT} or APP_{swe} sequence between the GFP fragments. We co-transfected the EGxxFP-APP_{WT} or EGxxFP-APP_{swe} with Cas9-SW1 or Cas9-SW2 into HEK 293T cells. Thus, administration of the Cas9 targeting the *APP* sequence results in the reconstitution of the GFP expression cassette and generate a GFP signal. We observed this GFP signal in the EGxxFP-APP_{swe} reporter after co-transfection of Cas9-SW1 or Cas9-SW2 but not in the cells that expressed the EGxxFP-APP_{WT}

reporter. This indicates that both SW1 and SW2 confer high allele specificity for the APP_{swe} mutation.

- 3. For the 5xFAD mice, the authors are targeting the Swedish site. But as also two other APP mutations are present, the authors should acknowledge the fact that they are simultaneously disrupting the effect of also these other mutations.

We thank the reviewer for this comment. The CRISPR/Cas9-mediated targeting of the *APP* Swedish mutation would disrupt the expression of mutated *APP*, thereby simultaneously disrupting the effect of the I716V [Florida] and V717I [London] mutations on the same *APP* transgene. Accordingly, we have revised the text and acknowledge that during the editing of the *APP* Swedish mutation in 5XFAD mice, we had simultaneously disrupted the effects of the other *APP* mutations (**Pages 6–7**). Nonetheless, we also observed a significant decrease in amyloid plaque burden in the APP/PS1 mice, which only carry the Swedish mutation in the *APP* transgene.

- 4. Figure 4 (and elsewhere): How have the authors made sure that their calculations were done on tissue sections from representative areas?

We thank the reviewer for raising this question. We selected brain sections with similar positions. We used nuclear staining to identify representative areas that clearly showed the structures of the subiculum and cornu ammonis. We also took 2–4 images for each position and obtained an average calculation to ensure that the quantification reflected the average change in each mouse.

- 5. Figure 4C: The control section seems to have been unevenly stained, which could result in a falsely positive treatment effect.

We apologize for not including the appropriate representative images. We have replaced the representative images accordingly (**new Fig. 4c**). We performed quantification for 2–4 images of each brain section in 4 mice to eliminate any potential false-positive treatment effects.

- 6. Figure 4F: The authors should explain and motivate why LAMP-1, a lysosomal marker, here is used as a marker for neuronal dystrophy.

We thank the reviewer for this comment. The lysosomal protein LAMP1 is a well-characterized marker of dystrophic neurites at amyloid plaque sites in transgenic mouse models of Aβ pathology, as it is highly enriched at amyloid plaque sites in dystrophic neurites. (**Gowrishankar et al., *Proceedings of the National Academy of Sciences of the U S A*, 2015**). Accordingly, we have revised the manuscript by changing "neuronal dystrophy" to "$LAMP1^+$ dystrophic neurites" in the **Fig. 4f** legend. We hope the reviewer agrees that $LAMP1^+$ dystrophic neurites serve as an *in vivo* readout of neurite dystrophy.

- 7. Page 18 / Line 292 (and elsewhere): The authors describe that the treatment results in reduced pathology, but should use another terminology. What they in fact demonstrate is that the treated mice do not develop as much pathology as the control-treated mice.

As suggested by the reviewer, in the revised manuscript, we have changed "reduce" to "not develop as much as the control mice", "ameliorate", or "alleviate", where appropriate. These changes are highlighted in the text.

- 8. For figure 2 and 3, it is not described in the legends what staining method that was used for Aβ visualization. For fig. 5 the 4G8 antibody was used and for fig. 6 the X34 dye, whereas IHC was used again for fig. 7 (but with no ab stated). Please provide the lacking details and explain why different methods were used for the different mice/CRISPR administrations.

We thank the reviewer for this comment. We have provided (and highlighted) the details of Aβ staining in the *Figure legends* accordingly.

We used the 4G8 antibody for Aβ staining shown in most figures except those in **Fig. 6e–g**. Systemic administration of Cas9-SW1 resulted in relatively low HA expression. When we used the same immunohistochemistry protocol to co-stain HA and Aβ with antigen retrieval, the HA staining signal was too weak for imaging. Therefore, we labeled the amyloid plaques with X34 dye together with HA staining in the Cas9-SW1 systemic administration experiment (**Fig. 6e–l**). Nonetheless, we included the results of Aβ antibody immunohistochemistry analysis for amyloid plaques in **Fig. 6b–d**, which confirmed that systemic administration of Cas9-SW1 resulted in decreased amyloid deposition.

- 9. Discussion: The authors should not use the term "AD transgenic models" but instead "transgenic models for Aβ pathology", or similar. In the same way, they should not describe the pathologies as "AD pathologies" but instead "Aβ-related pathologies, similar to what can be seen in AD brain", or similar.

We thank the reviewer for this comment. We have revised "AD transgenic model(s)" to "transgenic models of Aβ pathology" or "Aβ-deposition transgenic mice" where appropriate. We have also revised "AD pathologies" to "Aβ-related pathologies". These changes are highlighted in the text.

- 10. Supplemental figure 1: Please explain in which genes these potential off target sequences are found.

We thank the reviewer for this comment. As suggested, we conducted an additional analysis of the whole-genome sequencing data to identify potential off-target

sequences (**Pages 7 and 29**; **New Supplementary Fig. 1g**). We identified 257 somatic mutations as potential off-target events (All potential off-target mutations and their associated genes were listed in **new Supplementary Dataset**). Among them, 17 genes marked by 23 somatic mutations in exons or untranslated regions were identified that might exert potential biological functions in modifying gene expression and function (**new Supplementary Table 2**). Three of these genes—*Sp110*, *Sp140*, and *Abcg2*—were expressed in the mouse brain. Sp110 and Sp140 belong to the nuclear body protein family and potentially regulate gene transcription (**Bloch et al., *Molecular and Cellular Biology,* 2000**), whereas Abcg2 belongs to the ATP-binding cassette (ABC) transporter superfamily, which serves as a xenobiotic exporter (**Mo et al., *International Journal of Biochemistry and Molecular Biology,* 2012**). Notably, *Sp110* and *Sp140* are only expressed in brain myeloid and endothelial cells, whereas *Abcg2* is mainly expressed in endothelial cells. Given that these genes were not prominently expressed in neurons (**Zhang et al., *Journal of Neuroscience,* 2014**) whereas the synapsin-driven Cas9 system can only target the neurons, these mutations are unlikely to affect the expression and hence functions of these genes in neurons.

- 11. Please make sure that all the figure legends state clearly if the images relate to locally or systemically treated animals.

As requested, we have included new descriptions to state whether animals were treated locally or systemically in all figure legends.

- 12. For the statistical analyses, a separate section should be added to the Material and Methods, in which it is properly explained what methods that were used for which analyses.

As suggested, we have included a separate section for statistical analyses in the *Methods* (**Page 35**).

- 13. As for T-testing, the authors have used both unpaired and paired tests. However, only the unpaired variant should have been used since the control mice belong to a different population than the treated mice. Paired T-tests should be reserved to situations where the same mice are investigated before and after treatment.

We thank the reviewer for this comment. We used paired *t*-tests to analyze intrahippocampal injection data, because we compared the left and right hippocampal regions of the same mouse brain: we injected the Cas9-SW1 into the left hippocampal region and compared this region with the uninjected right hippocampal region. As suggested, we reanalyzed the data from the systemic injection experiment (**Figs. 6 and 7**).

- 14. Please state more clearly how control mice were treated. Empty vector? Scrambled gRNA sequences?

We thank the reviewer for this comment. Regarding systemic administration, although the controls were uninjected mice, we have conducted a new experiment that included control mice injected with scrambled gRNA, which did not affect Aβ-related phenotypes in 5XFAD mice (**new Supplementary Fig. 8**). For intrahippocampal injection, it is well documented that the virus does not cause any obvious phenotypic changes in mice (**Sun et al., *Nature communications*, 2019; Hudry et al., *Neuron*, 2019; Hocquemiller et al., *Human gene therapy*, 2016**); we have cited these reports in the revised text (**Page 31**).

- 15. Supplementary figure 7c: Please explain why there was much less HA staining in the control mice. That should not have been the case If they were injected with an AAV vector that did not include the target specific gRNA but that still expressed HA. And if the control vector did not express HA there should be no such staining at all.

We thank the reviewer for this comment. In the original figure, the control mice were uninjected controls that did not express HA. Accordingly, we have conducted a new experiment that included AAV-PHP.eB:Con-Syn::Cas9-mWPRE injection in both WT and 5XFAD mice. The control virus resulted in similar HA expression as that in 5XFAD mice injected with AAV-PHP.eB:SW1-Syn-mWPRE (**new Supplementary Fig. 8**).

Minor points:
- 1. Mention gender of animals used for the experiments

We thank the reviewer for this comment. As requested, we have included the gender of the animals in the *Methods* (**Page 30**). To clarify, we used male 5XFAD mice and female APP/PS1 mice.

- 2. Page 4: “Tau” should be “tau”.

We have revised the manuscript accordingly.

- 3. Page 18 / Line 282: Please point out that the injections were made intracerebrally.

We have revised the sentence in question to, “…we injected AAV-EFS::Cas9-SW1 into the hippocampus of APP/PS1 mice.”

- 4. Page 21-22 / Line 343-350: This part should instead be included in the Discussion.

We have replaced the sentence, “therefore, our results using systemic administration represent a significant improvement over intraparenchymal injection into the hippocampus, which only prevents the amyloid plaque burden in specific

hippocampal regions," with, "therefore, we conclude that systemic delivery of Cas9-SW1 globally decreases Aβ pathologies in 5XFAD mice."

- 5. Page 27 / Line 400: "is" should be "was"

We have revised the manuscript accordingly.

- 6. Supplementary figure 5: Please state in the legend that the effects were seen only in CAS9-expressing areas (similar to supplementary figures 2 and 5)?

We have revised the text in question to "in the Cas9-expressing areas."

Reviewer #3 (Report for the authors (Required)):

Summary
In the manuscript provided by Duan et al, the authors report on their work to develop and deploy adeno-associated virus (AAV) vectors to deliver CRISPR genome editing activity for the targeted disruption of a disease-causing allele of amyloid precursor protein (APPswe) in a transgenic mouse model of Alzheimer's Disease. They explain their rationale for choosing this particular allele, validate the specificity of their novel reagents in vitro, and demonstrate that disruption of APPswe reduces the severity of the disease phenotypes histologically and electrophysiologically. The use of two different mouse models allowed them to demonstrate that these vectors could be used as a therapy to improve outcomes after the onset of clinical symptoms. Finally, the application of the recently developed serotype "PHP.eB" facilitates the vectors' delivery to large regions of the brain without invasive intracranial surgery. In conclusion, this type of approach has potential for translation into a clinical therapy with several caveats. These include 1) this approach is not broadly applicable, in that it requires specific conditions for the target alleles, i.e. dominant alleles with base change in the seed, and 2) currently there is little/no evidence that PHP.eB serotype works to bypass the BBB in humans/NHP models (Matsuzaki et al. Neuroscience Letters, 2017; Liguore et al. Mol Therapy, 2019). These caveats are disclosed in the main text.

We thank the reviewer for succinctly summarizing our work and posing insightful questions. We believe the approach can be widely applicable based on the following reasons.

i. Although allele-specific targeting strategy requires dominate missense mutations in the seed or PAM sequence, the development of a SaCas9 variant with alternative PAM compatibility can further extend the targeting range. Besides, other AD mutations such as *PSEN1* L166P are also amenable to allele-specific gene disruption. Moreover, this approach can be applied to other brain diseases caused by dominant mutations—for example, the

TARDBP G287S mutation in familial amyotrophic lateral sclerosis. Even if the allele-specific targeting strategy requires mutations in the seed or PAM sequence, which would limit the approach to a subset of inherited diseases, this approach could have significant efficacy in affected people.

ii. Although there is little evidence that PHP.eB works in humans or NHP models, we have engineered AAV.CAP-B10 and AAV.CAP-B22 vectors that can bypass the blood–brain barrier in marmosets (**Flytzanis et al., *bioRxiv*, 2020**). With these ongoing developments, our study represents a proof-of-concept towards therapies using noninvasive systemic delivery vectors for brain diseases caused by dominant mutations. We have included the above information in the *Discussion* (**Page 28**).

* Your reasoned opinion on the degree of advance (fundamental, mechanistic, methodological, technological, therapeutic, translational and/or clinical) of the work with respect to the state of the art. If the results or conclusions are not original, please provide relevant references.

The individual components mostly represent established tools and techniques, however, it is the combined usage of these components that make this a significant advancement. Specifically, the work demonstrates a concise and well executed workflow of design choices and methods to non-invasively deliver gene therapy in the rodent brain.

We appreciate that the reviewer acknowledges how combining established techniques represents a significant advancement.

* Your reasoned opinion on the broad implications of the findings.

The present work is encouraging for those who work in the field of gene therapy by demonstrating what is possible once certain tools (i.e. BBB-bypassing serotypes of AAV, "small" Cas9 orthologs, etc.) are available for clinical use. Even if the targeting constraints limit the field to a subset of inherited diseases, this approach can have a significant impact for those afflicted.

We thank the reviewer for commenting on the significant impact of our study.

Major Technical
MAJOR: The effects of injecting AAVcas9 alone or AAVcas9+ nonspecific gRNA have not been included as a negative control. There are too many uncontrolled variables between the groups (uninjected vs injected with AAV-Cas9+Sw1). A control injection of a control gRNA would be more appropriate to control for surgery, inflammation, overexpression of CRISPR/Cas9, etc. If evidence supports that there is no difference from injected control virus to uninjected animals that data should be

referenced.

As suggested, we have referred to the literature in which intrahippocampal injection of empty AAV9 vector (alone or with PHP.B) has been widely used in mice without generating adverse effects (**Sun et al., *Nature communications*, 2019; Hudry et al., *Neuron*, 2019; Hocquemiller et al., *Human gene therapy*, 2016**).

As the first study to examine the effects of systemic injection of AAV-PHP.eB:SW1-Syn::Cas9-mWPRE in familial AD mouse models, we conducted a new experiment to include AAV-PHP.eB:Con-Syn::Cas9-mWPRE in both WT and 5XFAD mice. The control virus neither induced gliosis nor any behavioral deficits in WT mice. Moreover, the control virus did not affect Aβ-related phenotypes in 5XFAD mice, as amyloid plaque burden remains unchanged upon injection of Cas9-Con virus (**new Supplementary Fig. 8**).

No behavioral outcomes were examined. Although "improvements" in pathology were observed, were they enough to overcome cognitive impairment?

We thank the reviewer for this constructive comment. As suggested, we have performed additional behavioral experiments to evaluate the cognitive functions and memory performance of 5XFAD mice injected with Cas9-SW1.

In previous studies, *APP* transgenic model mice exhibited impaired habituation and working memory as well as increased risk-taking behavior (**Fu et al., *Proceedings of the National Academy of Sciences of the U S A*, 2016; Oakley et al., *Journal of Neuroscience*, 2006; Jawhar et al., *Neurobiology of Aging*, 2012; Tible et al., *Aging Cell*, 2019**). Concordant with the alleviation of amyloid-associated pathologies in 5XFAD mice, the present study provides new findings showing that systemic delivery of Cas9-SW1 improves the performance of the transgenic mice in different behavioral tests—specifically habituation ability, working memory, and risk-taking behavior in the exploratory open field test, Y-maze spontaneous alternation test, and elevated plus maze test, respectively. Accordingly, we have included descriptions of these tests in the *Methods* along with new findings in the revised manuscript (highlighted in text; **Pages 24 and 34–35**; **new Fig. 7e–g**).

The use of the paired t-test are not appropriate as used and authors should consult statistician. The group sizes also appear to be small for the types of assays performed.

We thank the reviewer for this comment. We used paired *t*-tests to analyze intrahippocampal injection data, because we compared the left and right hippocampal regions of the same mouse brain: we injected the Cas9-SW1 into the left hippocampal region and compared that region with the uninjected right hippocampal region. As suggested, we reanalyzed the data from the systemic injection experiment (**Figs. 6 and 7**).

Regarding the group size, we used $n = 4$ for most of immunohistochemistry analysis as the difference in immunohistochemistry was obvious and the sample size was sufficient for statistical significance. We used $n = 12$ for the behavioral tests to overcome the individual variation on the behavioral performance. The group size is similar to that reported in previous studies (**Park et al., *Nature neuroscience*, 2019**).

Minor Technical

Line 165 – There is no evidence specific to NHEJ function that is shown in figure 1. Statement should be removed or qualified to reflect the test performed.

We thank the reviewer for raising this issue. As the NHEJ function is not critical for our study, we removed the description from **Fig. 1** accordingly.

Missing/unclear details

Line 162 Figure 1e – "Con" is displayed in the graphic, but is not defined anywhere until Sup Fig 1. Please modify legend in Figure 1 to include (Con) definition. What method was used to obtain the biopsies used for genomic DNA isolation? It is unclear whether these are dissected hippocampal biopsies or whole punch.

We thank the reviewer for this comment. We would like to clarify that the "Con" in **Fig. 1e** is the hippocampal tissue from the uninjected controls, whereas that in **Supplementary Fig. 1f** is uninfected brain regions of the same animal. We dissected the subiculum and cornu ammonis regions from 300-μm-thick hippocampal slices for genomic DNA isolation and analysis. We have added the above information in the revised manuscript (highlighted in the text; **Page 31**).

Line 276 Fig 4h – Please explain what the basis for the analysis of "volume" is in the y-axis. If this is based on images, then I would expect it to be "area". In any case, the criteria for quantification of "Total dystrophy" should be explained in more detail.

We thank the reviewer for this comment. We quantified the "total dystrophic volume" by determining the percentage of the $LAMP1^+$ area within the area of interest. Therefore, we revised the y-axis to "$LAMP1^+$ dystrophic neurites (% area)" accordingly.

Line 328 – Please elaborate on the provenance and/or divulge the actual sequence of the "truncated WPRE" that was appended to the vector genome. The cited reference (#36, Patricio et al) discusses the effect of WPRE on AAV expression/safety, but does not appear to contain details that would indicate characterization of a truncated variant. This presumably novel element has value to those who design vectors for gene

therapy. How does it differ from the deletion mutants that are characterized in this reference: 10.1261/rna.061192.117?

We thank the reviewer for this comment. WPRE (589 bp) is a tripartite regulatory element with γ (303 bp), α (80 bp), and β (206 bp) subdomains. The "truncated WPRE" (247 bp) used in our study was adapted from **Choi et al.** (***Molecular Brain*, 2014**; Addgene #61463) and harbors minimal γ (i.e., the first 167 bp) and α subdomains. This truncated WPRE enables protein expression comparably efficient to that with the commonly used full-length WPRE in AAV vectors. Meanwhile, **Hollensen et al.** (***RNA*, 2017**; cited by the reviewer) identified a truncated WPRE variant harboring the γ and β subdomains that can enhance microRNA suppression. Therefore, the WPRE deletion mutants in these 2 studies serve different purposes.

Line 380 (As in Fig4) please explain what the basis for "volume" is in the y-axis. Criteria for quantification of "Total dystrophy" should be explained in more detail.

We thank the reviewer for this comment. We quantified neurite dystrophy by determining the percentage of the $LAMP1^+$ area within the area of interest. The lysosomal protein LAMP1 is a well-characterized marker of dystrophic neurites at amyloid plaque sites in transgenic mouse models of Aβ pathology, as it is highly enriched at amyloid plaque sites in dystrophic neuritis (**Gowrishankar et al., *Proceedings of the National Academy of Sciences of the U S A*, 2015**). We have revised the y-axis to "$LAMP1^+$ dystrophic neurites (% area)" accordingly.

Missing citations
Line 328 – Include citation or information pertaining to the provenance of "truncated WPRE".

We added the citation for **Choi et al.** (***Molecular Brain*, 2014**) accordingly.

Optional suggestions
Line 312 Fig 5g – As presented, the GFAP images in (g) are qualitatively different between samples, in that Con astrocytes show "thicker" processes than those in SW1. Does the quantification of (GFAP+ area (% area)), as in Fig 3e, reveal any quantifiable difference

We thank the reviewer for the suggestion. We have included the quantification of the percentage of the $GFAP^+$ area in **Fig. 5g**, which decreased upon injection of AAV-mediated Cas9-SW1 (**new Fig. 5i**).

Stylistic suggestions
Line 271 Fig 4e – consider relabeling the y-axis with something that does not include a "/" symbol. "Syn/Psd95 contacts") implies a ratio of two values.

As suggested by the reviewer, we have revised the y-axis to “synaptophysin–PSD-95 contacts.”

Response to reviewers (nBME-20-0510A)

We thank the reviewers for their positive and constructive comments about our manuscript and for supporting the publication of our manuscript in *Nature Biomedical Engineering*. Our responses to the comments of reviewer 2 are as follows.

Reviewer #2 (Report for the authors (Required)):

The manuscript by Duan et al has been much improved compared to the original submission. The addition of behavioral assessments have strengthened and made the study even more interesting. However, there are still some questions and concerns that need to be addressed.

Major remarks:
Please reconsider how to express and interpret the data. In the results section it should be described that treated mice "displayed lower Abeta levels compared to control mice" etc. In the discussion section it can be stated that the treatment "ameliorated", "alleviated", "prevented" or "reduced" the different features, but please then carefully consider which such interpretations that the data support.

We thank the reviewer for this comment. As suggested by the reviewer, we have carefully reconsidered the use of the terms in question. Accordingly, we have avoided using "ameliorated," "alleviated," "prevented," and "reduced" to describe the observations in the *Results* section.

Whereas the authors provide some estimates on the degree of gene editing it would be important to have such assessments performed on both mouse models used. If possible, it would also be desirable to analyze tissues from individual mice with next generation sequencing in order to see whether mice that were more efficiently edited also displayed lesser pathology or less cognitive impairment than non-treated mice.

We thank the reviewer for this comment. Accordingly, we have conducted additional experiments to examine the degree of gene editing in the APP/PS1 mouse model. We found that the gene editing rate in APP/PS1 mice is 24%, which is similar to 27% observed in 5XFAD mice. We have now included the new data in **new Supplementary Fig. 5a, b**.

While it would be interesting to investigate whether the mice with higher editing efficiency also exhibited less pathology or less cognitive impairment, the difference observed in gene editing among each gene-edited mouse might not be sufficiently large to lead to a significant change in the pathological changes or cognitive functions.

For the part of the study based on intracranial injections the authors used the contralateral side as control. However, AAVs have been described to spread to the contralateral side upon intracranial injection. Did they ascertain that this did not occur in their models?

We thank the reviewer for the comment. We showed that AAV did not spread to the contralateral side upon intracranial injection per the absence of HA staining for the virus at the contralateral side (**Fig. 2 and Supplementary Fig. 2**).

For the part of the study based on peripheral administration the authors used non-injected mice as controls. A better control would have been to use mice injected with empty vector or vector expressing a scrambled gRNA sequence.

We thank the reviewer for the comment. As suggested by the reviewers in the previous round of review, we conducted a new experiment and included AAV-PHP.eB-WPRE with a scrambled sgRNA in both WT and 5XFAD mice as controls to demonstrate the effects of AAV-PHP.eB:SW1-Syn::Cas9-mWPRE (highlighted in **Supplementary Fig. 8 on page 21**). Injection of the control virus did not apparently cause adverse effects in either WT or 5XFAD mice. Specifically, the control virus induced neither gliosis nor behavioral deficits in WT mice, nor did it affect the amyloid plaque burden in 5XFAD mice.

The authors used AAV with higher titers than what can generally be obtained. Please include a description on how the capsids could be generated with such impressive yields.

We thank the reviewer for this comment. The capsids were generated by following the method described by **Challis et al. (*Nature Protocols*, 2019)**, which has been cited in the *Methods* section (highlighted on **page 33**).

Please make more clear that dominantly inherited familial AD only represents less than 5% and that the current treatment paradigm can not be transferred to the vast majority of AD patients.

As suggested, we have included the percentage of dominantly inherited familial AD in the *Introduction* and *Discussion* sections (highlighted text on **pages 4** and **28**).

Please include more reflections to the discussion section regarding the treatment effects of the non-amyloid pathology parameters that were investigated.

We thank the reviewer for this suggestion. We have added to the *Discussion* section regarding the treatment effects of the non-amyloid pathology parameters (highlighted text on **page 29**).

CRISPR/Cas9-mediated disruption of the APP_{swe} allele alleviates not only amyloid plaque deposition but also neuroinflammation and neuronal dysfunctions as well as improving cognitive functions.

Minor remarks

Title:
Shouldn't the treatment effects on behavior also be reflected in the title?

We thank the reviewer for this suggestion. We hope the reviewer agrees that the current title provides a succinct overview of the major findings of the current study.

Abstract:
Row 33: The abbreviation for the amyloid precursor protein (APP) should be introduced, as it is used further down the abstract.

We have revised the manuscript accordingly.

Rows 33-34: It is unclear what is meant by "which affect multiple brain regions". Maybe this part of the sentence could be left out?

We have revised the sentence in question as follows: "Pathological features including extracellular amyloid plaques and neurofibrillary tangles are present in multiple brain regions."

Row 35: "brain-wide amelioration of Alzheimer's disease phenotypes" should be changed to: "amelioration of Alzheimer's disease-related phenotypes".

We thank the reviewer for this comment. However, we prefer to keep "brain-wide amelioration of Alzheimer's disease phenotypes," because we would like to emphasize that brain-wide effect of genome editing on the Alzheimer's disease phenotype has not been reported to date. As the pathology of Alzheimer's disease affects multiple brain regions, our study effectively addresses one major challenge of developing CRISPR/Cas9-mediated therapeutic intervention for the disease: the need for brain-wide genome editing and amelioration of Alzheimer's disease phenotypes.

Row 41: "AAV-Cas9 virus" should be changed to "AAV-Cas9", as "virus" is already included in the AAV abbreviation.

We have revised the manuscript accordingly.

Rows 42-43: "amyloid-beta–associated pathologies throughout the brain and alleviates the cognitive dysfunctions" should be changed to: "amyloid-beta–associated brain pathology and alleviates cognitive dysfunction".

We have revised the manuscript accordingly.

Introduction:
General: Gene names should be written in italic.

We have revised the manuscript accordingly and confirmed that all gene names are written in italic font.

Row 60: "Microbial" could be removed.

We have revised the manuscript accordingly.

Row 82: "heterozygous disease mutation" should be changed to "heterozygous disease causing mutation".

We have revised the manuscript accordingly.

Rows 102-103: "other nervous system disorders caused by autosomal dominant mutations and affect multiple brain regions" should be: "other central nervous system disorders caused by autosomal dominant mutations and affecting multiple brain regions".

We have revised the manuscript accordingly.

Results:
General: Please express more clearly that three months old mice were used for the treatment intervention.

We have reviewed the manuscript accordingly and ensured that the ages of the mice used for the treatment intervention are stated in the manuscript (highlighted in the text).

General: Please consider changing "virus infected" to "transduced" throughout the text.

We have revised the manuscript accordingly.

General: Please make sure to use the correct abbreviations for proteins and genes, respectively. For example, PS1 = protein, PSEN1= gene.

We thank the reviewer for raising this issue. We have revised the manuscript accordingly.

Row 135: “into the hippocampus in 3-month-old” should be changed to “into the hippocampus of 3-month-old”.

We have revised the manuscript accordingly.

Row 138: No protein expression data are shown to support that “the mutation abolishes the expression”.

We thank the reviewer for this comment. As suggested, we have revised the sentence in question as follows: “The CRISPR-mediated genome editing of the *APP* Swedish mutation can abolish the expression of full-length mutated APP and disrupt the effects of APP mutations.”

Row 155-157: The authors should specify if they refer to their own or to already published data. Also, they should make clear if what they state is true for mouse neurons, human neurons or both.

We thank the reviewer for raising this question. The cellular expressions of the 17 candidate genes were checked by using a published cell-type-specific transcriptome dataset (https://www.brainrnaseq.org/; **Zhang et al., *Journal of Neuroscience*, 2014**); we have added this citation accordingly. *Sp110*, *Sp140*, and *Abcg2* are not prominently expressed in mouse or human neurons. As suggested by the reviewer, we have stated that the identified mutations did not affect biological functions in the mouse brain (**page 7**).

Figure 1: Row 6: “Cas9” should be changed to “SaCas9”.

We have revised the manuscript accordingly.

Row 179: “To examine whether and how the disruption of the APPswe mutation in 5XFAD mice regulates Aβ-associated pathologies” should be changed to: “To examine if the disruption of the APPswe mutation in 5XFAD mice can alleviate Aβ-associated pathologies”.

We have revised the manuscript accordingly.

Row 181: “injection into the hippocampus in 3-month-old” should be changed to “injection into the hippocampus of 3-month-old”

We have revised the manuscript accordingly.

Rows 192-193: The text suggests that comparisons were made in injected and non-injected mice, while the statistic test used is paired. Please specify whether the percentages and staining shown refer to the injected and non-injected areas of the

same mice (paired t test) or if they refer to injected and non-injected mice (unpaired t test).

We thank the reviewer for raising this question. The comparisons were made between the staining of the injected and noninjected areas in the same mouse. We have revised the manuscript accordingly.

Row 224: "APPswe mutation" should be changed to "APPswe allele".

We have revised the manuscript accordingly.

Figure 3: "ameliorate" should be replaced with: "was associated with lower" (as the data do not reflect whether the effects were due to reduction or prevention).

We have revised "ameliorate" to "decrease," which is applicable to both reduction and prevention.

Row 247: "ameliorate hippocampal" should be: "affect hippocampal".

We have revised the manuscript accordingly.

Fig. 4: Please clarify what microscopy method that was used to make these images and reconsider if you can claim that these images provide support that PSD95 and synaptophysin interact with each other.

We thank the reviewer for raising this issue. These images were taken with the Leica TCS SP8 confocal system using a 63× objective and 4× digital zoom. The co-labeling with PSD-95 and synaptophysin is a well-established method to indicate the number of excitatory synapses in mouse brain sections (**Hong et al., *Science*, 2016; Gupta et al., *Neuropharmacology*, 2015**). To make the point clearer, we have revised the y-axis label from "Synaptophysin-PSD95 contacts" to "Synaptophysin-PSD95 colocalized puncta."

Row 326: "deliver Cas9-SW1 specifically into the adult brain via noninvasive systemic administration." should be changed to: "achieve a higher degree of Cas9-SW1 delivery into the adult brain via systemic administration."

We have revised, "…deliver Cas9-SW1 specifically into the adult brain via noninvasive systemic administration," to, "…efficiently deliver Cas9-SW1 into the adult brain via systemic administration."

Row 347: "any adverse effects" should be changed to: "any apparent adverse effects".

We have revised “did not cause any adverse effects” to “did not cause any apparent adverse effects.”

Row 351: “globally” can be removed.

We have revised the manuscript accordingly.

Row 375: “Aβ-deposition transgenic mice exhibit impaired” should be changed to: “APP transgenic mice exhibit impaired”

We thank the reviewer for raising this issue. However, as the APP/PS1 and 5XFAD transgenic mice carry both *APP* and *PSEN1* genes, using “APP transgenic mice” might not accurately describe the mouse models. Therefore, we have revised “Aβ-deposition transgenic mice” to “transgenic mouse models of Aβ deposition.”

Figure 7: The inflammatory and cognitive measures should be divided into two separate figures. A statistic comparison of Con (WT) and 5xFAD (SW1) would also be good to include.

We thank the reviewer for raising this issue. However, we prefer to keep the non-amyloid outcomes including the inflammatory and cognitive measures in one figure.
We performed statistic comparison of Con (WT) and 5XFAD (SW1) and found no significant difference between them in the open field, Y-maze, or elevated plus maze tests.

Row 421: “Aβ-deposition transgenic mice” should be changed to: ”APP transgenic mice”

We thank the reviewer for raising this issue. As mentioned above, we have revised “Aβ-deposition transgenic mice” to “transgenic mouse model of Aβ deposition.”

Rows 451-452: Please consider changing “However, it might not be possible to translate germline editing to humans.” to: “However, it might not be possible to translate germline editing to a viable AD treatment approach.”

We have revised “translate germline editing to humans” to “translate germline editing to an intervention strategy for AD.”

Methods:
Please explain why AAV9 was used for the intracerebral injections.

We thank the reviewer for this comment. We selected AAV9 for the intracerebral injections because the AAV9 system is widely used for intrahippocampal injection in

mouse brains and injection of empty AAV9 vector does not generate adverse effects (**Sun et al.,** ***Nature Communications*****, 2019; Hudry et al.,** ***Neuron*****, 2019; Hocquemiller et al.,** ***Human Gene Therapy*****, 2016**).