nature portfolio

Peer Review File

Microglial Serine Racemase Knockout Alleviates Alzheimer-like Neuropathology and Behavioral Deficit via Lactylation-Mediated Anti-inflammation

Corresponding Author: Professor Shengzhou Wu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is an interesting study that investigates the role of microglial SR in regulating A-beta levels via phagocytosis. The authors use in vitro cell models and knockout mice. There is a clear improvement in learning when SR is deleted in microglia, and this is closely correlated with A-beta burden. This is clearly novel and of general interest.
Specific points:
-The authors previously reported a dimer of SR in microglia. Now they show only a single band. Please clarify or show the dimer bands.
-The evidence for increased phagocytosis in primary microglia from SR knockout is weak- It occurs only at one single point (6h). Then it decreases and later increases, not different from the control. Confirmation is needed with another substrate of phagocytosis.
-Nuclear localization of SR in neurons is not supported by the literature.
-In the Introduction, the authors do not cite the original papers on the biochemical and electrophysiological roles of SR, and the reader is referred to much later, unrelated articles. I also suggest a discussion/correlation of this study with recent papers on the role of reactive microglia in TBI (PMID: 40796363, PMID: 35195906)

Reviewer #2

(Remarks to the Author)
In this study, the authors investigated whether serine racemase (SR) regulates microglial phagocytosis, inflammatory responses, and learning & memory behavior in AD-related models. Specifically, the findings demonstrate that Srr knockdown in BV-2 cells or Srr-/- microglia cultured cells showed increased phagocytosis, including A oligomers. The anti-inflammatory genes, IL-10 and Arg1, were also enhance following LPS stimulation of cultured Srr-/- microglia, which were shown to be P300 regulated. This study also employed constitutive Srr-floxed mice crossed with lysozyme cre mice (Lyz2cre) to knockdown Srr in myeloid cells and then crossed with 5xFAD AD-mice to examine learning and memory behavior and A plaque formation where sex difference was observed. Finally, this study examined the levels of histone lactylation in AD mice to determine whether H3K18 lactylation contribute to brain pathology. These results are interesting, however, there are a number of major and minor concerns that should be addressed.
1. The studies focus on 3 semi-distinct roles for SR: microglial phagocytosis, histone lactylation, and learning and memory behavior. However, other than their involvements in AD-pathologies, the present studies fell short of actually connecting these findings. For examine, studies could have examined whether blocking H3K18 would specially affect learning and memory behavior in 5xFAD:Lyzcre:Srrfl/fl mice as compared to control mice. Is A plaque phagocytosis responsible for learning and memory differences. To name a few.
2. The studies mainly suggest that deficiencies in SR lead to increased phagocytosis (including A phagocytosis), however, the link between decreased SR and enhanced phagocytosis is not clearly established since its mostly correlative. This link would be strengthened if the investigators performed gain-of-function studies to increase SR levels in vitro. Would that lead to reduced A oligomer phagocytosis?
3. The mechanism of serine racemase actions is largely uninvestigated. Are these findings dependent or independent of L-serine, D-serine, pyruvate levels or function? Basically, these studies fall short of providing a link between SR,

phagocytosis, behavior, and AD-pathologies. The authors discuss a potential metabolic effect due to potential L-serine accumulation, however, L-serine levels or other metabolites are not studied. Similarly, the authors discuss how sex-specific changes in D-amino acid oxidase may regulate D-serine levels to affect spatial learning, but again, neither D-serine or DAAO are examined.
4. Lyz2 cre-mediated deletion in adult microglia is not ideal since Lys2cre mice have shown to only affect a subset of microglia. The current study does not demonstrate the amount of SR knockdown in microglia or specific brain regions. It is also not clear why the investigators did not employ more classic microglial-cre mice (i.e. TMEM119cre or Cx3Cr1cre) that have been well-characterized in cortical and hippocampal microglial cells. Furthermore, both TMEM119creErt2 and CX3CR1creErt2 mice are commercially available to enable tamoxifen-induced functions at specific stages of AD progression.
5. The investigators seem to be unaware of previously published microglial SR studies, especially considering those studies examined the role of SR and D-serine in pathological learning and memory.
6. The in vivo experiments were performed at 9-11 months of age, but there is no discussion on how the timing of AD pathologies attribute to Srr deficiencies. Would similar effects be observed in 5xFAD:Lyzcre:Srrfl/fl mice prior to formation of AD plaques? In other words, are the behavioral alterations directly correlated to AD progression.
7. The lack of differences on Trem2 was interesting considering most of the findings associate SR in regulating phagocytosis. The studies seem to fall short of thoroughly investigating mechanism of phagocytosis as it relates to SR expression.
8. The authors state that Arg1 and IL-10 mRNA levels are increased in cultured Srr-/- microglia, suggesting that SR functions to regulate histone lactylation, which is not highly convincing. Basically, the author used an artificial inflammatory stimulator (i.e. LPS) in mixed microglia cultures to make their claims, however, there is no cooperating in vivo evidence. Furthermore, the pro-inflammatory cytokine, IL-1 , is arguably one of the most important cytokines in regulating histone lactylation but isn't studied, which leads the authors to claim a specific role for anti-inflammatory mechanisms.
9. The sex difference in learning and memory behavior and A plaque expression are described but no studies are performed to address why these differences are observed. In addition, the studies fall short of examining SR expression, phagocytosis and/or histone lactylation between sexes.
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1. There are several figures that use line graphs (i.e. Fig. 2B, Fig 2D) but these studies were not performed as repeated-measure experiments, which is the case for the Morris water maze (Fig. 3B). Furthermore, many of the bar-graph figures using different styles (scatterplot bars, bar only, no bars). The investigators should be consistent with their figure design to simplify the readers understanding of the results.
2. It was unclear whether the transfection process itself made the BV-2 or microglial cells more phagocytotic in nature and whether the lentiviral transfection efficiency differed between experimental and control vectors. I would be helpful to describe the effects of viral infection on phagocytosis capacity. At a minimum these differences or lack of differences should be discussed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors answered all my queries.

Reviewer #2

(Remarks to the Author)
This study investigated whether serine racemase (SR) regulates microglial phagocytosis, inflammatory responses, and learning & memory behavior in AD-related models. There were a number of concerns following the initial review that were adequately address by the authors through either addition of new studies or textual additional to introduction, results and/or discussion. Although, this study still lack some cohesion between the role of Serine racemase in regulating the distinct function in AD pathology, and determination of whether this is a D-serine mediated response, the authors did address most of the original concerns raised by both reviewers. In summary, the present manuscript to provide unique and interesting roles for microglial serine racemase and will add to our current knowledge in the field.

Reviewer #1 (Remarks to the Author):

Q1. This is an interesting study that investigates the role of microglial SR in regulating A-beta levels via phagocytosis. The authors use in vitro cell models and knockout mice. There is a clear improvement in learning when SR is deleted in microglia, and this is closely correlated with A-beta burden. This is clearly novel and of general interest.
Response: We are grateful for your kind comments.

Q2. Specific points:
-The authors previously reported a dimer of SR in microglia. Now they show only a single band. Please clarify or show the dimer bands.

Response: SR dimer in microglial cell culture, as examined with western blot, is clearly evident under inflammatory stimuli, whereas the dimer band is weak or nearly undetectable under sham treatment. Nitrosylation promotes the formation of SR dimer in microglia under inflammatory stimuli. Please refer to previous publications (PMIDs: 15285800, 15681805, 22354542) for further details. In figure1, micrgolial cell cultures are not under inflammatory stimuli and no dimer bands are present in Western blot. Additionally, denaturation-resistant dimers have been observed in many blotted proteins such as rhodopsin (PMID: 34805789), RTN3 protein (PMID: 38497356), and many others.

Q3. The evidence for increased phagocytosis in primary microglia from SR knockout is weak- It occurs only at one single point (6h). Then it decreases and later increases, not different from the control. Confirmation is needed with another substrate of phagocytosis.
Response: We add additional data examining the phagocytosis of fluorescence-labeled latex beads by microglia. SR deficiency in primary murine microglial cell cultures significantly increased phagocytosis of latex beads (Figure 2). We also overexpressed SR in BV2 cells which exhibited inhibited phagocytosis of microbeads (Figures 1D and 1E).

Q4. Nuclear localization of SR in neurons is not supported by the literature.

Response: Cytoplasm-to-nucleus shuttling of SR in neurons under apoptotic insult was reported by Dr. Herman Wolosker's group (PMID 26851299). In our study, we investigated how SR deficiency regulates microglial phagocytosis and inflammatory response. To my understanding, we did not examine SR in cultured neurons in this manuscript.

Q5. In the Introduction, the authors do not cite the original papers on the biochemical and electrophysiological roles of SR, and the reader is referred to much later,

unrelated articles. I also suggest a discussion/correlation of this study with recent papers on the role of reactive microglia in TBI (PMID: 40796363, PMID: 35195906)
Response: We added the two papers in the Introduction. We also discuss the sex-specific differences in cognitive function following deletion of microglial SR between amyloid-accumulated and traumatic brain injury model, as is copied below.

"Regarding the sex-specific effects on cognitive function, we found that deletion of microglial SR exerted a more pronounced improvement in female mice than in male mice within an AD-related mouse model. In contrast, in the traumatic brain injury (TBI) model, the beneficial effect of microglial SR deletion was more prominent in male mice [33]. A plausible explanation for this discrepancy lies in the distinct characteristics of the inflammatory responses in the two models: TBI involves an acute inflammatory reaction, in which estrogen exerts a prominent neuroprotective effect. By comparison, the AD model is associated with a slow-progressing, chronic inflammatory process, whereas the neuroprotective role of estrogen is less pronounced. Thus, the basal neuroprotective capacity of estrogen may underlie the sex-specific differences observed between these two models. Consistent with its more pronounced beneficial effects specifically in female mice within chronic inflammatory disease models, SR deletion in a schizophrenia mouse model—given that chronic inflammation is widely recognized to be a risk factor for schizophrenia—results in attenuated photoreceptor responses in male mice, whereas female mice exhibit no such alterations [45]. This cross-model consistency further validates the notion that estrogen exerts robust neuroprotective effects in female mice, a mechanism that underpins the sex-specific phenotypic outcomes induced by SR ablation."

Reviewer #2 (Remarks to the Author):

In this study, the authors investigated whether serine racemase (SR) regulates microglial phagocytosis, inflammatory responses, and learning & memory behavior in AD-related models. Specifically, the findings demonstrate that Srr knockdown in BV-2 cells or Srr-/- microglia cultured cells showed increased phagocytosis, including A oligomers. The anti-inflammatory genes, IL-10 and Arg1, were also enhance following LPS stimulation of cultured Srr-/- microglia, which were shown to be P300 regulated. This study also employed constitutive Srr-floxed mice crossed with lysozyme cre mice (Lyz2cre) to knockdown Srr in myeloid cells and then crossed with 5xFAD AD-mice to examine learning and memory behavior and Aβ plaque formation where sex difference was observed. Finally, this study examined the levels of histone lactylation in AD mice to determine whether H3K18 lactylation contribute to brain pathology. These results are interesting, however, there are a number of major and minor concerns that should be addressed.

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these findings. For examine, studies could have examined whether blocking H3K18 would specially affect learning and memory behavior in 5xFAD:Lyzcre:Srrfl/fl mice as compared to control mice. Is Aβ plaque phagocytosis responsible for learning and memory differences. To name a few.

Response: We value your suggestions.

SR deletion augmented the production of anti-inflammatory mediators (Arg1 and IL-10) and concomitantly enhanced phagocytic capacity in primary microglial cultures. Mechanistically, blocking histone lactylation and lactate production restored *Arg1* mRNA levels following LPS stimulation (Figure 3). Consistent with this in vitro observation, in vivo analysis revealed elevated expression of arginase 1—a downstream target gene of H3K18 lactylation—in SR-deficient microglia from 5×FAD mice. These integrated data strongly suggest that the dual beneficial effects of SR deletion—anti-inflammatory activity and improved phagocytosis—are mechanistically driven by H3K18la induction. However, we cannot specifically block H3K18 *in vivo* because p300, a typical histone acetyltransferase (HAT), also promotes lactylation of H3K18 under SR deficiency. Blockade or knockdown of p300 may lead to a wide range of nonspecific and potentially harmful effects due to interference of HAT activity. Similarly, blockade of lactate production by use of an inhibitor of lactate dehydrogenase A *in vivo* interferes with lactate metabolism in astrocytes as well, most likely producing non-specific effects as well.

We found that spatial learning improved dramatically in female mice but showed limited improvement in male mice following microglial SR deletion. Conversely, Aβ plaque deposition was not improved in females but decreased in males. We discuss the underlying mechanism for the lack of improvement in Aβ plaque deposition in female mice in the fifth paragraph in the Discussion section. An excerpt is provided below.

"Notably, amyloid plaque burden increases during the menopausal transition, attributed to the stimulatory effect of follicle-stimulating hormone on the C/EBPβ-δ-secretase pathway [49, 50]. Thus, the true extent of amyloid plaque burden may also be masked by these hormonal regulatory effects, as the mice utilized for spatial memory assessments were in the premenopausal stage."

In addition, Aβ plaque burden is not very closely correlated with learning and memory, which is consistent with numerous previous reports indicating that tau pathology is more tightly associated with cognitive decline, while Aβ deposition is also detectable in the brains of healthy individuals (PMID: 22487856, 40522652, 26401775). Thus, tau deposition is utilized to define the severity of AD, as outlined in the Braak staging.

Q2. The studies mainly suggest that deficiencies in SR lead to increased phagocytosis (including Aβ phagocytosis), however, the link between decreased SR and enhanced phagocytosis is not clearly established since its mostly correlative. This link would be

strengthened if the investigators performed gain-of-function studies to increase SR levels in vitro. Would that lead to reduced Aβ oligomer phagocytosis?

Response: We transfected BV2 microglial cells with an SR-overexpressing plasmid; in parallel, cells transfected with an empty control plasmid served as the negative control. Fluorescence microscopic analyses revealed a significant decrease in the phagocytic uptake of fluorescently labeled microspheres in SR-overexpressing cells relative to controls (Figures. 1D and 1E). In primary microglial cultures, increased uptake of microspheres is also observed in SR-deleted microglia (Figure 2). Collectively, our integrated loss-of-function and gain-of-function assays provide compelling evidence that altered SR expression levels in microglia modulate their phagocytic activity.

Q3. The mechanism of serine racemase actions is largely uninvestigated. Are these findings dependent or independent of L-serine, D-serine, pyruvate levels or function? Basically, these studies fall short of providing a link between SR, phagocytosis, behavior, and AD-pathologies. The authors discuss a potential metabolic effect due to potential L-serine accumulation, however, L-serine levels or other metabolites are not studied. Similarly, the authors discuss how sex-specific changes in D-amino acid oxidase may regulate D-serine levels to affect spatial learning, but again, neither D-serine or DAAO are examined.

Response: We lack access to a Seahorse analyzer for direct assessment of the metabolic profile. However, we measured lactate levels and found that microglial SR deficiency reduced lactate in response to LPS stimulation (newly added Supplemental figure 8). Combined with the in vivo data from Alzheimer's disease (AD)-like mice—where microglial PKM2 expression was elevated following SR deletion (Figure 6c)—these findings suggest that a greater proportion of pyruvate is directed toward oxidative phosphorylation rather than lactate production, as evidenced by the enhanced dynamics of pyruvate synthesis and a concomitant reduction in lactate levels. Further, our studies show that deletion or inhibition of SR in diverse cell types and tissues—including the retinal pigment epithelium (PMID: 30917905) and the retina (39136959)—leads to the accumulation of L-serine, an agonist of PKM2. Given that lactate generation is a hallmark of anaerobic glycolysis, we consider that SR deletion shifts microglial metabolic phenotype from anaerobic glycolysis toward oxidative phosphorylation in response to LPS treatment or in AD mice. These considerations have been incorporated into the first paragraph of the Discussion section.

We have also discussed the seemingly discrepancy regarding enhanced H3K18 lactylation and reduced lactate synthesis by microglia-specific deletion of SR in the second paragraph in Discussion section. The excerpt is provided below.

"Targeted deletion of SR in microglia elevates H3K18 lactylation, likely through the relief of steric hindrance imposed on p300 by endogenous SR. Notably, this

observation stands in contrast to the diminished lactate synthesis associated with microglial SR ablation. We assume that the stimulatory effect of SR deletion on p300-dependent lactylation activity overrides the impact of reduced lactate availability."

Taken together with the upregulation of anti-inflammatory factors and enhanced phagocytic activity detected in LPS-challenged SR-deficient microglia, these results lead us to propose that SR deficiency reprograms microglia toward an anti-inflammatory phenotype and enhanced phagocytic capacity.

We also conducted DAAO immunostaining. As shown in figure 7, DAAO staining intensity is much stronger in the cerebral cortex of AD male mice compared to that in AD female mice.

Q4. Lyz2 cre-mediated deletion in adult microglia is not ideal since Lys2cre mice have shown to only affect a subset of microglia. The current study does not demonstrate the amount of SR knockdown in microglia or specific brain regions. It is also not clear why the investigators did not employ more classic microglial-cre mice (i.e. TMEM119cre or Cx3Cr1cre) that have been well-characterized in cortical and hippocampal microglial cells. Furthermore, both TMEM119creErt2 and CX3CR1creErt2 mice are commercially available to enable tamoxifen-induced functions at specific stages of AD progression.

Response: Thank you for your valuable suggestions. We have validated that Lyz2-Cre mediates efficient SR knockout specifically in microglia, as shown in the newly added Figure 4A. Notably, Lyz2-Cre mice have been widely employed to achieve microglia-specific gene ablation in multiple well-established studies (PMID: 37300531, 34811872).

Purchased Lyz2-Cre mice are C57BL/6J, while available Cx3CR1-Cre mice are C57BL/6N. Our *Srr*$^{flox/flox}$ and 5×FAD mice are C57BL/6J. Lyz2-Cre mice were selected to maintain genomic consistency. For differences between C57BL/6J and C57BL/6N, refer to the Jackson Laboratory website at the following link: https://www.jax.org/news-and-insights/2013/april/a-tool-for-telling-apart-c57bl-6j-versus-c57bl-6n-substrains.

Q5. The investigators seem to be unaware of previously published microglial SR studies, especially considering those studies examined the role of SR and D-serine in pathological learning and memory.
Response: We have included the relevant information in both the Background and Discussion sections.

Q6. The in vivo experiments were performed at 9-11 months of age, but there is no

discussion on how the timing of AD pathologies attribute to Srr deficiencies. Would similar effects be observed in 5xFAD:Lyzcre:Srr$^{fl/fl}$ mice prior to formation of AD plaques? In other words, are the behavioral alterations directly correlated to AD progression.

Response: A previous study showed that SR deletion alone does not affect spatial learning in the water maze assay, as SR ablation relieves the inhibition of GABAergic neurons in the CA1 network (PMID: 33322577). Accordingly, the behavioral differences between 5×FAD;*Lyz2*^{cre};*Srr*$^{fl/fl}$ and 5×FAD;*Srr*$^{fl/fl}$ mice are associated with AD pathogenesis. We used 9–11-month-old mice because Aβ plaque deposition begins in 5×FAD mice at 2–3 months, and this age range mirrors the mid-to-late stage of human AD.

Q7. The lack of differences on Trem2 was interesting considering most of the findings associate SR in regulating phagocytosis. The studies seem to fall short of thoroughly investigating mechanism of phagocytosis as it relates to SR expression.

Response: We thank you for your insightful comments. A preliminary mechanistic exploration has been carried out, showing that SR knockdown increases CD36 mRNA and protein levels, while CD36 knockdown did not reverse the phagocytic phenotype in BV2 cells (Supplemental Figure 1). Based on the current data, our results further support that SR deletion enhances microglial phagocytosis, whereas SR overexpression inhibits this process. Future work will involve RNA sequencing coupled with downstream functional assays, though these experiments would extend the scope and workload of the present manuscript.

Q8. The authors state that Arg1 and IL-10 mRNA levels are increased in cultured Srr-/- microglia, suggesting that SR functions to regulate histone lactylation, which is not highly convincing. Basically, the author used an artificial inflammatory stimulator (i.e. LPS) in mixed microglia cultures to make their claims, however, there is no cooperating in vivo evidence. Furthermore, the pro-inflammatory cytokine, IL-1β, is arguably one of the most important cytokines in regulating histone lactylation but isn't studied, which leads the authors to claim a specific role for anti-inflammatory mechanisms.

Response: We used microglial cultures with ≥99% purity. Lipopolysaccharide was chosen as the stimulant because contaminating astrocytes (if any) are unresponsive to LPS, given their lack of Toll-like receptor 4 (TLR4). Additionally, IL-1β treatment induced a non-significant trend toward increased *Il10* mRNA levels in *Srr*$^{-/-}$ microglia (newly added Supplemental Figure 3). We also conducted immunostaining of Arg1 added to figure 6B. Arg1 staining is increased in the brain of 5xFAD:Lyz2^{cre}:Srr$^{fl/fl}$ compared to 5xFAD: Srr$^{fl/fl}$.

Q9. The sex difference in learning and memory behavior and Aβ plaque expression are described but no studies are performed to address why these differences are

observed. In addition, the studies fall short of examining SR expression, phagocytosis and/or histone lactylation between sexes.

Response: We have added SR, DAAO, H3K18la expression profile in figure7, which may explain sex difference in learning and memory behavior. We also discussed the possible mechanism of the sex-specific difference of amyloid deposition, as copied below.

"amyloid plaque burden increases during the menopausal transition, attributed to the stimulatory effect of follicle-stimulating hormone on the C/EBPβ-δ-secretase pathway [49, 50]. Thus, the true extent of amyloid plaque burden may also be masked by these hormonal regulatory effects, as the mice utilized for spatial memory assessments were in the premenopausal stage".

We also examined microglial SR expression and histone lactylation in male and female mice. We have substantially examined phagocytosis *in vitro* but can not examine in vivo phagocytosis due to technology challenge. Through the supplemental materials and discussion, we can explain the sex-specific effect in AD mice. We summary the main points as below.

"As shown in figure 7, DAAO staining intensity is much stronger in the cerebral cortex of AD male mice compared to that in AD female mice. Elevated DAAO activity has been consistently found in male mice across six different strains when compared to their female counterparts [42-44]."

Collectively, our supplementary findings, together with published peer work, delineate the mechanistic basis underlying sex-specific responses in AD mouse models harboring microglial SR knockout.

"Notably, amyloid plaque burden increases during the menopausal transition, attributed to the stimulatory effect of follicle-stimulating hormone on the C/EBPβ-δ-secretase pathway [49, 50]. Thus, the true extent of amyloid plaque burden may also be masked by these hormonal regulatory effects, as the mice utilized for spatial memory assessments were in the premenopausal stage."

The above statement, incorporated into the Discussion section, clarifies the apparent discrepancy between improved cognitive function and unaltered amyloid plaque burden in our model. Please refer to previous studies regarding sex-specific differences in microglial phagocytic activity. We have also included a dedicated discussion of this topic in the Discussion section, and an excerpt is provided below.

"Sex-dependent differences in several aspects of microglial activity have been widely documented, particularly in the context of AD [46, 47]. Specifically, male microglia *in vitro* exhibit greater migratory capacity than female microglia under both basal and stimulated conditions. Conversely, female microglia demonstrate higher levels of basal and stimulated phagocytic activity compared to male microglia in response to interferon-γ [48]."

Q10. Figure 4 is not described in the results.
Response: currently, figure 4 is changed to figure5. we have added more detail.

Minor
Q1. There are several figures that use line graphs (i.e. Fig. 2B, Fig 2D) but these studies were not performed as repeated-measure experiments, which is the case for the Morris water maze (Fig. 3B). Furthermore, many of the bar-graph figures using different styles (scatterplot bars, bar only, no bars). The investigators should be consistent with their figure design to simplify the readers understanding of the results.
Response: It is my understanding that the reviewer's comment is intended to suggest that **Fig. 1B and Fig. 1D** should be revised to scatter plots. For clarification, **Fig. 2B and Fig. 2D** are scatter plots in their current form, not line graphs.

Q2. It was unclear whether the transfection process itself made the BV-2 or microglial cells more phagocytotic in nature and whether the lentiviral transfection efficiency differed between experimental and control vectors. I would be helpful to describe the effects of viral infection on phagocytosis capacity. At a minimum these differences or lack of differences should be discussed.
Response: All the transfection processes between NC and SR-specific siRNA are the same. Lentivirus-packaged scrambled siRNA was transfected in NC group and lentivirus-packaged SR-specific siRNA was transfected in 336. The related information was described at the end of the Method section: Construction of a BV2 cell line with stable knockdown of Srr: …The mCherry fluorescence intensity was comparable between BV2 cells transfected with scrambled siRNA and those transfected with SR-specific siRNA.

Reviewer #1 (Remarks to the Author):

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Response: We are grateful for your kind comments.

Q2. Specific points:
-The authors previously reported a dimer of SR in microglia. Now they show only a single band. Please clarify or show the dimer bands.

Response: SR dimer in microglial cell culture, as examined with western blot, is clearly evident under inflammatory stimuli, whereas the dimer band is weak or nearly undetectable under sham treatment. Nitrosylation promotes the formation of SR dimer in microglia under inflammatory stimuli. Please refer to previous publications (PMIDs: 15285800, 15681805, 22354542) for further details. In figure1, micrgolial cell cultures are not under inflammatory stimuli and no dimer bands are present in Western blot. Additionally, denaturation-resistant dimers have been observed in many blotted proteins such as rhodopsin (PMID: 34805789), RTN3 protein (PMID: 38497356), and many others.

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unrelated articles. I also suggest a discussion/correlation of this study with recent papers on the role of reactive microglia in TBI (PMID: 40796363, PMID: 35195906)
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Reviewer #2 (Remarks to the Author):

In this study, the authors investigated whether serine racemase (SR) regulates microglial phagocytosis, inflammatory responses, and learning & memory behavior in AD-related models. Specifically, the findings demonstrate that Srr knockdown in BV-2 cells or Srr-/- microglia cultured cells showed increased phagocytosis, including A oligomers. The anti-inflammatory genes, IL-10 and Arg1, were also enhance following LPS stimulation of cultured Srr-/- microglia, which were shown to be P300 regulated. This study also employed constitutive Srr-floxed mice crossed with lysozyme cre mice (Lyz2cre) to knockdown Srr in myeloid cells and then crossed with 5xFAD AD-mice to examine learning and memory behavior and Aβ plaque formation where sex difference was observed. Finally, this study examined the levels of histone lactylation in AD mice to determine whether H3K18 lactylation contribute to brain pathology. These results are interesting, however, there are a number of major and minor concerns that should be addressed.

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these findings. For examine, studies could have examined whether blocking H3K18 would specially affect learning and memory behavior in 5xFAD:Lyzcre:Srrfl/fl mice as compared to control mice. Is Aβ plaque phagocytosis responsible for learning and memory differences. To name a few.

Response: We value your suggestions.

SR deletion augmented the production of anti-inflammatory mediators (Arg1 and IL-10) and concomitantly enhanced phagocytic capacity in primary microglial cultures. Mechanistically, blocking histone lactylation and lactate production restored *Arg1* mRNA levels following LPS stimulation (Figure 3). Consistent with this in vitro observation, in vivo analysis revealed elevated expression of arginase 1—a downstream target gene of H3K18 lactylation—in SR-deficient microglia from 5×FAD mice. These integrated data strongly suggest that the dual beneficial effects of SR deletion—anti-inflammatory activity and improved phagocytosis—are mechanistically driven by H3K18la induction. However, we cannot specifically block H3K18 *in vivo* because p300, a typical histone acetyltransferase (HAT), also promotes lactylation of H3K18 under SR deficiency. Blockade or knockdown of p300 may lead to a wide range of nonspecific and potentially harmful effects due to interference of HAT activity. Similarly, blockade of lactate production by use of an inhibitor of lactate dehydrogenase A *in vivo* interferes with lactate metabolism in astrocytes as well, most likely producing non-specific effects as well.

We found that spatial learning improved dramatically in female mice but showed limited improvement in male mice following microglial SR deletion. Conversely, Aβ plaque deposition was not improved in females but decreased in males. We discuss the underlying mechanism for the lack of improvement in Aβ plaque deposition in female mice in the fifth paragraph in the Discussion section. An excerpt is provided below.

"Notably, amyloid plaque burden increases during the menopausal transition, attributed to the stimulatory effect of follicle-stimulating hormone on the C/EBPβ-δ-secretase pathway [49, 50]. Thus, the true extent of amyloid plaque burden may also be masked by these hormonal regulatory effects, as the mice utilized for spatial memory assessments were in the premenopausal stage."

In addition, Aβ plaque burden is not very closely correlated with learning and memory, which is consistent with numerous previous reports indicating that tau pathology is more tightly associated with cognitive decline, while Aβ deposition is also detectable in the brains of healthy individuals (PMID: 22487856, 40522652, 26401775). Thus, tau deposition is utilized to define the severity of AD, as outlined in the Braak staging.

Q2. The studies mainly suggest that deficiencies in SR lead to increased phagocytosis (including Aβ phagocytosis), however, the link between decreased SR and enhanced phagocytosis is not clearly established since its mostly correlative. This link would be

strengthened if the investigators performed gain-of-function studies to increase SR levels in vitro. Would that lead to reduced Aβ oligomer phagocytosis?

Response: We transfected BV2 microglial cells with an SR-overexpressing plasmid; in parallel, cells transfected with an empty control plasmid served as the negative control. Fluorescence microscopic analyses revealed a significant decrease in the phagocytic uptake of fluorescently labeled microspheres in SR-overexpressing cells relative to controls (Figures. 1D and 1E). In primary microglial cultures, increased uptake of microspheres is also observed in SR-deleted microglia (Figure 2). Collectively, our integrated loss-of-function and gain-of-function assays provide compelling evidence that altered SR expression levels in microglia modulate their phagocytic activity.

Q3. The mechanism of serine racemase actions is largely uninvestigated. Are these findings dependent or independent of L-serine, D-serine, pyruvate levels or function? Basically, these studies fall short of providing a link between SR, phagocytosis, behavior, and AD-pathologies. The authors discuss a potential metabolic effect due to potential L-serine accumulation, however, L-serine levels or other metabolites are not studied. Similarly, the authors discuss how sex-specific changes in D-amino acid oxidase may regulate D-serine levels to affect spatial learning, but again, neither D-serine or DAAO are examined.

Response: We lack access to a Seahorse analyzer for direct assessment of the metabolic profile. However, we measured lactate levels and found that microglial SR deficiency reduced lactate in response to LPS stimulation (newly added Supplemental figure 8). Combined with the in vivo data from Alzheimer's disease (AD)-like mice—where microglial PKM2 expression was elevated following SR deletion (Figure 6c)—these findings suggest that a greater proportion of pyruvate is directed toward oxidative phosphorylation rather than lactate production, as evidenced by the enhanced dynamics of pyruvate synthesis and a concomitant reduction in lactate levels. Further, our studies show that deletion or inhibition of SR in diverse cell types and tissues—including the retinal pigment epithelium (PMID: 30917905) and the retina (39136959)—leads to the accumulation of L-serine, an agonist of PKM2. Given that lactate generation is a hallmark of anaerobic glycolysis, we consider that SR deletion shifts microglial metabolic phenotype from anaerobic glycolysis toward oxidative phosphorylation in response to LPS treatment or in AD mice. These considerations have been incorporated into the first paragraph of the Discussion section.

We have also discussed the seemingly discrepancy regarding enhanced H3K18 lactylation and reduced lactate synthesis by microglia-specific deletion of SR in the second paragraph in Discussion section. The excerpt is provided below.

"Targeted deletion of SR in microglia elevates H3K18 lactylation, likely through the relief of steric hindrance imposed on p300 by endogenous SR. Notably, this

observation stands in contrast to the diminished lactate synthesis associated with microglial SR ablation. We assume that the stimulatory effect of SR deletion on p300-dependent lactylation activity overrides the impact of reduced lactate availability."

Taken together with the upregulation of anti-inflammatory factors and enhanced phagocytic activity detected in LPS-challenged SR-deficient microglia, these results lead us to propose that SR deficiency reprograms microglia toward an anti-inflammatory phenotype and enhanced phagocytic capacity.

We also conducted DAAO immunostaining. As shown in figure 7, DAAO staining intensity is much stronger in the cerebral cortex of AD male mice compared to that in AD female mice.

Q4. Lyz2 cre-mediated deletion in adult microglia is not ideal since Lys2cre mice have shown to only affect a subset of microglia. The current study does not demonstrate the amount of SR knockdown in microglia or specific brain regions. It is also not clear why the investigators did not employ more classic microglial-cre mice (i.e. TMEM119cre or Cx3Cr1cre) that have been well-characterized in cortical and hippocampal microglial cells. Furthermore, both TMEM119creErt2 and CX3CR1creErt2 mice are commercially available to enable tamoxifen-induced functions at specific stages of AD progression.

Response: Thank you for your valuable suggestions. We have validated that Lyz2-Cre mediates efficient SR knockout specifically in microglia, as shown in the newly added Figure 4A. Notably, Lyz2-Cre mice have been widely employed to achieve microglia-specific gene ablation in multiple well-established studies (PMID: 37300531, 34811872).

Purchased Lyz2-Cre mice are C57BL/6J, while available Cx3CR1-Cre mice are C57BL/6N. Our *Srr*$^{flox/flox}$ and 5×FAD mice are C57BL/6J. Lyz2-Cre mice were selected to maintain genomic consistency. For differences between C57BL/6J and C57BL/6N, refer to the Jackson Laboratory website at the following link: https://www.jax.org/news-and-insights/2013/april/a-tool-for-telling-apart-c57bl-6j-versus-c57bl-6n-substrains.

Q5. The investigators seem to be unaware of previously published microglial SR studies, especially considering those studies examined the role of SR and D-serine in pathological learning and memory.
Response: We have included the relevant information in both the Background and Discussion sections.

Q6. The in vivo experiments were performed at 9-11 months of age, but there is no

discussion on how the timing of AD pathologies attribute to Srr deficiencies. Would similar effects be observed in 5xFAD:Lyzcre:Srr$^{fl/fl}$ mice prior to formation of AD plaques? In other words, are the behavioral alterations directly correlated to AD progression.

Response: A previous study showed that SR deletion alone does not affect spatial learning in the water maze assay, as SR ablation relieves the inhibition of GABAergic neurons in the CA1 network (PMID: 33322577). Accordingly, the behavioral differences between 5×FAD;*Lyz2*^{cre};*Srr*$^{fl/fl}$ and 5×FAD;*Srr*$^{fl/fl}$ mice are associated with AD pathogenesis. We used 9–11-month-old mice because Aβ plaque deposition begins in 5×FAD mice at 2–3 months, and this age range mirrors the mid-to-late stage of human AD.

Q7. The lack of differences on Trem2 was interesting considering most of the findings associate SR in regulating phagocytosis. The studies seem to fall short of thoroughly investigating mechanism of phagocytosis as it relates to SR expression.

Response: We thank you for your insightful comments. A preliminary mechanistic exploration has been carried out, showing that SR knockdown increases CD36 mRNA and protein levels, while CD36 knockdown did not reverse the phagocytic phenotype in BV2 cells (Supplemental Figure 1). Based on the current data, our results further support that SR deletion enhances microglial phagocytosis, whereas SR overexpression inhibits this process. Future work will involve RNA sequencing coupled with downstream functional assays, though these experiments would extend the scope and workload of the present manuscript.

Q8. The authors state that Arg1 and IL-10 mRNA levels are increased in cultured Srr-/- microglia, suggesting that SR functions to regulate histone lactylation, which is not highly convincing. Basically, the author used an artificial inflammatory stimulator (i.e. LPS) in mixed microglia cultures to make their claims, however, there is no cooperating in vivo evidence. Furthermore, the pro-inflammatory cytokine, IL-1β, is arguably one of the most important cytokines in regulating histone lactylation but isn't studied, which leads the authors to claim a specific role for anti-inflammatory mechanisms.

Response: We used microglial cultures with ≥99% purity. Lipopolysaccharide was chosen as the stimulant because contaminating astrocytes (if any) are unresponsive to LPS, given their lack of Toll-like receptor 4 (TLR4). Additionally, IL-1β treatment induced a non-significant trend toward increased *Il10* mRNA levels in *Srr*$^{-/-}$ microglia (newly added Supplemental Figure 3). We also conducted immunostaining of Arg1 added to figure 6B. Arg1 staining is increased in the brain of 5xFAD:Lyz2^{cre}:Srr$^{fl/fl}$ compared to 5xFAD: Srr$^{fl/fl}$.

Q9. The sex difference in learning and memory behavior and Aβ plaque expression are described but no studies are performed to address why these differences are

observed. In addition, the studies fall short of examining SR expression, phagocytosis and/or histone lactylation between sexes.

Response: We have added SR, DAAO, H3K18la expression profile in figure7, which may explain sex difference in learning and memory behavior. We also discussed the possible mechanism of the sex-specific difference of amyloid deposition, as copied below.

"amyloid plaque burden increases during the menopausal transition, attributed to the stimulatory effect of follicle-stimulating hormone on the C/EBPβ-δ-secretase pathway [49, 50]. Thus, the true extent of amyloid plaque burden may also be masked by these hormonal regulatory effects, as the mice utilized for spatial memory assessments were in the premenopausal stage".

We also examined microglial SR expression and histone lactylation in male and female mice. We have substantially examined phagocytosis *in vitro* but can not examine in vivo phagocytosis due to technology challenge. Through the supplemental materials and discussion, we can explain the sex-specific effect in AD mice. We summary the main points as below.

"As shown in figure 7, DAAO staining intensity is much stronger in the cerebral cortex of AD male mice compared to that in AD female mice. Elevated DAAO activity has been consistently found in male mice across six different strains when compared to their female counterparts [42-44]."

Collectively, our supplementary findings, together with published peer work, delineate the mechanistic basis underlying sex-specific responses in AD mouse models harboring microglial SR knockout.

"Notably, amyloid plaque burden increases during the menopausal transition, attributed to the stimulatory effect of follicle-stimulating hormone on the C/EBPβ-δ-secretase pathway [49, 50]. Thus, the true extent of amyloid plaque burden may also be masked by these hormonal regulatory effects, as the mice utilized for spatial memory assessments were in the premenopausal stage."

The above statement, incorporated into the Discussion section, clarifies the apparent discrepancy between improved cognitive function and unaltered amyloid plaque burden in our model. Please refer to previous studies regarding sex-specific differences in microglial phagocytic activity. We have also included a dedicated discussion of this topic in the Discussion section, and an excerpt is provided below.

"Sex-dependent differences in several aspects of microglial activity have been widely documented, particularly in the context of AD [46, 47]. Specifically, male microglia *in vitro* exhibit greater migratory capacity than female microglia under both basal and stimulated conditions. Conversely, female microglia demonstrate higher levels of basal and stimulated phagocytic activity compared to male microglia in response to interferon-γ [48]."

Q10. Figure 4 is not described in the results.
Response: currently, figure 4 is changed to figure5. we have added more detail.

Minor
Q1. There are several figures that use line graphs (i.e. Fig. 2B, Fig 2D) but these studies were not performed as repeated-measure experiments, which is the case for the Morris water maze (Fig. 3B). Furthermore, many of the bar-graph figures using different styles (scatterplot bars, bar only, no bars). The investigators should be consistent with their figure design to simplify the readers understanding of the results.
Response: It is my understanding that the reviewer's comment is intended to suggest that **Fig. 1B and Fig. 1D** should be revised to scatter plots. For clarification, **Fig. 2B and Fig. 2D** are scatter plots in their current form, not line graphs.

Q2. It was unclear whether the transfection process itself made the BV-2 or microglial cells more phagocytotic in nature and whether the lentiviral transfection efficiency differed between experimental and control vectors. I would be helpful to describe the effects of viral infection on phagocytosis capacity. At a minimum these differences or lack of differences should be discussed.
Response: All the transfection processes between NC and SR-specific siRNA are the same. Lentivirus-packaged scrambled siRNA was transfected in NC group and lentivirus-packaged SR-specific siRNA was transfected in 336. The related information was described at the end of the Method section: Construction of a BV2 cell line with stable knockdown of Srr: …The mCherry fluorescence intensity was comparable between BV2 cells transfected with scrambled siRNA and those transfected with SR-specific siRNA.