Peer Review File

Manuscript Title: CRISPR-Cas9 screens reveal regulators of aging in neural stem cells

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

This manuscript, entitled “In vitro and in vivo CRISPR-Cas9 screens reveal drivers of aging in neural stem cells of the brain”, used CRISPR-Cas9 screens to identify genes responsible for increased neural stem cell (NSC) quiescence in aging. First, CRISPR-Cas9 screens were conducted on subventricular zone NSC cultures from young and old Cas9 mice and identified 300 gene knockouts that boost old NSC activation. Then combinations of 10-gene libraries of sgRNAs were packaged into lentivirus and injected into the brain in vivo to identify genes that boost the activation of old NSCs in vivo. One of the genes identified in both the in vitro and in vivo screens was the glucose transporter Slc2a4 (GLUT4). Knockout of Slc2a4 in the subventricular zone boosted neurogenesis in old mice. Old NSC cultures exhibited enhanced glucose uptake compared to young NSC cultures and knocking out Slc2a4 in old NSC cultures reduced glucose uptake. Finally, knocking out Slc2a4 or conditions of glucose starvation promoted old NSC culture activation. This manuscript is the first study to use a genome-wide knockout screen to identify genes that impact aging-related decline of NSC activation. The identification of glucose transporter as a potent regulator of NSC activation in the aged SVZ is interesting, but not surprisingly as several studies already show its role in regulating NSC proliferation. The authors claimed that they developed new methods for in vitro and in vivo screen of stem cell activation, therefore it will be very important to know the validation rate of their screen hits with analysis of individual hits. It is not sufficient to focus on one for detailed validation and without sufficient validation, it could be very misleading for the field to claim that they have identify over 300 genes that regulate NSC activation.

Major Concerns

1. The authors did a large scale in vitro screen for NSC reactivation using young and old NSCs, but there is not sufficient validation in vitro. The authors need to pick a number of hits to validate them individually to conform the knockout efficacy and effect on NSC reactivation in vitro. This will be very important to know the validation rate. Without knowing the validation rate, the majority results in Fig. 1 (f-i) and associated supplementary tables are not meaningful.

2. The author should provide sample images to show mCherry+ cells in the olfactory bulb with different pools of sgRNA.

3. Because they authors did bulk sequencing for in vivo screen, not single-cell, the number of guides is dependent on not only the number of cells expressing the sgRNA, but also expression levels of the sRNA in different cells due to their integration site. The authors need to validate some of the results with counting of neuronal numbers in the olfactory bulb with sgRNA targeting an individual gene by

histology, instead of purely relying on sequencing and gene score (Fig. 2e). It is also important to show cells in the olfactory bulb are neurons, not other cell types.

4. The “Depleted (Young/Old)” library with genes that when knocked out blocked young or old NSC activation in the in vitro screens did not impair the activation of old NSCs in vivo (Fig. 2E). The hypothesis would be that at least some of these genes would reduce the capacity for old NSCs to activate in vivo, but the effect for most of these genes is no change compared to Control. Can the authors comment on this unexpected result?

5. The validation of GLUT4 knockout was done in the olfactory bulb. It should be examined in the SVZ. In addition to look at EdU+ cell numbers in the olfactory bulb, it is important to validate that knockout GLUT4 indeed promotes activation of NSCs in the SVZ by immunostaining. Look at in the olfactory bulb has the complication of impact on neuronal migration and survival by the genetic manipulation.

6. It is known that there is region heterogeneity for NSCS along different sides of ventrical walls (e.g. PMID: 34112692). Does GLUT4 knockout have a similar effect on different regions based on immunohistolology to look at proliferation status of NSCs?

7. In Fig. 3c, it does not look like NeuN and mCherry are co-localized at all. The mCherry cells also do not show any neuronal morphology. This is the only evidence the authors have for neurogenesis. The authors need to use additional markers (may for oligodendrocytes as well) and show much better images with NeuN, mCherry and DAPI and 3D reconstruction.

8. GFAP alone cannot differentiate SVZ neural stem cells and astrocytes. The authors need to co-stain with additional markers, such as nestin (Fig. 3F).

9. GLUT4 protein expression is higher in aNSCs than qNSCs in vivo (Fig. 3F), while GLUT4 expression is lower in aNSCs than qNSCs in vitro (Fig. 4D). Can the authors comment on this discrepancy? Are there other differences between the in vivo niche and the in vitro cultures that might impact the interpretation of the in vitro results?

10. The ultimate goal of the CRISPR-Cas9 screens is to identify genes that can be manipulated to activate old NSCs to generate larger numbers of new neurons (Fig. 4I). Fig. 3B shows quantification of mCherry+ EdU+ cells in Control, Slc2a4 KO or Vmn1r107 KO conditions, but these cells could be neurons or glia. To show that neurogenesis is increased when Slc2a4 is knocked out, please quantify mCherry+ EdU+ NeuN+ newborn neurons, similar to quantification in Fig. 3B. This is necessary to claim that Slc2a4 knockout “boosts neurogenesis”.

11. It will be useful if the authors can confirm the results on increased NSC activation in old mice with Stx4a knockout to provide support that this pathway is important.

12. Mean fluorescent intensity was used as a measurement in multiple experiments. Each n was

from a single mouse or biological replicate. Immunostaining is not very quantitative, especially between samples. What conditions or parameters were put in place to try to control for technical variability in fluorescent intensity between samples that was not due to experimental conditions?

Minor Concerns

13. In the introduction, Statement "The ability of NSCs to activate and form newborn neurons is severely impaired in the aging brain, and this can contribute to deficits in cognition and regeneration" about cognition could be true for hippocampal NSCs, but SVZ NSCs.

14. Legend of Figure 1A grammatical error: "Genomic DNA is prepared and sgRNA constructs are PCR amplified rom Day 4 or Day 14 prior to sequencing on an Illumina Novaseq S4 system."

15. In the in vitro screen, the authors present the list of both depleted and enriched genes. Genes from the enriched list suggest that knockout of them will promote reaction. Some of the genes from the depleted list could be interesting as well and it is possible overexpression of them can promote reactivation. The authors can test some of them by overexpression or CRISPRa.

16. The authors mentioned that their in vitro screen identifies some of previous known NSC regulators. How about those NSC regulators tested in Fig. 2 in vivo?

17. The subtitle: "Development of an in vivo CRISPR-Cas9 screening platform to rapidly screen gene knockouts for their ability to rejuvenate NSC activity in the old brain". It will be better to state that this is a targeted screen.

18. What's the reason to have reads for sgRNA in cerebellum in the in vivo screen?

19. Figure 4F: There appear to be multiple error bars on each bar in the graph, one set that is gray and one set that is colored.

20. Figure 4H: The error bar on Old, Normal Glucose, GLUT4 bar is not even in the up and down.

Referee #2 (Remarks to the Author):

This interesting study examines the ability of neuronal stem cells to undergo proliferation in adult brain. A genome-wide screen was performed to identify genes in older mice that might prevent regeneration, and thus provide insights into why old animals have defects in neuronal activation. An elegant in vivo screen was developed, yielding a number of new genes that restored NSC activation. Among these was Glut4, the insulin-stimulated facilitative glucose transporter. Knockout of Glut4 in vivo represented the top rejuvenating intervention, and this was reinforced by measurements of glucose uptake, suggesting that glucose metabolism per se provides the inhibiting signal for regeneration.

This is an elegant study that sheds new light on an important question, and identifies a new site of

potential therapeutic intervention. My questions are mainly focused on the regulation of Glut4 and subsequent energy metabolism in these neuronal stem cells. Studies on Glut4 have revealed its restricted and unique role as an insulin-regulated protein, due to changes in its trafficking. Indeed, Glut4 generally resides in specific intracellular vesicles, and traffics to the plasma membrane only after receiving cues from the insulin receptor, or in some cases AMPK, which is itself activated due to depletion of energy. What is regulating Glut4 in these cells? Aging is generally associated with peripheral insulin resistance but higher circulating levels of insulin. Perhaps the small increase n Glut4 protein levels is less important than higher exposure to insulin? Was insulin used in the glucose uptake assays? It will be critical to demonstrate whether Glut4 is regulated in these cultured cells by either insulin (or IGF1) or an AMPK activator.

A second issue concerns the source of energy in young cells. Presumably there are other glucose transporters, esp Glut1 and 2. Are these changed in aging? What about fatty acid metabolism, and does this recede in aging? It will be relatively simple to perform seahorse experiments on young and old NSCs to determine whether there is a switch in substrate preference and if this is completely due to Glut4 expression.

Minor point: need validation of the Glut4 KO by western blot.

Referee #3 (Remarks to the Author):

In this manuscript, Ruetz and colleagues use an in vitro and in vivo screening approach (based on CRISPR-Cas9 knockout technology), to identify genetic pathways that regulate the age-associated block of neural stem cell (NSC) activation in the subventricular zone (SVZ) of aged mice. Starting with an in vitro screen using primary NSCs isolated from young and aged mice, they identify numerous genes whose knock-out induces NSC activation in young NSCs, in old NSCs, or in both. Over 300 genes were found to suppress quiescence exit in aged NSCs alone, including genes involved in cilium organization, ribonucleoprotein structures and glucose transport. Importantly, while some of these hits were already known to influence NSC behaviour (such as glucose metabolism), many genes had not been associated with NSC activation before. To assess the in vivo relevance of their screen's top hits, the authors adapted their methodology to investigate which genes are able to activate NSCs in the aging mouse brain. Through a lower-scale in vivo CRISPR-Cas9 screen, they tested a total of 50 genes and identified 23 gene knockouts that stimulated SVZ/OB neurogenesis (as measured by enhanced representation of KO cells in the olfactory bulb, OB) in old mice. Probing in more detail the importance of glucose metabolism in aged NSCs, the authors show that depleting the gene Slc2a4, which codes for the transmembrane glucose transporter GLUT4, significantly increased the rate of neurogenesis in the aged OB. Moreover, they show that GLUT4 protein expression is increased with age and correlated in vitro with a 2-fold greater rate in glucose uptake by old quiescent NSCs compared to young cells. Finally, genetically reducing glucose import into cells through GLUT4 knockout or through transient glucose starvation was able to recover old NSCs' ability to exit quiescence, suggesting the reversibility of reduced activation properties in aged NSCs. Together, the manuscript by Ruetz et al. identified several genes/pathways involved in the age-associated inhibition of SVZ NSC activation, including a notable shift in glucose transport dynamics.

This study represents a technical tour de force and provides a novel resource for the field. The genome-wide screen aiming to identify genes that regulate the exit from quiescence in cultured NSCs (that is affected with age) will be valuable to others (as this is one of the behavioural features of NSCs that is affected by age). The authors validate their screening approach by studying one identified gene, Slc2a4, in more detail. The identification of age-regulated glucose transport is novel and interesting - but conceptually not really unexpected (as somewhat discussed by the authors) given that glucose metabolism has been previously implicated in NSC behaviour. Therefore, the study rather represents a resource (and an elegant technical advance!) but is limited in its advance in terms of understanding age-dependent biology of SVZ NSCs.

Main comments:

Screening:
The data shown in Figure 2E are obtained from 2 mice, which is not ideal considering the huge variability found for the same target gene. This is particularly evident for quite a few genes (incl. Slc45a4 or Bmpr2), where one mouse showed strong enrichment of sgRNAs, whereas the other showed a depletion or at least no enrichment for the same target gene. Adding more mice would reassure the reader of the gene's effect and directionality on NSC activation. The large variability also makes us questioning the efficiency of the KO system in the Cas9 mice. With exception of the EGFP KO, this manuscript does not demonstrate that the CRISPR-Cas9 is actively knocking out target genes in vivo. Secondly, the 4th group of genes targeted in vivo (referred to as 'Depleted') did not show any significant sgRNA depletion in old NSCs as seen in Figure 2E. This is very surprising and does not recapitulate the in vitro results (or the literature on targeted genes). The authors should clarify the absence of phenotype in the 'Depleted' group. To more systematically show that gene knockouts are indeed occurring, immunofluorescence against at least 3 genes/proteins should be included to show that targets are indeed efficiently reduced.

Glucose transport:
The in vitro screen assesses activation of qNSCs. The in vivo experiments focus on the progeny of NSCs in the olfactory bulb, which makes sense. However, it is unclear why many of the analyses (e.g., showing the KO of Slc2a4) are performed in the OB and not in the SVZ. Furthermore, the analyses of the Slc2a4 is very superficial and very much weakens the relevance of the findings. It is unclear whether the results presented in Fig. 3 are due to actual activation of old qNSCs, or whether already active NSCs were simply encouraged to proliferate more, or if more cells survived…. The authors need to answer the following questions: Does the global proportion of quiescent and active NSCs in the SVZ differ between control and GLUT4 KO mice? Hence, are quiescent NSCs pushed towards an active state by GLUT4 KO in vivo? What is the proportion of NSCs that are induced to activate (i.e. proliferate) due to GLUT4 KO compared to control? Does this increase in old NSC activation also lead to their accelerated exhaustion? Or is GLUT4 KO a sustainable activation model? The authors should quantify the number of mCherry+ NSCs at 1 day and 5 weeks post-injection in control and sgRNA brains to determine whether old NSCs are consequently depleted post-activation. Further, the authors need to show that the sgRNAs targeting GLUT4 do in fact lead to a decrease in this glucose transporter in vivo in the SVZ NSCs (the in vitro data are not sufficient here). Performing immunofluorescence on brain slices is required. The authors claim that increasing GLUT4 expression in old NSCs is causally linked to the inhibition of quiescence exit in the aging brain. Yet, little

evidence supports the causality of the phenotype. The authors should overexpress GLUT4 in young NSCs and quantify stem cell activation. If the hypothesis holds true, then this simple experiment should show a block in young NSC activation.

Specific comments:

1. Based on the data shown in Fig. 1C, it is surprising that only 3 samples from the young NSCs showed higher activation levels compared to old NSCs. More replicates would better support the authors' claim, as there is clearly high variability in the activation capacity of young NSCs. Furthermore, the authors should add growth rate/proliferation data of young and aged NSCs (e.g., it remains unclear how many passages were required to obtain the starting populations that were induced for quiescence). Were there any changes in proliferation/initial growth between young and aged NSCs isolated from the SVZ?

2. Reactivation rate of old NSCs displayed in Fig. 1E seems to be very low when compared to the data shown in Fig. 1C. Please explain why the knockout of your top 10 hits seems to be comparable or even below the activation rate of old NSCs shown in Fig. 1C. Additionally, it would be of interest to add young NSCs to Figure 1E, to show at what extent the KO in old NSCs can recapitulate the young NSC activation levels.

3. The authors show that GLUT4 expression increases with age in qNSCs and aNSCs, as shown in Fig. 3E and 3F. Using established markers, the authors need to separate qNSCs from astrocytes (that are now grouped together). Further, this finding is only supported by immunofluorescence. Being one of the major statements of this manuscript, further complementary experiments may be added to clearly show the age-associated increase in GLUT4. For example, a western blot quantifying protein amounts should be considered. Alternatively, publicly available or lab-based RNA-seq data from aging SVZ NSCs may be used to confirm the increase in glucose transporters in aging NSCs (anyways: it will be interesting to analyse if only protein or also mRNA levels are altered with age).

4. Similar to point 2: the very low proportion of Ki67+ cells in the young samples shown in Fig. 4H is surprising and many fold less compared to the data shown in Fig. 1C. Please explain this discrepancy. The dramatic differences between experiments somewhat questions the robustness of the used assays.

5. Throughout the manuscript, there is some contradiction between the proposed role of GLUT4 in old NSC activation inhibition and the measured levels of GLUT4 in SVZ stem cells. Based on Fig. 3F and Ext. Fig. 6C glucose transport protein levels (GLUT4 and STX4A) seem to be higher in old active NSCs than in old quiescent NSCs. This observation somewhat contradicts the author's interpretation that high levels of GLUT4 (or STX4A) block the quiescent-to-active transition (as active NSCs seem to have higher levels of glucose transporters). In addition, there is a disagreement between in vivo and in vitro data concerning GLUT4 levels in quiescent and active NSCs. Indeed, Fig. 3F shows that in vivo, active NSCs seem to have higher levels of GLUT4 (irrespective of age) compared to quiescent NSCs. However, Fig. 4D clearly depicts the opposite: in vitro quiescent NSCs seem to have higher GLUT4 levels compared to active NSCs in vitro. This needs to be clarified.

6. It is suprising that the knockout of 1 glucose transporter (out of 12) can have a significant effect on global glucose uptake levels in NSCs, considering that cells express several glucose import channels and that rapid compensation for the loss of 1 transporter is likely to occur. It is somewhat unclear if the minor decrease in glucose import shown in Fig. 4G at day 8 can have an actual physiological impact on NSC behaviour. This question is particularly relevant considering the experimental design of Fig. 4H is based on complete extremes: either the total absence of glucose ('Glucose Starvation') or glucose presence ('Normal Glucose'). Yet, this hardly reflects physiological conditions of the SVZ stem cell niche. It may be much more convincing to see how the activation of old quiescent NSCs is affected by increments of glucose concentrations. Furthermore, the timing of the proliferation/activation assays (e.g., using Ki67) should be correlated with the glucose uptake measurements (at least the same timing for these experiments should be used).

Additional comments

1. The title of the manuscript does not reflect its content. The use of the term "drivers" is not appropriate, unless new experiments can better prove the mechanistic relationship between GLUT4 and the inhibition of NSC activation.

Results & Figures

2. In Fig. 2B, the mCherry signal is not really convincing. Why are mostly processes labelled? The authors should clearly illustrate NSCs and their morphology in the SVZ upon transduction.

3. In Fig. 3, Vmn1r107 is used throughout to represent the 'depleted' sgRNA condition that is hypothesized to cause less NSC activation. Yet, none of the data points support that deleting this gene has any negative impact on NSC proliferation or OB neurogenesis. This example questions again a little bit the robustness of the in vivo screen (with none of the "depleted" guide RNAs showing indeed depletion; Fig. 2E).

4. In Fig. 3C, it is very hard to discern the nuclei that correspond to the mCherry+ NeuN+ cells. Using more contrasting colours and adding the DAPI channel would clarify this. Also, images of the control sgRNA sample should be added.

5. In Fig. 3E, it is not possible to see individual cells and the reader cannot interpret that old NSCs have increased GLUT4 expression. Images with a higher magnification of the stem cell niche or inserting a zoom of the cells should be shown. In order to clearly depict an increase in GLUT4, example images of each cell type quantified should be displayed. The analyses of GLUT4 in NSCs need to be substantially improved (see above).

6. In Fig. 4C and Ext. Fig. 6A, the images of active NSCs (young and old) are not really comparable: roughly the same number of cells as in the quiescent samples should be used for the active population (the DAPI signal in aNSCs should be improved).

7. In Fig. 4D and 4E, the same p value (0.029) is displayed for GLUT4 and STX4A between young and old qNSC samples (the data shown suggest that the difference is larger for GLUT4). Statistics should

be checked.

8. Showing the KO of EGFP in olfactory bulb neurons (Ext. Fig. 3B and 3C) is fine. But the authors also need to show the KO of EGFP in NSCs of the SVZ (plus additional genes/proteins as suggested above).

9. In Ext. Fig. 6B and 6C, the representative images do not convincingly show that STX4A is increased in old NSCs. Images with a higher magnification of the stem cell niche or an insert zooming in on the cells should be shown as it is impossible to see individual cells currently. In addition, the same analyses as suggested for GLUT4 should be done for STX4A. For Ext. Fig. 6A (same as for 4C): the images of active NSCs (young and old) are not really comparable: roughly the same number of cells as in the quiescent samples should be used for the active population.

Methods

10. Two glucose uptake kits are mentioned in the methods section. Yet, it is not clear where and for which experiment either of the kits was used. Please clarify this point in your methods and results sections.

Discussion

11. We thought it was interesting to observe that 210 gene KO specifically improved NSC activation in young NSCs (but nor in old) as shown in Fig. 1I and 1J. Although the GO terms are illustrated, the authors may comment on these genes and their meaning for NSC behaviour. Any speculation and comparison to previously published pathways would be of interest to the readers. Furthermore, the authors may add a short section that genes not only will be upregulated with age but that indeed the age-dependent down-regulation of genes with age (as shown in NSCs but also other somatic stem cells) may play an important role.

12. The term “rejuvenation” that is used throughout the manuscript is not correct or appropriate. The authors show that the transition from quiescence to activation can be induced in old NSCs through various knockouts (which is one previously described feature of aged NSCs, among several others changes that may occur with age. Yet, (partially) recapitulating one phenotype of young NSCs is not enough to claim that the aged cells have rejuvenated.

13. The authors should speculate in the discussion on the reasons why only 2 out of 12 glucose transporters boost old NSC activation. Is it due to Slc2a4 (GLUT4) and Slc2a12 (GLUT12) being the most highly expressed in SVZ NSCs? Is there a switch in isoform preference with age? Is their effect on NSC activation solely linked to their sensitivity to insulin? Please expand the discussion section on the role of these two glucose transporters.

Referee #4 (Remarks to the Author):

This manuscript by Ruetz and colleagues aims to use unbiased genetic screens to identify factors controlling the activation of neural stem cells (NSCs) for neuronal regeneration. NSC activation is impaired in the brain of old mice, which is thought to contribute to aging-associated deficits in brain function. The authors establish a highly innovative CRISPR-based screening approach, first in primary mouse NSCs, and then, as a secondary validation screen, in mouse brains. They identify several pathways knockdown of which enhances NSC activation and neurogenesis, including glucose uptake, primary cilia, and (somewhat more vaguely) cytoplasmic ribonucleoprotein structures. The authors focus on one particular hit, the insulin-sensitive glucose uptake channel GLUT4, which is more highly expressed old NSCs than in young NSCs, suggesting a possible role in altering NSC function with aging. Indeed, the authors validate in vitro that glucose uptake is increased in old NSCs, and that GLUT4 knockout reduces it to levels found in young NSCs. Furthermore, glucose starvation enhances the activation potential of old NSCs to match that of young NSCs. However, in GLUT4 knockout cells, there is no additional benefit of glucose starvation, supporting the notion that the effect of GLUT4 knockout on NSC activation is through the reduction of glucose uptake.

This work should be of interest to the broad audience of Nature for two reasons: first, it delineates novel pathways with a role in NSC activation, and second, it establishes an elegant paradigm to uncover such pathways using CRISPR screens in vitro and in vivo. Very few CRISPR screens have so far been conducted in vivo, and those were mostly done in the context of cancer, enhancing the innovative aspect of this manuscript.

There are several minor issues with the manuscript that should be addressed before it is suitable for publication:

- Introduction: The authors claim that “In addition, in vivo genetic screens are challenging in mammals and have been so far limited to cancer and development.“ While this is mostly correct, there it at least one example of a screen conducted in vivo in adult mouse brains (PMID: 32004439)
- Fig. 1d: The PCA shows that Young 1 is a big outlier. Did authors look into underlying reasons? If technical artifacts drive this, would exclusion of Young 1 lead to cleaner results?
- Extended Data Fig. 1c: A lot of variance between Day 4 samples (PC 1, 2, 4) is technical, not biological. In particular, bottlenecking of Young 1 and Old 1 samples (ED Fig. 1a) seems to affect those samples, but other sources of variation seem to drive PC2 and PC4. It would be worth to investigate the loadings of the PCs to obtain clues about potential artefacts in the screen results.
- Fig 2f,g: why is the distribution of control sgRNAs bimodal, with a subset that is strongly enriched (on par sgRNAs for the strongest hit genes)?
- Day 14 screen will also pick up genes affecting survival. This could be addressed experimentally by transducing activated NSCs and expanding them. At least, authors should compare with known essential genes (e.g. from depmap.org or crisprbrain.org).
- Fig. 4g: even control sgRNA infection reduces glucose uptake – lentiviral infection per se affects the physiology of NSCs (this is a caveat that should be pointed out). Therefore in 4h, the authors should also test uninfected cells in parallel.
- Fig. 4i should only include genes that were validated in vivo.

Author Rebuttals to Initial Comments:

Referees' comments:

Referee #1 (Remarks to the Author):

This manuscript, entitled "In vitro and in vivo CRISPR-Cas9 screens reveal drivers of aging in neural stem cells of the brain", used CRISPR-Cas9 screens to identify genes responsible for increased neural stem cell (NSC) quiescence in aging. First, CRISPR-Cas9 screens were conducted on subventricular zone NSC cultures from young and old Cas9 mice and identified 300 gene knockouts that boost old NSC activation. Then combinations of 10-gene libraries of sgRNAs were packaged into lentivirus and injected into the brain in vivo to identify genes that boost the activation of old NSCs in vivo. One of the genes identified in both the in vitro and in vivo screens was the glucose transporter Slc2a4 (GLUT4). Knockout of Slc2a4 in the subventricular zone boosted neurogenesis in old mice. Old NSC cultures exhibited enhanced glucose uptake compared to young NSC cultures and knocking out Slc2a4 in old NSC cultures reduced glucose uptake. Finally, knocking out Slc2a4 or conditions of glucose starvation promoted old NSC culture activation. This manuscript is the first study to use a genome-wide knockout screen to identify genes that impact aging-related decline of NSC activation. The identification of glucose transporter as a potent regulator of NSC activation in the aged SVZ is interesting, but not surprisingly as several studies already show its role in regulating NSC proliferation. The authors claimed that they developed new methods for in vitro and in vivo screen of stem cell activation, therefore it will be very important to know the validation rate of their screen hits with analysis of individual hits. It is not sufficient to focus on one for detailed validation and without sufficient validation, it could be very misleading for the field to claim that they have identify over 300 genes that regulate NSC activation.

We thank the Reviewer for their interest in our manuscript and their very interesting and helpful suggestions. The Reviewer has excellent points regarding the validation rate of the screens. Our responses are detailed below in our replies to Major Concerns. Briefly, we have now performed functional validation experiments for 10 individual genes *in vitro* and conducted additional screens *in vivo*. *In vitro*, we find that 10 out of the 10 individual hits tested indeed validate for boosting the activation of old NSCs. These results corroborate the screen results and indicate that the hit rate is likely high, at least for top hits (**new Fig. 1j, new Extended Data Fig. 1n**). *In vivo*, we have also now repeated the 5 targeted screens in additional mice, with results that are in line with our previous screens (**revised Fig. 2e**). Additionally, we also modified the Discussion section to clearly indicate that these 300 genes were identified in a pooled CRISPR-Cas9 screen and represent candidates that would also need to be independently validated *in vitro* (in addition to the 10 we have now validated) and *in vivo*. We have also modified the summary scheme indicating in bold the hits that were independently validated *in vivo*.

Major Concerns

1. The authors did a large scale in vitro screen for NSC reactivation using young and old NSCs, but there is not sufficient validation in vitro. The authors need to pick a number of hits to validate them individually to conform the knockout efficacy and effect on NSC reactivation in vitro. This will be very important to know the validation rate. Without knowing the validation rate, the majority results in Fig. 1 (f-i) and associated supplementary tables are not meaningful.

This is a great point and we agree it is important. We have now validated hits individually to confirm NSC reactivation and knock-out efficiency *in vitro*:

i) We have now independently tested 10 individual gene knockouts for their effects on young and old NSC reactivation efficiency. Using n=8 independent cultures of old NSCs, we found that 10 out of the 10 individual top gene knockouts significantly boosted old NSC activation. These results strengthen the confidence in the genome-wide screens, at least for top hits, and their ability to identify gene knockout interventions that can individually boost old NSC activation. We have added these results as **new Fig. 1j.**

ii) To validate the knockout efficiency, we lentivirally infected primary cultures of Cas9 NSCs with pools of 5 sgRNAs targeting a single gene (4 of the 10 genes tested above, as well as *Slc2a4*) and performed indel analysis on genomic DNA using the Decodr indel analysis program. We found that the percentage of indels ranged from 7 to 60% for individual sgRNAs. For each gene, there was at least one sgRNA that led to 20% of knockout or higher, and because there are 5 sgRNAs per genes, the percentage of knockout is likely higher for each gene. We have added these results in **new Extended Data Fig. 1n.**

2. The author should provide sample images to show mCherry+ cells in the olfactory bulb with different pools of sgRNA.

Thank you for this suggestion. We have now added immunofluorescent sample images of mCherry+ cells in olfactory bulbs of mice treated with different lentivirus pools of sgRNA. We have added these sample images as **revised Extended Data Fig. 3b**.

3. Because they authors did bulk sequencing for in vivo screen, not single-cell, the number of guides is dependent on not only the number of cells expressing the sgRNA, but also expression levels of the sRNA in different cells due to their integration site. The authors need to validate some of the results with counting of neuronal numbers in the olfactory bulb with sgRNA targeting an individual gene by histology, instead of purely relying on sequencing and gene score (Fig. 2e). It is also important to show cells in the olfactory bulb are neurons, not other cell types.

We agree with the Reviewer that this is an important point.

As suggested by the Reviewer, we have now counted the number of newborn neurons (NeuN+EdU+) in the olfactory bulbs of old mice treated with sgRNAs targeting individual genes using immunostaining on brain sections. These experiments confirm that the knockout of *Slc2a4* (GLUT4) increases the number of neurons in the olfactory bulb of old mice, corroborating the results of our targeted *in vivo* screen. We have included these new results as **new Fig. 3e-g**.

As suggested by the Reviewer, we have also now added other cell type markers in the olfactory bulb: Tuj1 (new neurons), Dcx (immature neuronal progenitors [neuroblasts]), calretinin (very mature neurons), astrocytes/glial cells (GFAP), or oligodendrocyte cells (Olig2, Sox10). These

experiments show that most targeted cells (mCherry+) in the olfactory bulb were indeed newborn neurons (positive for Tuj1 and NeuN), and not other cell types (astrocytes, oligodendrocytes, immature neuronal progenitors). We also observe that a few targeted cells stain for a marker of very mature neurons (Calretinin$^+$). This low number of targeted Calretinin$^+$ cells could be expected because the 5 week time point following virus injection is probably not sufficient time to allow for knockout, migration to olfactory bulb, differentiation, and neuronal maturation (and also because Calretinin is only expressed in a subset of neurons). We have included these new data as **new Extended Data Fig 5f-i.**

4. The "Depleted (Young/Old)" library with genes that when knocked out blocked young or old NSC activation in the in vitro screens did not impair the activation of old NSCs in vivo (Fig. 2E). The hypothesis would be that at least some of these genes would reduce the capacity for old NSCs to activate in vivo, but the effect for most of these genes is no change compared to Control. Can the authors comment on this unexpected result?

Yes, we were also surprised that we did not detect depleted genes in the old brain *in vivo* screen experiments. We speculated that this could be due in part to the low levels of neurogenesis in the old brain, making it harder to detect further impaired neurogenesis in a sensitive manner in old mice. To test this, we have now performed an *in vivo* targeted screen of the depleted gene list in a young mouse, as young mice have high neurogenesis. We observed 3 of the 5 gene knockouts that were predicted to specifically block young NSC activation based on the genome-wide *in vitro* screen (in blue), were also significantly depleted in a young mouse screen *in vivo*. In contrast, none of the 4 genes that were predicted to specifically block old NSC activation *in vitro* (in red) were significantly depleted in the young mouse screen *in vivo.* These results are consistent with the notion that depleted genes are easier to observe in the young brain. Our *in vivo* screening system is better suited to identify gene knockouts that boost activation than genes knockouts that impair activation in old mice. We have included the young mouse screen *in vivo* as **new Extended Data Fig. 4h** and have also clarified this point in the revised text.

5. The validation of GLUT4 knockout was done in the olfactory bulb. It should be examined in the SVZ. In addition to look at EdU+ cell numbers in the olfactory bulb, it is important to validate that knockout GLUT4 indeed promotes activation of NSCs in the SVZ by immunostaining. Look at in the olfactory bulb has the complication of impact on neuronal migration and survival by the genetic manipulation.

The Reviewer has a series of excellent suggestions.

i) We have now examined and confirmed the *Slc2a4* (GLUT4) knockout in the subventricular zone (SVZ) neural stem cell niche. To this end, we infected the NSC niche in old Cas9-expressing mice with lentivirus to express sgRNA targeting GLUT4, and then performed immunostaining for GLUT4 (Green), together with markers of NSCs/astrocytes (GFAP) and markers of infection (mCherry$^+$, Red). These experiments indicated that infected NSCs/astrocytes showed a decrease in GLUT4 staining in comparison to uninfected NSCs/astrocytes. We have included these data as **new Fig. 3a,b**.

ii) We have also determined the impact of *Slc2a4* (GLUT4) knockout on NSCs in the SVZ niche. We performed immunostaining for markers of different cells in brain sections from old Cas9

mice injected with lentiviruses expressing sgRNAs targeting *Slc2a4* (GLUT4) or control sgRNAs (Quiescent NSCs: $GFAP^+S100a6^+Ki67^-$; Activated NSCs: $GFAP^+S100a6^+Ki67^+$; Neuroblasts: $GFAP^-Ki67^+$; Astrocytes: $GFAP^+S100a6^-$). We observed a significant increase in activated NSCs and neuroblasts in the GLUT4 knockout treated old brains as compared to control, which is consistent with an increase in NSC activation (**new Fig. 3h,i**). We note that we also observe an increased in quiescent NSCs, which could be due to increase in NSC self-renewal or an increase in activation followed by a return to quiescence. These data indicate that *Slc2a4* (GLUT4) knockout boosts NSC number in the niche, which is consistent with the notion that increased neurogenesis in response to *Scl2a4* knockout is due to increased activation of NSCs. We have included these data as **new Fig. 3h,i**.

6. It is known that there is region heterogeneity for NSCS along different sides of ventrical walls (e.g. PMID: 34112692). Does GLUT4 knockout have a similar effect on different regions based on immunohistolology to look at proliferation status of NSCs?

This is another very interesting question. For our present analysis, we captured images of the lateral wall of the lateral ventricle, but we did not take tiled images down the entire length of the SVZ niche. To investigate regional heterogeneity of NSCs with GLUT4 knockout accurately, we would need to perform additional injections and image the entire SVZ region. Given the relatively low levels of virus infection in general, we feel that quantitative interpretation from this experiment could be difficult. We are definitely interested in pursuing this direction in the lab, as we agree it would be interesting to combine genetic knockout with regional and spatial impact. However, we believe it would be outside of the scope of the present study. We have mentioned this paper and interesting future direction in the **Discussion section**.

7. In Fig. 3c, it does not look like NeuN and mCherry are co-localized at all. The mCherry cells also do not show any neuronal morphology. This is the only evidence the authors have for neurogenesis. The authors need to use additional markers (may for oligodendrocytes as well) and show much better images with NeuN, mCherry and DAPI and 3D reconstruction.

We agree with the Reviewer that it is important to add additional neuronal markers and include better images.

i) We have now included better magnification images of NeuN+mCherry+ cells, with z-stack reconstructed images in new **Fig. 3e**. We note that NeuN is a nuclear protein whereas mCherry is a mostly cytoplasmic protein (though it does not completely fill the cells). So we were not expecting a direct overlap of NeuN and mCherry (or that mCherry would completely fill in dendrites).

ii) As suggested by the Reviewer, we have now performed immunostaining for additional markers of neurons (as well as oligodendrocytes and astrocytes) in the olfactory bulb. We find that $mCherry^+$ cells in the olfactory bulb stained positive for NeuN, a marker of neuronal nuclei, and Tuj1, a marker of newborn neurons. By contrast, $mCherry^+$ cells were negative for markers of other cells types: astrocytes/glial cells (GFAP) and oligodendrocytes (Olig2, Sox10). We have included these new data as **new Extended Data Fig 5f-i.**

8. GFAP alone cannot differentiate SVZ neural stem cells and astrocytes. The authors need to co-stain with additional markers, such as nestin (Fig. 3F).

This is another great suggestion. For an additional NSC marker, we have now stained with the marker S100a6, which can help distinguish NSCs from astrocytes in the NSC niche[1] (we have found that antibodies to Nestin do not work very well to mark NSCs in the SVZ *in vivo*). We now use this S100a6 marker to assess the effects of GLUT4 knockout on NSC activation (**new Fig. 3h,i**) and to quantify GLUT4 levels in young and old NSCs (**new Extended Data Fig. 5l,m)**.

9. GLUT4 protein expression is higher in aNSCs than qNSCs in vivo (Fig. 3F), while GLUT4 expression is lower in aNSCs than qNSCs in vitro (Fig. 4D). Can the authors comment on this discrepancy? Are there other differences between the in vivo niche and the in vitro cultures that might impact the interpretation of the in vitro results?

These are all great questions.

i) We have tested the statistical significance for differences between qNSCs and aNSCs *in vivo* and *in vitro* and **indicated this in revised Fig. 3k and revised Fig. 4d**. *In vivo*, GLUT4 protein levels between is higher in young aNSCs than qNSCs/astrocytes (though there is no statistical difference in old). *In vitro*, GLUT4 protein levels appear lower in aNSCs than in qNSCs (in both young and old). However, we feel that it is difficult to make strong claims about protein levels when comparing different cell types by immunofluorescence, in part because qNSCs and aNSCs have different sizes. We have now commented on the discrepancy between GLUT4 protein expression in aNSCs and qNSCs between *in vivo* and *in vitro* experiments, indicating that they could be due to cell cycle state heterogeneity (see also below).

ii) We now also analyze published single cell and bulk transcriptomic data for *Slc2a4* (GLUT4) RNA levels both *in vitro* and *in vivo*. *In vivo*, *Slc2a4* RNA levels tended to have higher expression in aNSCs/NPCs (compared to qNSCs/astrocytes), mainly due to a higher proportion of cells having non-zero expression but of the cells that do express the gene, aNSCs/NPCs have lower expression than qNSCs/astrocytes (**new Extended Data Fig. 5k**). These differences could be due to cell cycle states. *In vitro*, we observed that *Slc2a4* (GLUT4) RNA levels are lower in aNSCs compared to qNSCs (in young) – in agreement with our immunofluorescence results on GLUT4 protein *in vitro*. We have included these data as **new Extended Data Fig. 5k** (*in vivo*) and **new Extended Data Fig. 6e** (*in vitro*).

iii) We have now better highlighted that GLUT4 expression is higher in old quiescent NSCs than in young quiescent NSCs, both *in vitro* and *in vivo* and that we therefore focus on quiescent NSCs for downstream analyses (**page 10**).

iv) While the *in vitro* system recapitulates many *in vivo* phenomena[2-8], we have also clarified there may also be differences that could contribute to some divergence (e.g. cell cycle status, cell-cell interaction, cell-matrix interaction, etc) (**page 8**).

10. The ultimate goal of the CRISPR-Cas9 screens is to identify genes that can be manipulated to activate old NSCs to generate larger numbers of new neurons (Fig. 4I). Fig. 3B shows quantification of mCherry+ EdU+ cells in Control, Slc2a4 KO or Vmn1r107 KO conditions, but these cells could be neurons or glia. To show that neurogenesis is increased when Slc2a4 is knocked out, please quantify mCherry+ EdU+ NeuN+ newborn neurons, similar to quantification in Fig. 3B. This is necessary to claim that Slc2a4 knockout "boosts neurogenesis".

We agree with the Reviewer that this is an important point. We have now quantified the number of mCherry+EdU+NeuN+ newborn neurons and we find that *Slc2a4* (GLUT4) knockout, but not *Vmn1r107* knockout, leads to an increase in the number of newborn neurons (**new Fig. 3e-g**).

11. It will be useful if the authors can confirm the results on increased NSC activation in old mice with Stx4a knockout to provide support that this pathway is important.

The suggestion regarding STX4A is interesting, and this is something we are indeed interested in pursuing. STX4A is a Syntaxin involved in exocytosis and it has been involved not only in the exocytosis of GLUT4 but also in the exocytosis of other proteins (synaptic proteins[9], sphingomyelinase[10] and inflammatory cytokines[11]). We believe that dissecting the effects of STX4A that are specifically related to glucose receptor exocytosis would be outside of the scope of the present manuscript, and we have now indicated this as an interesting future direction in the **Discussion section** (**page 13**).

Nevertheless, we agree that this is an important question and to independently test the importance of the glucose pathway for NSCs, we have now performed new experiments. We have treated young and old qNSCs with 2-Deoxy-D-glucose (2-DG), a glucose analog that inhibits glycolysis (and also activates AMPK). We found that 2-DG treatment specifically boosted old (but not young) NSC activation. We believe that these results provide additional support for increased glucose uptake as an impediment to old NSC activation. We have added these data as **new Extended Data Fig. 6j.**

12. Mean fluorescent intensity was used as a measurement in multiple experiments. Each n was from a single mouse or biological replicate. Immunostaining is not very quantitative, especially between samples. What conditions or parameters were put in place to try to control for technical variability in fluorescent intensity between samples that was not due to experimental conditions?

This is another great point. We agree that it is challenging to compare immunofluorescent images between samples and that the method itself is not very quantitative. For this reason, we took great care when processing samples and acquiring images for analysis. All samples were stained at the same time. For confocal microscopy, the exposure and gain settings for each channel/antibody were set at the beginning of each imaging session and remained the same for all animals and treatments. We randomized the order in which we imaged the slides, and we ensured that different treatments and age groups were all imaged in the same session on the same day. For automated quantification of the images, we used the open-source software QuPath. This approach allowed us to set the thresholds and quantification parameters on training images, and then run the same analysis across all sections, samples and treatments in an automated and unbiased manner. For all experiments, the output numbers displayed on the graphs were derived

from the average of all serial section images across a biological replicate (1 mouse), biological sample values were then analyzed for significance by two-tailed Mann-Whitney test. We now have added these specific points to the **Methods** section.

Minor Concerns

13. In the introduction, Statement "The ability of NSCs to activate and form newborn neurons is severely impaired in the aging brain, and this can contribute to deficits in cognition and regeneration" about cognition could be true for hippocampal NSCs, but SVZ NSCs.

Thank you for this comment. We have now modified the sentence in the **introduction** to highlight that NSCs have been proposed to be important for sensory and cognitive function (for example the ability to remember which odors are associated with food or water sources), and we have added the relevant references[12,13].

14. Legend of Figure 1A grammatical error: "Genomic DNA is prepared and sgRNA constructs are PCR amplified rom Day 4 or Day 14 prior to sequencing on an Illumina Novaseq S4 system."

Thank you for pointing this out. We have now corrected the grammar in the **legend of Figure 1a**.

15. In the in vitro screen, the authors present the list of both depleted and enriched genes. Genes from the enriched list suggest that knockout of them will promote reaction. Some of the genes from the depleted list could be interesting as well and it is possible overexpression of them can promote reactivation. The authors can test some of them by overexpression or CRISPRa.

This is a great idea and indeed something we are very interested in pursuing. However, we have not yet established an efficient system (e.g. CRISPRa) for controlled overexpression in old NSCs of the hits that were depleted. This will require additional tool development, as we likely will need to achieve optimal levels of gene (and protein) expression in old NSCs. We have added this interesting idea in the **Discussion section (page 13).**

16. The authors mentioned that their in vitro screen identifies some of previous known NSC regulators. How about those NSC regulators tested in Fig. 2 in vivo?

Thank you for this great suggestion. We have now specifically examined genes in the "Published NSC regulators" (Fig. 2) in our *in vitro* screens. We find that some hits (*Cdkn1a*, *Sox2*, and *Bmpr1a*) were significantly enriched both *in vitro* and *in vivo*. Other enriched *in vivo* hits were not enriched *in vitro* (*Ascl1*, *Igfr1*, *GFAP*, *Hes1*, *Olig2*, *Bmpr2* (and *Gfap* and *Id4* were depleted). Differences between *in vivo* and *in vitro* may be due to differences such as cell cycle state, cell-cell interactions, or cell-matrix interactions in the primary culture system. This highlights the importance of screening both *in vitro* and *in vivo* for specific hits, especially when they are not high on the list. We have included this figure in **new Extended Data Fig. 4i and have indicated these points in the revised text (pages 8 and 12)**.

17. The subtitle: "Development of an in vivo CRISPR-Cas9 screening platform to rapidly screen

gene knockouts for their ability to rejuvenate NSC activity in the old brain". It will be better to state that this is a targeted screen.

We agree and we have now stated that this is a targeted screen in the **subtitle**.

18. What's the reason to have reads for sgRNA in cerebellum in the in vivo screen?

The cerebellum was chosen as a control brain region, far away from stereotactic lentiviral injection site, where we expected low guide RNA detection and lack of any guide enrichment. Indeed, we detected very few guides in that region (Fig. 2c,d). We have added a sentence in **the text** to clarify this (**page 6**).

19. Figure 4F: There appear to be multiple error bars on each bar in the graph, one set that is gray and one set that is colored.

Thank you for pointing this out. We have now fixed the figure.

20. Figure 4H: The error bar on Old, Normal Glucose, GLUT4 bar is not even in the up and down.

Thank you for highlighting this. We have now fixed the figure.

Referee #2 (Remarks to the Author):

This interesting study examines the ability of neuronal stem cells to undergo proliferation in adult brain. A genome-wide screen was performed to identify genes in older mice that might prevent regeneration, and thus provide insights into why old animals have defects in neuronal activation. An elegant in vivo screen was developed, yielding a number of new genes that restored NSC activation. Among these was Glut4, the insulin-stimulated facilitative glucose transporter. Knockout of Glut4 in vivo represented the top rejuvenating intervention, and this was reinforced by measurements of glucose uptake, suggesting that glucose metabolism per se provides the inhibiting signal for regeneration.

This is an elegant study that sheds new light on an important question, and identifies a new site of potential therapeutic intervention. My questions are mainly focused on the regulation of Glut4 and subsequent energy metabolism in these neuronal stem cells. Studies on Glut4 have revealed its restricted and unique role as an insulin-regulated protein, due to changes in its trafficking. Indeed, Glut4 generally resides in specific intracellular vesicles, and traffics to the plasma membrane only after receiving cues from the insulin receptor, or in some cases AMPK, which is itself activated due to depletion of energy. What is regulating Glut4 in these cells? Aging is generally associated with peripheral insulin resistance but higher circulating levels of insulin. Perhaps the small increase n Glut4 protein levels is less important than higher exposure to insulin? Was insulin used in the glucose uptake assays? It will be critical to demonstrate whether Glut4 is regulated in these cultured cells by either insulin (or IGF1) or an AMPK activator.

The Reviewer makes a series of excellent points on the regulation of GLUT4 in NSC aging. During the revision, we have now further examined the regulation of *Slc2a4* (GLUT4) and glucose uptake in this system:

i) By analyzing our single cell RNA-seq data, we now show that old quiescent NSCs exhibit an increase in the mRNA levels of *Slc2a4* (GLUT4). This increase in *Slc2a4* (GLUT4) transcripts may be one way in which the GLUT4 protein is upregulated during aging. We have included these new data as **new Extended Data Fig. 5k and new Extended Data Fig. 6a** and have mentioned these results in the revised text.

ii) As suggested by the Reviewer, it is also possible that insulin levels are changing with age and that this influences GLUT4 trafficking to the membrane. Unfortunately, we found that

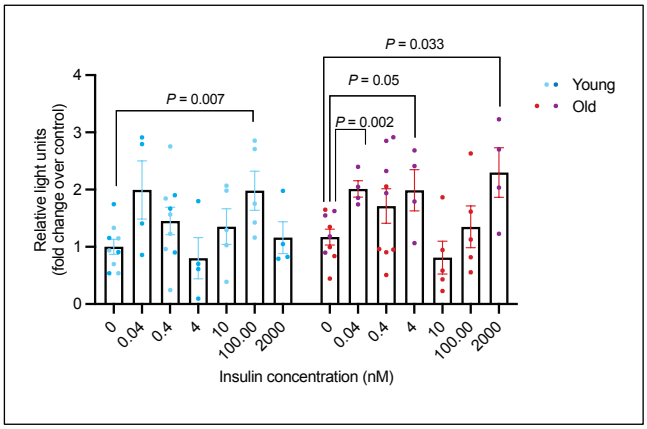

Figure R1. Bioluminescent glucose uptake assay on quiescent NSCs from young (3-4 months old) or old (18-21 months old) mice. Dot plot showing mean +/- SEM of fold change over control (Insulin = 0 nM in young) from 4-9 NSC cultures for each age, each from a single mouse. Note that 7 of the cultures overlapped between both experiments (4 from young, 3 from old), and 4 were independent. Each dot represents the average value from technical triplicates for a single culture (from a single mouse). Light blue and light red: Experiment 1. Dark blue and dark red: Experiment 2. *P*-values determined by two-tailed Mann-Whitney test. Only the significant *P*-values are plotted.

GLUT4 staining was not sensitive enough in NSCs to observe trafficking changes. As a proxy for GLUT4 trafficking, we have attempted to measure glucose uptake in cultured NSCs in response to varying concentrations of insulin (0-2000 nM). In our previous experiments, glucose uptake assays were done in glucose-free NSC media, which contains ~2000nM insulin. To test insulin dependency, young and old quiescent NSCs were incubated in glucose-free and insulin-free media supplemented with different concentrations of insulin (0, 0.4, 4, 10, 100, or 2000 nM) for 1 hour, then glucose uptake was measured. The variability of these experiments was high, perhaps due to their acute nature (**Figure R1**). With this limitation in mind, we observe increased glucose uptake in response to some concentrations of insulin, especially in old qNSCs (**Figure R1**). We also observe an increased glucose uptake in old NSCs compared to young NSCs at 2000 nM insulin (the concentration we had used previously, in 'steady-state' assays) (**Figure R1**). We believe that a complete understanding of the regulation of GLUT4 in NSCs, particularly during aging, would require more sensitive and accurate tools and assays. We have now mentioned the possibility of differential insulin regulation and sensitivity, in addition to mRNA changes, as an interesting direction in the **Discussion section (page 13)**.

A second issue concerns the source of energy in young cells. Presumably there are other glucose transporters, esp Glut1 and 2. Are these changed in aging?

This is another excellent point. We used our single cell *in vivo* NSC transcript data to determine the log-fold change with age of all glucose transporter transcripts expressed in in quiescent NSCs (*Slc2a1,3,4,6,8,10,12,13*). We found that *Slc2a4* (GLUT4) transcripts were upregulated with age (consistent with GLUT4 protein upregulation). *Slc2a3* (GLUT3) transcripts were also upregulated with age but not as much as *Scl2a4* (GLUT4). By contrast, *Slc2a1* (GLUT1)*, Scl2a8*, *Slc2a10* and *Slc2a13*) were not changed with age. As for *Scl2a6* and *Slc2a12* transcripts, they were decreased with age. All the other glucose transporters were not expressed at detectable level in NSCs. Together, these data indicate that *Slc2a4* (GLUT4) transcript levels are the most upregulated with age. These data are in line with our genome-wide screen data, where knockout of the other glucose transporters did not show age-related functional effect on NSC activation (see Fig. 4b). We have included these analyses in **new Extended Data Fig. 6a**.

What about fatty acid metabolism, and does this recede in aging? It will be relatively simple to perform seahorse experiments on young and old NSCs to determine whether there is a switch in substrate preference and if this is completely due to Glut4 expression.

We thank the Reviewer for these very helpful suggestions.

i) We have now assessed ECAR (extracellular acidification rate) – which mainly reflects glycolysis – and OCR (oxygen consumption rate) – which reflects mitochondria respiration. We find that old quiescent NSCs have a significantly higher ECAR and lower OCR compared to young quiescent NSCs (**new Fig. 4g**). Together, these results suggest that old quiescent NSCs have increased glycolysis and decreased mitochondrial respiration. This is also consistent with our findings that old quiescent NSCs uptake ~2-

fold more glucose with age. We have included the ECAR and OCR data in the manuscript as **new Fig. 4g.**

ii) Fatty acid oxidation (FAO) has been previously shown to be high in young NSCs[14], and we find that FAO signature genes decrease in expression with age in quiescent NSCs (**new Extended Data Fig. 6g**). We have not directly tested other substrates (e.g. palmitate or other substrates) in old quiescent NSCs, though this is definitely a direction of interest in the future and **we have added a sentence on this in the discussion (page 13)**. Together with our metabolism analyses, these observations are consistent with the notion that old quiescent NSCs use more glucose as a substrate (rather than other substrates) compared to young quiescent NSCs.

iii) Finally, as suggested by the Reviewer, we also tested how much these metabolic readouts are dependent on GLUT4 expression by knocking out *Slc2a4* (GLUT4). We find that *Slc2a4* (GLUT4) knockout significantly decreased ECAR (but not OCR) in old NSCs. We note that in young NSCs, *Slc2a4* (GLUT4) knockout decreases both ECAR and OCR. These data raise the possibility that *Slc2a4* (GLUT4) knockout boosts old NSC activation by decreasing glycolysis. We have included the data on the impact of *Slc2a4* (GLUT4) knockout on ECAR and OCR in **new Fig. 4g** in the revised manuscript.

Minor point: need validation of the Glut4 KO by western blot.

We agree with the Reviewer. We have now validated the *Slc2a4* (GLUT4) knockout *in vivo* in the subventricular zone (SVZ) NSC niche using immunofluorescence with a GLUT4 antibody (the number of NSCs per mouse, particularly upon viral injection, was too small for a western blot). We indeed observe that quiescent NSCs/astrocyte cells (GFAP+) infected with lentivirus with sgRNA to *Slc2a4* (GLUT4) (mCherry+) had lower levels of GLUT4 as compared to uninfected neighbor GFAP+ cells in the SVZ. These experiments are included as **new Fig. 3a,b**.

Referee #3 (Remarks to the Author):

In this manuscript, Ruetz and colleagues use an in vitro and in vivo screening approach (based on CRISPR-Cas9 knockout technology), to identify genetic pathways that regulate the age-associated block of neural stem cell (NSC) activation in the subventricular zone (SVZ) of aged mice. Starting with an in vitro screen using primary NSCs isolated from young and aged mice, they identify numerous genes whose knock-out induces NSC activation in young NSCs, in old NSCs, or in both. Over 300 genes were found to suppress quiescence exit in aged NSCs alone, including genes involved in cilium organization, ribonucleoprotein structures and glucose transport. Importantly, while some of these hits were already known to influence NSC behaviour (such as glucose metabolism), many genes had not been associated with NSC activation before. To assess the in vivo relevance of their screen's top hits, the authors adapted their methodology to investigate which genes are able to activate NSCs in the
aging mouse brain. Through a lower-scale in vivo CRISPR-Cas9 screen, they tested a total of 50 genes and identified 23 gene knockouts that stimulated SVZ/OB neurogenesis (as measured by enhanced representation of KO cells in the olfactory bulb, OB) in old mice. Probing in more detail the importance of glucose metabolism in aged NSCs, the authors show that depleting the gene Slc2a4, which codes for the transmembrane glucose transporter GLUT4, significantly increased the rate of neurogenesis in the aged OB. Moreover, they show that GLUT4 protein expression is increased with age and correlated in vitro with a 2-fold greater rate in glucose uptake by old quiescent NSCs compared to young cells. Finally, genetically reducing glucose import into cells through GLUT4 knockout or through transient glucose starvation was able to recover old NSCs' ability to exit quiescence, suggesting the reversibility of reduced activation properties in aged NSCs. Together, the manuscript by Ruetz et al.
identified several genes/pathways involved in the age-associated inhibition of SVZ NSC activation, including a notable shift in glucose transport dynamics.
This study represents a technical tour de force and provides a novel resource for the field. The genome-wide screen aiming to identify genes that regulate the exit from quiescence in cultured NSCs (that is affected with age) will be valuable to others (as this is one of the behavioural features of NSCs that is affected by age). The authors validate their screening approach by studying one identified gene, Slc2a4, in more detail. The identification of age-regulated glucose transport is novel and interesting - but conceptually not really unexpected (as somewhat discussed by the authors) given that glucose metabolism has been previously implicated in NSC behaviour. Therefore, the study rather represents a resource (and an elegant technical advance!) but is limited in its advance in terms of understanding age-dependent biology of SVZ NSCs.

We thank the Reviewer for their interest in our study as a resource and technical advance, and for their supportive and excellent suggestions. We have now provided additional experiments to bolster the biology part of our manuscript, notably the importance of glucose transport *in vivo* in subventricular zone NSCs, the regulation of glucose transporter transcripts during aging, and subsequent energy metabolism (**new Fig. 3h,i, new Fig. 4g, new Extended Data Fig. 5k, new Extended Data Fig. 6a,g**). We have also now better delineated the advances of our study for NSC biology (e.g. the role for the rate-limiting step of glucose import in old NSCs and more generally the implication of glucose metabolism in NSCs during aging) (**pages 5, 8, 13**).

Main comments:

Screening:
The data shown in Figure 2E are obtained from 2 mice, which is not ideal considering the huge variability found for the same target gene. This is particularly evident for quite a few genes (incl. Slc45a4 or Bmpr2), where one mouse showed strong enrichment of sgRNAs, whereas the other showed a depletion or at least no enrichment for the same target gene. Adding more mice would reassure the reader of the gene's effect and directionality on NSC activation.

We thank the Reviewer for this great suggestion. We have now performed an additional *in vivo* screen for each of the 5 library of 10 genes, bringing the total mice tested to 3 for each library. The results of this additional *in vivo* screen were in line with our previous screens and helped clarify the gene effect and directionality of some of the subtler gene knockout effects, including *Bmpr2*. We have now included these data as **revised Fig. 2e**.

The large variability also makes us questioning the efficiency of the KO system in the Cas9 mice. With exception of the EGFP KO, this manuscript does not demonstrate that the CRISPR-Cas9 is actively knocking out target genes in vivo.

This is another terrific point. We have now used immunofluorescence to validate the *Slc2a4* (GLUT4) knockout in the NSC niche and the olfactory bulb *in vivo*. We observed significant depletion of the GLUT4 protein in cells infected with lentiviruses that express sgRNA to knockout *Slc2a4* (GLUT4) in the subventricular zone (SVZ). Notably, we found that $GFAP^+$ (qNSCs/astrocyte cells in niche), $mCherry^+$ (*Slc2a4* sgRNA expressing lentiviral infected cells) had lower levels of the GLUT4 protein as compared to uninfected neighboring $GFAP^+$ cells in the subventricular zone (SVZ). We also found lower levels of the GLUT4 protein in lentiviral infected cells in the olfactory bulb (OB) *in vivo*. We have included these data as **new Fig. 3a-d.**

Secondly, the 4th group of genes targeted in vivo (referred to as 'Depleted') did not show any significant sgRNA depletion in old NSCs as seen in Figure 2E. This is very surprising and does not recapitulate the in vitro results (or the literature on targeted genes). The authors should clarify the absence of phenotype in the 'Depleted' group.

Yes, we were also surprised that we did not detect significantly depleted genes when injecting the "Depleted" library in the old brain screen experiments *in vivo*. We believe this is at least in part due to the low levels of SVZ neurogenesis in the old mice, which makes it hard to detect further detrimental effect of knockouts. We have now performed an additional screen of the "Depleted" gene library in a young mouse *in vivo*, and we observed depletion of some of the genes. Notably, 3 out of the 5 gene knockouts that specifically impeded young NSC activation *in vitro* were also significantly depleted *in vivo* (and the other 2 were trending for depletion). These results suggest that in old mice, our *in vivo* screening system is likely better suited to identify gene knockouts that boost (rather than impede) NSC activation. We have included these results as **new Extended Data Fig. 4h** and provide more explanation in the corresponding text.

To more systematically show that gene knockouts are indeed occurring, immunofluorescence against at least 3 genes/proteins should be included to show that targets are indeed efficiently reduced.

We agree with the Reviewer that this is an important point. *In vivo*, in addition to GFP, we have now confirmed knockout of GLUT4 by immunofluorescence with antibodies to the GLUT4 protein in the subventricular zone (SVZ) and the olfactory bulb (OB) (**new Fig. 3a-d).** *In vitro*, we have also validated that GLUT4 knockout decreased GLUT4 protein by FACS in cultured NSCs (**Extended Data Fig. 6h**). Importantly, we have also now confirmed that 5 out of 5 gene knockouts we selected were occurring (at the individual gene locus level) and that they indeed functionally boost old NSC activation we have now included these experiments in **new Extended Data Fig. 1n and new Fig. 1j**. Collectively, these results indicate that knockout is occurring in this system and is efficient.

Glucose transport:
The in vitro screen assesses activation of qNSCs. The in vivo experiments focus on the progeny of NSCs in the olfactory bulb, which makes sense. However, it is unclear why many of the analyses (e.g., showing the KO of Slc2a4) are performed in the OB and not in the SVZ. Furthermore, the analyses of the Slc2a4 is very superficial and very much weakens the relevance of the findings. It is unclear whether the results presented in Fig. 3 are due to actual activation of old qNSCs, or whether already active NSCs were simply encouraged to proliferate more, or if more cells survived…. The authors need to answer the following questions: Does the global proportion of quiescent and active NSCs in the SVZ differ between control and GLUT4 KO mice? Hence, are quiescent NSCs pushed towards an active state by GLUT4 KO in vivo? What is the proportion of NSCs that are induced to activate (i.e. proliferate) due to GLUT4 KO compared to control? Does this increase in old NSC activation also lead to their accelerated exhaustion? Or is GLUT4 KO a sustainable activation model? The authors should quantify the number of mCherry+ NSCs at 1 day and 5 weeks post-injection in control and sgRNA brains to determine whether old NSCs are consequently depleted post-activation.

These are all excellent points. We have now validated the knockout of *Slc2a4* (GLUT4) in the SVZ neurogenic niche (**new Fig. 3a,b**). As suggested by the Reviewer, we have also examined the effects of *Slc2a4* (GLUT4) knockout on NSC proportion in the SVZ by immunostaining. Interestingly, we observed a significant increase in the percentage of quiescent NSCs, activated NSCs and neuroblasts in the *Slc2a4* (GLUT4) sgRNA treated old brains as compared to control sgRNAs. The increase in quiescent NSCs in the *Slc2a4* (GLUT4) knockout condition could be due to increased self-renewal of NSCs or some return to quiescence of activated cells. Furthermore, there does not appear to be NSC exhaustion in the niche, 5 weeks after the knockout treatment. We believe that these results are consistent with the notion that the increase in neurogenesis in response to *Slc2a4* (GLUT4) knockout is due to NSC being activated. We have included these data **as new Fig. 3h,i** and discuss them in the text.

Further, the authors need to show that the sgRNAs targeting GLUT4 do in fact lead to a decrease in this glucose transporter in vivo in the SVZ NSCs (the in vitro data are not sufficient here). Performing immunofluorescence on brain slices is required.

This is another important point. We have now validated the *Slc2a4* (GLUT4) knockout *in vivo* in the NSC niche by immunofluorescence with antibodies to GLUT4. We observed $GFAP^+$ (qNSCs/astrocyte cells in niche), $mCherry^+$ (GLUT4 gRNA expressing lentiviral infected cells) had lower levels of GLUT4 as compared to uninfected neighboring GFAP+ cells. These data

indicate that *Slc2a4* (GLUT4) knockout is occurring in the subventricular zone (SVZ) *in vivo.* We have included these data as **new Fig. 3a,b**.

The authors claim that increasing GLUT4 expression in old NSCs is causally linked to the inhibition of quiescence exit in the aging brain. Yet, little evidence supports the causality of the phenotype. The authors should overexpress GLUT4 in young NSCs and quantify stem cell activation. If the hypothesis holds true, then this simple experiment should show a block in young NSC activation.

We thank the Reviewer raising this interesting point. We have now done the following to address it:

i) GLUT4 overexpression in young NSCs is definitely a direction that we are interested in. However, in pilot overexpression experiments, we have found that it was difficult to reach adequate levels of overexpression in young NSCs, and some of the overexpressed GLUT4 protein appeared stuck in trafficking vesicles. We believe that the optimization of GLUT4 levels would likely require co-expression of vesicle transport proteins and/or altering insulin signaling, which we feel would be outside the scope of the present study. We have thus toned down our claim for increased GLUT4 expression as a causal "driver" of aging in the text (**page 10**) and the title of our manuscript.

ii) Nonetheless, we agree with the Reviewer that it would be important to perform additional analyses to understand the link between GLUT4 and aging. To further assess GLUT4 levels during aging, we examined *Scl2a4* (GLUT4) gene expression during aging using our published single cell RNA-seq of neurogenic niches *in vivo*[15], and we found that the *Scl2a4* (GLUT4) transcript levels (but not those of other glucose transporters) increase with age, consistent with the increased protein levels. We have included these experiments as **new Extended Data Fig. 5k and new Extended Data Fig. 6a**.

iii) Finally, we have examined the importance of glucose in young and old NSC activation. We performed experiments to test the impact of 2-Deoxy-D-glucose (2-DG, a non-hydrolyzable form of glucose) in young vs. old NSCs activation. We find that 2-DG boosted old, but not young NSC activation, in line with our findings that *Slc2a4* (GLUT4) knockout and glucose depletion ameliorate old NSC activation. We have included these experiments as **new Extended Data Fig. 6j**.

Specific comments:

1. Based on the data shown in Fig. 1C, it is surprising that only 3 samples from the young NSCs showed higher activation levels compared to old NSCs. More replicates would better support the authors' claim, as there is clearly high variability in the activation capacity of young NSCs.

Thank you for this comment. This variability is due to baseline differences between each independent experiment pooled in this figure, which obscures part of the differences in activation between the paired young and old NSC primary cultures. We have now presented the data normalized to the average reactivation of the young sample for each experiment in **revised Fig.**

1c. These normalized data confirm that there is a consistent decrease in old NSC activation compared to young counterparts (**revised Fig. 1c**).

Furthermore, the authors should add growth rate/proliferation data of young and aged NSCs (e.g., it remains unclear how many passages were required to obtain the starting populations that were induced for quiescence). Were there any changes in proliferation/initial growth between young and aged NSCs isolated from the SVZ?

We thank the Reviewer for these important questions. For the genome wide *in vitro* screens, the young and old NSC culture passage numbers were kept the same between age groups and we have now included the passage number in **the legend of Fig. 1a**: Screen 1 - Passage 8; Screen 2 - Passage 7; Screen 3 - Passage 12. As suggested by the Reviewer, we have now also added a plot depicting the growth rate comparisons between the young and old cells at each passage for the cells that were used in the genome-wide screens. These experiments indicate that young and old NSCs have a similar growth rate once in culture (though their activation potential is different). We have included this as **new Extended Data Fig. 1c.**

2. Reactivation rate of old NSCs displayed in Fig. 1E seems to be very low when compared to the data shown in Fig. 1C. Please explain why the knockout of your top 10 hits seems to be comparable or even below the activation rate of old NSCs shown in Fig. 1C. Additionally, it would be of interest to add young NSCs to Figure 1E, to show at what extent the KO in old NSCs can recapitulate the young NSC activation levels.

The Reviewer has another great point. In Fig. 1c, the reactivation experiments were performed on uninfected NSCs, whereas in Fig. 1e, the reactivation experiments were conducted on NSCs infected with lentiviruses expressing control or Top10 sgRNAs. While lentiviral infection leads to an overall reduction in activation ability, the age-dependent difference in activation ability between young and old NSCs is preserved in infected cells (with control sgRNAs). The top 10 sgRNA restores old NSC activation to ~70% of the activation levels in young NSCs. We have included the activation in young NSCs data as a comparison and added this new panel as **revised Fig. 1e**.

In addition, to better understand the validation rate of our *in vitro* genome-wide screens and the specificity for young vs. old NSCs, we have now tested the Top 10 gene knockouts individually for their ability to boost old NSC activation. Using n=8 independent cultures of young and old NSCs, we found that 10 of the 10 individual gene knockouts tested (but not control guides or sgRNA to GFP) resulted in a significant boost in old NSC activation. These results strengthen the confidence in the genome-wide screens and their ability to identify gene knockout interventions that can specifically boost old NSC activation. We have included these new results as **new Fig. 1j.**

3. The authors show that GLUT4 expression increases with age in qNSCs and aNSCs, as shown in Fig. 3E and 3F. Using established markers, the authors need to separate qNSCs from astrocytes (that are now grouped together). Further, this finding is only supported by immunofluorescence. Being one of the major statements of this manuscript, further complementary experiments may be added to clearly show the age-associated increase in GLUT4. For example, a western blot quantifying protein amounts should be considered.

Alternatively, publicly available or lab-based RNA-seq data from aging SVZ NSCs may be used to confirm the increase in glucose transporters in aging NSCs (anyways: it will be interesting to analyse if only protein or also mRNA levels are altered with age).

We thank the Reviewer for helpful suggestion. We have now performed additional experiments and analyses to address this point. We have used the marker S100a6, which has been proposed to distinguish NSCs from astrocytes in the niche[1]. We observed that GLUT4 levels were also higher with age in the $S100a^{+}/GFAP^{+}$ NSC cell populations in the niche. We have included these data as **new Extended Data Fig. 5l,m.**

To independently test GLUT4 increase with age, as suggested by the Reviewer, we checked published single cell RNA-seq datasets. We found that *Slc2a4* (GLUT4) transcripts were significantly upregulated with age in quiescent NSCs/astrocytes (no significant changes in aNSCs/NPCs). We have included these data as **new Extended Data Fig. 5k and new Extended Data Fig. 6a**.

4. Similar to point 2: the very low proportion of Ki67+ cells in the young samples shown in Fig. 4H is surprising and many fold less compared to the data shown in Fig. 1C. Please explain this discrepancy. The dramatic differences between experiments somewhat questions the robustness of the used assays.

The Reviewer has another great point. The cells in Fig. 4h (and Fig. 1e) were infected by lentiviruses whereas cells in Fig. 1c were not, and we have found that lentiviral infection decreases overall efficiency of activation. We now added statistical testing indicating that the difference between young vs. old for activation efficiency is still preserved under lentiviral infection conditions (**revised Fig. 1e and revised Fig. 4i)**. We have also now indicated more clearly that although overall activation is decreased, the difference in reactivation between young and old NSCs is maintained when the cells are infected with lentiviruses in the Methods section.

5. Throughout the manuscript, there is some contradiction between the proposed role of GLUT4 in old NSC activation inhibition and the measured levels of GLUT4 in SVZ stem cells. Based on Fig. 3F and Ext. Fig. 6C glucose transport protein levels (GLUT4 and STX4A) seem to be higher in old active NSCs than in old quiescent NSCs. This observation somewhat contradicts the author's interpretation that high levels of GLUT4 (or STX4A) block the quiescent-to-active transition (as active NSCs seem to have higher levels of glucose transporters). In addition, there is a disagreement between in vivo and in vitro data concerning GLUT4 levels in quiescent and active NSCs. Indeed, Fig. 3F shows that in vivo, active NSCs seem to have higher levels of GLUT4 (irrespective of age) compared to quiescent NSCs. However, Fig. 4D clearly depicts the opposite: in vitro quiescent NSCs seem to have higher GLUT4 levels compared to active NSCs in vitro. This needs to be clarified.

This is another great point.

We have tested the statistical significance for differences between qNSCs and aNSCs *in vivo* and *in vitro* and **indicated this in revised Fig. 3k and Fig. 4d**. *In vivo*, GLUT4 protein levels is higher in young aNSCs than in young qNSCs/astrocytes (there was no statistically significant

difference in old mice). *In vitro*, GLUT4 protein levels appear lower in aNSCs than in qNSCs *in vitro* (in both young and old).

We now also analyze published bulk and single cell transcriptomic data for *Slc2a4* (GLUT4) RNA levels both *in vitro* and *in vivo*. *In vivo*, *Slc2a4* RNA levels tended to have higher expression in aNSCs than in qNSCs/astrocytes (in both young and old). *In vitro*, we observed that *Slc2a4* (GLUT4) RNA levels are lower in aNSCs compared to qNSCs (in young) – in agreement with our immunofluorescence results on GLUT4 protein *in vitro*. We have included these data as **new Extended Data Fig. 5k** and **new Extended Data Fig. 6e**. We have now commented on the discrepancy between GLUT4 protein expression in aNSCs and qNSCs between *in vivo* and *in vitro* experiments, indicating that they could be due to cell cycle state heterogeneity.

Given that the main focus is on quiescent NSCs, we have now better highlighted in the text that the findings that GLUT4 is higher in old quiescent NSCs than their young counterparts is observed both *in vitro* and *in vivo*, and at both RNA and protein levels.

6. It is surprising that the knockout of 1 glucose transporter (out of 12) can have a significant effect on global glucose uptake levels in NSCs, considering that cells express several glucose import channels and that rapid compensation for the loss of 1 transporter is likely to occur. It is somewhat unclear if the minor decrease in glucose import shown in Fig. 4G at day 8 can have an actual physiological impact on NSC behaviour. This question is particularly relevant considering the experimental design of Fig. 4H is based on complete extremes: either the total absence of glucose ('Glucose Starvation') or glucose presence ('Normal Glucose'). Yet, this hardly reflects physiological conditions of the SVZ stem cell niche. It may be much more convincing to see how the activation of old quiescent NSCs is affected by increments of glucose concentrations. Furthermore, the timing of the proliferation/activation assays (e.g., using Ki67) should be correlated with the glucose uptake measurements (at least the same timing for these experiments should be used).

The Reviewer has a series of great questions.

Yes, quiescent NSCs express several other glucose transporters and two of them – *Slc2a1* (GLUT1) and *Slc2a10* (GLUT10) – are expressed at similar levels as *Slc2a4* (GLUT4) according to our single cell transcriptomics data. However, unlike *Slc2a4* (GLUT4) knockout, *Slc2a1* (GLUT1) or *Slc2a10* (GLUT10) knockouts did not show any effect on NSC activation, irrespective of age, in our *in vitro* screens. In addition, among glucose transporters, the transcript levels of *Slc2a4* (GLUT4) are the only ones that strongly increase with age. We have included these expression data as **new Extended Data Fig. 6a**. It is also possible that the unique effect of *Slc2a4* (GLUT4) knockout on old NSCs is linked to the insulin-dependency of this glucose transporter (compared to other glucose transporters that are not insulin dependent). We have included these new data as **new Extended Data Fig. 6a** in the revised manuscript and have also indicated this more clearly in the text.

We agree with the Reviewer that it would be interesting to test glucose increments or more physiological changes than zero glucose. While we have not directly done this, we have now tested the effect of 2-Deoxy-D-glucose (2-DG, a non-hydrolyzable form of glucose). We find

that 2-DG specifically increases old NSCs activation, consistent with the possibility that increase in (hydrolyzable) glucose entry is detrimental for old NSC activation (and that preventing downstream glucose utilization could have benefits). Together with our glucose starvation data, these results are consistent with increased glucose being detrimental for old NSCs. We have now included these data in **new Extended Data Fig. 6j**.

Yes, we have tried to do the glucose uptake measurement and the activation assays following a similar timing. We have performed glucose import at 4 or 8 days after lentiviral infection (10 and 14 days after plating cells) and we have done activation assays (Ki67) at 6 days after lentiviral infection (10 days after plating cells) and then followed the cells for 4 more days (14 days total). This time point roughly corresponds to the glucose import time point of 8 days and we have clarified this in the figure legend.

Additional comments

1. The title of the manuscript does not reflect its content. The use of the term "drivers" is not appropriate, unless new experiments can better prove the mechanistic relationship between GLUT4 and the inhibition of NSC activation.

We agree and have replaced "drivers" by "regulators" in the **title** of the manuscript.

Results & Figures

2. In Fig. 2B, the mCherry signal is not really convincing. Why are mostly processes labelled? The authors should clearly illustrate NSCs and their morphology in the SVZ upon transduction.

We have now included additional close up images that show more clearly $mCherry^{+}GFAP^{+}$ cells in the SVZ in **new Fig. 3a**.

3. In Fig. 3, Vmn1r107 is used throughout to represent the 'depleted' sgRNA condition that is hypothesized to cause less NSC activation. Yet, none of the data points support that deleting this gene has any negative impact on NSC proliferation or OB neurogenesis. This example questions again a little bit the robustness of the in vivo screen (with none of the "depleted" guide RNAs showing indeed depletion; Fig. 2E).

The Reviewer raises another great point. We think that the detection of significantly depleted genes in *in vivo* screen experiments is due in part to the low levels of neurogenesis in the old brain, which makes it harder to detect knockout depletion phenotypes (floor effect). To test that possibility, we performed a screen of the depleted gene list in a young mouse, with higher rates of neurogenesis, and indeed observed significant depletion of some of the genes (**new Extended Data Fig. 4h**). Of the 5 gene knockouts that were predicted to specifically block young NSC activation *in vitro*, 3 of them significantly depleted in a young mouse screen (and the other 2 trended for depletion). These results are compatible with the notion that our *in vivo* screening system is better suited to identify gene knockouts that boost (rather than impede) activation in the old brain.

4. In Fig. 3C, it is very hard to discern the nuclei that correspond to the mCherry+ NeuN+ cells. Using more contrasting colours and adding the DAPI channel would clarify this. Also, images of the control sgRNA sample should be added.

Thank you for this suggestion. We have added the DAPI channel to Fig. 3c as well as zoomed in images (**now revised Fig. 3f**). We have also added a new panel (**new Fig. 3e**), which displays the NeuN channel in a z-stack with mCherry.

5. In Fig. 3E, it is not possible to see individual cells and the reader cannot interpret that old NSCs have increased GLUT4 expression. Images with a higher magnification of the stem cell niche or inserting a zoom of the cells should be shown. In order to clearly depict an increase in GLUT4, example images of each cell type quantified should be displayed. The analyses of GLUT4 in NSCs need to be substantially improved (see above).

We thank the Reviewer for these suggestions. We have now added arrows to point to each cell type in Fig. 3e (**now revised Fig. 3j**), and have also added close up images in **new Extended Data Fig. 5l**.

6. In Fig. 4C and Ext. Fig. 6A, the images of active NSCs (young and old) are not really comparable: roughly the same number of cells as in the quiescent samples should be used for the active population (the DAPI signal in aNSCs should be improved).

Yes, the culture conditions and cell confluency were indeed different between quiescent NSCs and active NSCs - these were empirically determined for optimal culture maintenance of quiescent NSCs (non-dividing) and active NSCs in the proliferating state. We believe that changing cell densities would likely result in non-optimal culture conditions for one of the cell types and would be different than the conditions used in other assays throughout the manuscript. We have now de-emphasized comparisons across cell states and have focused on the comparison between young versus old within a cell state (**page 11**). We have also better highlighted in the **Methods** section that we used an automated QuPath pipeline for image analysis.

7. In Fig. 4D and 4E, the same p value (0.029) is displayed for GLUT4 and STX4A between young and old qNSC samples (the data shown suggest that the difference is larger for GLUT4). Statistics should be checked.

Thank you for pointing this out. We have now checked the statistics and they are accurate.

8. Showing the KO of EGFP in olfactory bulb neurons (Ext. Fig. 3B and 3C) is fine. But the authors also need to show the KO of EGFP in NSCs of the SVZ (plus additional genes/proteins as suggested above).

We agree with the Reviewer that this is an important point. As described above, we have now validated the *Slc2a4* (GLUT4) knockout *in vivo* in the SVZ niche by immunofluorescence with antibodies to GLUT4. We observed significant depletion of GLUT4 in cells infected with lentivirus to express guide RNA to knockout *Slc2a4* (GLUT4) in the subventricular zone (SVZ). Notably, we observed $GFAP^+$ (qNSCs/astrocyte cells in niche), $mCherry^+$ (GLUT4 gRNA

expressing lentiviral infected cells) had lower levels of GLUT4 as compared to uninfected neighbor GFAP$^+$ cells in the subventricular zone (SVZ) (**new Fig. 3a,b**).

9. In Ext. Fig. 6B and 6C, the representative images do not convincingly show that STX4A is increased in old NSCs. Images with a higher magnification of the stem cell niche or an insert zooming in on the cells should be shown as it is impossible to see individual cells currently. In addition, the same analyses as suggested for GLUT4 should be done for STX4A. For Ext. Fig. 6A (same as for 4C): the images of active NSCs (young and old) are not really comparable: roughly the same number of cells as in the quiescent samples should be used for the active population.

We have now clarified that the quantification of STX4A (and GLUT4) staining *in vivo* and *in vitro* is done using an automated QuPath pipeline and we have now better highlighted this in the **Methods** section. *In vitro*, the cell density was different between quiescent NSCs and active NSCs - these were empirically determined for optimal culture maintenance of quiescent NSCs (non-dividing) and active NSCs in the proliferating state. We have de-emphasized the comparison between different cell states in the text and focused on comparisons with aging (**page 11**).

Methods

10. Two glucose uptake kits are mentioned in the methods section. Yet, it is not clear where and for which experiment either of the kits was used. Please clarify this point in your methods and results sections.

We have now clarified the glucose uptake kits that were used for each experiment in the **Methods** and **Results** sections.

Discussion

11. We thought it was interesting to observe that 210 gene KO specifically improved NSC activation in young NSCs (but nor in old) as shown in Fig. 1I and 1J. Although the GO terms are illustrated, the authors may comment on these genes and their meaning for NSC behaviour. Any speculation and comparison to previously published pathways would be of interest to the readers. Furthermore, the authors may add a short section that genes not only will be upregulated with age but that indeed the age-dependent down-regulation of genes with age (as shown in NSCs but also other somatic stem cells) may play an important role.

We thank the Reviewer for their helpful suggestion. We have edited the text to add this in the **result and discussion sections (pages 5 and 12)**.

12. The term “rejuvenation” that is used throughout the manuscript is not correct or appropriate. The authors show that the transition from quiescence to activation can be induced in old NSCs through various knockouts (which is one previously described feature of aged NSCs, among

several others changes that may occur with age. Yet, (partially) recapitulating one phenotype of young NSCs is not enough to claim that the aged cells have rejuvenated.

Yes, we have edited the text to tone this down.

13. The authors should speculate in the discussion on the reasons why only 2 out of 12 glucose transporters boost old NSC activation. Is it due to Slc2a4 (GLUT4) and Slc2a12 (GLUT12) being the most highly expressed in SVZ NSCs? Is there a switch in isoform preference with age? Is their effect on NSC activation solely linked to their sensitivity to insulin? Please expand the discussion section on the role of these two glucose transporters.

Thank you for this interesting suggestion. We have now included speculation on this point in the discussion (**page 13**).

Referee #4 (Remarks to the Author):

This manuscript by Ruetz and colleagues aims to use unbiased genetic screens to identify factors controlling the activation of neural stem cells (NSCs) for neuronal regeneration. NSC activation is impaired in the brain of old mice, which is thought to contribute to aging-associated deficits in brain function. The authors establish a highly innovative CRISPR-based screening approach, first in primary mouse NSCs, and then, as a secondary validation screen, in mouse brains. They identify several pathways knockdown of which enhances NSC activation and neurogenesis, including glucose uptake, primary cilia, and (somewhat more vaguely) cytoplasmic ribonucleoprotein structures. The authors focus on one particular hit, the insulin-sensitive glucose uptake channel GLUT4, which is more highly expressed old NSCs than in young NSCs, suggesting a possible role in altering NSC function with aging. Indeed, the authors validate in vitro that glucose uptake is increased in old NSCs, and that GLUT4 knockout reduces it to levels found in young NSCs. Furthermore, glucose starvation enhances the activation potential of old NSCs to match that of young NSCs. However, in GLUT4 knockout cells, there is no additional benefit of glucose starvation, supporting the notion that the effect of GLUT4 knockout on NSC activation is through the reduction of glucose uptake.

This work should be of interest to the broad audience of Nature for two reasons: first, it delineates novel pathways with a role in NSC activation, and second, it establishes an elegant paradigm to uncover such pathways using CRISPR screens in vitro and in vivo. Very few CRISPR screens have so far been conducted in vivo, and those were mostly done in the context of cancer, enhancing the innovative aspect of this manuscript.

We thank the Reviewer for their interest in our work and for their supportive comments.

There are several minor issues with the manuscript that should be addressed before it is suitable for publication:

- Introduction: The authors claim that “In addition, in vivo genetic screens are challenging in mammals and have been so far limited to cancer and development.“ While this is mostly correct, there it at least one example of a screen conducted in vivo in adult mouse brains (PMID: 32004439)

Thank you for pointing this paper out, this is very helpful! We have now edited the sentence in the text and added this paper, as well as a recent *bioRxiv* manuscript, as references.

- Fig. 1d: The PCA shows that Young 1 is a big outlier. Did authors look into underlying reasons? If technical artifacts drive this, would exclusion of Young 1 lead to cleaner results?

We thank the Reviewer for these important questions. We now include the genes that underlie the PC axes as **new Supplementary Table 5**. GO term analysis indicates that the genes underlying the PC2 axis on Young 1 Day 14 sample are enriched for genes implicated in the ubiquitin proteasome system pathways. Notably, these GO terms are distinct from those identified for the gene knockouts that boost or impede NSC activation.

To further investigate the possibility that Young Day 14 is an outlier, we have also plotted the correlation of CasTLE scores between each *in vitro* screen, as was done for other CRISPR/Cas9 screens[1]. We find that overall, the correlation values between Young 1 and Young 2 screens and between Young 1 and Young 3 screens were similar to those between Young 2 and Young 3 screens. We have included these data as **new Extended Fig. 1d-f**.

Together, these results indicate that while Young 1 Day 14 is an outlier, as the Reviewer noticed, it is unlikely to strongly impact the results and we have now indicated this in the text and methods. We have also further clarified that we chose hits from the screen that were significant in 2 or 3 replicates to avoid having one of the screens skew the data.

- Extended Data Fig. 1c: A lot of variance between Day 4 samples (PC 1, 2, 4) is technical, not biological. In particular, bottlenecking of Young 1 and Old 1 samples (ED Fig. 1a) seems to affect those samples, but other sources of variation seem to drive PC2 and PC4. It would be worth to investigate the loadings of the PCs to obtain clues about potential artefacts in the screen results.

This is another excellent point. We have now investigated the loading of all PCs for both Day 4 and Day 14, and included them in new Supplementary Table 5. We also performed a GO term analysis on the genes underlying all PCs for both Day 4 and Day 14 and we have also included these GO terms in **new Supplementary Table 5**. We find that genes and GO terms underlying the technical variance for Day 4 samples (PC1, 2, 4) are involved in cell division, proteostasis, and transcription/translation. We hypothesize that one source of variance in this system could be due to lentiviral infection (impacting cell survival/cell proliferation) or bottlenecking during passaging as the Reviewer suggests. We have discussed these results in the **Methods section**.

- Fig 2f,g: why is the distribution of control sgRNAs bimodal, with a subset that is strongly enriched (on par sgRNAs for the strongest hit genes)?

This is another great question. We believe that the bimodal distribution of control sgRNAs is most likely due to a control sgRNA infecting an NSC that is, at the time, highly active and that will naturally enrich in the olfactory bulb. We have now clarified this in the **figure legend and the Methods**.

- Day 14 screen will also pick up genes affecting survival. This could be addressed experimentally by transducing activated NSCs and expanding them. At least, authors should compare with known essential genes (e.g. from depmap.org or crisprbrain.org)

We thank the Reviewer for these suggestions. We have now generated a list of significantly depleted genes (FDR < 0.1) between 2 or 3 screen at the Day 14 time point and included it in Supplementary Table 1. As the reviewer suggests, this list contains some essential genes that would be shared with other cell types. We compared this list of significantly depleted genes with published lists of known essential genes (compiled from 581 cell lines from the Online GEne Essentiality database, OGEE[16], and from a separate database of 17 reanalyzed knockout screens in cancer cell lines from Core Essential Genes 2, CEG2[17]). We found that there was a small but statistically significant overlap. We have added these Venn diagrams as **new Extended Data Fig. 1l,m** and comment on them in the text.

- Fig. 4g: even control sgRNA infection reduces glucose uptake – lentiviral infection per se affects the physiology of NSCs (this is a caveat that should be pointed out). Therefore in 4h, the authors should also test uninfected cells in parallel.

The Reviewer's point is well taken. We have now pointed out this caveat more explicitly in the text (**page 11**). We have not tested NSC activation side-by-side (infected, not infected) in the same experiment, but from comparing across independent experiments overall activation is indeed lower in infected cells. Nonetheless, the reduced activation ability in old NSCs compared to young counterparts is still preserved in infected NSCs and we have added statistical tests for this (**revised Fig. 4i**).

To perform an additional experiment in non-infected cells, we have now examined the importance of glucose metabolism in uninfected NSCs by treating young and old NSCs with 2-Deoxy-D-glucose (2-DG), a non-hydrolyzable form of glucose). We find that 2-DG boosts old (but not young) activation of uninfected NSCs, in line with our finding that lowering glucose boosts old infected NSC activation. We have now included this as **new Extended Data Fig. 6k**.

- Fig. 4i should only include genes that were validated in vivo.

We thank the Reviewer for this suggestion. We have now included a **revised version of Fig. 4i (now revised Fig. 4j)** where only the genes that were validated *in vivo* are in bold.

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16 Gurumayum, S. *et al.* OGEE v3: Online GEne Essentiality database with increased coverage of organisms and human cell lines. *Nucleic Acids Res* **49**, D998-D1003, doi:10.1093/nar/gkaa884 (2021).
17 Hart, T. *et al.* Evaluation and Design of Genome-Wide CRISPR/SpCas9 Knockout Screens. *G3 (Bethesda)* **7**, 2719-2727, doi:10.1534/g3.117.041277 (2017).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors tried to address my concerns with new functional validation results of several positive hits in vitro, along with new knockout validation, immunostaining, and quantifications. While the manuscript is improved, the author did not address the comments regarding the effects of GLUT4 knockout on the old NSC activation at different regions of SVZ, the effect of Stx4a knockout, and the effect of overexpression or CRISPRa of some depleted genes on old NSC activation. While the CRISPR screening is very powerful, after the screening, the general readers are still more interested in the functional validation of positive hits and the underlying mechanisms.

Remaining concerns:
Although the authors presented the immunostaining results and indel percentage assessment to confirm the knockout efficiency of the gRNAs targeting several genes, including GLUT4, this evidence is not enough and convincing. Notably, the overall indel percentage of most examined genes is around 20%, which may be considered insufficient. In addition to immunofluorescence measurements, independent qPCR and western blotting assays with the young and old NSCs in vitro infected with gRNA of these genes (especially GLUT4) are necessary to convince the readers the good knockout efficiency at the mRNA and protein levels within this system. Similarly, the observed increase in GLUT4 protein levels in old NSCs compared to young NSCs in vitro need be validated using qPCR and western blotting assays. These experiments are easily doable.

The quality of some images in Figure 3 and Extended Figure 5 are still not good enough for the publication in Nature, which should be replaced with images with better resolution, lower background, higher magnification, and clearer color display.
For example, in Figure 3a&c, it is essential to include GLUT4 staining in mcherry+ cells infected with control gRNA. Additionally, a GLUT4 staining image with better resolution (possibly with a 100X objective) is needed to more clearly depict the distribution pattern of GLUT4 in both control and knockout cells in both the SVZ and olfactory bulb. The background of GLUT4 in Figure 3c is too high, and the magnification is not high enough.
In Figure 3f, the EdU signal is not clear enough and the background is too high. A better image with higher resolution is needed. Besides, the sample images for control and Vmn1r107 KO sections are also needed.
In Figure 3h and Extended Figure 5a, it is necessary to include mCherry staining at the same time to demonstrate the lentivirus infection efficiency in these SVZ regions.
In Figure 3g, the images for the GLUT4 staining in the four types of cells with higher resolution (with 100X objective?) and higher magnification needs to be shown in separate insets.
In Extended Figure 5h, the background of mCherry is too high, and a better image is need to be provided.
In Extended Figure 5l, it is difficult to tell which S100a6+GFAP+cell the GLUT4 signal comes from. Better images with higher resolution and higher magnification to show the GLUT4 distribution pattern in an individual S100a6+GFAP+ NSC is needed to be shown in the insets.

Overall, it is strange that the mcherry signal is mainly enriched in the cytosol of soma (maybe except in Extended Figure 5h), making colocalization assessment less straightforward. Did the author try other mcherry antibodies to improve the mcherry staining signal? How about the mcherry signal in infected NSCs cultured in vitro?

Minor point:
At the “sgRNA PCR amplification and sequencing”, there is an error: “In optimizing this PCR reaction, we found that Herculase II Polymerase was outperformed by Q5 polymerase outperformed Herculase II Polymerase,”

Referee #3 (Remarks to the Author):

The authors have now submitted a revised version of their manuscript. They added a substantial amount of new data and modified the interpretation/discussion of obtained results. The key advance of the study is indeed the use of an in vitro/in vivo screening approach for the identification of novel regulators of stem cell activation in the aged SVZ (the data on GLUT4 relevance have improved; however, the conceptual advance provided by GLUT4 data alone is not that substantial or "exciting"). However, we do see how the revised study will be of interest to the field - and given its screening approach also to a broader audience.

Referee #4 (Remarks to the Author):

The revised manuscript addresses my concerns, and I now recommend it for publication.

Author Rebuttals to Initial Comments:

Point-by-point reply

Reviewer 1

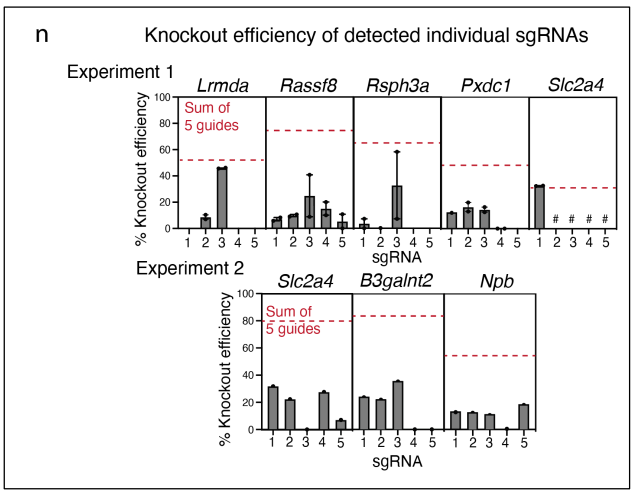
The authors tried to address my concerns with new functional validation results of several positive hits in vitro, along with new knockout validation, immunostaining, and quantifications. While the manuscript is improved, the author did not address the comments regarding the effects of GLUT4 knockout on the old NSC activation at different regions of SVZ, the effect of Stx4a knockout, and the effect of overexpression or CRISPRa of some depleted genes on old NSC activation. While the CRISPR screening is very powerful, after the screening, the general readers are still more interested in the functional validation of positive hits and the underlying mechanisms.

Remaining concerns:

Although the authors presented the immunostaining results and indel percentage assessment to confirm the knockout efficiency of the gRNAs targeting several genes, including GLUT4, this evidence is not enough and convincing. Notably, the overall indel percentage of most examined genes is around 20%, which may be considered insufficient.

We thank the Reviewer for raising this important point. We have now done the following experiments and changes to address it:

i) We have now clarified that the 20% knockout efficiency is per sgRNA (rather than per gene). As we used pools of 5 sgRNAs for each gene in these validation experiments and as the infection rate is quite high, the knockout efficiency per gene is likely higher than that per sgRNA and closer to the sum of the knockout efficiencies of the individual sgRNAs. In addition, for some sgRNAs, knockout was detected but at lower confidence by DECODR and we did not plot these knockout values, which further underestimates the actual knockout efficiency. Finally, we note that the percentage of knockout per gene is likely also underestimated due to the fact that larger indels that span sgRNA cutting sites are not taken into account by DECODR. **To clarify these points, we have now added a Supplementary Table S4 that indicates knockout efficiency per sgRNA for each gene as well as the level of confidence of knockout by DECODR. We have also plotted the knockout efficiency**

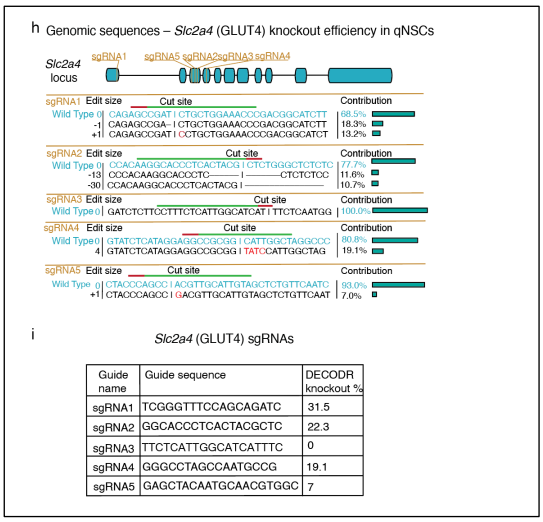


Excerpt from Extended Data Fig. 1 in revised manuscript:
n, Validation of gene knockout efficiency at the genomic level. qNSCs were infected with lentivirus expressing sgRNAs targeting individual genes (5 sgRNAs per gene) and genomic DNA was extracted. Top: Experiment 1. Bottom: Experiment 2. Percentage of knockout was quantified by sequencing PCR products followed by DECODRv3.0. Each dot represents the percentage knockout for one sgRNA. #: knockout detected by DECODRv3.0, but with low confidence ($r^2 < 0.6$) (see Supplementary Table 4). No data point: knockout not detected by DECODRv3.0 (see Supplementary Table 4). Dotted red line: sum of knockout percentages for high confidence and detected knockouts. See Extended Data Fig. 6h-m for genomic knockout examples and knockout efficiency by western blot and FACS for *Slc2a4* (GLUT4).

data in a clearer manner with a line denoting the sum of knockout efficiency of each sgRNA and a # to indicate knockout with low confidence (revised Extended Data Fig. 1n, see excerpt below). We have also clarified these points in the figure legends and Methods section.

ii) We have now also performed additional genomic experiments (conducted by an independent investigator) for 3 genes (5 sgRNAs per gene): *Slc2a4* and 2 additional genes, *B3galnt2*, and *Npb*. **We find that the knockout efficiency in this experiment is also around 20% per sgRNA, with the sum of knockout efficiency per gene being ~56-80%. We have included these new results in revised Extended Data Fig. 1n, bottom panel (see excerpt above).**

iii) For *Slc2a4* (GLUT4), we now show the detailed indel contributions from each of the 5 sgRNAs (**new Extended Data Fig. 6h,i, see excerpt below**. We find that each sgRNA edits the *Slc2a4* (GLUT4) locus with an efficiency of 7 – 31.5%. As a pool, the contributions from the 5 sgRNAs sum to a genomic knockout of ~80%.



Guide name	Guide sequence	DECODR knockout %
sgRNA1	TCGGGTTTCCAGCAGATC	31.5
sgRNA2	GGCACCCTCACTACGCTC	22.3
sgRNA3	TTCTCATTGGCATCATTTC	0
sgRNA4	GGGCCTAGCCAATGCCG	19.1
sgRNA5	GAGCTACAATGCAACGTGGC	7

Excerpt from Extended Data Fig. 6 in revised manuscript:

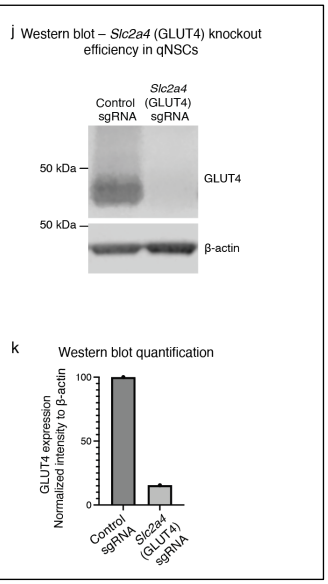
h, Top panel: scheme of the *Slc2a4* (GLUT4) locus with the location of sgRNAs 1-5. Bottom panel: genomic sequences for *Slc2a4* (GLUT4) sgRNAs 1-5 from DECODRv3.0 analysis tool indicating the sgRNA target and cut site and indel distribution. **i,** Guide sequences used for *Slc2a4* (GLUT4) sgRNAs 1-5 and the DECODRv3.0 knockout percentage for each sgRNA.

iv) Importantly, we have also performed new validation experiments at the protein level by western blot as well as quantified validation experiments at the protein levels by FACS (see detailed response below). We find that the sum of the percentage of knockout efficiency for *Slc2a4* (GLUT4) sgRNAs at the genomic level is consistent with that of GLUT4 at the protein level (quantified by FACS or western blot) (**new Extended Data Fig. 6j,k,m, see excerpts below**).

In addition to immunofluorescence measurements, independent qPCR and western blotting assays with the young and old NSCs in vitro infected with gRNA of these genes (especially GLUT4) are necessary to convince the readers the good knockout efficiency at the mRNA and protein levels within this system.

The Reviewer makes a great suggestion and we have now performed the following experiments and analyses:

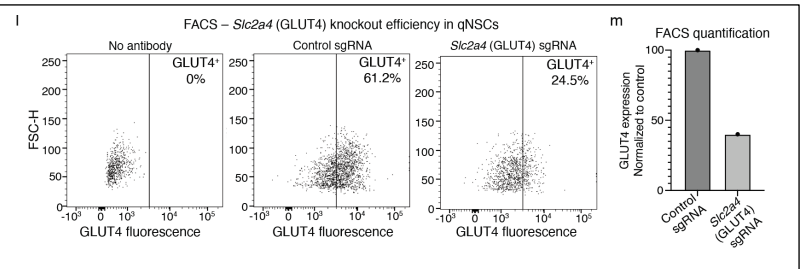
i) We have now used western blot experiments to assess the efficiency of the knockout for endogenous GLUT4 in quiescent NSCs infected with lentiviruses expressing control sgRNAs versus *Slc2a4* (GLUT4) sgRNAs (pool of 5 sgRNAs). Quantification of the western blot shows that GLUT4 protein levels were reduced by ~85%. This percentage of knockout is consistent with the sum of the percentages of each sgRNA knockout at the genomic level. **We have included these new western blot data as new Extended Data Fig. 6j,k (see excerpt below).**



Excerpt from Extended Data Fig. 6 in revised manuscript:

j, GLUT4 knockout efficiency in primary qNSC cultures by western blot. Western blot analysis of GLUT4 levels in qNSCs infected with control sgRNA (targeting unannotated regions of the genome) or sgRNA targeting *Slc2a4* (GLUT4), 10 days after infection by lentivirus and 3 days of selection with puromycin. β-actin is used as a loading control. **k**, Quantification of western blot: GLUT4 intensity, normalized to β-actin intensity.

ii) We have also quantified the percentage of knockout at the protein level by FACS (60%) from a previous experiment. **We have included the quantification of previous FACS data as new Extended Data Fig. 6m (see excerpt below).**



Excerpt from Extended Data Fig. 6 in revised manuscript:

l, GLUT4 knockout efficiency in primary qNSC cultures by FACS. Intracellular FACS analysis of GLUT4 levels in fixed qNSCs treated with control sgRNA or sgRNA targeting *Slc2a4* (GLUT4), 10 days after lentivirus infection to express sgRNA. No antibody control panel is on the left. Plots show mCherry^{+} gated cells, GLUT4 fluorescence. **m,** Quantification of FACS data, normalized to control.

iii) As suggested by the Reviewer, we have also used RT-qPCR to evaluate knockdown for *Slc2a4* RNA (**Figure R1 below**). The CRISPR-Cas9 cutting system induces indels in the genomic DNA and premature stop codons, which decreases the level of the protein but does not necessarily reduce levels of the corresponding mRNA. Using a primer set downstream of the sgRNA cut sites, we did not detect a decrease in total mRNA levels of *Slc2a4* (note that the levels of *Slc2a4* RNA are very low and the RT-qPCR are variable likely as a consequence of the very low levels). Interestingly, however, using a primer set that directly overlaps with the sgRNA cut sites for *Slc2a4*, we indeed detected a decrease in mRNA levels in the knockout

condition. This is characteristic of indel accumulation in the edited mRNA transcripts, as primers overlapping the cut sites preferentially amplify wild type transcripts[1]. This result confirms that there is editing at the transcript level for *Slc2a4* (GLUT4).

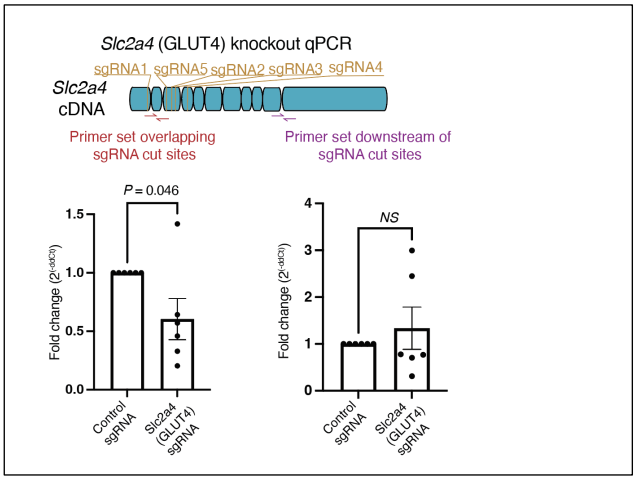


Figure R1. *Slc2a4* (GLUT4) mRNA levels upon *Slc2a4* knockout in quiescent NSCs.
Top panel: Scheme of sgRNAs for the *Slc2a4* (GLUT4) locus, exons indicated by the blue rectangles. Primer sets are indicated below. Bottom panels: RT-qPCR of *Slc2a4* (GLUT4) expression in primary cultures of quiescent NSCs treated with control sgRNA or sgRNA targeting *Slc2a4* (GLUT4) 10 days after lentiviral infection to express sgRNA. Primer sets overlapping *Slc2a4* sgRNA cut sites and downstream of cut sites are indicated. Dot plot showing mean +/-SEM of results from 5 independent NSC cultures. Relative gene expression fold changes were calculated as 2^(-ddCt), normalizing to the geometric mean of housekeeping genes (*Gapdh*, *Hrpt1* and *Actb*). *P*-values determined by two-tailed Mann Whitney test.

Taken together, these different experiments (genome sequencing, RT-qPCR with primers overlapping the sgRNA cutting sites, FACS, and western blot) confirm efficient *Slc2a4* (GLUT4) knockout **and we have now indicated that we have observed efficient knockout at the genomic and protein level in the revised text.**

Similarly, the observed increase in GLUT4 protein levels in old NSCs compared to young NSCs in vitro need be validated using qPCR and western blotting assays. These experiments are easily doable.-

The Reviewer's point is well taken, and we have now conducted the following experiments:

i) We have performed RT-qPCR to detect *Slc2a4* mRNA levels in primary cultures of young and old quiescent NSCs. These experiments showed a trend (not significant) for upregulation of *Slc2a4* mRNA with age (**Figure R2 below**). We note that the levels of *Slc2a4* mRNA were overall very low and that, likely as a consequence, the RT-qPCR results were variable (**Figure R2 below**).

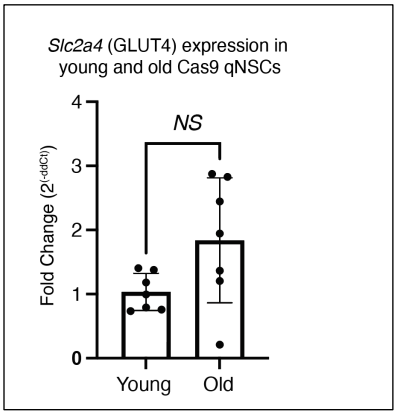


Figure R2. RT-qPCR of *Slc2a4* (GLUT4) expression in primary cultures of quiescent NSCs from young and old Cas9 mice. The primer set used was the "downstream" primer set indicated in Figure R1. Bar graph showing mean +/-SEM of results from 7 independent NSC cultures per age group. Relative gene expression fold changes were calculated as 2^(-ddCt), normalizing to the geometric mean of housekeeping genes (*Gapdh*, *Hrpt1* and *Actb*). *P*-values determined by two-tailed Mann Whitney test.

ii) We have also performed western blot experiments to assess the protein levels of GLUT4 *in vitro* in primary cultures of young and old quiescent NSCs. Western blot experiments did not

detect an increase in GLUT4 protein levels in old quiescent NSCs compared to young counterparts (**Figure R3 below**). The lack of detection in western blot (compared to immunostaining) could be due to differences in sensitivity of bulk vs. single cell assays or in differences in the pool of GLUT4 protein recognized by the antibodies used for immunofluorescence vs. for western blot (the antibodies we used for immunofluorescence did not work for western blot).

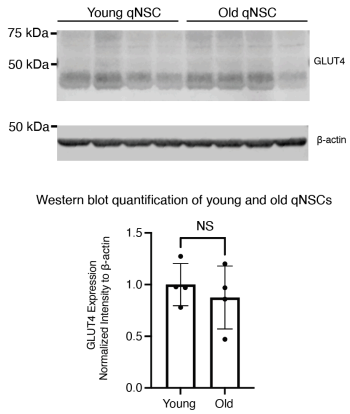


Figure R3. Western blot of GLUT4 protein levels in primary cultures of quiescent NSCs (qNSCs) from young and old Cas9 mice. GLUT4 and β-actin bands quantified using Image J. GLUT4 band intensity normalized to β -actin. Bar graph showing +/- SEM from 4 independent cultures per age group. *P*-values determined by two-tailed Mann Whitney test.

Overall, in the manuscript, we present evidence that: i) GLUT4 protein is increased in old quiescent NSCs by immunofluorescence *in vivo* (**Fig. 3j,k; Extended Data Fig. 5m,n**); ii) GLUT4 protein is increased in old quiescent NSCs by immunofluorescence *in vitro* (**Fig. 4c,d**) (not detected by western blot *in vitro*); iii) *Scl2a4* (GLUT4) mRNA, among the 12 mouse glucose transporter transcripts, is the only one to be increased in old quiescent NSCs by single cell RNA-seq *in vivo* (**Extended Data Fig. 6a**) (increasing trend, but not significant, by RT-qPCR *in vitro*); iv) Glucose uptake is increased in old quiescent NSCs *in vitro* (and this reduced upon *Slc2a4* (GLUT4) knockout) (**Fig. 4f,h; Extended Data Fig. 6f**). Collectively, these experiments are consistent with the notion that GLUT4 levels increase with age.

We have now indicated **in the Methods section that we did not detect significant changes by western blot and RT-qPCR, likely because of detection/sensitivity issues. We have also clarified in the main text that our observations of differences in GLUT4 expression in qNSCs with age are based primarily on immunofluorescence staining**.

The quality of some images in Figure 3 and Extended Figure 5 are still not good enough for the publication in Nature, which should be replaced with images with better resolution, lower background, higher magnification, and clearer color display.

For example, in Figure 3a&c *[now Fig. 3a&b]*, it is essential to include GLUT4 staining in mcherry+ cells infected with control gRNA-

Thank you for this important suggestion. We have now included GLUT4 staining in mCherry+ cells infected with control gRNA in **revised Fig. 3a and b**.

Additionally, a GLUT4 staining image with better resolution (possibly with a 100X objective) is needed to more clearly depict the distribution pattern of GLUT4 in both control and knockout cells in both the SVZ and olfactory bulb. –

This is another great point. We have now generated insets for GLUT4 staining in **revised Fig. 3a and b.**

The background of GLUT4 in Figure 3c *[now Fig. 3b]* is too high, and the magnification is not high enough.—

We agree and we have now included new images for GLUT4 staining, which have reduced background and better magnification, in **revised Fig. 3b**.

In Figure 3f, the EdU signal is not clear enough and the background is too high. A better image with higher resolution is needed.

We agree and we have now included an inset with a better resolution image for the EdU signal in **revised Fig. 3f**.

Besides, the sample images for control and Vmn1r107 KO sections are also needed.-

This is another great point. We have now included sample images for control and *Vmn1r107* KO in **revised Extended Data Fig. 5d.**

In Figure 3h and Extended Figure 5a, it is necessary to include mCherry staining at the same time to demonstrate the lentivirus infection efficiency in these SVZ regions.-

The Reviewer's point is well taken. We did not perform mCherry staining in the same exact section because of lack of distinct channels. We now provide mCherry staining in adjacent sections in **revised Extended Fig. 5a (bottom panels).**

In Figure 3g *[now Fig. 3j]*, the images for the GLUT4 staining in the four types of cells with higher resolution (with 100X objective?) and higher magnification needs to be shown in separate insets.—

We now provide insets with GLUT4 staining in different cells from young and old mice at higher magnification in **revised Fig. 3j**.

In Extended Figure 5h *[now Extended Data Fig. 5i]*, the background of mCherry is too high, and a better image is need to be provided.

We agree: we have now reduced the background for mCherry in revised **Extended Data Fig. 5i**.

In Extended Figure 5l *[now Extended Data Fig. 5m]*, it is difficult to tell which S100a6+GFAP+cell the GLUT4 signal comes from. Better images with higher resolution and

higher magnification to show the GLUT4 distribution pattern in an individual S100a6+GFAP+ NSC is needed to be shown in the insets.

We now provide insets with GLUT4 staining in different cells from young and old mice at higher magnification in **revised Extended Data Fig. 5m**.

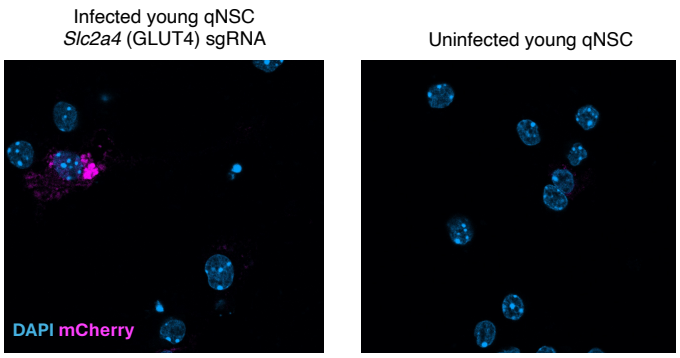


Figure R4. Immunofluorescence images of young *in vitro* Cas9 qNSCs 5 days after infection with lentiviruses expressing *Slc2a4* (GLUT4) sgRNAs or uninfected cells. Markers: mCherry (lentivirus-infected cells, magenta), DAPI (nuclei, blue).

Overall, it is strange that the mcherry signal is mainly enriched in the cytosol of soma (maybe except in Extended Figure 5h), making colocalization assessment less straightforward. Did the author try other mcherry antibodies to improve the mcherry staining signal?-

We thank the Reviewer for raising this point. We did try another mCherry antibody, and this particular one was the best (among the two we tested) for staining mouse brain sections. **We have now indicated this in Methods to help further studies**.

How about the mcherry signal in infected NSCs cultured in vitro?

We have looked at the mCherry signal in infected NSCs cultured *in vitro* and we find that it was also mainly enriched in the cytosol of soma (**Figure R4 above**).

Minor point:

At the "sgRNA PCR amplification and sequencing", there is an error: "In optimizing this PCR reaction, we found that Herculase II Polymerase was outperformed by Q5 polymerase outperformed Herculase II Polymerase,"-

Thank you! We have now fixed this error in the Methods section.

Reference

1. Li, B., Ren, N., Yang, L. *et al.* A qPCR method for genome editing efficiency determination and single-cell clone screening in human cells. *Sci Rep* **9**, 18877 (2019). https://doi.org/10.1038/s41598-019-55463-6

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have made significant effort and majority of the concerns have been addressed with better sample images. The manuscript is significantly improved.

It is disappointing to see that the authors were not able to confirm increased Glut4 expression at the protein level with westernblot analysis of NSCs from old animals, which is the most quantitative approach at the protein level. If the antibodies are not sensitive to westernblot, how to believe the immunohistology data, which is not as quantitative and was performed in different sections. As a minimal, these results suggest that the difference is not as dramatic. The single cell RNA-seq data only showed the mRNA or the protein level. Can the authors show increased protein levels of Stx4a by Westernblot in old NSC? The authors do have glucose uptake data to show functionally higher uptake in older NSCs. Nonetheless, these results raise questions about the specificity of regulators for NSC activation in different ages, as claimed in the abstract and main conclusion of the screen. Does Slc2a4 knockout also increase NSC activation of young NSCs in vitro and in vivo? Does restricting glucose uptake also affect young NSCs? As this pathway is the main validation and main conclusion of the manuscript, this information is critically needed to let readers to have confidence of the whole screen and gene list that were not validated.

The higher number of quiescent stem cells with Slc2a4 knockout can only explained by increased symmetric division of NSCs and then return to quiescence, not by other types of self-renewal or return to quiescence. To ensure there is no quantification bias, the authors need to directly demonstrate this with pulsing with EdU and quantified EdU+Ki67-GFAP+ NSCs with Slc2a4 knockout (Ideally, to see pairs of these NSCs next to each other).

As the authors did not examine adult NSCs and neurogenesis in the hippocampus, which is more associated with cognitive functions, the authors need to avoid making speculative statements at the end of the abstract.

Reviewer #2 on remaining concerns

Our initial main concern with the manuscript - that has now been submitted in a 2nd revised version - questioned the novelty and/or importance of the mechanisms identified using an in vivo Crispr screen-based approach (the screening approach by itself is novel in the context of adult neural stem cells (NSCs) and without any doubt novel and interesting).

We had voiced also major concerns regarding GLUT4 expression levels in young vs. aged NSCs and the relative lack of evidence for in vivo knockdown efficiency (and functional relevance of GLUT4 deletion).
Previously, we had indicated that the authors had addressed most of our initial concerns (with the exception of our major concern regarding importance of a potential age-dependent regulation of GLUT4 in SVZ NSCs or throughout the brain as the potential regulation does not seem to be selective for NSCs given the data of S100b-labeled astrocytes - if the antibody data hold true; unfortunate, that the authors did not include additional antibodies to support their main claims; e.g., RRID: AB_2631197 used by Ashrafi et al., 2017 Neuron in vivo and in vitro cultured neurons). It remains surprising that the antibody that is sufficient in cultured NSCs to detect the complete deletion of GLUT4 using Western blots (suggesting specificity..?) does not detect any differences in the levels of GLUT4 protein (see figure R3) in young versus old cells using the same technique (indeed, the IHC-detected upregulation of GLUT4 protein in S100b-labeled astrocytes also weakens the previous Extended Data 5k and 6a where – seemingly very low levels of Slc2a4 – had been analyzed in combined qNSCs and astrocytes). We are not sure what had been shown in R4 (there is not GLUT4 signal in all conditions….?).

The interpretation of the findings claiming to show that GLUT4 is upregulated with age in NSCs in the SVZ and that deletion of GLUT4 selectively enhances activation of aged NSCs is not fully supported by experimental evidence (at least not to a level that I would hope for and that would be fully convincing). Given that this is the key finding of the study (I understand that the experimental “approach” using an in vivo screen is the “exciting” part of the paper) I would have hoped for more (but I also feel that these concerns could have been more explicitly voiced by myself after the first round of revisions). Thus, I feel that it is not fully appropriate to make a more explicit recommendation.

Author Rebuttals to Second Revision:

Point-by-point reply

REFEREE 1

The authors have made significant effort and majority of the concerns have been addressed with better sample images. The manuscript is significantly improved.

We thank the Reviewer for their suggestions and for stating that our revised manuscript is significantly improved.

It is disappointing to see that the authors were not able to confirm increased Glut4 expression at the protein level with westernblot analysis of NSCs from old animals, which is the most quantitative approach at the protein level. If the antibodies are not sensitive to westernblot, how to believe the immunohistology data, which is not as quantitative and was performed in different sections.

The Reviewer's point is well taken. We believe that our immunofluorescence data *in vitro* and *in vivo* still represent trustworthy results, even though the changes in GLUT4 expression with age are not large (**and we now better acknowledge this in the text**).

- The observation that antibodies used for immunofluorescence experiments do not work for western blot experiments is a common occurrence and is likely due to the fact that proteins are in their native form in immunofluorescence experiments but are denatured in western blot experiments.
- We independently validated both types of GLUT4 antibodies (for immunofluorescence in vivo and western blot) using knockout (Fig. 3a-d and Extended Data Fig 6j). Thus, we believe that antibody specificity is not a major issue.
- Immunofluorescence (and single cell RNA-seq), which are single cell-based assays, can be more sensitive than western blot (and RT-qPCR), which are bulk assays, in capturing small differences in protein (or gene) expression.
- In our western blot experiments, the protocol enriches for membranes whereas in immunofluorescence experiments, the protocol does not. Together with the different antibodies used (which could recognize different fractions of the GLUT4 proteins) and especially the single cell vs. bulk assays, these differences could contribute to the lack of detection of an increase in protein by western blot.
- To best ensure our ability to quantify the immunofluorescence staining, when conducting our immunofluorescence *in vitro* and *in vivo*, we paid special attention to stain all sections and coverslips in the same way and at the same time. In addition, many cells (>100 cells per section in vivo) and many sections (>50 sections per age group in vivo) were counted in an unbiased manner using an automated pipeline (in Qupath).

We have now made all these points clearer in the Methods section of our revised manuscript and we state that the age-dependent increase in GLUT4 by immunofluorescence is not large.

As a minimal, these results suggest that the difference is not as dramatic.

We agree with the Reviewer, and we **have now indicated that GLUT4 expression change with age is not large and that other factors could contribute to the specific sensitivity of old NSCs to GLUT4 knockout, both in the Text and Discussion section.**

The single cell RNA-seq data only showed the mRNA or the protein level.

We agree that the single cell RNA-seq data only shows the mRNA, and not the protein level. The increase in *Scl2a4* (GLUT4) mRNA in a single cell RNA-seq assay is consistent with the increase in GLUT4 protein in single cell-based protein assay (immunofluorescence).

Can the authors show increased protein levels of Stx4a by Westernblot in old NSC?

This is interesting for future direction, though we feel that this would be outside the scope of the present manuscript, which is focused on GLUT4.

The authors do have glucose uptake data to show functionally higher uptake in older NSCs.

We thank the Reviewer for highlighting that our data show glucose uptake increase, which is consistent with GLUT4 increase by immunofluorescence *in vitro* and *in vivo*. We would also like to point out that glucose uptake is reduced in GLUT4 knockout in old quiescent NSCs (**see Fig. 4h**). The increase in glucose uptake coupled with the GLUT4 dependency of glucose uptake is consistent with the increase in GLUT4 expression with age (captured by single cell RNA-seq *in vivo* and by immunofluorescence *in vitro* and *in vivo*). **We have made this point clearer in the revised text.**

Nonetheless, these results raise questions about the specificity of regulators for NSC activation in different ages, as claimed in the abstract and main conclusion of the screen.

We understand the Reviewer's point. However, we would like to respectfully disagree that our lack of ability to detect an increase in GLUT4 protein level by western blot raises question about the general specificity of NSCs' sensitivity to gene knockouts (KO) at different ages. In fact, we believe that changes in mRNA or protein of interest with age may not be the only factor responsible for the specificity of the KO of that gene of interest for old vs. young NSCs. In the case of GLUT4, changes in gene expression may contribute (with the acknowledgement that other factors could also participate). For other genes, it could be that the state (e.g. cellular network) of the old NSCs is different compared to that of the young NSCs, and as such old NSCs could be more sensitive than young counterparts to the KO of a gene of interest. **We have now clarified that other factors (e.g. differences in cell network) could also contribute to the specific effects of GLUT4 knockout, and more generally to the specificity of other knockouts, in old vs. young NSCs in the Discussion section.**

Does Slc2a4 knockout also increase NSC activation of young NSCs in vitro and in vivo? Does restricting glucose uptake also affect young NSCs? As this pathway is the main validation and main conclusion of the manuscript, this information is critically needed to let readers have confidence of the whole screen and gene list that were not validated.

The Reviewer raises interesting questions:
In vitro: yes, both *Scl2a4* (GLUT4) knockout and glucose restriction specifically boost old NSCs, but not young NSCs (**Fig. 4i, blue data points**).
In vivo: this would indeed be very interesting to do, but we feel that these experiments would be beyond the scope of the present manuscript. **We have modified the text to make it clearer that *Slc2a4* (GLUT4) knockout and glucose deprivation *in vitro* both increase old NSC activation, but not young, NSC activation.**

The higher number of quiescent stem cells with Slc2a4 knockout can only explained by increased symmetric division of NSCs and then return to quiescence, not by other types of self-renewal or return to quiescence. To ensure there is no quantification bias, the authors need to directly demonstrate this with pulsing with EdU and quantified EdU+Ki67-. GFAP+ NSCs with Slc2a4 knockout (Ideally, to see pairs of these NSCs next to each other).

This is another interesting point. However, we feel that the exact mechanism by which the number of quiescent stem cells increases upon GLUT4 KO would be beyond the scope of the present manuscript. **We have now included the possibility of increased symmetric division followed by return to quiescence as a possible mechanism for increased quiescent NSC number in the Text.**

As the authors did not examine adult NSCs and neurogenesis in the hippocampus, which is more associated with cognitive functions, the authors need to avoid making speculative statements at the end of the abstract.

Thank you for this suggestion. **We have now toned down the statement regarding cognitive function at the end of the abstract.**

REFEREE 2

Our initial main concern with the manuscript - that has now been submitted in a 2nd revised version - questioned the novelty and/or importance of the mechanisms identified using an in vivo Crispr screen-based approach (the screening approach by itself is novel in the context of adult neural stem cells (NSCs) and without any doubt novel and interesting).

We thank the Reviewer for their interest in our study.

We had voiced also major concerns regarding GLUT4 expression levels in young vs. aged NSCs and the relative lack of evidence for in vivo knockdown efficiency (and functional relevance of GLUT4 deletion).

We thank the Reviewer for raising these important points. We believe that our *in vivo* knockdown experiments (**Fig. 3a-d**) and our quantification of NSC numbers (**Fig. 3h,i**) have helped to provide additional evidence for the in vivo knockdown efficiency and functional relevance. **We have now toned down the text to indicate that the increase in GLUT4 expression levels *in vivo* and *in vitro* is not large and that other factors may contribute to**

the selectivity of GLUT4 knockout to old NSCs.

Previously, we had indicated that the authors had addressed most of our initial concerns (with the exception of our major concern regarding importance of a potential age-dependent regulation of GLUT4 in SVZ NSCs or throughout the brain as the potential regulation does not seem to be selective for NSCs given the data of S100b-labeled astrocytes - if the antibody data hold true; unfortunate, that the authors did not include additional antibodies to support their main claims; e.g., RRID: AB_2631197 used by Ashrafi et al., 2017 Neuron in vivo and in vitro cultured neurons). It remains surprising that the antibody that is sufficient in cultured NSCs to detect the complete deletion of GLUT4 using Western blots (suggesting specificity..?) does not detect any differences in the levels of GLUT4 protein (see figure R3) in young versus old cells using the same technique (indeed, the IHC-detected upregulation of GLUT4 protein in S100b-labeled astrocytes also weakens the previous Extended Data 5k and 6a where – seemingly very low levels of Slc2a4 – had been analyzed in combined qNSCs and astrocytes). We are not sure what had been shown in R4 (there is not GLUT4 signal in all conditions….?).

The Reviewer's points are all well taken.

The marker we have used for NSCs is the S100a6 marker (different from S100b), and this S100a6 marker has been shown to be relatively specific for NSCs in the niche[1]. We observed a significant increase in GLUT4 levels in S100a6 immunofluorescence in SVZ sections *in vivo* (**Extended Data Fig. 5m,n**). **We have now made it clearer that S100a6 marker is relatively specific for NSCs in the adult SVZ niche and included the corresponding reference.**

We have now better validated the GLUT4 antibodies used for immunofluorescence on brain section *in vivo* by knockout (**Fig. 3a-d**).

We would like to apologize for the lack of clarity regarding Figure R4: this was a control asked by Reviewer 1 to test if the mCherry antibody staining also showed a puncta pattern in mCherry-infected cultured NSCs (which it does).

The interpretation of the findings claiming to show that GLUT4 is upregulated with age in NSCs in the SVZ and that deletion of GLUT4 selectively enhances activation of aged NSCs is not fully supported by experimental evidence (at least not to a level that I would hope for and that would be fully convincing). Given that this is the key finding of the study (I understand that the experimental "approach" using an in vivo screen is the "exciting" part of the paper) I would have hoped for more (but I also feel that these concerns could have been more explicitly voiced by myself after the first round of revisions). Thus, I feel that it is not fully appropriate to make a more explicit recommendation.

The Reviewer's point is well taken. **We have now toned down the conclusion regarding the age-dependent upregulation of GLUT4 in the revised manuscript.**

Reference

1 Kjell, J. *et al.* Defining the Adult Neural Stem Cell Niche Proteome Identifies Key Regulators of Adult Neurogenesis. *Cell Stem Cell* **26**, 277-293 e278 (2020). https://doi.org/10.1016/j.stem.2020.01.002