

Astrogenesis in the murine dentate gyrus is a life-long and dynamic process

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DOI: 10.15252/emboj.2021110409

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Review Timeline:

Submission Date:	10th Dec 21
Editorial Decision:	26th Jan 22
Revision Received:	15th Feb 22
Editorial Decision:	17th Mar 22
Revision Received:	25th Mar 22
Accepted:	30th Mar 22

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Ruth,

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision, but I have now received the comments from two referees. I am still waiting to hear back from a third referee, but at this stage I don't think that I will receive the comments. I will therefore go with the two reports on hand.

As you can see below, the referees appreciate the analysis and find the insights gained important. I would therefore like to ask you to submit a revised version. The referees raise relative minor concerns that should be fairly straightforward to address. Let me know if we need to discuss any issues further. I am happy to do so via email or a video call.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: <https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess>

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF with helpful tips on how to prepare the revised version.

Further information is available in our Guide For Authors: <https://www.embopress.org/page/journal/14602075/authorguide>

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (26th Apr 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

<https://emboj.msubmit.net/cgi-bin/main.plex>

Referee #1:

**Astrogenesis in the murine dentate gyrus is a life-long and dynamic process.
Schneider et al.**

Astrocytes are a major non-neuronal cell type in the mammalian brain and their functions are now widely acknowledged to be indispensable for all aspects of CNS development, adult function and ageing. However, our understanding of astrocyte development is superficial at best. The dentate gyrus (DG) of the mouse brain is a specialized region, which retains neurogenic potential into adulthood and is an excellent model system to study the process of astrogenesis from development to ageing. In this study, Schneider and colleagues apply a variety of techniques, including genetic fate mapping, behavioral paradigms, single cell transcriptomics and *in vivo* two-photon imaging to assess astrogenesis across the life of a mouse. Key observations include (i) that astrogenesis in the DG is initiated by radial glial-like neural stem cells, with local proliferation of the resulting astrocytes responsible for enlarging the astrocyte compartment in an outside-in-pattern mirroring neurogenesis, (ii) that astrogenesis also occurs in the adult DG with the majority of astrocytes also produced by local proliferation and (iii) that local astrocyte proliferation is plastic and adapts to environmental and behavioral stimuli. Based on their findings, the authors conclude that (adult) astrocytes are plastic elements in DG circuits, implying a vital role for them in hippocampal plasticity.

The increasing realization that astrocytes play key roles in all aspect of CNS development, function and ageing mean that this study is both timely and relevant. In my opinion, the study was well designed and executed. Considerable time and effort have obviously gone into the writing process, with both a scholarly introduction to the problem under study and thorough discussion of the results in relation to others work. Results are presented with a structured, logical progression and (within the limitations of the techniques used) are convincing. Important areas for follow up are noted and discussed.

Conceptually, I think there are two issues which do need to be noted. First, the issue of adult neurogenesis is contentious. Although adult neurogenesis has been reported across various mammalian models, its actual existence in the human brain is still hotly debated, with various evidences for and against. Second, there is a lack of direct data to support a significant functional role for the adult born astrocytes observed by the authors: single cell data is used to draw conclusions which, by their very nature, are largely circumstantial. Irrespective of these issues (which the authors go to great lengths to discuss), the manuscript does provide interesting insights into astrocyte development and how it impacts circuit formation, while also hinting at astrocyte adaptation during ageing to support CNS function. Such concepts are important for the field and may well be more generally applicable and will be of interest to the astrocyte biologists and neuroscientists in general. Overall, therefore, I am convinced the study is a significant addition to the literature and consider it a strong candidate for EMBO Journal.

Major issues:

In general, I think the overall conclusions of the work, backed by the use of multiple independent approaches, are solid. It is unclear to me that asking for extensive follow up experiments, such as assessing astrocyte maturity at the functional level (see, for example, electrophysiological measurements of ionic conductances in Ge et al., Nature, 2012), would necessarily strengthen the conclusions of the paper, and would just serve to impose an unnecessary burden on the authors, delaying publication.

Rather, my major concerns rest on the data obtained to date - and specifically on how it is presented.

1. The text and figures are extremely dense making the manuscript (in my opinion) difficult to read in places.

While the "Abstract" is correct in stating that the manuscript uses various techniques, including genetic fate mapping, behavioral paradigms, single cell transcriptomics and *in vivo* two-photon imaging, the actual balance of work is heavily weighted towards analysis of marker stainings (including proliferation measured with BrdU) to provide molecular, morphological and positional information on cell populations of interest.

As it stands, however, I think it is sometimes unclear exactly the criteria which distinguishes between the different populations: NSC-derived, local proliferation etc. Definitions could be clearer and cell populations marked explicitly on the images.

Cell populations tend to be defined by multiple markers. These are often shown as "merged" images at low magnification. This means it can be difficult to fully distinguish the different cell markers (e.g. Figure 3K). In this respect, separate panels, such as used in Figure 2G, are much more convincing. Cells with overlapping markers should be indicated consistently with arrows. Points at which morphological criteria are used to distinguish radial glial cells from astrocytes need to be clearly indicated.

While I appreciate this is a lot of work and would require significant thought to go into figure redrafting, in my opinion it would be much more convincing and greatly strengthen the claims in the manuscript.

2. Key methodologies are sometimes skipped over, which is a problem for non-specialists. For example, how is the lineage tree in Figure EV2I constructed? I could find no details at all in the "Figure Legend" or "Methods".

3. Would a final summary figure be of use?

Minor issues:

1. Page 5: The authors write "Due to their high density, it was impossible to reliably count the number of NESTIN+ cells during early postnatal DG stages." In this case, how was figure EV1A generated at P3?

2. Page 5: In the discussion of proliferative cells, I think corresponding markers should be mentioned.

3. Page 6: The authors claim that they observe different astrocyte subtypes in different layers of adult DG based on morphological, molecular, and functional differences. On which data is this conclusion based?

4. Page 6: "Co-staining of ACSBG1 in combination with proliferation markers (P3/P7: KI67; P10/P14: MCM2) revealed that the developing DG harboured locally proliferating astrocytes which did not express the rNSC marker NESTIN." It would be nice to have the staining of NESTIN in Figure 1G, to see that it does not overlap with the other markers.

5. The markers used to define astrocytes change during the study: at the beginning, the authors use ACSBG1 and S100beta; later, they switch to using SOX2. Can the authors confirm the expression of these markers at corresponding ages/levels of maturation in publicly available single cell datasets to make a stronger argument? In addition, can they back up their claim that "early post-natal astrocytes predominantly expressed ACSBG1, while GFAP and S100beta expression was initiated later, accompanied by the development of the typical astrocyte morphology"? Without an experimental time-course or appropriate reference this seems a claim too far.

6. Page 9: Figures do seem to be cited out of order

7. Figure 3G, H: What do authors mean by "astrocyte subtypes"? Do they mean astrocytes in the various DG layers?

8. Page 13: If genes are not significantly enriched markers, it is unclear to me how the authors could use them for pathway analysis? Are there any other criteria used to select genes?
9. Page 14: If I remember correctly, Batiuk et al. present single cell sequencing data showing a population of astrocytes in cortex enriched in transcripts relating to cell cycle and proliferation. I am not sure that this is proof for ongoing astrogenesis. In comparison, Ohlig and colleagues did report clonal analysis experiments strongly arguing for ongoing astrogenesis (albeit at a very low level).
10. Page 20: How was running distance measured? Was there a correlation between the distance covered in individual animals and the degree of astrogenesis?
11. Page 21: What is the "rev mut" primer and why is it used?
12. Page 22: "If BrdU-incorporation into the DNA was examined, pre-treatment of sections in 2 N HCl was performed for 10 min at 37 degrees C after the staining for the other used primary and secondary antibodies was completed". I always thought that low pH was used to elute antibodies from peptide columns during affinity purifications. As such, are the authors sure that this acid treatment is not affecting their antibody staining?
13. Page 23: Please give more details on the microscopy parameters used. What was the NA of the objective used? Were images acquired using complete confocality? How thick were image sections? Why were different z-stack thicknesses used at various points in the study?
14. Were specific plugins used in ImageJ? If so, please state.
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19. Page 37: Figure 4: In my opinion, Figure Legends should stand alone from each other and the main text: please list the molecular, morphological and positional criteria used for cell classification.
20. Page 38: "8 week old.....were administered with tamoxifen for 10x at five consecutive days". To my mind, this is a slightly odd 'definition' and appears to be lab shorthand: please correct.

Referee #3:

This paper is extremely impactful and rigorous, it provides essential piece of information regarding astrogenesis in the dentate gyrus of the hippocampus. By using genetic fate-mapping, proliferation analysis, retrospective birth-dating experiments and suitable mouse models the authors demonstrate that most astrocytes in DG are locally proliferating astrocytes thus forming a pool of cells able to produce new astrocytes during all the lifespan of mouse. The manuscript is well organized and clearly written.

There are few points that in my opinion should be addressed.

Use also the ubiquitous astrocyte marker ALDH1L1 to identify astrocytes derived from rNSCs and from non-stem cells (fig1). Adult neurogenesis in the DG is strongly modulated by neurovascular coupling, what about astrogenesis?

scRNAseq analysis of astrocytes transcriptomic profile was done by 10X genomics which, if I'm not wrong, is able to capture only highly expressed genes, this aspect should be discussed.

In the discussion, the sentence "Especially in the adult brain, astrocytes have always been considered to be postmitotic under physiological conditions" is misleading since it is known that local production of glia is a major source of astrocytes in the cerebral cortex (Ge et al 2012 already quoted in the manuscript) but also the review by Ge and Jia 2016.

We want to cordially thank the editor and the reviewers for their careful and constructive comments. All changes in the revised manuscript are marked by underlines. In the following, we have listed a point-by-point response to the reviewer's suggestions.

Reviewer 1:

Major issues

1. The text and figures are extremely dense making the manuscript (in my opinion) difficult to read in places.

While the "Abstract" is correct in stating that the manuscript uses various techniques, including genetic fate mapping, behavioral paradigms, single cell transcriptomics and in vivo two-photon imaging, the actual balance of work is heavily weighted towards analysis of marker stainings (including proliferation measured with BrdU) to provide molecular, morphological and positional information on cell populations of interest.

Response: We thank the reviewer for his/her comment. We agree that analysis by immunohistochemistry is the most used technique in this manuscript, which is backed up by genetic fate mapping, behavioral paradigms, single cell transcriptomics and in vivo two-photon imaging. We rephrased the sentence in the abstract accordingly to clarify this balance more precisely.

As it stands, however, I think it is sometimes unclear exactly the criteria which distinguishes between the different populations: NSC-derived, local proliferation etc. Definitions could be clearer and cell populations marked explicitly on the images.

Response: We thank the reviewer for his/her comment and added definitions in the text and legends when necessary. Local proliferation of astrocytes was always assessed by the expression of cell cycle markers (KI67/MCM2). Many proliferating astrocytes derived from rNSCs as assessed by *Nestin*CreER^{T2}-mediated fate-mapping. However, the recombination efficiency is lower than 100%, i.e. we do not label the progeny of every rNSCs and are therefore not able to specify for each astrocytes whether or not it is rNSC-derived.

Furthermore, we have now included arrows and arrowheads to better mark specific cell population in our images and indicated their meaning in the Figure Legends. If not specifically stated otherwise, cells belonging to the neuronal lineage are always marked with arrows, while astrocytes are highlighted by arrowheads throughout the manuscript.

Cell populations tend to be defined by multiple markers. These are often shown as "merged" images at low magnification. This means it can be difficult to fully distinguish the different cell markers (e.g. Figure 3K). In this respect, separate panels, such as used in Figure 2G, are much more convincing. Cells with overlapping markers should be indicated consistently with arrows. Points at which morphological criteria are used to distinguish radial glial cells from astrocytes need to be clearly indicated.

While I appreciate this is a lot of work and would require significant thought to go into figure redrafting, in my opinion it would be much more convincing and greatly strengthen the claims in the manuscript.

Response: We thank the reviewer for his/her thoughtful comment. Indeed, the figures were very compact and potentially difficult to understand in some parts. In order to improve this, we redrafted the figures accordingly. Furthermore, we consistently labelled astrocytes by arrowheads, and neuronal progeny by arrows in all new Figures. Morphological criteria by which cell type identity was determined are specified in the Figure Legends.

- Figure 1: As requested by this reviewer, we integrated new images into Figure 1H showing a ACSBG1⁺/MCM2⁺ proliferating astrocyte that does NOT express Nestin.
- EV Figure 1: We added images of ALDL1H1, another marker for astrocytes, which was requested by reviewer 2. Using *Nestin*CreER^{T2}; GFP fate mapping, we show here that many recombined GFP⁺ astrocytes express ALDH1L1 through postnatal development (EV 1E) as a further readout for their astrocytic identity. Equivalent to the other

astrocyte markers shown in the previous version of the manuscript, proliferating astrocytes can be additionally labelled with ALDH1L1 (new confocal images in EV1F).

- As noted by the reviewer, cell type-specific markers can be difficult to distinguish in merged images at low magnification. Therefore, we incorporated separate panels showing single markers in the corresponding EV Figures. For Figure 3B, single images are shown in EV Figure 2E; the merge in Figure 3D is displayed singly in EV Figure 2F, and Figure 3K is shown in EV Figure 2L. All arrows and arrowheads of the main figure images are displayed also in the single panel images in the EV Figures.
- Especially Figure 4 was very dense and is now separate into two distinct figures, one showing the effects of running on astrogenesis (Figure 4), the other one the effects of aging (Figure 5). This allowed us to better illustrate our finding by showing single marker panels in addition to merged triple/quadruple IHC images of the key stainings: (i) the generation of new cells (BrdU/DCX/GFAP/SOX2), (ii) proliferation of rNSCs, IPC/neuroblasts and astrocytes (MCM2/NESTIN/SOX2), and (iii) on fate-mapping experiments using *Nestin*CreER^{T2}; GFP mice (GFP/DCX/NESTIN/SOX2). Please note that the single panels of proliferation of rNSCs, IPC/neuroblasts and astrocytes (MCM2/NESTIN/SOX2) for the running wheel paradigm are now displayed in EV Figure 3D.

2. Key methodologies are sometimes skipped over, which is a problem for non-specialists. For example, how is the lineage tree in Figure EV2I constructed? I could find no details at all in the "Figure Legend" or "Methods".

Response: We thank the reviewer for his/her comment and apologize for our mistake. All key technologies were now carefully checked to guarantee that they are sufficiently explained to non-specialists in the text (Figure Legends and/or Methods).

3. Would a final summary figure be of use?

Response: We agree with the reviewer and added a final Figure 7 summarizing our results on astrogenesis in the DG from development to age.

Minor issues

1. Page 5: The authors write "Due to their high density, it was impossible to reliably count the number of NESTIN+ cells during early postnatal DG stages." In this case, how was figure EV1A generated at P3?

Response: We thank the reviewer for his/her comment and apologize for our mistake. Indeed, the graph of EV1B wrongly indicated that we counted GFP⁺/KI67⁺ cell over all NESTIN⁺ cells. We changed the wrong labelling in EV1B, where we now state the number of KI67⁺/NESTIN⁺ over all proliferating cells as a readout for recombination efficiency.

2. Page 5: In the discussion of proliferative cells, I think corresponding markers should be mentioned.

Response: In the revised version of the manuscript, we now included that the cell cycle marker KI67 was used as a marker for proliferating cells (EV1B, C).

3. Page 6: The authors claim that they observe different astrocytes subtypes in different layers of adult DG based on morphological, molecular, and functional differences. On which data is this conclusion based?

Response: We thank the reviewer for this comment. Astrocyte diversity in the adult DG was carefully investigated in another project of our group. In this very comprehensive analysis, we observed that the distinct DG compartments (molecular layer, granule layer, SGZ, hilus) are populated by astrocyte subtypes that differ in their molecular, morphological and physiological features. Our data revealed that the functional separation of DG compartments is not limited to neuronal structures but also applies for astrocytes, indicating that the composition of neural networks is determined by both neuronal and glial functional

specification. This work is at present under consideration, and we are happy to share the unpublished manuscript with the reviewers if requested.

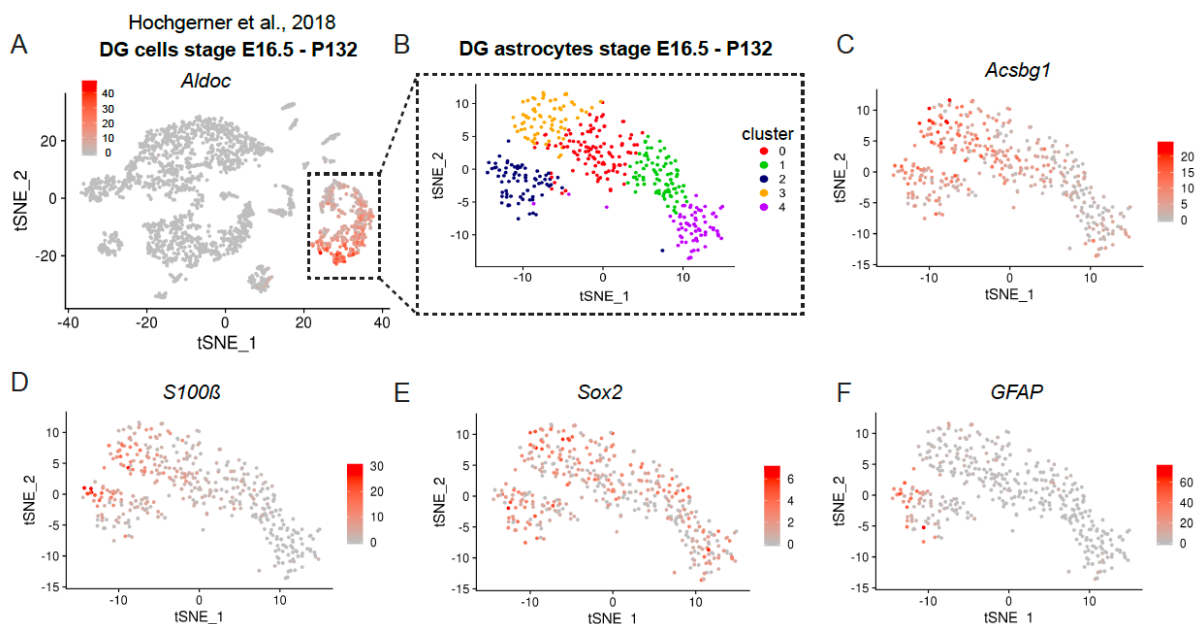
4. Page 6: "Co-staining of ACSBG1 in combination with proliferation markers (P3/P7: KI67; P10/P14: MCM2) revealed that the developing DG harboured locally proliferating astrocytes which did not express the rNSC marker NESTIN." It would be nice to have the staining of NESTIN in Figure 1G, to see that it does not overlap with the other markers.

Response: We agree with the reviewer and added an image in Figure 1H showing an example of a proliferating astrocyte at P7 that does not express NESTIN.

5. The markers used to define astrocytes change during the study: at the beginning, the authors use ACSBG1 and S100beta; later, they switch to using SOX2. Can the authors confirm the expression of these markers at corresponding ages/levels of maturation in publicly available single cell datasets to make a stronger argument?

Response: We thank the reviewer for his/her comment. ACSBG1 is one of the firstly expressed astrocyte markers, and is detectable as early as P3 already. In contrast, GFAP and S100 β start to be expressed around P7 and P10, respectively. Upon expression start, all markers are consistently expressed in astrocytes throughout life. In the adult hippocampus, SOX2 is expressed in all astrocytes, including stem- and progenitor cells. As a transcription factor, SOX2 is localized to the nucleus, which made it easier to assess co-localization with cell cycle markers MCM2/KI67 and BrdU. Before doing these countings, we confirmed that SOX2 co-localizes with all other astrocyte markers used in this study (ACSBG1, S100 β , GFAP). Furthermore, as requested by reviewer 2, we have now also included an additional pan-astrocyte marker ALDH1L1.

For our study of astrocyte diversity (mentioned above), we used a published single cell (sc)RNA-seq data set of mouse DG cells derived from early developmental stages until adulthood (Hochgerner et al., 2018). Including only cells expressing astrocyte markers for reanalysis (Rebuttal Figure 1A), clustering revealed that the astrocytes of the DG shared many similarities in their overall transcriptomic profiles. Still, they could be discriminated due to relevant molecular differences (Rebuttal Figure 1B; Karpf et al., submitted). As visible from the tSNE plots below, ACSBG1, S100 β and SOX2 are expressed at comparable levels in all astrocyte subgroups (Rebuttal Figure 1C-E). Notably, GFAP (Rebuttal Figure) mRNA levels were unexpectedly low in mature astrocytes (clusters 0, 1, 3) and radial glia-like NSCs (cluster 4), and much higher in proliferating astrocytes (cluster 2). In a preliminary project, we are now comparing the molecular fingerprints of mature versus proliferating astrocytes.



Rebuttal Figure: scRNA-seq of DG astrocytes derived from E16.5 – P132

(A) Astrocytes were identified by the expression of astrocyte markers as exemplified by *Aldoc* in a published scRNA-seq data set of DG cells (Hochgerner et al., 2018). (B) Cluster analysis of astrocytes identified in (A) according to molecular similarities revealed five molecularly distinct astrocyte subgroups. (C-F) tSNE plot illustrating the transcriptional levels of *Acsbg1*, *S100β*, *Sox2* and *GFAP*. Figure adapted from Karpf et al., submitted.

In addition, can they back up their claim that "early post-natal astrocytes predominantly expressed ACSBG1, while GFAP and S100beta expression was initiated later, accompanied by the development of the typical astrocyte morphology"? Without an experimental time-course or appropriate reference this seems a claim too far.

Response: We thank the reviewer for his/her comment. We agree that based on the presented experimental evidence, the claim is too strong to put it into a causative correlation. Therefore, we omitted the speculative link between the expression of GFAP/S100β and the development of a typical astrocytic morphology. Still, we would like to note here that analysis at four different time points during postnatal development (P3, P7, P10 and P14) revealed that the expression of GFAP and S100β starts around P7/10. Notably, this is also the time in which astrocytes develop their typical morphology (and also their subtype-specific morphology).

6. Page 9: Figures do seem to be cited out of order.

Response: We thank the reviewer for his/her comment. We have carefully checked the order of the cited figures.

7. Figure 3G, H: What do authors mean by "astrocyte subtypes"? Do they mean astrocytes in the various DG layers?

Response: Yes, we meant astrocytes located to distinct DG layers. The text is now changed accordingly.

8. Page 13: If genes are not significantly enriched markers, it is unclear to me how the authors could use them for pathway analysis? Are there any other criteria used to select genes?

Response: We thank the reviewer for his/her comment. As stated in the manuscript, we found only very few significantly differentially expressed genes (DEGs) as determined by calculation of the adjusted p-values. Therefore, for pathway analysis, genes were included based on their average logarithmic fold change (*avg_logFC*). In the comparison of both young non-runners (ctrl) to young runners as well as young non-runner (ctrl) to aged non-runners, we selected genes with an *avg_logFC* ≥ 0,25 as being expressed at higher levels in astrocytes of ctrl animals, while genes with an *avg_logFC* ≤ -0,25 as being expressed at higher levels in astrocytes of running/aged mice. This is now also clarified in Material and Method.

9. Page 14: If I remember correctly, Batiuk et al. present single cell sequencing data showing a population of astrocytes in cortex enriched in transcripts relating to cell cycle and proliferation. I am not sure that this is proof for ongoing astrogenesis. In comparison, Ohlig and colleagues did report clonal analysis experiments strongly arguing for ongoing astrogenesis (albeit at a very low level).

Response: It is correct that Batiuk et al. identified astrocytes expressing cell cycle markers in the adult cortex (and hippocampus) by scRNA seq, while Ohlig et al. used both scRNA seq and clonal analysis to show astrogenesis in the diencephalon. To be more specific here, we rephrased that sentence in the discussion of our revised manuscript.

10. Page 20: How was running distance measured? Was there a correlation between the distance covered in individual animals and the degree of astrogenesis?

Response: We thank the reviewer for his/her comment. Running distance was measured using tachometers attached to the running wheels as now also stated in the Methods. Interestingly, and corresponding to what has been reported for neurogenesis, the distance

ran did not influence the effects on astrogenesis. As seen in the data, all animals show a comparable response, while the distance ran ranged between 20km and 180 km in total (12 days). It appears therefore, that it does not matter how long an animal runs as long as it runs at all.

11. Page 21: What is the "rev mut" primer and why is it used?

Response: We apologize that we did not specify the name correctly. YFP-reporter mice (<http://www.informatics.jax.org/allele/key/22442>) carry a YFP inserted via homologous recombination into the Gt(ROSA)26SOR locus. A STOP codon flanked by loxP site is located before the YFP, which will be floxed out by Cre recombinase, leading to the expression of the YFP reporter. The mut rev primer detects the shorter allele, in which the STOP codon has been removed by Cre recombinase, while the rev primer detects the allele carrying the STOP codon (before recombination occurs). Since the genotyping of the experimental animals was performed before recombination was induced by administration of Tamoxifen, the rev mut primer is not necessary for the genotyping reported here. Therefore, we omitted this primer for the Method part.

12. Page 22: "If BrdU-incorporation into the DNA was examined, pre-treatment of sections in 2 N HCl was performed for 10 min at 37 degrees C after the staining for the other used primary and secondary antibodies was completed". I always thought that low pH was used to elute antibodies from peptide columns during affinity purifications. As such, are the authors sure that this acid treatment is not affecting their antibody staining?

Response: We thank the reviewer for his/her comment. All markers used in combination with BrdU have been established before without BrdU pretreatment. Comparing IHC results with and without BrdU treatment did not result in changes of the signal as long as the marker stainings have been completed (primary and secondary antibodies) BEFORE the treatment.

13. Page 23: Please give more details on the microscopy parameters used. What was the NA of the objective used? Were images acquired using complete confocality? How thick were image sections? Why were different z-stack thicknesses used at various points in the study?

Response: We thank the reviewer for his/her comment. All requested additional information can now be found in the microscopy section of the revised Methods.

14. Were specific plugins used in ImageJ? If so, please state.

Response: No, we have not used specific plugins from ImageJ and specify this now in the revised manuscript.

15. The protocols given for immunocytochemistry are a bit superficial. Please give full details.

Response: We thank the reviewer for his/her comment and have carefully amended the protocol for immunohistochemistry.

16. Page 24: Were movies supplied? I did not find them in the documents provided for review.

Response: We thank the reviewer for his/her comment. In the revised version we now provide a EV Movie of the *in vitro* imaging of SEZ-derived NSC-culture.

17. What were the viabilities of the single cell suspensions created? How was the potential stress response (Van den Brink et al., Nat Methods, 2017) accounted for?

Response: We thank the reviewer for his/her careful comment, raising a very important point. As shown in Van den Brink et al., Nat Methods, 2017 for skeletal muscle cells, the dissection and dissociation of solid tissue such as muscles and brain may impose changes in transcription or even activation of cell types within the dissected tissue. Indeed, the dissection and dissociation of adult brain tissue is very harmful for neurons. It is hypothesized that especially neurons increasingly die during dissection due to cutting off their axons and dendrites. This is indeed reflected by the numerical underrepresentation of neurons in the DG

scRNA-seq data. To avoid this effect, single nuclei RNA sequencing is now widely used to investigate the transcriptome of neurons on single cell level. However, rNSCs and astrocytes appear to survive the procedure quite well. Continuous live imaging of adult neural stem cell and astrocytes (Costa, Ortega et al., 2011, Development) revealed that rNSCs isolated from the adult brain progress through a stereotypic lineage, retaining their neurogenic nature and division pattern. The *in vitro* cultures furthermore showed that astrocytes are only insignificantly impaired in their behavior by the dissociation protocol. However, this does not rule out that changes in their transcriptome occur, such as upregulation of factors induced by the injury. We have not accurately assessed these effects in our study. However, we would like to point out that all experimental conditions compared to each other underwent the exact same treatments, assuming that the transcriptional changes imposed would be the same in each condition. Nevertheless, we cannot rule out effects at the transcriptional level by the dissection and dissociation. We have now integrated a sentence in the revised version of our discussion, in which we advert the reader to this problem, emphasizing the need to validate candidate factors by orthogonal methods such as *in situ* hybridization.

18. Page 25: Reporting rpm without specifying the centrifuge rotor used is meaningless. Please either insert the rotor type used or convert to average g-force.

Response: As the reviewer correctly requested, we exchanged rpm to g-force in the revised Methods chapter.

19. Page 37: Figure 4: In my opinion, Figure Legends should stand alone from each other and the main text: please list the molecular, morphological and positional criteria used for cell classification.

Response: We thank the reviewer for his/her comment and have carefully revised the Figure Legends, including now the molecular, morphological and positional criteria used to classify distinct cell types.

20. Page 38: "8 week old.....were administered with tamoxifen for 10x at five consecutive days". To my mind, this is a slightly odd 'definition' and appears to be lab shorthand: please correct.

Response: We thank the reviewer for his/her comment. All requested additional information can now be found in the Figure Legends and the revised Methods.

Reviewer 2:

Use also the ubiquitous astrocyte marker ALDH1L1 to identify astrocytes derived from rNSCs and from non-stem cells (fig1).

Response: We thank the reviewer for his/her suggestion. Indeed, ALDH1L1 evolved as a useful marker that is highly specific to astrocytes throughout the brain. The revised version of our manuscript now contains two new picture panels showing that rNSC-derived astrocytes express ALDH1L1 from early postnatal stages already (EV 1E). Furthermore, proliferating astrocytes labelled by cell cycle markers KI67/MCM2 all co-express ALDH1L1 (EV1F). These statements can be also found in the revised results part.

Adult neurogenesis in the DG is strongly modulated by neurovascular coupling, what about astrogenesis?

Response: We thank the reviewer for his/her comment. This is indeed a very interesting question that we just started to tackle in a new project. As the reviewer mentioned neurogenesis is strongly modulated by the vasculature. It has been shown that NSCs, which will divide, are in closer proximity to blood vessels than non-dividing NSCs. Furthermore, upon aging the decline in NSCs divisions has been also hypothesized to be, at least partly, due to decreased angiogenesis leading to the greater distance of NSCs to blood vessels. In our new study, we started out to assess if proliferating astrocytes are in closer contact to

blood vessels that non-proliferating one. So, we cannot answer this reviewer's question yet, but hopefully in the future.

scRNAseq analysis of astrocytes transcriptomic profile was done by 10X genomics which, if I'm not wrong, is able to capture only highly expressed genes, this aspect should be discussed.

Response: We thank the reviewer for his/her comment and agree that the analysis by 10x genomics may miss genes that are only weakly expressed. We are now discussing this in the text.

In the discussion, the sentence "Especially in the adult brain, astrocytes have always been considered to be postmitotic under physiological conditions" is misleading since it is known that local production of glia is a major source of astrocytes in the cerebral cortex (Ge et al 2012 already quoted in the manuscript) but also the review by Ge and Jia 2016.

Response: We thank the reviewer for his/her comment. The local production of astrocytes has been impressively demonstrated by Ge et al, however they studied the postnatal cerebral cortex and did not include the adult brain. Despite that, we agreed that the sentence may be misleading and have omitted it from the revised manuscript.

Dear Ruth,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee # 1.

As you can see below, the referee appreciates the introduced changes and have just a few minor remaining concerns. I have also looked at the manuscript and have a few comments as well:

- One of concern raised during initial review was the use of low magnification image. I think it would be good to show all independent channels as source data. I see that you do it for some figures, but not for others like Figs 3B&D.
- when you upload the source data, please upload as one file per figure
- In your point-by-point response I see that you mention a related manuscript. Any related manuscript should be provided at time of initial submission so that the editor and/or referees can look for potential overlap. Can you send me the MS file so that I can look at the manuscript?
- Also, if I understand it correctly the statement 'we observed that distinct astrocyte subtypes populate different DG layers (ML, GZ and hilus)' is based on unpublished data which we don't allow.
- we only allow 3-5 keywords
- COI should be labelled as "Disclosure statement and competing interests" please also see guide to authors
- For the EV tables, please add names to the files and add legend as a separate tab.
- The movie needs to be zipped with the legend.
- For the reagent table, please remove template instructions and unnecessary fields.
- Please double check that the manuscript sections are in the right order.
- Heading "Expanded View Figure Legends" needs adding.
- EV tables, movie and appendix table legends should be removed from the manuscript file.

That should be all. You can use the link below to upload the revised version.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Guide For Authors: <https://www.embopress.org/page/journal/14602075/authorguide>

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (15th Jun 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

<https://emboj.msubmit.net/cgi-bin/main.plex>

Referee #1:

Astrogenesis in the murine dentate gyrus is a life-long and dynamic process.
Schneider et al.

Astrocytes are a major non-neuronal cell type in the mammalian brain and their functions are now widely acknowledged to be indispensable for all aspects of CNS development, adult function and ageing. However, our understanding of astrocyte development is superficial at best. The dentate gyrus (DG) of the mouse brain is a specialized region which retains neurogenic potential into adulthood and is an excellent model system to study the processes of astrogenesis from development to ageing. In this study, Schneider and colleagues apply a variety of techniques, including genetic fate mapping, behavioral paradigms, single cell transcriptomics and *in vivo* two-photon imaging to assess astrogenesis across the life of a mouse. Key observations include (i) that astrogenesis in the DG is initiated by radial glial-like neural stem cells, with local proliferation of the resulting astrocytes responsible for enlarging the astrocyte compartment in an outside-in-pattern mirroring neurogenesis, (ii) that astrogenesis also occurs in the adult DG with the majority of astrocytes also produced by local proliferation and (iii) that local astrocyte proliferation is plastic and adapts to environmental and behavioral stimuli. Based on their findings, the authors conclude that (adult) astrocytes are plastic elements in DG circuits, implying a vital role for them in hippocampal plasticity.

This is a review of a resubmitted article. In my original review, I was enthusiastic about the manuscript and considered it a strong candidate for publication in EMBO J, subject to some textual changes. The majority of the requested changes (although not all) were made by the authors. As always, which changes are absolutely necessary and which are not is a moot point. For example, personally I am still not convinced that Fig. 1C adequately addresses the issue of temporally distinct expression of marker proteins. Irrespective of this, however, the overall conclusions of the story are sound and, in my opinion, will be of interest to the field. As such, I see no reason to unnecessarily delay publication. In fact, the authors should be congratulated on what is a very nice piece of work.

Minor points:

Before publication, the authors may want to address the following small points:

(i) Page 10: 'Even though few BrdU-incorporating cells in the SGZ expressed astrocyte markers, the vast majority of newly generated as well as proliferating cells in the SGZ belonged to the neuronal lineage. Hence, astrogenesis was only quantified within the hilus, GZ and ML of the adult DG'.

I understand the authors' general meaning but the text reads poorly. Is it necessary to say 'even though'? If the majority of new cells in the SGZ are neurons then why would you quantify astrocyte numbers?

(ii) Page 12: Fig 3C or Fig 4C?

(iii) Page 21: Competing interests: alter to 'performed without any'.

(iv) Page 25: It is not immediately clear what 'BrdU pretreatment' refers to.

(v) Page 27: The section of 'Single cell tracking' does not make sense to me as written. Which channels were used for which fluorophores? Is Channel 1 or Channel 3 used for DAPI? Does it matter?

(vi) The figure legends were extensively rewritten. However, there are some issues which need to be taken care of.

In the main, these consist of small typos (Figure 1D: 'at during DG development'?) and inconsistencies with tense (Figure 3D: 'harbored astrocyte which have(?) been newly generated'?).

However, in at least one case, I think the rewrite means the legend no longer stands alone as an accurate description of the figure. Expanded View Figure 2: In all other figures a description of the experimental paradigm is given. This is missing in EV2, which I think is an issue.

I would strongly recommend the authors to revisit all the figure legends before publication to ensure that (small) issues such as these are addressed.



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Erlangen, 24.03.2022

Dear Karin,

Enclosed please find our revised version of the manuscript "Astrogenesis in the murine dentate gyrus is a life-long and dynamic process", which we are resubmitting for your consideration for publication in *The EMBO Journal*.

We were very happy to read that both you and reviewer 1 are still convinced about our manuscript and we want to explicitly thank you both again for your careful and thorough suggestions. We changed the manuscript, the EV Tables and EV Movie as well as the source files according to your suggestions.

- Manuscript:

All changes in the text are tracked.

- As mentioned by you and reviewer 1, we carefully checked and rewrote the legends to make sure that each legend stands alone as an accurate description of the Figure. Therefore, we added experimental details and paradigms that are necessary to fully understand the Figure, and corrected small typos and inconsistencies with tense.
- We made sure that the manuscript sections are in the right order according to the guidelines of EMBO Journal.
- Only 5 keywords are now included.
- COI is now labelled as "Disclosure statement and competing interest".
- Heading "Expanded View Figure legends" was added.
- All EV Tables, Movie and Appendix Table legends were removed from the manuscript and either added to the EV Table files in a separate tab or included in a zip-file together with the EV Movie.
- We removed the statement "We observed that distinct astrocyte subtypes populate different DG layers", since it was based on our unpublished data from the manuscript from Karpf *et al* (which I send to you). In the present manuscript we instead refer to it as astrocytes localized to distinct DG layers/compartments and do not cite unpublished findings anymore.

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- We revised the text according to the reviewer' suggestion on page 10, 12, 21. On page 25 the BrdU pretreatment is now explained better and we rewrote the single-cell tracking part of the materials and method section (page 27) to clarify, which channels were used for which fluorophore, which was indeed misleading.

- Source data:

According to your suggestions, we now present single channel images for all images shown in the Figures and EV Figures in the source data files. These files are organized as one file per figure and all images are labelled according to the guidelines of *The EMBO Journal*.

- EV Tables:

All EV Tables are named and we included the legend in a separate tab.

- EV Movie:

The EV Movie can be now found in a zip-file together with the revised legend.

- Reagent Table:

All template instructions and unnecessary fields have been removed.

We hope that we could address all comments and want to thank you again for your support in publishing our study in *The EMBO Journal*.

With best regards,



Ruth Beckervordersandforth

Dear Ruth,

Thank you for submitting your revised manuscript To The EMBO Journal. I have now had a chance to take a look at everything and I appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](https://doi.org/10.31222/osf.io/9sm4x)). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➡ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➡ ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- ➡ plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➡ if $n < 5$, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- ➡ Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➡ a specification of the experimental system investigated (eg cell line, species name).
- ➡ the assay(s) and method(s) used to carry out the reported observations and measurements.
- ➡ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➡ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- ➡ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➡ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➡ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➡ definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Method section: Genotyping
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Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	NA
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Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	NA
Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if external detailed step-by-step protocols are available.	Yes	Material and Methods: Single-Cell Isolation of DG tissue: DOI: 10.1038/nprot.2011.412
Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about sample size estimate even if no statistical methods were used.	Not Applicable	Typical sample sizes were chosen in accordance with previous publications and are similar to those generally employed in the field. Sample size (number of animals) is provided in the figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, have they been described?	Yes	Material and Methods: Experimental model and subjected details
Include a statement about blinding even if no blinding was done.	Yes	Material and Methods: Quantification of total cell numbers
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not Applicable	NA
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.		
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Yes, statistical tests are appropriate. Statistical tests were chosen appropriate to sample sizes, distribution, variance and whether results had to be coreted for multiple comparison. General information about statistical tests can be found in the methods section (Statistical analysis). Detailed information about the used statistical test are stated in each figure. Following normal distribution, two-sample comparisons were conducted by using unpaired student's t-test
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Reporting

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For phase II and III randomized controlled trials , please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	NA

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Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	NA
Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	NA
If publicly available data were reused, provide the respective data citations in the reference list .	Not Applicable	NA