

Confined migration induces nonlethal DNA damage in developing neurons

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The study by Zhang et al. evaluates the relationship between neuronal cell migration (especially by cerebellar granule neurons (CGNs) during development in mice. They report that γ H2AX-marked DSBs can be observed in neural precursors and the CGNs, especially following CGNs' migration from the Inner EGL (external granule layer) to the IGL (internal granule layer). They also report the appearance of transient 53BP1-mNG-marked DSBs in CGNs subjected to confined migration in vitro. Unlike HeLa cells, CGNs did not appear to exhibit nuclear envelope rupture during confined migration in vitro. In addition, in vitro, migration through confining 3- μ m pores, but not through non-confining 8- μ m pores, induces γ H2AX-marked DSBs that are mostly resolved within 12-24 hr from inoculation. They also show that confined migration induces TOP2-DNA complexes (TOP2-cc), which are known to be eventually converted into DSBs. They report that the resolution of γ H2AX foci post-confined-migration was abrogated by the inhibition of Top2 (using etoposide or doxorubicin) but not by the inhibition of Top1 (using Topotecan).

Similarly, pharmacological inhibition of the NHEJ-mediating Lig4 (using SCR7) or DNA-PK (using Nu7441), but not of the HR-mediating Rad51 (using B02), stalled γ H2AX focus resolution. CGNs from Lig4(ND1-Cre) mice also showed delayed γ H2AX foci resolution in confined migration assays in vitro. In addition, in vivo, the accumulation of γ H2AX-marked DSBs in the IGL of Lig4(ND1-Cre) and Lig4(Gabra6-Cre) mice was easily detectable, with the ND1-Cre showing increased γ H2AX levels from P15 while the Gabra6-Cre showing increases at later stages of development (3 Mo+). No major structural cerebellar development issues were observed.

The authors then conducted END-seq to assess DSB formation across the genome in migrated vs. non-migrated cells. The authors claimed that migration showed more END-seq peaks in active introns, transcriptionally inactive, and heterochromatin LADs, and at repetitive regions (LINEs, SINEs, and LTRs). Differential gene expression analysis was then conducted to compare cerebellar tissue from control and Lig4(ND1-Cre) mice, and some potentially interesting changes were noted; however, most observations were presented here without further detailed follow-up experiments, and the usefulness of such data is debatable. Finally, Lig4-deficient mice showed elevated brain γ H2AX/DSBs, especially within the cerebellum. The Lig4-deficient mice also showed some progressive motor discoordination phenotypes consistent with the potential negative impact of cerebellar DSB accumulations on cerebellar function.

Taken together, this is a potentially intriguing, timely, and arresting study that addresses a technically challenging but important question related to the physiological induction, role, and resolution of DSBs during neuronal brain development. This study is novel and would be of interest to scientists and clinicians in the field of neurodegeneration, neuronal migration, brain development, DNA repair, nuclear organization, mechanotransduction, and possibly brain neoplasia. Overall, the methodology is valid and impressive (e.g., several in vitro migration models and in vivo mouse models) and the results are mostly well presented. That said, the study has some major weaknesses that must be convincingly addressed. Particularly, the proposed mechanisms are somewhat vague and open to various interpretations that should be addressed experimentally and clarified in the text.

Major points:

1* Overall, it is unclear what aspect of CGNs' confinement leads to increased DSB levels in the absence of NE rupture. More specifically, it is uncertain whether confined migration can both cause DSBs and facilitate their repair. Also, how the confined migration of CGNs triggers Top2-related DSBs is unclear.

For instance, etoposide/DSB-induced mechanical reshaping and invagination of the nuclear envelope promote DSB repair and is controlled by DDR kinases (PMIDs 38632359, 40527886, 40097581). Could the accumulation of DSBs during CGN migration be related to the disruption of DNA repair caused by such guided nuclear envelope remodeling, which is also especially important for TOP2-related, NHEJ-dependent repair?

On the other hand, some studies have proposed that mechanical stress can activate DDR kinases at the nuclear envelope (PMIDs 25083873, 32973141, 38088930). Therefore, could confined migration first compromise DSB repair by disrupting the guided restructuring of the nuclear envelope above, which is also accompanied or followed by a confinement-based boost in DDR signaling to restore guided nuclear restructuring and enhance the DDR more broadly for repair post-migration?

It is also possible that increased nuclear/genome contortion during cell migration alters the coiling nature of chromatin, requiring more Top2 to resolve such issues post-migration. More direct evidence for such a model would be helpful. If demonstrated, then how would such a model intersect with the mechanosensing and nuclear reshaping processes mentioned above? This is especially intriguing since the data in Fig. 4e showed that myosin and tubulin proteins, which can be involved in mechanosensing-based DSB repair, were induced by migration in wild-type but not in Lig4-deficient cells.

Overall, more experiments and clearer interpretations that better place the herein findings within the literature would be helpful in deciphering the role of confinement in inducing vs. repairing DSBs during and after migration.

2* Would the knockdown of factors that directly promote or limit CGN migration influence the induction and resolution of DSB/γH2AX foci?

3* An alternative explanation related to the data in Fig. 1b,c may suggest that DSBs are indeed acquired de novo during neuronal migration, or that DSBs induced before or early during migration may not mount a full activation of the DDR until confined migration is at a more advanced stage. How can the authors rule out the latter possibility?

4* Regarding the apparent transient formation of 53BP1 foci in CGNs undergoing radial migration, how can the authors rule out the possibility that they are simply imaging 53BP1 foci moving in and out of the imaging plane?

5* Related to Fig. 2d,e and ED Fig. 2d,e, why did the authors start the analysis at 6 hr post-migration? NHEJ is a rapid repair mechanism and can mediate the repair of most breaks in certain cell types within 1-3 hr of DSB induction. Could this be why the increase in DSB induction through the 3 μm barrier only increased the percentage of γH2AX-positive cells from the controls' 7-8% of cells to only about 20% of cells?

6* Regarding the etoposide experiments in Fig. 2, it seems intriguing/unexpected that etoposide did not induce DSBs at the 6 or 12 hr time points (e.g., Fig. 2j,k). Please clarify why that may be the case and precisely what drug dose was used, as well as how the treatments were performed (the text, methods, and legends were insufficient). Also, an essential missing control to add here would be to conduct these etoposide treatment experiments after allowing the cells to migrate through the non-confining pores (i.e., 8 μm). As shown, one cannot evaluate the level of overlap/interaction between etoposide treatment and confined migration.

Additionally, as the authors appear to propose repair mechanisms focused on the rapidly acting NHEJ pathway, the relatively slow kinetics of γH2AX resolution shown (i.e., ~12-24 hr) suggest the involvement of slow-progressing repair mechanisms, at least in part. Please address/clarify this point.

7* Regarding the use of small-molecule drugs to inhibit various proteins/enzymes, experiments are needed to confirm that the intended target has indeed been efficiently and significantly inhibited. This is especially important when the drug treatments are claimed not to influence γH2AX foci resolution post-confined-migration, as is the case with Topotecan (Top1) and B02 (Rad51).

8* There is a need to assess the relative degree of overlap or genetic interaction of Top2 with LigIV or DNA-PK and of Top2 with Rad51 in γH2AX focus resolution after confined migration. This is necessary to confidently place Top2 and NHEJ factors in the same post-confinement DNA repair process studied here.

9* Regarding the use of Lig4(ND1-Cre) and Lig4(Gabra6-Cre), add data showing the protein expression level of Lig4 at different stages (covering P4 to 12 mo), ideally via imaging. This is necessary to ensure the accurate interpretation of a large fraction of the results.

10* It is again very odd that the Lig4(ND1-Cre) CGNs did not show a higher level of γH2AX foci at 12 hr (e.g., Fig. 3c); please explain. Also, missing and necessary are more timepoints and the addition of control conditions where the cells are migrating through non-confining pores.

11* Based on the data shown, one still does not know the exact effect of disrupting NHEJ/Lig4 on the levels of DSBs in

migrating CGNs at the developmental stages where damage is usually naturally induced and repaired (as shown in Fig. 1b). For instance, why are the P4-P8 stages not shown in Fig. 3d? Can it be done, or does the Lig4(Nd1-Cre) not allow for testing the effect of Lig4 deficiency at the P4, P6, and other early developmental stages, limiting the analysis to P15 and beyond (Fig. 3d)?

12* More evidence is needed to better support the claim that NHEJ is directing DSB repair in CGNs during/after confined migration. To more directly confirm that DNA repair during or after confined migration is conducted by NHEJ, staining for at least one NHEJ (Ku70) and one HR (Rad51, RPA, CtIP) protein should be conducted on migrating CGNs/developing brains. For example, would the localization of Ku70, but not Rad51, correlate with γ H2AX as shown in Fig. 1a,b?

13* Regarding the End-seq experiments in Fig. 4, why were CGNs migrated through non-confining pores not included here as controls instead? Wouldn't these be more appropriate? Is it possible that comparing CGNs subjected to confined migration to other CGNs that did not undergo any migration at all may have even masked some of the potential/expected differences?

14* It is difficult to claim that the transwell is significantly higher vs. the control for the active introns, transcriptionally inactive and unlabeled euchromatin, and the heterochromatin LADs, based on the data in Fig. 4b. Possibly, the only substantial difference this reviewer sees between the control and the transwell is that the transwell has substantially fewer peaks compared to the control at active promoters. At the very least, the interpretation of such results should be toned down, and their limitations should be more thoroughly discussed to better reflect the data. This may eliminate the need for additional experimental replicates that would allow for more robust statistical analyses. Similar concerns apply to Fig. 4c and ED Fig. 5b,c.

Also related to this point, and in the absence of substantiating statistical analyses, why is the slight difference in ED Fig. 4d dismissed, but similarly minor differences were taken at face value in Fig. 4b,c, and ED Fig. 5b,c?

Similarly, on line 336, the statement that "Although overall differences between control and Lig4ND1-Cre were not huge," what does that mean? Consider using more scientific and accurately interpretable descriptions of the results.

Overall, more care should be taken to ensure the accurate interpretation of the results. The discussion section should then also be revised accordingly (e.g., lines 423-427, which refer to the issues mentioned right above in this point).

15* Related to the DEGs heatmap in Fig. 4e, please clarify what is shown exactly. For instance, Nrep is upregulated in the control and downregulated in the Lig4ND1-Cre compared to what exactly? The text, legends, and methods were not clear enough.

Additional points:

16* Line 73: In "We first observed DNA damage in the developing cerebellar cortex at postnatal day 6 (P6) by immunofluorescence for γ H2AX", replace "first observed" with "first studied", since DNA damage is also present at P4 in Fig. 1b.

17* Line 80: In "a significant number of γ H2AX foci were", remove "significant" since no statistical analysis is conducted or presented in Fig. 1a.

18* Line 87: Regarding "DSB repair occurs in a few hours in normal cells", please note that the kinetics of DSB repair can be anywhere from a few hours (as noted here by the authors) to a day or a couple of days depending on the precise nature/position of the DSB and the type of DNA repair mechanism involved. Therefore, this sentence, at this stage of the manuscript, can be misleading and should be revised with more accurate language. For example, classical NHEJ is generally faster than HR, and HR subtypes can also vary significantly in terms of repair kinetics.

19* Lines 155-159 and 415-417: Related to the repeated statements throughout the paper that cancer cells commonly undergo nuclear envelope rupture under mechanical stress, this is not accurate. For instance, cancer and non-cancer cells treated with etoposide undergo a rapid cytoskeleton-dependent nuclear reshaping process that exerts considerable mechanical force on the nucleus, dramatically reshaping it (PMID 38632359). Yet, under such mechanically stressful conditions, nuclear rupture was reported to be very rare, and such nuclear reshaping was found to promote DSB repair. Nuclear envelope invaginations and deformations without rupture were also observed in cancer cells treated with various DNA lesion-inducing agents, such as PARP inhibitors (PMIDs 38632359 and 40527886). Even studies that more dramatically stressed the nucleus of wild-type cancer cells, only a small percentage of cells (~10%) exhibited nuclear envelope rupture (PMID 37832547). So, at the most, one may postulate that NE rupture is rare in cancer cells and may be even more rare in neurons. Finally, related to the data in Fig. 2a,b, what percentage of HeLa cells and granule cells exhibit rupture within the in vitro confined migration assay used here?

20* Lines 208-209: The sentence here is somewhat unclear. Clarify if this sentence is meant to state that DSBs in this context are thought to result from mechanical strain on the chromatin structure by the authors, or by the field more broadly, and according to which prior studies/citations.

21* Line 227: Should the sentence "Neither drug altered DSB formation in transmigrated CGNs at 6 h and 12 h." have a callout to Fig. 2j?

- 22* Line 237: Update the figure callout from Fig. 2d to Fig. 2e,f to better match the sentence.
- 23* Line 244: In "decay of γ H2AX foci", change "decay" to "resolution".
- 24* Line 275: In "another Lig4 deficient mice"; "another" is singular, but "mice" is plural.
- 25* Lines 283-285: Regarding "These results suggest that mechanostress during confined migration is a primary trigger of DNA damage in juvenile CGNs during development, rather than other stressors like transcription or neural activity", this statement is not supported by the data at all and should be removed.
- 26* Line 345: In "for the genes showed", add "that" before "showed".
- 27* Line 387: In "may affect to motor coordination", remove "to".
- 28* Line 423: Correct "mechanostess" to "mechanostress".
- 29* Quantification of the data in Fig. 2a-c with statistical analysis is missing.
- 30* Fig. 4 is more of an FYI-type figure and the authors should consider making it an Extended Data figure coupled with a shortening of its related description in the results section.
- 31* For ED Fig. 7a,b, the figure panels indicate 3 and 12 mo, while the legend states that 6 and 12 mo. Please correct/clarify.
- 32* The references cited fall short on covering the minimum set of studies related to the relationship between mechanical stress, the nuclear envelope, the DNA damage response, and DSB repair. Some of these studies have been mentioned throughout this report.
- 33* The title could be more informative; for example, why should the reader care that "Mechanostress-induced DNA damage in migrating neurons during brain development" is reported here?
- 34* Once the issues raised in this review have been addressed, the abstract should be revised to ensure that only fully supported claims are mentioned. For instance, lines 33-34, this reviewer is not convinced that "Genome sequencing revealed that DSBs tend to occur outside of protein-coding regions and transcription regulatory regions", considering the very mild differences and absence of statistical analyses.
- 35* The statistical analysis for most data appeared to be appropriate, but there were a few issues. Why is a t-test used for multiple comparisons in Fig. 2h? What statistical information can be presented to corroborate the conclusions drawn based on data such as those in Fig. 4? Additionally, the presentation of the statistics at the end of the legend of ED Fig. 2 was confusing, and the text was misplaced (e.g., which statistical tests were conducted in ED Fig. 2d,e?).

Referee #2

(Remarks to the Author)

The authors have submitted a well-written manuscript describing the novel finding that migration of neurons in the cerebral and cerebellar cortices during development results in increased DNA damage, which is repaired by non-homologous end joining (NHEJ) pathways. Using complementary *in vivo* and *in vitro* assays, the authors further postulate that the DNA damage results from confined migration and is mediated by topoisomerase II-beta (Top IIb). Additionally, they suggest that inhibiting DNA damage repair by deleting DNA ligase IV leads to mild changes in gene expression and motor defects.

The data supporting increased, transient DNA damage in the neurons are convincing; in contrast, some of the authors' other conclusions, including that the *in vivo* DNA damage is caused by nuclear deformation and mediated by Top IIb, currently appear somewhat preliminary and not sufficiently supported by experimental data. Several experiments lack essential controls and/or statistical analyses to rule out alternative explanations.

The authors need to address the following concerns:

1. The authors argue that the observed DNA damage results from nuclear deformation during confined migration. While this argument is reasonable and consistent with previous studies in various other *in vitro* and *in vivo* systems, experimental support for this conclusion is limited, and the authors do not sufficiently address other explanations. For example, based on the Ki67 staining in Fig. 1a, it appears that some of the migrating neurons might still be capable of proliferation or replicating their DNA, which could result in DNA damage, particularly in the presence of topoisomerase inhibitors in later experiments. The authors should include additional controls to demonstrate that the observed DNA damage in migrating occurs in the absence of DNA replication. In addition, immunofluorescence images should include separate channels for each fluorophore, in addition to the merged color channel, to allow for better assessment of individual signals.
2. Related to the above concern, another explanation is that the DNA damage is associated with migration *per se*, regardless of nuclear deformation, for example, by increased mitochondrial activity and production of reactive oxygen species. Most experiments (e.g., those described in Fig. 1e) lack controls for cells migrating through larger channels that do

not require nuclear deformation, and most transwell assays compare cells that migrated through 3 μm pores to cells that did not migrate at all. The authors should instead include comparisons to cells that migrated through larger 8 μm pores, which do not require substantial nuclear deformation, to determine whether the observed DNA damage is specific to cells migrating through small pores. This applies to both the measurements of DNA damage itself and the subsequent experiments to determine the underlying mechanism (e.g., Fig. 2j-k, etc.).

3. Several reported findings lack sufficient quantification and/or statistical analysis. For example, in vivo image sequences of DNA damage (Fig. 1d) lack quantification and comparison to cells in different zones, giving them an anecdotal appearance without robust documentation. The same applies to the images shown in Figs 1e, 2c, Ext. Data Fig. 1e,f, and many other figure panels. Furthermore, the authors should clarify whether they performed 3D analysis of these cells to rule out that the appearance of new 53BP1 foci does not result from the movement of existing foci into the focal plane, rather than new DNA damage.

4. The authors state that the DNA damage clustered around a single spot of Ku70 (Ext. Data Fig. 2f). However, the co-localization is not particularly convincing, given that the nuclei also contain many Ku70 foci without gH2AX staining, and the large number of Ku70 foci makes overlap, even if by chance, very likely. The authors should perform a more thorough analysis to support this claim.

5. It appears that the authors included etoposide for all experimental conditions in their END-seq analysis, which could substantially confound the experiments. The authors should include transwell migration experiments in the absence of etoposide, and, as stated before, include controls with larger pores. The argument that etoposide is needed to trap Top2b at the cleavage site is not sufficient, as alternative approaches are available.

6. The authors state confined migration results in an enrichment of dsDNA breaks in introns, transcriptionally inactive euchromatin, and LADs, referring to Fig. 4b. However, based on the graph in Fig. 4b, it does not appear that the transwell results differ statistically from the controls in those regions, and the graph (as well as several others, such as the one shown in Fig. 4c) lacks a statistical analysis and comparison between groups.

7. To determine the functional consequences of preventing DNA damage repair by deleting Lig4, the authors perform bulk-RNA seq and confirm some results by immunofluorescence analysis. However, neither approach provides cell-type-specific information, and the immunofluorescence analysis does not include markers to identify specific cell types. Thus, it appears unclear if any of the detected changes relate to neurons with DNA damage.

8. The authors speculate that the lack of DNA damage repair might induce some broader inflammatory responses. The authors should support this interpretation by performing immunofluorescence labeling for inflammatory markers in the tissues, particularly at early time points in the Lig4 NDI-Cre mice.

9. Representative images of nuclei with DNA damage or other markers often show a single nucleus, making it difficult to determine how generalizable the findings are. The authors should include less-cropped images that show multiple cells and nuclei, and, if necessary, display the zoomed-in nuclei as insets or additional panels.

10. As mentioned earlier, all immunofluorescence images should include separate channels for each fluorophore, in addition to the merged color channel, to allow for better assessment of individual signals.

Minor comments:

11. The authors state that confined migration-induced DNA damage, occurring independent of NE rupture, is distinct from previous findings in cancer cells. However, previous studies had already reported that some cancer cells, such as MDA-MB-231 breast cancer cells, along with some non-cancerous cells, can exhibit DNA damage caused by nuclear deformation even in the absence of nuclear envelope rupture (e.g., Shah et al., Curr. Biol., 2021, PMID 33326770; Nader et al., Cell, 2021, PMID 34551315). The authors should acknowledge this previous work. The Shah et al. publication might be particularly relevant to the authors findings, given that it reports increased replication stress caused by nuclear deformation, which might further support the idea of a role of topoisomerase in the DNA damage formation.

12. The authors provide information on the width of their microfluidic channels (e.g., those used in Fig. 1e) but not on the height of the constrictions, which is important to determine the nuclear deformation.

13. The authors should add a label to Fig. 3d to indicate what is stained here. Presumably, this is gH2AX?

14. The analysis in Ext. Data Fig. 3c should include a counterstain for nuclei and quantification.

Referee #3

(Remarks to the Author)

In this article, Zhang and colleagues investigate the impact of the migration of neuronal cells in the developing brain, on the integrity of their DNA. They find the presence of large amounts of DNA damage in several types of migratory neurons, and they nicely show, in the more specific system of cerebellar granule neurons, the focus of their study: that DNA damage is found in post-mitotic neurons in a region that can be reached only after migration and strong nuclear deformations, suggesting that DNA damage was induced by migration and mechanical deformation of the nucleus. They also use in vitro

systems of microchannels and transwells to confirm the induction of DNA damage by constricted migration, and to dissect the mechanism that leads to this damage. They find very little nuclear envelope ruptures in migrating neurons and they propose that the main driver of DNA damage in this system is topoisomerase 2, which gets recruited to DNA upon nuclear deformation and could generate such DNA damage. They investigate in detail, with all the up-to-date tools, whether there is a specific type of DNA damage induced by neuronal migration, and they find no specific sites, but on the contrary, even less specificity than the type of damage produced by Topoisomerase II, suggesting that nuclear deformation could induce action of this enzyme in unusual regions due to mechanical stress on the chromatin. They then use some genetic tools in mice to investigate the potential impact of this DNA damage for brain development, when it is not repaired, comparing mice that have a defect in repair occurring either before or after the neuronal migration stage. They find significant by mild defects and they are very careful about their conclusions.

Overall, the article is very well written, clear and mostly convincing. I agree with the authors that there is so far little study of the implication of mechanically induced DNA damage in developmental contexts, while it is more and more clear that cell migration, as well as other types of constraints, induce DNA damage in a large variety of contexts, including during immune response and cancer. Their work thus represents a very significant advance, including a novel mechanism for mechanically-induced DNA damage.

Before listing my concerns, I want to first disclose that I am not a specialist of neuronal development and to apologize in case some of my concerns are based on misunderstandings. The authors should not refrain from contradicting me in case I did not understand correctly some aspects of their work, especially related to the biological system and the associated specific techniques.

My main concerns, which I hope the authors can address in a revised manuscript are:

1) In several instances, important experiments are shown only via images of a single representative cell. This is the case for Fig. 1d,e, Fig. 2a-c. I understand that Fig. 1d might be hard to quantify, but the authors should at least tell (maybe in the figure legend) how many cells they observed and how many times the experiment was done. For Figs 1e and 2a-c, it is less understandable because they are done *in vitro* and quantification should be easy: what % of cells showed the phenotypes, how many cells are observed, and how many experiments? In Fig. 2d,e, the authors nicely show the quantification, so it should be possible to do it also for the other experiments, that are also important, even if they are meant to show the absence of nuclear envelope rupture.

2) I feel that some important controls are missing (but here I might have misunderstood some aspects of these experiments): in the experiments with the inhibition of TOP2 by various drugs, the authors see a long-term maintenance of the DNA damage after the transwell migration, and they take that as proof that TOP2 is responsible for the DNA damage produced during the migration. This is actually I think the only result they show to support the role of TOP2 in generating DNA damage during constricted migration. I must say that for me the reasoning is not obvious. I understand why TOP2 could be a candidate (it is well explained in the discussion of the article), but why inhibiting TOP2 would lead to a sustained DNA damage after the migration, without changing at all the amount of DNA damage caused by the migration is less obvious to me. DNA damage is not affected at all at the 6-hr timepoint upon etoposide (or doxorubicin) treatment. One way to clarify the reason for sustained DNA damage at long times would be to show the data for non-migrating cells (the control with the 0.4 microns constrictions, or just on a simple plate, as the authors do for the experiments in Fig. 4). This would tell whether there is just a background level of DNA damage induced by the sustained inhibition of TOP2, leading to an elevated fraction of γ H2AX positive cells even at long times. This is what is usually observed (and I think it is also the case in Fig. 4) when cells are treated with etoposide or doxorubicin, even when they are not migrating at all. What is hard to explain, I agree, is that this level seem the same as the level induced by migration, while it could also be different, so it is tempting to interpret as the authors do, that it is a 'fixation' of the migration induced DNA damage. Nevertheless, I think that the control of non-migrating cells is important here, as well as in Fig. 3 for the role of the NHEJ pathway in the repair, and for the same reason.

Even if maybe unnecessary here, it is often clearer to analyze the number of foci, in addition to the number of positive cells, because 'positive' is not very quantitative and here it might be important to be quantitative to distinguish the effect of the inhibition of repair versus a constant production of additional damage due to the drugs. When possible, it is even better to follow the individual foci and quantify their rate of appearance and their lifetime, which is really the most direct way to estimate the rate of new DNA damage and the repair efficiency. But this might not be feasible here in conjunction with the transmigration assay.

I am also wondering, for the same reason of interpreting this key experiment, whether it could be interesting to combine for example the etoposide treatment with the disruption of the NHEJ pathway, in the cells undergoing migration in the transwell assay. If the maintenance of DNA damage for longer times in etoposide is due to constant production of DNA breaks in this context, and constantly repaired, then inhibiting the repair pathway should lead to accumulation of more DNA damage in time, and this would contradict the interpretation of the results from Fig. 3i-k proposed by the authors; While if there is no accumulation of DNA damage post-migration, then it would argue for the fact that all the damage was done during the migration step.

3) I am not sure of the feasibility of the experiment I propose here, as I am not an expert of brain development: are there pools of neurons that do not significantly migrate (and do not proliferate) at a similar stage of development? Would it be possible, if they exist, to then produce similar experiments in such neurons (for the mice experiments) and check whether there is DNA damage in these neurons or not during development and whether the Lig4 mutants show any sort of defects? This would also be a nice control for the specific impact of migration on DNA damage.

Some minor comments:

1) Line 76: When the authors state that many Ki67 positive cells are also positive for gH2AX, did they check that these are not in mitosis? Mitotic cells are usually positive for gH2AX even in the absence of DNA damage.

2) In many cell types, the confinement size defines the amount of nuclear envelope rupture (NER) observed; even in cancer and immune cells, at 3 micron, the fraction of NER is not so high, compared to 2 microns for example, where a majority of cells display NER. Here the authors mostly investigated the 3-micron constriction size. It would be interesting to know whether migrating neurons are totally proof to NER, or if, like other cells, they mostly display NER at 2-micron constrictions. Then another question would be whether there are contexts in which such deformations can be seen in neurons in development. It does not change the message of the authors here, because they show that at 3 microns, they observe DNA damage and no NER in neurons, so their reasoning holds. But for the cells in vivo, it is maybe less clear that there is no NER at all (the authors show no evidence for that), especially if cells in vivo undergo deformation of nuclei down to 2 microns. Maybe they could give some numbers for the typical nuclear deformations they observe in the developing brain? And at least discuss this limitation of their work more directly. It is also in general complicated to rule out NER, because it is a transient event lasting just a few min, that can in turn cause DNA damage for long periods of time (hr). I think the authors might be right that is mostly TOP2-induced DNA damage in the brain, because it would occur for less nuclear deformation and in the absence of NER, but since they actually show no direct proof of that and instead propose this hypothesis as a conclusion from their in vitro experiments, they could maybe leave open the possibility, in their discussion, that stronger nuclear deformations could also cause NER and DNA damage in neurons (except if they add new experiments to show that this is not the case).

Referee #4

(Remarks to the Author)

In this study, Zhang et al. aim to study DNA damage induced by mechanical stress due to migration of neurons during brain development.

The authors show that newly-born neurons show immunoreactivity to gH2AX in relation to migration. In cerebellum, gH2AX immunoreactivity is common in the outer EGL where cells proliferate, but then is uncommon in migrating cells in the inner EGL, but then is more common in migratory cells in the ML, and then in post-migratory cells in the IGL. They interpret this spatial correlation as suggesting that DNA double-strand breaks (DSBs) due to mechanostress occur during their migration. They perform live imaging after electroporation of a fluorescent reporter (53BP1-mNeonGreen) that monitors p53 activity, which accumulates at DSBs, and provide some limited movies suggesting increased fluorescence when cells traverse narrow channels. Videos are provided that show increased 53BP1-mNeonGreen signal in migrating cells, but some of the videos are more convincing than others, and they are presented as examples without quantification. Some sort of quantitative analysis of the relation of 53BP1-fluorescence in relation to migration in vivo or in relation to narrow channels would strengthen their argument. These transient breaks are effectively repaired as part of normal brain development, without leading to significant defects in the mature brain that can be detected with gH2AX staining later in life.

Interestingly, unlike cancer cells that often undergo nuclear envelope rupture, cerebellar neurons form DSBs through a TOP2 mechanism that helps relieve torsional stress in DNA. They establish by using a transwell assay that deforms the nuclei, that there is increased covalent binding of TOP2 to DNA (TOP2cc) after transiting the micropores in the transwell, which then decreases with time as the 53BP1 signal also decreases.

The authors used gH2AX to confirm DSBs, and showed that gH2AX staining was slower to disappear in the presence of inhibitors of NHEJ, or in Lig4 conditional mutants, which have a defect in NHEJ.

The group further investigated the locations where these DSBs occur and found that they tend to form in transcriptionally-inactive chromatin and non-coding regions, which may suggest safe zones that minimize impact on genome stability and function. Their transcriptomic analysis of Lig4ND1-Cre mice, which have defective DSB repair, showed only minor changes in gene expression. These changes typically involve downregulation of genes related to neuronal function and an upregulation of those associated with stress responses, but they do not appear to result directly from DNA breaks at crucial regulatory regions. This study suggests a connection between persistent DSBs and mild inflammatory responses in brain tissues, possibly contributing to subtle changes in neuronal gene expression seen in certain mouse models. These findings align with the idea that migration-induced DNA damage mainly affects less critical genomic regions, thereby preserving overall neuronal function despite these perturbations. They also see few morphological defects in the Lig4ND1-Cre mice, suggesting that the DSBs accumulated during migration don't cause major abnormalities.

So in summary I find this to be an interesting and very well done study. I find it convincing that DNA breaks are induced in migrating neurons, apparently by nuclear deformation as the neurons migrate close spaces. On the other hand, the functional impact of these DNA breaks appears to be modest and so their greater significance is less clear. The breaks appear to be repaired accurately, and so that even in the presence of defective DSB repair (Lig4 conditional mutant) there are few defects at the cellular or organismic role.

I have a few technical considerations:

1] Adding western blot images will help in understanding how consistent the levels of gH2AX detected across different

experimental conditions and setups (in vivo vs. transwell) are. Assessing reproducibility ensures the reliability of gH2AX as a marker for DSBs (for example, Fig. 2b shown in this study: https://www.researchgate.net/figure/Radiation-induced-DNA-damage-A-DNA-double-strand-breaks-were-visualized-with_fig1_328227387).

2] It would be helpful to provide some sort of quantification of the videos of 53BP1-mNeonGreen in migrating cells, rather than just show a few examples.

3] They examine long-term behavioral effects of persistent DSBs in the Lig4 ND1-Cre mice, and see only modest effects, albeit likely referable to the cerebellum, which is where most of the unrepaired DSBs are found in these mice. They attribute these effects to effects of unrepaired DSBs. Given LIG4's known functions (primarily in NHEJ repair), it is reasonable to attribute these behavioral disorders to some defect in DSB repair. However, we don't really know from these experiments whether the non-repaired DSBs are caused by the developmental mechanism of neuronal migration. It is also possible, perhaps even to my mind more likely, that the progressive, age-related phenotypes reflect some unrelated formation of DSBs in neurons through their lifetime (for example, in the DSBs that are claimed to occur in relationship to gene expression). It would seem to be plausible that this might occur at a level sufficiently low as not to be captured by the gH2AX staining that they perform at later developmental stages. So that while the phenotypes likely relate to unrepaired DSBs, I am not convinced they relate strictly to unrepaired DSBs that originate during neuronal migration, which is the focus of the argument.

Minor comments:

Fig. 1b,c shows DNA damage in P4 itself, but it is mentioned that the authors first observed DNA Damage at P6?

Line 875: A word is missing. I think it should read "Once they had a firm [grasp], then they were slowly...."

Referee #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

Overall, the authors have conducted thorough revisions and have satisfactorily addressed most of my original comments and concerns. I commend the authors on conducting a substantial and genuine revision. This remains a technically challenging and impressive study, and the reviewer's enthusiasm for it remains.

That said, some responses to my original concerns were partial or unsatisfactory, requiring further revision and clarification. In particular, the manuscript still lacks clear mechanistic coherence because it remains unclear how confined migration-induced TOP2cc/DSBs intersect with DDR kinases and the nuclear envelope in the short term, and with genome instability and brain/animal function in the long term. The manuscript may be considered suitable for publication if these significant weaknesses and other points below are convincingly addressed.

Specific points:

* In the longer term, it remains unclear how the early onset of DSB accumulation in migrating CGNs contributes to elevated DSB levels later (up to 12 Mo) and whether the initial DSB accumulation in migrating neurons caused the defects observed in animals. This might also explain why it appears difficult to find a title that captures the study's overall functional significance.

* In the shorter term, the authors present new data showing that the % of cells positive for NE tubules/invaginations increases following confined migration, phenocopying 50 μ M ETO treatment in their system and suggesting that this mechanotransduction-based DSB repair mechanism remains functional/activated and thus not disrupted in constricted/migrating neurons. The authors' conclusion here is logical and plausible.

However, the authors also show that treatment with nocodazole prevents tubule formation without inducing detectable increases in the % of DSB-positive cells at 12 h or 24 h. It is also claimed that ATM inhibition, which can disrupt NE tubules, did not increase DSB levels. The nocodazole results are suggestive, but it is difficult to assess the effects of such a microtubule-disrupting drug, especially in neurons, which are highly dependent on microtubules and motors for the transport of critical cargo throughout the cell body. In addition, the cited studies show that NE tubules depend especially on DNA-PKcs and ATM and contribute mainly to NHEJ-dependent repair in mitotically dividing cells. However, DNA repair processes in postmitotic neurons are generally more dependent on DNA-PKcs than ATM.

Therefore, it is likely that DNA-PKcs inhibition disrupts the NE tubules induced by confined migration in neurons. This should be addressed experimentally by quantifying both the % of cells with NE tubules and possibly the surface area of the

nuclear envelope. It would also be informative to disrupt in CGNs (or possibly in a surrogate and more tractable neuronal system) the LINC complex or other factor(s) that are reported to be important for tubules/invaginations and DSB-NE interactions in mitotically dividing cells before measuring tubules/invaginations and DSB levels using 53BP1 and gH2AX (% of cells, foci numbers, and more intermediate timepoints). Such mechanistic clarifications could strengthen the coherence of the authors' model, in which confined migration phenocopies TOP2 inhibition, leading to TOP2cc and activating NE tubules/invaginations-dependent NHEJ repair, thereby facilitating repair without NE rupture. Moreover, this could explain why the END-seq data revealed that confined migration yielded the largest increases in absolute DSB peaks at heterochromatic LADs, thereby potentially establishing a stronger connection between Fig. 4 and the remainder of the manuscript. Alternatively, the authors may present other observations to fill the gaps and establish mechanistic coherence.

* Related to Fig. 4: First, the text section related to Fig. 4 and its Extended Data is of potential interest, but still appears to be out of place. I suggest significantly shortening this text section, moving 1-2 small panels to another main figure, and moving the remainder of Fig. 4 to the Extended Data. Second, the statement regarding Fig. 4b, that confined migration shifts DSBs from promoters toward heterochromatin at LADs and non-LADs, does not appear to be statistically incorrect but is somewhat misleading. In terms of the largest increase following confined migration, it appears to be primarily at heterochromatic LADs. The text should be revised to clarify this point. Third, the conclusion at lines 549 to 552 is not convincingly supported and should be removed. Overall, this section stands out as being relatively weak compared to the remainder of the study. It might even be worth considering removing it, unless the heterochromatic LADs phenotype becomes relevant and can be better integrated into the paper's main mechanism and message.

* Lines 520-522: Including the key references intended here again would ensure clarity for the readers: "These results suggest that DSBs generated during confined migration are topologically and functionally different from the typical TOP2 β hotspots (?references?), which include regulatory elements and chromatin loop anchors."

*Related to the discussion:

- The discussion was too wordy and inefficient.

- The authors begin this section with the following text: "In this study, we demonstrate that newly born neurons inevitably undergo DNA damage by mechanostress during migration. The transient DSBs are faithfully repaired during normal brain development without causing obvious defects in the mature brain." This is neither effective nor sufficient. The discussion should begin with a paragraph that presents a model coherently linking all key elements of the study.

- Lines 617-620: "Recent studies have demonstrated that phosphorylation of LMNA by DSB-activated ATR induces lamina disassembly and NE rupture, suggesting that low Lamin A expression in neurons may contribute to the NE robustness." That statement is illogical and inaccurate. DSB-induced LMNA phosphorylation destabilizes the LMNA, leading to rupture. In cells where LMNA expression is already "low", rupture would be expected to become more common, not less common. Therefore, it is much more likely that the absence of rupture is linked with the increased ability of cells to tolerate NE deformation and form NE tubules under DNA-PKcs control, though this remains to be experimentally tested in the experimental systems used by the authors.

* Related to the manuscript's title:

- This revised title still does not clearly convey the functional significance of the authors' observations.

* Other comments:

- The general text had a good reading flow except for the results section related to Figure 4 and the discussion.

- The figure legends were concise and clear, and included appropriate statistical analyses.

- The methods and references were improved.

- The data were of a higher quality, and the higher resolution images were helpful.

Referee #2

(Remarks to the Author)

The authors are commended on the thorough revisions and additional experiments they have performed to address the reviewers' comments. The revised manuscript addresses all my previous comments. There are only a few minor items the authors should address to further improve the manuscript.

1) The authors should consider including the overview of the cerebellar structure, shown currently in ED Fig. 1a, as part of Fig. 1 to illustrate where the outer/inner EGL, ML, and IGL regions referred in Fig. 1 are located. This would make the figure more accessible to a broader readership.

2) The supplemental videos using fluorescent markers should include labeling of the different color channels in the videos themselves to make it easier to interpret them. This was done for some of the videos but omitted in most other videos.

3) In several sections of the text, the authors refer to “micropatterned substrates” when referring to the microfabricated constriction channels. This term is confusing and potentially misleading, as micropatterning typically refers to patterned deposition on 2D surfaces. Assuming that the experiments were conducted on substrates with 3D constrictions, as indicated in ED Fig. 1g, the authors should revise their terminology accordingly.

4) The supplementary materials appear to be missing descriptions of the supplementary videos.

Referee #3

(Remarks to the Author)

In their revised article, the authors have provided a large number of additional experiments and, importantly, have now robustly quantified the phenotypes that they report. Although, due to the complexity of the system, they still do not have a very direct proof that in vivo, it is really the nuclear deformation associated to neuronal migration that causes the observed DNA damage, they now better acknowledge this limitation. I think the article, with the combination of in vitro and in vivo systems, and the clever mouse models to assess the impact of the DNA damage occurring at this specific stage of development, makes a strong and original contribution; I would thus recommend publication of this very nice article.

Referee #4

(Remarks to the Author)

The authors have conducted more live-imaging experiments in organotypic slice cultures and acquired additional results showing de novo DSB formation in migrating CGNs and cortical neurons. The authors have repeated transwell assays using 3 μ m and 8 μ m pores, confirming that confined migration leads to DSB formation rather than interfering the repair of DSBs generated independently of confined migration. They have added additional data using the END-seq protocol to identify specific sites of DSBs in developing GCNs. These newly added data address our major concerns.

Minor Points

- In the newly added scRNA-seq analysis of cerebellar cell types, the authors need to specify clearly if no other filtering was performed. Include the R version, Seurat sub version for better reproducibility. Along with this, please add what type of normalization was used.

- Line 1429: Each category represents what (e.g., promoter, enhancer, intergenic...)? Please clarify.

- Line 542: Which “are” primarily expressed.

Conclusions

The newly added γ H2AX western blot analysis helps clarify the “transient” increase in the gH2AX signal after confined migration.

Live imaging quantification using improved resolution and measuring the occurrence rate by pore diameter helps quantify the cell passage rate and DSB occurrence rate better.

Referee #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

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Referee #1

Thank you for giving us the opportunity to submit a revised manuscript. We really appreciate your thorough and thoughtful consideration of our manuscript. Your review has provided many helpful suggestions, which have greatly improved our paper. In response to these comments, as well as those of the other referees, we have added several new analyses and experiments. Major revisions are as follows:

1) With high resolution imaging of the nuclear membrane and additional transwell assays, we have reinforced the demonstration that nuclear migration through confined spaces induces DSB formation rather than delaying or hindering the repair of DSBs by interfering nuclear envelope dynamics.

2) With additional Top2cc assays and transwell assays, we have solidified the evidence that confined migration induces the accumulation of stalled Top2-DNA cleavage complexes (Top2ccs), which are removed by proteasomal degradation and subsequently religated through the NHEJ pathway.

3) More live imaging analyses have been performed for quantitative assessment of dynamics of 53BP1 foci formation and nuclear envelope rupture during confined migration in vivo and in vitro. We have reinforced the evidence that migrating neurons generate DSBs independent of nuclear envelope rupture and nuclear entry of cytoplasmic nucleases, mechanisms that have been demonstrated in multiple cancer cells.

4) the END-seq analysis has been replaced with new data comparing the patterns after confined and non-confined migration without etoposide treatment with statistical analyses.

We hope that these as well as our point-by-point responses below will answer any uncertainties and be to the satisfaction of the Referee.

Taken together, this is a potentially intriguing, timely, and arresting study that addresses a technically challenging but important question related to the physiological induction, role, and resolution of DSBs during neuronal brain development. This study is novel and would be of interest to scientists and clinicians in the field of neurodegeneration, neuronal migration, brain development, DNA repair, nuclear organization, mechanotransduction, and possibly brain neoplasia. Overall, the methodology is valid and impressive (e.g., several in vitro migration models and in vivo mouse models) and the results are mostly well presented. That said, the study has some major weaknesses that must be convincingly addressed. Particularly, the proposed mechanisms are somewhat vague and open to various

interpretations that should be addressed experimentally and clarified in the text.

Response: We sincerely appreciate positive and encouraging comments by the Referee.

Major point 1-1: Overall, it is unclear what aspect of CGNs' confinement leads to increased DSB levels in the absence of NE rupture. More specifically, it is uncertain whether confined migration can both cause DSBs and facilitate their repair. Also, how the confined migration of CGNs triggers Top2-related DSBs is unclear.

For instance, etoposide/DSB-induced mechanical reshaping and invagination of the nuclear envelope promote DSB repair and is controlled by DDR kinases (PMIDs 38632359, 40527886, 40097581). Could the accumulation of DSBs during CGN migration be related to the disruption of DNA repair caused by such guided nuclear envelope remodeling, which is also especially important for TOP2-related, NHEJ-dependent repair?

Response: Thank you for indicating important papers demonstrating that DSBs generated by drugs or ionizing radiation induce nuclear envelope (NE) invagination toward the damaged sites, promoting DDR through the recruitment of the NE-associated repair factors. Using CGNs, we have reproduced the observation that DSBs induced by high concentration (50 μ M) of etoposide accompany the formation of tubular NE invagination (new ED Fig.4c). We also found that CGNs generated NE invagination after confined migration, which was abolished by nocodazole treatment in agreement with previous studies (PMID 38632359)(new ED Fig. 3i). These observations suggest that confined migration does not interfere with the guided restructuring of the NE. Moreover, the inhibition of NE invagination by nocodazole treatment did not affect DSB repair after confined migration, suggesting that the migration-induced DSBs are repaired independently of guided NE remodeling (new ED Fig. 3j). The relevant parts in the Results section have been revised (line 219-230).

If confined migration interferes with DSB repair, rather than increasing the formation of DSBs, then DSBs should be generated during both confined and non-confined migration, yet they are more efficiently repaired via the DDR after non-confined migration. To test this possibility, we performed transwell assays through 8 μ m pores and examine if DDR inhibitors lead to the accumulation of DSBs. None of the inhibitors (etoposide, SCR7 and Nu7441) nor Lig4 loss, which disturbed DSB repair after 3 μ m transwell migration, increased γ H2AX foci after 8 μ m-pore migration at 12h and 24h (new Fig. 2j, 3b, 3c, ED Fig. 4e). We believe that these new data support that confined migration enhances DSB formation rather than suppress DSB repair, and that the DSBs induced by confined migration are repaired through DDR

independently of NE remodeling.

Major point 1-2: On the other hand, some studies have proposed that mechanical stress can activate DDR kinases at the nuclear envelope (PMIDs 25083873, 32973141, 38088930). Therefore, could confined migration first compromise DSB repair by disrupting the guided restructuring of the nuclear envelope above, which is also accompanied or followed by a confinement-based boost in DDR signaling to restore guided nuclear restructuring and enhance the DDR more broadly for repair post-migration?

Response: We thank the Referee for pointing out important issues with seminal papers demonstrating that DDR kinases ATR and ATM mediate mechanotransduction during confined migration. In these context, DDR kinase(s) is activated independently of DNA damage, and regulates downstream effectors regulating mechanical properties of the nucleus and chromatin remodeling rather than enhancing DDR response. As ATM is implicated in the NHEJ pathway in certain context, we have examined whether mechanosensing ATM directly or indirectly affects DSB dynamics in CGNs during and after confined migration, using a drug inhibitor of ATM (ATMi: Ku55933, 10 μ M) in transwell culture. We found no significant changes in the formation and decay of γ H2AX foci, supporting that activation of ATM is dispensable for the repair of migration-induced DSBs (ED Fig. 4k). ATM has also been implicated in the removal of stalled Top2ccs independently of the proteasome-TDP2-pathway (Nat Comm 2020, 32060399). The ineffectiveness of ATMi therefore also suggests that Top2ccs generated by confined migration is mainly resolved through the proteasome-dependent pathway (Please see the Response to Major point 6).

As Referee indicated, mechanostress-induced ATR activation at the NE has been shown to be critical for the structural integrity of the NE besides its function as a DDR factor. Other studies have demonstrated that activation of ATR by DNA damage induces NE rupture through the phosphorylation of Lamin A/C in RPE cells (Kovacs et al., Mol Cell 2023; 37832547). It is intriguing that cancer cell lines expressing low ATR undergo DSBs by nuclear deformation independently of NE rupture (Shah et al., Curr Biol 2021, 33326770). By contrast, the low incidence of NE rupture in neurons may be associated with their very low expression of the ATR phosphorylation target LMNA. We have examined the effect of an ATR inhibitor (berzosertib, 5 μ M) in transwell assay, but it strongly inhibited neuronal migration in agreement with previous studies and made further investigation difficult (Kidiyoor et al., Nat Comm 2020; 32973141). We aim to further investigate the mechanism of NE rupture and the

role of mechanosensitive ATR in this process in future studies.

Major point 1-3: It is also possible that increased nuclear/genome contortion during cell migration alters the coiling nature of chromatin, requiring more Top2 to resolve such issues post-migration. More direct evidence for such a model would be helpful. If demonstrated, then how would such a model intersect with the mechanosensing and nuclear reshaping processes mentioned above? This is especially intriguing since the data in Fig. 4e showed that myosin and tubulin proteins, which can be involved in mechanosensing-based DSB repair, were induced by migration in wild-type but not in Lig4-deficient cells.

Response: We agree that nuclear deformation and/or mechanical stress alters chromatin structure, causing the formation of TOP2ccs and subsequent DSBs. In the revised manuscript, we have added experiments solidifying the evidence that confined migration specifically leads to accumulation of stalled TOP2ccs, which are removed by proteasomal degradation and subsequently processed through the NHEJ pathway. Please see the response to the Major point 6.

It is intriguing that TOP2 is an ATM/ATR phosphorylation target, and upon irradiation ATM is known to phosphorylate TOP2A at serine 1512 and increase the stability of the protein (23163762), suggesting the possibility that mechanosensing DDR kinases may be involved in the regulation of TOP2 turnover and activity (Kidiyoor et al., Nat Comm 2020; 32973141) to resolve torsional stress post migration. However, our results using transwell assays do not support an ATM-dependent role in either the formation or repair of the DSBs after confined migration. Instead, the results are more consistent with TOP2cc caused from direct mechanical effects on DNA, stretching or elevated torsional stress that interferes with religation, rather than from ATM's mechanosensory function. The function of ATR is needed to be further investigated.

It has also been shown that ATM/ATR interact with cytoskeletal proteins, implying their potential functions in sensing cytoskeletal stress and promoting cytoskeletal remodeling. It is an interesting hypothesis that the DSB repair pathway might also be involved in the transcriptional control of cytoskeletal remodeling in response to mechanical stress. However, the RNA-seq analysis for the comparison between wildtype and Lig4^{ND1-cre} was performed using 2-month-old mice, making it unlikely that the observed differences are directly related to the processes of DSB formation and repair, which are completed within 1–2 weeks after birth. We infer that these gene expression changes were induced by DSBs that remained

unrepaired over the long term. From GO analysis, we assume that these downregulated cytoskeletal molecules are involved in neuronal morphogenesis and function.

As noted by the Referee, many outstanding studies have shown that the repair of pre-existing DSBs involves dynamic structural changes of the NE and nucleoplasm. On the other hand, there is also accumulating evidence—based on studies using cancer cell lines and primary immune cells—that nuclear deformation during confined migration can itself induce DSB formation (e.g, Science 2016, 27013426; Science 2016, 27013428; Current Biology 2021, 33326770; Cell 2021, 34551315). Our present study is the first to demonstrate that mechanical stress during confined migration causes transient DSB formation in the living brain and uncovered part of the molecular mechanisms underlying their formation and repair. We have performed additional experiments to reinforce the conclusion in the revised version. We have also revised Discussion to provide more comprehensive view of nuclear damage during confined migration and clarify the limitation of this study (lines 615-622).

Major point 2: [Would the knockdown of factors that directly promote or limit CGN migration influence the induction and resolution of DSB/γH2AX foci?](#)

Response: As far as I know, no guidance molecules have been identified that accelerate CGN migration, and there are currently no means to promote it. On the other hand, inhibiting CGN migration using drugs that target cytoskeletons and their regulatory proteins suppresses passage through the pores and subsequent formation of DSBs: For inhibition of NE restructuring after confined migration (new ED Fig. 3j), nocodazole was added only to the bottom chamber to avoid its inhibitory effect on neuronal migration.

We have performed almost all transwell assays using 3 μm and 8 μm pores to compare confined and non-confined migration (new Fig. 2j, 2k, 3b, 3c). We observed that DSB formation was specific to cells migrating through 3 μm pores, but not increased after migration through 8 μm pores. It is thus unlikely that the formation and resolution of DSBs are associated with migration per se, regardless of nuclear deformation.

Major point 3: [An alternative explanation related to the data in Fig. 1b,c may suggest that DSBs are indeed acquired de novo during neuronal migration, or that DSBs induced before or early during migration may not mount a full activation of the DDR until confined migration is at a more advanced stage. How can the authors rule out the latter possibility?](#)

Response: We conducted live imaging to visualize the formation of 53BP1 foci in migrating

neurons in slice and in vitro. Similarly to previous studies demonstrating that DSBs are generated by confined migration (e.g, Science 2016, 27013426; Science 2016, 27013428; Current Biology 2021, 33326770; Cell 2021, 34551315), we observed the emergence of 53BP1 foci during and after passage through confinement. To further negate that DSBs are induced before migration, we performed transwell assays with different pore sizes using Lig4^{ND1-cre} cells. If DSBs are formed before migration and repaired after migration, then Lig4 loss should increase DSBs in non-migrated CGNs or those migrated through non-confined pores. We found little or no increment in γH2AX foci in cells after non-confined migration (8 μm) or non-migrated cells (0.4 μm), supporting the idea that confined migration induces DNA damage directly, rather than interfering with the DNA-damage sensing or repair pathways (new Fig. 3c).

Major point 4: Regarding the apparent transient formation of 53BP1 foci in CGNs undergoing radial migration, how can the authors rule out the possibility that they are simply imaging 53BP1 foci moving in and out of the imaging plane?

Response: To address the important concerns raised by this and other Referees, we have performed live imaging of 53BP1 foci formation in migrating CGNs on patterned substrates, covering the full cellular depth (0.6-μm z-step x 15 slices, instead of 1 μm z-steps x 5 slices). We were able to trace 53BP1 foci during migration through narrow constrictions. We have added new imaging data with quantitative assessment (new Fig. 1e-g, new Video1). We have also repeated live imaging of 53BP1 foci in brain slices for more quantitative assessment. Organotypic slices were observed using a 40× water-immersion objective (NA 0.8), under which individual foci were less clearly resolved than in dissociated cultures on glass dishes imaged with a 100x oil-immersion objective (NA 1.3). Furthermore, neurons migrate in 3D while changing their depth in tissue, unlike on planar substrates. Therefore, it is technically very challenging to achieve long-term, high-resolution imaging while finely scanning the z-axis to cover the entire depth. New imaging data and quantitation have been added as new ED Fig. 1e, 1f, 2c, 2d.

Major point 5: Related to Fig. 2d,e and ED Fig. 2d,e, why did the authors start the analysis at 6 hr post-migration? NHEJ is a rapid repair mechanism and can mediate the repair of most breaks in certain cell types within 1-3 hr of DSB induction. Could this be why the increase in DSB induction through the 3 μm barrier only increased the percentage of γH2AX-positive

cells from the controls' 7-8% of cells to only about 20% of cells?

Response: In our transwell assay, the dissociated CGNs inoculated on top attach to the filter and begin penetrating the pores after 2-3 h of inoculation (Fig.S3, Nakazawa et al., Cell Reports 2025, 40053456). The timing of passage of individual neurons through the pores is asynchronous rather than simultaneous, and some CGNs are still migrating through the pores at 12 h; we observe that some neurons even go back and forth across the filter. As the Referee indicated, NHEJ is a rapid mechanism, but could be an order of several hours for the bulk of repair in our setting and the residual DSBs demarcated by γ H2AX could last after 12 h.

We reason that the low proportion of DSB-positive cells, approximately 20%, observed in the transwells is partly due to the short distance of confined migration through the pore membrane, which is only $\sim 10\ \mu\text{m}$ in thickness.

Major point 6: Regarding the etoposide experiments in Fig. 2, it seems intriguing/unexpected that etoposide did not induce DSBs at the 6 or 12 hr time points (e.g., Fig. 2j,k). Please clarify why that may be the case and precisely what drug dose was used, as well as how the treatments were performed (the text, methods, and legends were insufficient). Also, an essential missing control to add here would be to conduct these etoposide treatment experiments after allowing the cells to migrate through the non-confining pores (i.e., $8\ \mu\text{m}$). As shown, one cannot evaluate the level of overlap/interaction between etoposide treatment and confined migration.

Additionally, as the authors appear to propose repair mechanisms focused on the rapidly acting NHEJ pathway, the relatively slow kinetics of γ H2AX resolution shown (i.e., ~ 12 -24 hr) suggest the involvement of slow-progressing repair mechanisms, at least in part. Please address/clarify this point.

Response: Thank you for pointing out that the original writing was misleading. As the Referee indicated, etoposide at several tens μM strongly traps TOP2 in the stalled TOP2cc state by preventing DNA ligation after cleavage and generates sustained DSBs (so-called ETO-breaks). We unintentionally omitted to state this explicitly, but we used a very low concentration of etoposide and doxorubicin (1 nM), a level that do not induce detectable γ H2AX foci in CGNs in this study. As you suggested, we have examined the effect of 1 nM etoposide in CGNs migrating through the non-confined pores ($8\ \mu\text{m}$) and observed that it causes little or no increase in γ H2AX foci up to 36 h (new Fig. 2j, k). We have also compared

the effect of high-dose (50 μ M) and low-dose (1 nM) etoposide using SIM imaging and found that only high-dose etoposide induced the accumulation of γ H2AX foci and NE invagination (new ED Fig. 4c). Likewise, etoposide strongly induced TOP2b trapped on DNA (TOP2cc) at 50 μ M, but only very weakly at 1 nM, in CGNs cultured on dishes (new ED Fig. 4b).

Using a TOP2cc assay, we observed that confined migration causes a significant accumulation of stalled TOP2cc, which is resolved within 12 h in transwell culture (new Fig. 2g). In the revised manuscript, we show that 1 nM etoposide strongly inhibits the resolution of TOP2ccs generated by confined migration, while producing only minimal TOP2cc formation in non-migrated CGNs (new Fig. 2h and ED Fig. 4b). These results are consistent with the observation that 1 nM etoposide does not affect γ H2AX foci formation by confined migration, but it causes γ H2AX foci to persist until 24 h and 36 h of transwell cultures (Fig. 2j, k): It is suggested that low etoposide stabilizes or slows the clearance of mechanically generated TOP2ccs, leading to sustained γ H2AX foci at later time points. We infer that nuclear deformation increases the mechanical torsion on the genome and physically traps TOP2ccs, which are ubiquitinated and degraded by the ubiquitin-proteasome system, cleaved by TDP2 into protein-free DSBs, and subsequently repaired by the NHEJ pathway (Mol Cell 2019, 31202577; Nat Rev Mol Cell Biol 2022, 35228717). To further test this hypothesis, we have examined the effect of a proteasome inhibitor epoxomicin (5 nM) and found that it causes prolonged accumulation of TOP2ccs and delayed the resolution of γ H2AX foci after confined migration (new Fig. 2h, 2k). These new results strengthen our model in which nuclear deformation during confined migration traps TOP2 on DNA, and proteasomal degradation is required for removal of these TOP2ccs, before the NHEJ-dependent repair can proceed (new ED Fig. 4g). The relevant parts of Main text have been revised (lines 254-353).

As the Referee perceptively pointed out, proteasome-dependent removal of TOP2cc is a slow process that requires 1~3 h (Science 2017, 28912134) before NHEJ can repair protein-free DSBs, and therefore the TOP2-dependent DSBs in migrating neurons are expected to persist longer than those directly repaired via NHEJ. Consistently, in our live imaging, the average lifetime of 53BP1 foci in CGNs migrating through the constricted pores was approximately 94 min ($n = 66$ cells), which is longer than those previously reported using RPE cells treated with high dose etoposide (~20 min) or after constricted migration (20~40 min)(Nadar et al., Cell 2021, 34551315) (new Fig. 1f). However, tracking fluorescent marker dynamics in moving nuclei – CGN nuclei even actively rotate during migration (Wu et al.

Development 2018, 29519888) – is technically challenging; therefore we consider this approach less suitable for precisely determining the duration of the repair process, and we would therefore like to refrain from discussing this point in the present manuscript.

Major point 7: Regarding the use of small-molecule drugs to inhibit various proteins/enzymes, experiments are needed to confirm that the intended target has indeed been efficiently and significantly inhibited. This is especially important when the drug treatments are claimed not to influence γ H2AX foci resolution post-confined-migration, as is the case with Topotecan (Top1) and B02 (Rad51).

Response: We determined the effective concentration based on previous studies and conducted our experiments accordingly. The working concentration of topotecan (300 nM) was determined according to a report using primary mouse neurons (King et al., Nature 2013; 23995680). We have performed Topoisomerase I cleavage complexes (TOP1cc) DUST assay (Detection of Ubiquitylated and SUMOylated TOP-DNA complexes, Sun et al., 2020) and detected marked increase in TOP1cc in the CGNs treated with 300 nM topotecan, indicating effective inhibition of the resolution of TOP1ccs (ED Fig. 4f). As homologous recombination (HR) is a major pathway for the repair of replication-induced DSBs in mitotic cells, RAD51 foci are not detected in postmitotic CGNs (Please also see the Response to Major point 8). We have examined the effect of 1.2 μ M B02 on cultured cerebellar granule neuron precursors (CGPs) according to a previous report using cultured glioblastoma cells (King et al., Stem Cell Rep. 2017; 28076755). We have confirmed the inhibition of RAD51 foci formation in CGPs treated with 1.2 μ M B02 (ED Fig. 5b).

Major point 8: There is a need to assess the relative degree of overlap or genetic interaction of Top2 with LigIV or DNA-PK and of Top2 with Rad51 in γ H2AX focus resolution after confined migration. This is necessary to confidently place Top2 and NHEJ factors in the same post-confinement DNA repair process studied here.

Response: Thank you for your insightful suggestion for this important experiment. We have examined the effects of 1 nM etoposide – which stabilizes and inhibits TOP2cc resolution without increasing the formation of new TOP2cc – on Lig4^{ND1-cre} cells. While etoposide alone strongly inhibited the decay of γ H2AX foci in wildtype CGNs after confined migration, it had no additional effect on Lig4^{ND1-cre} cells. Similarly, inhibition of proteasomal degradation of TOP2cc by epoxomicin did not increase γ H2AX foci in Lig4^{ND1-cre} cells (ED Fig. 5c). These

results are consistent with our view of an epistatic relationship, in which TOP2ccs generated during confined migration are processed by proteasomal degradation and subsequently repaired by Lig4-dependent NHEJ pathway.

This study focuses on postmitotic CGNs in which the HR is inactive. Using SIM, we have also observed that CGNs do not form RAD51 foci that overlap with γH2AX foci (ED Fig. 3e, f).

Major point 9: Regarding the use of Lig4(ND1-Cre) and Lig4(Gabra6-Cre), add data showing the protein expression level of Lig4 at different stages (covering P4 to 12 mo), ideally via imaging. This is necessary to ensure the accurate interpretation of a large fraction of the results.

Response: We have added immunofluorescence staining against LIG4 in the cerebella from Lig4^{ND1-Cre} and Lig4^{Gabra6-Cre} mice at different stages. In Lig4^{ND1-Cre} mice, Lig4 loss is evident in postmitotic CGNs in the inner EGL, ML and IGL from P6. In contrast, in Lig4^{Gabra6-Cre} mice, Lig4 loss is seen in CGNs in the IGL at P15 and thereafter (ED Fig. 5e).

Major point 10: It is again very odd that the Lig4(ND1-Cre) CGNs did not show a higher level of γH2AX foci at 12 hr (e.g., Fig. 3c); please explain. Also, missing and necessary are more timepoints and the addition of control conditions where the cells are migrating through non-confining pores.

Response: We have performed time-course analysis of γH2AX signals in Lig4^{ND1-cre} cells (new Fig. 3c). We have found that Lig4 loss does not affect the formation of DSBs at 6 and 12 h but it inhibits the repair of DSBs at 24 and 36 h. In contrast, Lig4 loss does not increase DSBs at any time points after non-confined migration. These observations are in line with our conclusion that confined migration induces the formation of DSBs rather than affecting the subsequent repair pathway.

Major point 11: Based on the data shown, one still does not know the exact effect of disrupting NHEJ/Lig4 on the levels of DSBs in migrating CGNs at the developmental stages where damage is usually naturally induced and repaired (as shown in Fig. 1b). For instance, why are the P4-P8 stages not shown in Fig. 3d? Can it be done, or does the Lig4(Nd1-Cre) not allow for testing the effect of Lig4 deficiency at the P4, P6, and other early developmental stages, limiting the analysis to P15 and beyond (Fig. 3d)?

Response: We did not show DSB staining of cKO mouse brains at earlier stages because there are naturally induced DSBs in many dividing cells even in wildtype animals, making it difficult to evaluate which cell types bear gH2AX signals. We have included gH2AX staining at P4, P6, P8 and P11 in the revised version. At P4 and P6, the number of postmigratory CGNs is low in the nascent IGL which contains actively dividing glial cells exhibiting replication-induced DSBs. gH2AX signals in CGNs appeared to increase in Lig4^{ND1-Cre} mice at P8 and thereafter, while no difference was observed in Lig4^{Gabra6-Cre} mice in early stages before 3 mo (Fig. 3d, 3e, ED Fig. 5d).

Major point 12: More evidence is needed to better support the claim that NHEJ is directing DSB repair in CGNs during/after confined migration. To more directly confirm that DNA repair during or after confined migration is conducted by NHEJ, staining for at least one NHEJ (Ku70) and one HR (Rad51, RPA, CtIP) protein should be conducted on migrating CGNs/developing brains. For example, would the localization of Ku70, but not Rad51, correlate with yH2AX as shown in Fig. 1a,b?

Response: We have increased the sample number of SIM imaging for gH2AX and Ku70 in CGNs after confined migration. Quantitative analysis indicates significant overlap of Ku70 and gH2AX signals (ED Fig. 3e, f). We have also added SIM imaging for gH2AX and Rad51. We did not detect the formation of clear Rad51 foci in postmitotic CGNs, as expected. High resolution imaging of Ku70 requires RNase and detergent-based pre-extraction (Britton et al., JCB 2019, 23897892). It is therefore difficult to apply the method of observing Ku70 signals with low noise to tissue preparation.

Major point 13: Regarding the End-seq experiments in Fig. 4, why were CGNs migrated through non-confining pores not included here as controls instead? Wouldn't these be more appropriate? Is it possible that comparing CGNs subjected to confined migration to other CGNs that did not undergo any migration at all may have even masked some of the potential/expected differences?

Response: We agree that CGNs after non-confined migration would serve as a better control. In the original version, however, we used cells cultured on flat dishes as control because of the very low recovery rate from transwells; only 20–30% of the seeded cells. Therefore primary neurons were prepared in 60 transwell dishes for one reaction of END-seq (10⁶ cells). As we were concerned that increasing the number of transwell samples and handling

time might compromise the stability of the results, we used neurons cultured on flat dishes as a control, as they can be collected more easily and efficiently. Other referees also suggested that reanalysis should be done without etoposide, which we added to stabilize and enhance DSB signals.

In view of critical importance of the Referee's suggestion, we have repeated the END-seq analysis using CGNs after migration through 3 μm pores or 8 μm pores without etoposide treatment. We obtained similar results, showing that DSBs generated specifically by confined migration were excluded from active promoters, while they were enriched in heterochromatin (LADs and non LADs) and retrotransposon LINEs. In contrast from previous results, DSBs in transcriptionally inactive euchromatin was not different between CGNs after confined migration and unconfined migration. The relevant parts of Result sections have been revised accordingly.

Major point 14-1: It is difficult to claim that the transwell is significantly higher vs. the control for the active introns, transcriptionally inactive and unlabeled euchromatin, and the heterochromatin LADs, based on the data in Fig. 4b. Possibly, the only substantial difference this reviewer sees between the control and the transwell is that the transwell has substantially fewer peaks compared to the control at active promoters. At the very least, the interpretation of such results should be toned down, and their limitations should be more thoroughly discussed to better reflect the data. This may eliminate the need for additional experimental replicates that would allow for more robust statistical analyses. Similar concerns apply to Fig. 4c and ED Fig. 5b,c.

Also related to this point, and in the absence of substantiating statistical analyses, why is the slight difference in ED Fig. 4d dismissed, but similarly minor differences were taken at face value in Fig. 4b,c, and ED Fig. 5b,c?

Response: We have replaced the END-seq results and added statistical analyses. In addition to parametric Welch's t-test and a permutation (randomization) test, we applied a non-parametric bootstrap analysis because the sample size was limited and the data distribution did not meet the assumptions required for parametric tests. Therefore we report bootstrap 95% confidence intervals (CIs) for the difference in means. The bootstrap estimates the mean difference of the 2 groups by resampling the observed values many times (5,000 resamples) and finally determines the 95% confidence interval of the distribution of all the differences (2.5th–97.5th percentiles). If CI includes 0, it means that there is no

significant difference; if the CI lies above or below 0, the two groups are significantly different. As indicated in the new Fig. 4b, c and ED Fig. 7e,f, we found statistically supported difference in relative appearance of DSBs in active promoters and heterochromatin (LADs and non-LADS) in cultured CGNs after confined and non-confined migration, and active promoters and inactive euchromatin in the cerebellar tissues from *Lig4*^{ND1-cre} and control mice. We have revised the figure legends and methods to describe the tests, the number of resamples, and the exact sample sizes used.

Major point 14-2: Similarly, on line 336, the statement that “Although overall differences between control and *Lig4*ND1-Cre were not huge,” what does that mean? Consider using more scientific and accurately interpretable descriptions of the results.

Overall, more care should be taken to ensure the accurate interpretation of the results. The discussion section should then also be revised accordingly (e.g., lines 423-427, which refer to the issues mentioned right above in this point).

Response: Thank you for pointing out the imprecise sentence. We have revised the manuscript to use more quantitative language, ‘Although overall differences between control and *Lig4*^{ND1-Cre} were relatively modest (few genes showed log2 fold change >2.5)’. Discussion has been substantially rewritten to prevent imprecise wording and to clarify the limitation of this study.

Major point 15: Related to the DEGs heatmap in Fig. 4e, please clarify what is shown exactly. For instance, *Nrep* is upregulated in the control and downregulated in the *Lig4*ND1-Cre compared to what exactly? The text, legends, and methods were not clear enough.

Response: Heatmap of DEGs was generated based on RPKM values that were normalized by row-wise Z-score scaling. Colors indicate relative expression levels within the same gene across samples, with red representing expression above the gene’s mean RPKM and blue representing expression below the gene’s mean RPKM. Figure legend has been revised accordingly.

Additional point 18: Regarding “DSB repair occurs in a few hours in normal cells”, please note that the kinetics of DSB repair can be anywhere from a few hours (as noted here by the authors) to a day or a couple of days depending on the precise nature/position of the DSB and the type of DNA repair mechanism involved. Therefore, this sentence, at this stage of

the manuscript, can be misleading and should be revised with more accurate language. For example, classical NHEJ is generally faster than HR, and HR subtypes can also vary significantly in terms of repair kinetics.

Response: We thank the Referee for important suggestion. The DSBs in mitotic CGPs are thought to be repaired by HR and NHEJ pathways, but the foci clearance may require more than 24 hours as you rightly noted. We have deleted the sentence according to your suggestion.

Additional point 19: Lines 155-159 and 415-417: Related to the repeated statements throughout the paper that cancer cells commonly undergo nuclear envelope rupture under mechanical stress, this is not accurate. For instance, cancer and non-cancer cells treated with etoposide undergo a rapid cytoskeleton-dependent nuclear reshaping process that exerts considerable mechanical force on the nucleus, dramatically reshaping it (PMID 38632359). Yet, under such mechanically stressful conditions, nuclear rupture was reported to be very rare, and such nuclear reshaping was found to promote DSB repair. Nuclear envelope invaginations and deformations without rupture were also observed in cancer cells treated with various DNA lesion-inducing agents, such as PARP inhibitors (PMIDs 38632359 and 40527886). Even studies that more dramatically stressed the nucleus of wild-type cancer cells, only a small percentage of cells (~10%) exhibited nuclear envelope rupture (PMID 37832547). So, at the most, one may postulate that NE rupture is rare in cancer cells and may be even more rare in neurons. Finally, related to the data in Fig. 2a,b, what percentage of HeLa cells and granule cells exhibit rupture within the in vitro confined migration assay used here?

Response: We thank the Referee for indicating important papers demonstrating dynamic nuclear reshaping after DSB formation. These studies induced DSBs by ionizing radiation or drugs such as etoposide (high concentration) and a PARP inhibitor, and then observed NE shape changes in response to DSBs. In contrast, nuclear deformation during confined migration has been shown to induce NE rupture at a high frequency in multiple cancer cell lines and immune cells: up to 80% (HeLa, RPE, human dendritic cells) and 90% or more (MDA-MB-231, HT1080, MSF10A) undergo NE rupture by confined migration through 3 μ m or narrower channels, preceding 53BP1 foci formation (Raab et al., Science 2016, 27013426; Denais et al., Science 2016; 27013428). These reports also confirmed that NE rupture is rare (below 5%) in the cells migrating on glass or through wider channels, indicating that NE

rupture is specifically induced by confined migration.

We have performed more live-cell imaging for quantitative evaluation of NE rupture. In contrast to the above mentioned non neuronal cells, CGNs rarely undergo NE rupture even when passing through a narrow 2- μ m pores as shown in new Fig 1g. Probability of NE rupture in HeLa cells and CGNs have also been quantified (Fig. 2f).

Additional point 30: Fig. 4 is more of an FYI-type figure and the authors should consider making it an Extended Data figure coupled with a shortening of its related description in the results section.

Response: We thank the Referee for the suggestion. We have replaced END-seq and RNA-seq analyses in response to the suggestions by this and other Referees. Should the Referee consider that the entire or a part of revised Fig.4 ought to be moved to Extended Data, we would be pleased to make the revision accordingly.

Additional point 33: The title could be more informative; for example, why should the reader care that “Mechanostress-induced DNA damage in migrating neurons during brain development” is reported here?

Response: The title has been shortened as ‘Confined migration induces transient DNA damage in developing neurons’

All other additional points have been accommodated in the revised manuscript.

Referee #2

Thank you for giving us the opportunity to submit a revised manuscript. We really appreciate your thorough and thoughtful consideration of our manuscript. Your review has provided many helpful suggestions, which have greatly improved our paper. In response to these comments, as well as those of the other Referees, we have added several new analyses and experiments. Major revisions are as follows:

1) With high resolution imaging of the nuclear membrane and additional transwell assays, we have reinforced the demonstration that nuclear migration through confined spaces induces DSB formation rather than delaying or hindering the repair of DSBs that are already formed in CGNs with or without confined migration.

2) More live imaging analyses have been performed for quantitative assessment of dynamics of 53BP1 foci formation and nuclear envelope rupture during confined migration in vivo and in vitro. We have reinforced the evidence that migrating neurons generate DSBs independent of nuclear envelope rupture and nuclear entry of cytoplasmic nucleases, mechanisms that have been demonstrated in multiple cancer cells.

3) With additional Top2cc assays and transwell assays, we have solidified the evidence that confined migration induces the accumulation of stalled Top2-DNA cleavage complexes (Top2ccs), which are removed by proteasomal degradation and subsequently religated through the NHEJ pathway.

4) The END-seq analysis has been replaced with new data comparing the patterns after confined and non-confined migration without etoposide treatment with statistical analyses.

We hope that these as well as our point-by-point responses below will answer any uncertainties and be to the satisfaction of the Referee.

Major point 1: The authors argue that the observed DNA damage results from nuclear deformation during confined migration. While this argument is reasonable and consistent with previous studies in various other in vitro and in vivo systems, experimental support for this conclusion is limited, and the authors do not sufficiently address other explanations. For example, based on the Ki67 staining in Fig. 1a, it appears that some of the migrating neurons might still be capable of proliferation or replicating their DNA, which could result in DNA damage, particularly in the presence of topoisomerase inhibitors in later experiments. The authors should include additional controls to demonstrate that the observed DNA damage in migrating occurs in the absence of DNA replication. In addition, immunofluorescence images

should include separate channels for each fluorophore, in addition to the merged color channel, to allow for better assessment of individual signals.

Response: We thank the Referee for important suggestion. As the dissociated CGN culture inevitably includes approximately 20% granule cell precursors (GCPs) in transwell assays, we costained cells with Ki67 and counted DSB foci only in Ki67-negative cells in all assays. However, one could argue that those cells were Ki67+ during confined migration, generating replication-dependent DSBs, and became postmitotic after reaching the bottom surface. To negate this possibility, we have performed transwell assays after preincubating dissociated CGNs with a CDK4/6 inhibitor palbociclib which causes a G1 cell cycle arrest. After 24h of incubation with 5 μ M palbociclib, almost no GCPs remained, and the cells were collected and seeded onto transwell filters. Although the rate of transwell migration was decreased after 24h preincubation, the percentage of DSB formation after 3 μ m- and 8 μ m-pore migration was comparable to untreated CGNs that were directly inoculated after dissociation, as shown in ED Fig. 3g (Text lines 210-216). Although it is difficult to obtain direct evidence that the DSBs in migrating CGNs are newly formed after the complete resolution of replication-induced DSBs in vivo, we have conducted more live-imaging experiments in organotypic slice cultures and acquired additional results demonstrating de novo DSB formation in migrating CGNs and cortical neurons (ED Fig. 1e, 1f, 2c, 2d). We have added separate channel views for most immunofluorescence images, including new ED Fig. 1b (Fig. 1a), ED Fig. 1c, 2a.

Major point 2: Related to the above concern, another explanation is that the DNA damage is associated with migration per se, regardless of nuclear deformation, for example, by increased mitochondrial activity and production of reactive oxygen species. Most experiments (e.g., those described in Fig. 1e) lack controls for cells migrating through larger channels that do not require nuclear deformation, and most transwell assays compare cells that migrated through 3 μ m pores to cells that did not migrate at all. The authors should instead include comparisons to cells that migrated through larger 8 μ m pores, which do not require substantial nuclear deformation, to determine whether the observed DNA damage is specific to cells migrating through small pores. This applies to both the measurements of DNA damage itself and the subsequent experiments to determine the underlying mechanism (e.g., Fig. 2j-k, etc.).

Response: To address the important concerns raised by the Referee, we have repeated

almost all transwell assays using 3 μm and 8 μm pores to compare confined and non-confined migration (Fig. 2j, 2k, 3b, 3c). We observed that DSB formation was specific to cells migrating through 3 μm pores, but not increased after migration through 8 μm pores. None of the inhibitors, nor Lig4 loss, which disturbed DSB repair after 3 μm -pore migration, increased γH2AX foci after 8 μm -pore migration at 12h and 24h (new Fig. 2j, 3b, 3c, ED Fig. 3j, 3k, 4e). These results support the idea that confined migration leads to DSB formation rather than interfering the repair of DSBs generated independently of confined migration. We have also performed the live-imaging of CGNs migrating through pores of different sizes. We observed that the probability of DSB formation decreases as the pore size increases, suggesting that DSBs are induced by nuclear deformation during confined migration. Quantitative analysis of the probability of DSB formation has been added as new Fig. 1g.

Major point 3: Several reported findings lack sufficient quantification and/or statistical analysis. For example, in vivo image sequences of DNA damage (Fig. 1d) lack quantification and comparison to cells in different zones, giving them an anecdotal appearance without robust documentation. The same applies to the images shown in Figs 1e, 2c, Ext. Data Fig. 1e,f, and many other figure panels. Furthermore, the authors should clarify whether they performed 3D analysis of these cells to rule out that the appearance of new 53BP1 foci does not result from the movement of existing foci into the focal plane, rather than new DNA damage.

Response: To address the important concerns raised by this and other referees, we have performed live imaging of 53BP1 foci formation in migrating CGNs on patterned substrates, covering the full cellular depth (0.6- μm z-step x 15 slices, instead of 1 μm z-steps x 5 slices). We were able to trace 53BP1 foci during migration through narrow constrictions. New imaging data with quantitative assessment are shown as new Fig. 1e-g, 2f and new Video1. We have also repeated live imaging of 53BP1 foci in brain slices (both cerebrum and cerebellum) for more quantitative assessment. Organotypic slices were observed using a 40 $\times$ water-immersion objective (NA 0.8), under which individual foci were less clearly resolved than in dissociated cultures on glass dishes imaged with a 100 $\times$ oil-immersion objective (NA 1.3). In addition, because excessively high expression of 53BP1 increases background noise, while too low expression makes foci undetectable, we track foci only in cells that happen to express 53BP1 at an appropriate level. Although this allows us to identify the cells in which foci are formed in tissue, we must note that quantifying the proportion of

cells that express 53BP1 at suitable levels *and* undergo comparable migration, with or without foci formation, is inherently less accurate than doing so in vitro. New imaging data and quantitation have been added as new ED Fig. 1e, 1f, 2c, 2d.

Major point 4: The authors state that the DNA damage clustered around a single spot of Ku70 (Ext. Data Fig. 2f). However, the co-localization is not particularly convincing, given that the nuclei also contain many Ku70 foci without gH2AX staining, and the large number of Ku70 foci makes overlap, even if by chance, very likely. The authors should perform a more thorough analysis to support this claim.

Response: We have increased the sample number of SIM imaging for gH2AX and Ku70 in CGNs after confined migration. For colocalization analysis, random 3.5 μm x 3.5 μm squares in DAPI positive regions were cropped from central 5 images in z-stack images. Spots segmentation was performed using auto local threshold (MidGrey method). For quantification of the random colocalization as negative control, each square image was flipped horizontally and vertically. There was significant overlap of Ku70 and gH2AX signals (ED Fig. 3f). In response to other referees, we have also added SIM imaging for gH2AX and Rad51, a key protein in the homologous recombination pathway. We did not detect the formation of clear Rad51 foci in CGNs, as expected.

Major point 5: It appears that the authors included etoposide for all experimental conditions in their END-seq analysis, which could substantially confound the experiments. The authors should include transwell migration experiments in the absence of etoposide, and, as stated before, include controls with larger pores. The argument that etoposide is needed to trap Top2b at the cleavage site is not sufficient, as alternative approaches are available.

Response: We thank the Referee for important comments. We agree that CGNs after non-confined migration would serve as a better control. In the original version, however, we used cells cultured on flat dishes as control because of the very low recovery rate from transwells; only 20–30% of the seeded cells. Therefore primary neurons were prepared in 60 transwell dishes for one reaction of END-seq ($>10^6$ cells). As we were concerned that increasing the number of transwell samples and handling time might compromise the stability of the results, we used neurons cultured on flat dishes as a control, as they can be collected more easily and efficiently. Etoposide was added to stabilize and enhance DSB signals from a small number of samples.

In view of critical importance of the Referee's suggestion, we have repeated the END-seq analysis from CGNs after migration through 3 μ m pores or 8 μ m pores without etoposide treatment. We obtained similar results, showing that DSBs generated specifically by confined migration were excluded from active promoters, while they were enriched in heterochromatin (LADs and non LADs) and retrotransposon LINEs. In contrast from previous results, DSBs in transcriptionally inactive euchromatin was not significantly different between CGNs after confined migration and unconfined migration. The relevant parts of Result sections have been revised accordingly.

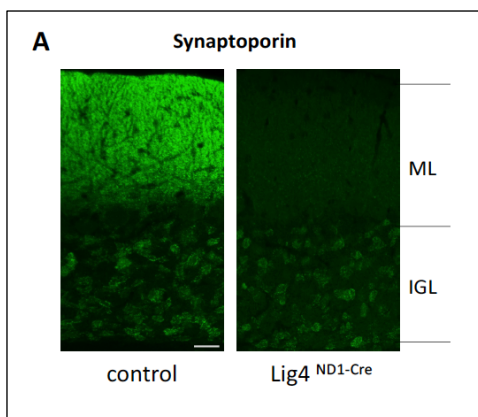
Major point 6: The authors state confined migration results in an enrichment of dsDNA breaks in introns, transcriptionally inactive euchromatin, and LADs, referring to Fig. 4b. However, based on the graph in Fig. 4b, it does not appear that the transwell results differ statistically from the controls in those regions, and the graph (as well as several others, such as the one shown in Fig. 4c) lacks a statistical analysis and comparison between groups.

Response: We have replaced the END-seq results and added statistical analyses. In addition to parametric Welch's t-test and a permutation (randomization) test, we applied a non-parametric bootstrap analysis because the sample size was limited and the data distribution did not meet the assumptions required for parametric tests. Therefore we report bootstrap 95% confidence intervals (CIs) for the difference in means. The bootstrap estimates the mean difference of the 2 groups by resampling the observed values many times (5,000 resamples) and finally determines the 95% confidence interval of the distribution of all the differences (2.5th–97.5th percentiles). If CI includes 0, it means that there is no significant difference; if the CI lies above or below 0, the two groups are significantly different. As indicated in the new Fig. 4b, c and ED Fig. 7e,f, we found statistically supported difference in relative appearance of DSBs in active promoters and heterochromatin (LADs and non-LADS) in cultured CGNs after confined and non-confined migration, and active promoters and inactive euchromatin in the cerebellar tissues from Lig4cKO and control mice. We have revised the figure legends and Methods to describe the tests, the number of resamples, and the exact sample sizes used.

Major point 7: To determine the functional consequences of preventing DNA damage repair by deleting Lig4, the authors perform bulk-RNA seq and confirm some results by immunofluorescence analysis. However, neither approach provides cell-type-specific

information, and the immunofluorescence analysis does not include markers to identify specific cell types. Thus, it appears unclear if any of the detected changes relate to neurons with DNA damage.

Response: We thank the Referee for critical comments. To assess the cell types expressing genes with marked expression changes, we have analyzed downregulated and upregulated genes using publicly available snRNA-seq datasets from mouse cerebellum (Kozareva et al., Nature 2021, 34616064). Neurons are arranged on the right, whereas immune cells and glial cell types involved in neuroinflammation (astrocytes and Bergmann glia) are shown on the left (ED Fig. 8a, b). Downregulated genes tend to be enriched in neurons on the right, while upregulated genes are predominantly observed in immune cells on the left. We have immunostained some of these genes with a granule cell marker p27Kip1 or microglia marker Iba1. The expression of the downregulated genes Myo15a is well overlapped with p27Kip1-positive cell body of CGNs, while Synaptoporin showed typical staining of parallel fiber axons of CGNs in the ML, indicating that sustained DSBs causes their downregulation in CGNs, although downregulation in other cell types cannot be excluded (Fig. 4g and attached Fig. A). In contrast, upregulated genes Cd86 was overlapped with Iba1, suggesting that sustained DSBs in CGNs causes activation of immune response genes in microglia (Fig. 4g).



Major point 8: The authors speculate that the lack of DNA damage repair might induce some broader inflammatory responses. The authors should support this interpretation by performing immunofluorescence labeling for inflammatory markers in the tissues, particularly at early time points in the Lig4 ND1-Cre mice.

As indicated above, the deletion of Lig4 in CGNs causes upregulation of immune response genes that are mainly expressed in immune cells and microglia. We have conducted IF on

cerebellar tissues from Lig4^{ND1-cre} and wildtype at P30 and 2 mo, but have not observed clear signs of neuroinflammation, such as microglial activation, as revealed by cell shape changes and increase in Iba1-positive cells, or glial scar formation by GFAP staining. At this stage, the speculation that persistent DSBs in neurons lead to mild inflammatory conditions in brain tissue is based solely on inference from the characteristics of the upregulated genes. Nevertheless, we have kept this statement, explicitly stating that no clear signs of neuroinflammation were detected in Results and Discussion (lines 546-547; 670-671). Should the Referee consider that this sentence ought to be removed, we would make the change accordingly.

Major point 9: Representative images of nuclei with DNA damage or other markers often show a single nucleus, making it difficult to determine how generalizable the findings are. The authors should include less-cropped images that show multiple cells and nuclei, and, if necessary, display the zoomed-in nuclei as insets or additional panels.

Response: We have added low magnified views covering multiple cells in relevant figures (Fig. 2a, 2i, 3a, Ext Fig. 4d, 5b).

Major point 10: As mentioned earlier, all immunofluorescence images should include separate channels for each fluorophore, in addition to the merged color channel, to allow for better assessment of individual signals.

Response: We have added single channel images in Ext Fig. 1b, 1c, 1d, 2a, 5e, Fig. 4g.

Minor point 11: The authors state that confined migration-induced DNA damage, occurring independent of NE rupture, is distinct from previous findings in cancer cells. However, previous studies had already reported that some cancer cells, such as MDA-MB-231 breast cancer cells, along with some non-cancerous cells, can exhibit DNA damage caused by nuclear deformation even in the absence of nuclear envelope rupture (e.g., Shah et al., Curr. Biol., 2021, PMID 33326770; Nader et al., Cell, 2021, PMID 34551315). The authors should acknowledge this previous work. The Shah et al. publication might be particularly relevant to the authors findings, given that it reports increased replication stress caused by nuclear deformation, which might further support the idea of a role of topoisomerase in the DNA damage formation.

Response: We thank the Referee for pointing out important issues with seminal papers

demonstrating DSB formation caused by nuclear deformation in the absence of NE rupture. Since especially the study by Shah et al. focused on replication-associated DSBs, we did not discuss this issue in the original manuscript. Nevertheless, as the Referee rightly noted, it is highly important and should have been addressed. Shah et al. demonstrated that nuclear deformation increases replication stress and induces DSBs at replication forks independent of NE rupture in some breast cancer cells (MDA-MB-231 and BT-549 cells). The authors assume that disfunction of Mus81, an endonuclease that cleaves stalled replication forks, or low expression of ATR, mechanosensitive regulator of replication stress, might cause excessive replication stress and subsequent DSBs at forks by nuclear deformation. As Top2 is known to be recruited to replication forks in response to replication stress, it might be involved in deformation-induced DSBs in these cells. We have amended Discussion to acknowledge these studies and to discuss a potential link between their findings and present study (lines 255-291; 625-628).

All other minor points have been accommodated in the revised manuscript.

Referee #3

Thank you for giving us the opportunity to submit a revised manuscript. We really appreciate your thorough and thoughtful consideration of our manuscript. Your review has provided many helpful suggestions, which have greatly improved our paper. In response to these comments, as well as those of the other Referees, we have added several new analyses and experiments. Major revisions are as follows:

1) More live imaging analyses have been performed for quantitative assessment of dynamics of 53BP1 foci formation and nuclear envelope rupture during confined migration in vivo and in vitro. We have reinforced the evidence that migrating neurons generate DSBs independent of nuclear envelope rupture and nuclear entry of cytoplasmic nucleases, mechanisms that have been demonstrated in multiple cancer cells.

2) With additional Top2cc assays and transwell assays, we have strengthened the evidence that confined migration induces the accumulation of stalled TOP2-DNA cleavage complexes (TOP2ccs), which are removed by proteasomal degradation and subsequently religated through the NHEJ pathway.

3) With high resolution imaging of the nuclear membrane and additional transwell assays, we have reinforced the demonstration that nuclear migration through confined spaces induces DSB formation rather than delaying or hindering the repair of DSBs that are already formed in CGNs with or without confined migration.

4) The END-seq analysis has been replaced with new data comparing the patterns after confined and non-confined migration without etoposide treatment with statistical analyses.

We hope that these as well as our point-by-point responses below will answer any uncertainties and be to the satisfaction of the Referee.

Overall, the article is very well written, clear and mostly convincing. I agree with the authors that there is so far little study of the implication of mechanically induced DNA damage in developmental contexts, while it is more and more clear that cell migration, as well as other types of constraints, induce DNA damage in a large variety of contexts, including during immune response and cancer. Their work thus represents a very significant advance, including a novel mechanism for mechanically-induced DNA damage.

Response: We sincerely appreciate positive and encouraging comments by the Referee.

Major point 1: In several instances, important experiments are shown only via images of a

single representative cell. This is the case for Fig. 1d,e, Fig. 2a-c. I understand that Fig. 1d might be hard to quantify, but the authors should at least tell (maybe in the figure legend) how many cells they observed and how many times the experiment was done. For Figs 1e and 2a-c, it is less understandable because they are done in vitro and quantification should be easy: what % of cells showed the phenotypes, how many cells are observed, and how many experiments? In Fig. 2d,e, the authors nicely show the quantification, so it should be possible to do it also for the other experiments, that are also important, even if they are meant to show the absence of nuclear envelope rupture.

Response: To address the important concerns raised by this and other referees, we have repeated live imaging of 53BP1 foci in cultured CGNs with improved resolution. We have measured the cell passage rate and DSB occurrence rate by pore diameter. New imaging data and quantitative assessment have been added as new Fig.1f, 2f and ED Fig. 1g. We have also repeated live imaging of 53BP1 foci in brain slices (both cerebrum and cerebellum) for more quantitative assessment. Because excessively high expression of 53BP1 increases background noise, while too low expression makes foci undetectable, we track foci only in cells that happen to express 53BP1 at an appropriate level. Although this allows us to identify the cells in which foci are formed in tissue, we must note that quantifying the proportion of cells that express 53BP1 at suitable levels *and* undergo comparable migration, with or without foci formation, is inherently less accurate than doing so in vitro. We hope the Referee would agree with our view. New imaging data and quantitation have been added as new ED Fig. 1e, 1f, 2c, 2d.

Major point 2-1: I feel that some important controls are missing (but here I might have misunderstood some aspects of these experiments): in the experiments with the inhibition of TOP2 by various drugs, the authors see a long-term maintenance of the DNA damage after the transwell migration, and they take that as proof that TOP2 is responsible for the DNA damage produced during the migration. This is actually I think the only result they show to support the role of TOP2 in generating DNA damage during constricted migration. I must say that for me the reasoning is not obvious. I understand why TOP2 could be a candidate (it is well explained in the discussion of the article), but why inhibiting TOP2 would lead to a sustained DNA damage after the migration, without changing at all the amount of DNA damage caused by the migration is less obvious to me. DNA damage is not affected at all at the 6-hr timepoint upon etoposide (or doxorubicin) treatment. One way to clarify the reason

for sustained DNA damage at long times would be to show the data for non-migrating cells (the control with the 0.4 microns constrictions, or just on a simple plate, as the authors do for the experiments in Fig. 4). This would tell whether there is just a background level of DNA damage induced by the sustained inhibition of TOP2, leading to an elevated fraction of gH2AX positive cells even at long times. This is what is usually observed (and I think it is also the case in Fig. 4) when cells are treated with etoposide or doxorubicin, even when they are not migrating at all. What is hard to explain, I agree, is that this level seem the same as the level induced by migration, while it could also be different, so it is tempting to interpret as the authors do, that it is a 'fixation' of the migration induced DNA damage. Nevertheless, I think that the control of non-migrating cells is important here, as well as in Fig. 3 for the role of the NHEJ pathway in the repair, and for the same reason.

Response: We are grateful that the Referee highlights the major weaknesses in our original version. As the Referee indicated, etoposide at several tens μM strongly traps TOP2 in the stalled TOP2cc state by preventing DNA ligation after cleavage and generates sustained DSBs (so-called ETO-breaks). We unintentionally omitted to state this explicitly, but we used a very low concentration of etoposide and doxorubicin (1 nM), a level that do not induce detectable gH2AX foci in CGNs in this study. As you suggested, we have examined the effect of 1 nM etoposide in CGNs migrating through the non-confined pores (8 μm) and confirmed that it causes little or no increase in gH2AX foci up to 36 h (new Fig. 2j, k). We have also compared the effect of high-dose (50 μM) and low-dose (1 nM) etoposide using SIM imaging and found that only high-dose etoposide induced the accumulation of gH2AX foci (new ED Fig. 4c). Likewise, etoposide strongly induces TOP2b trapped on DNA (TOP2cc) at 50 μM , but only very weakly at 1 nM, in CGNs cultured on dishes (new ED Fig. 4b).

Using a TOP2cc assay, we observed that confined migration causes a significant accumulation of stalled TOP2cc, which is resolved within 12 h in transwell culture (new Fig. 2g). In the revised manuscript, we show that 1 nM etoposide strongly inhibits the resolution of TOP2ccs generated by confined migration, while producing only minimal TOP2cc formation in non-migrated CGNs (new Fig. 2h and ED Fig. 4b). These results are consistent with the observation that 1 nM etoposide does not affect gH2AX foci formation by confined migration, but it causes gH2AX foci to persist until 24 h and 36 h of transwell cultures (Fig. 2j, k): It is suggested that low etoposide stabilizes or slows the clearance of mechanically generated TOP2ccs, leading to sustained gH2AX foci at later time points. We infer that nuclear deformation increases the mechanical torsion on the genome and physically traps

TOP2ccs, which are ubiquitinated and degraded by the ubiquitin-proteasome system, cleaved by TDP2 into protein-free DSBs, and subsequently repaired by the NHEJ pathway (Mol Cell 2019, 31202577; Nat Rev Mol Cell Biol 2022, 35228717). To further test this hypothesis, we have examined the effect of a proteasome inhibitor epoxomicin (5 nM) and found that it causes prolonged accumulation of TOP2ccs and delayed the resolution of gH2AX foci after confined migration (new Fig. 2h, 2k). These new results strengthen our model in which nuclear deformation during confined migration traps TOP2 on DNA, and proteasomal degradation is required for removal of these TOP2ccs, before the NHEJ-dependent repair can proceed. The relevant parts of Main text has been revised (lines 254-353).

Major point 2-2: Even if maybe unnecessary here, it is often clearer to analyze the number of foci, in addition to the number of positive cells, because 'positive' is not very quantitative and here it might be important to be quantitative to distinguish the effect of the inhibition of repair versus a constant production of additional damage due to the drugs. When possible, it is even better to follow the individual foci and quantify their rate of appearance and their lifetime, which is really the most direct way to estimate the rate of new DNA damage and the repair efficiency. But this might not be feasible here in conjunction with the transmigration assay.

Response: We appreciate the Referee for raising important points. When costained, gH2AX foci and 53BP1 foci are not identical, with gH2AX foci being more abundant than 53BP1 foci. As a few gH2AX foci were detected in most neurons with or without migration in culture, cells with 5 or more gH2AX foci were classified as positive. In contrast, 53BP1 foci were rarely observed in neurons after non-confined migration or in non-migrated neurons; therefore, cells with one or more 53BP1 foci were classified as positive. Basal foci numbers vary with cell type and state; A previous study using MCF10DCIS cells defined cells with 3 or more 53BP1 foci as positive (Nadar et al., Cell 2021, 34551315), whereas other report using U2OS cells or A549 cells observed more than 10 foci in the non-migrated state, with a twofold or greater increase after confined migration (Irianto et al. Curr Biol 2017, 27989676; Xia et al., JCB 2018, 31239284). We have compared the absolute number of gH2AX foci in neurons with and without confined migration and observed similar trends to that observed for the number of positive neurons (new Fig. 2c). We have also compared the number of gH2AX foci with or without etoposide and found that there was no increase in the number of foci at 12 h, but the

resolution of foci was significantly delayed at 24 h of confined migration (new Fig. 2k).

As it was technically challenging to adopt live-imaging to the transwell assays in our setup, we have observed CGNs migrating in micropatterns, covering the full cellular depth, to trace 53BP1 foci during migration through narrow constrictions. The average lifetime of 53BP1 foci in CGNs migrating through the constricted pores was approximately 94 min ($n = 66$ cells)(new Fig. 1f), which is longer than those previously reported using RPE cells treated with high dose etoposide (~20 min) or after constricted migration (20~40 min)(Nadar et al., Cell 2021, 34551315). This is consistent with previous studies showing that the removal of Top2ccs via the ubiquitin-proteasome system likely requires 1~3 h (Schellenberg et al., Science 2017, 28912134) before the initiation of NHEJ, and therefore the Top2-dependent DSB in migrating neurons should persist longer than those directly repaired via NHEJ. However, tracking fluorescent marker dynamics in moving nuclei – CGN nuclei even actively rotate during migration (Wu et al. Development 2018, 29519888) – is technically challenging; therefore we consider this approach less suitable for precisely determining the duration of the repair process, and we would therefore like to refrain from discussing this point in the present manuscript.

Major point 2-3: I am also wondering, for the same reason of interpreting this key experiment, whether it could be interesting to combine for example the etoposide treatment with the disruption of the NHEJ pathway, in the cells undergoing migration in the transwell assay. If the maintenance of DNA damage for longer times in etoposide is due to constant production of DNA breaks in this context, and constantly repaired, then inhibiting the repair pathway should lead to accumulation of more DNA damage in time, and this would contradict the interpretation of the results from Fig. 3i-k proposed by the authors; While if there is no accumulation of DNA damage post-migration, then it would argue for the fact that all the damage was done during the migration step.

Response: Thank you for your insightful suggestion for this important experiment. We have examined the effects of 1 nM etoposide – which stabilizes and inhibits TOP2cc resolution without increasing the formation of new TOP2cc – on Lig4^{ND1-cre} cells. While etoposide alone strongly inhibited the decay of gH2AX foci in wildtype CGNs after confined migration, it had no additional effect on Lig4^{ND1-cre} cells. Similarly, inhibition of proteasomal degradation of TOP2cc by epoxomicin did not increase gH2AX foci in Lig4^{ND1-cre} cells (ED Fig. 5c). These results are consistent with our view of an epistatic relationship, in which TOP2ccs generated

during confined migration are processed by proteasomal degradation and subsequently repaired by Lig4-dependent NHEJ pathway.

Major point 3: I am not sure of the feasibility of the experiment I propose here, as I am not an expert of brain development: are there pools of neurons that do not significantly migrate (and do not proliferate) at a similar stage of development? Would it be possible, if they exist, to then produce similar experiments in such neurons (for the mice experiments) and check whether there is DNA damage in these neurons or not during development and whether the Lig4 mutants show any sort of defects? This would also be a nice control for the specific impact of migration on DNA damage.

Response: We appreciate the Referee's suggestion. However, most CNS neurons are produced in germinal zones and migrate to the place they eventually function, albeit with varying distances. We have discussed with several specialists and learned that retinal neurons are produced near their eventual functional locations, undergoing only short distance migration. However, the fate commitment and differentiation of retinal neurons are complex, and the close proximity of postmitotic neurons to progenitor cells makes it challenging to exclude the possibility that the observed DSB signals represent residual replication-dependent DSBs generated in progenitors. We hope that the Referee would understand the difficulty in performing this experiment.

Minor point 1: Line 76: When the authors state that many Ki67 positive cells are also positive for gH2AX, did they check that these are not in mitosis? Mitotic cells are usually positive for gH2AX even in the absence of DNA damage.

Response: The outer EGL is populated with Ki67-positive proliferative granule cell precursors (GCPs). We confirmed that a subset of these Ki67-positive cells are in M phase by DAPI staining; however, approximately half of the outer EGL cells are gH2AX-positive, indicating that the signals we observe are not restricted to the cells undergoing mitosis.

To prove that gH2AX foci in postmitotic CGNs are not residual gH2AX foci generated in mitotic precursors but represent de novo DSBs acquired in postmitotic CGNs during migration, we have performed transwell assays after preincubating dissociated CGNs with a CDK4/6 inhibitor palbociclib which causes a G1 cell cycle arrest. After 24h of incubation with 5 μ M palbociclib, almost no GCPs remained, and the cells were collected and seeded onto transwell filters. Although the rate of transwell migration was decreased after 24h

preincubation, the percentage of DSB formation after 3 μm - and 8 μm -pore migration was comparable to untreated CGNs, as shown in ED Fig. 3g.

Minor point 2: In many cell types, the confinement size defines the amount of nuclear envelope rupture (NER) observed; even in cancer and immune cells, at 3 micron, the fraction of NER is not so high, compared to 2 microns for example, where a majority of cells display NER. Here the authors mostly investigated the 3-micron constriction size. It would be interesting to know whether migrating neurons are totally proof to NER, or if, like other cells, they mostly display NER at 2-micron constrictions. Then another question would be whether there are contexts in which such deformations can be seen in neurons in development. It does not change the message of the authors here, because they show that at 3 microns, they observe DNA damage and no NER in neurons, so their reasoning holds. But for the cells in vivo, it is maybe less clear that there is no NER at all (the authors show no evidence for that), especially if cells in vivo undergo deformation of nuclei down to 2 microns. Maybe they could give some numbers for the typical nuclear deformations they observe in the developing brain? And at least discuss this limitation of their work more directly. It is also in general complicated to rule out NER, because it is a transient event lasting just a few min, that can in turn cause DNA damage for long periods of time (hr). I think the authors might be right that is mostly TOP2-induced DNA damage in the brain, because it would occur for less nuclear deformation and in the absence of NER, but since they actually show no direct proof of that and instead propose this hypothesis as a conclusion from their in vitro experiments, they could maybe leave open the possibility, in their discussion, that stronger nuclear deformations could also cause NER and DNA damage in neurons (except if they add new experiments to show that this is not the case).

Response: We thank the Referee for important comments. We have performed live-imaging using the micropatterned substrate with arrays of pores with widths ranging from 1 to 5 μm , arranged at 1- μm steps (1, 2, 3, 4, 5 μm). Although the number was low, we have observed neurons that passed through 2 μm , as shown in the new ED Fig. 1g. NE rupture was never observed among neurons with severely deformed nuclei migrating through narrow 2 μm (29 cells) or 3 μm (63 cells) pores. Quantitative analysis of the probability of DSB formation and NE rupture in different pore sizes as shown in the new Fig.1f. As the Referee acknowledged, we believe that our data support the conclusion that neurons are less prone to sustain NE rupture and that they produce DSBs in the absence of NE rupture, at least in vitro.

During live imaging of neurons on the patterned substrate, we observe that even partial deformation of the nucleus, when passing through relatively wider channel, can induce 53BP1 foci, making it difficult to simply correlate damage with nuclear diameter. Shape dynamics of migrating CGNs are more complex in soft tissue, likely due to the irregular geometry of the tissue environment and the deformability and movement of surrounding cells. Using our current live-imaging microscope with a 40× water-immersion objective (NA 0.8), it is difficult to clearly detect NE rupture in organotypic slices. We therefore cannot rule out the possibility that neurons penetrate smaller spaces in tissue and undergo NE rupture during migration. If the NE ruptures and allows nonspecific DNA damage by cytoplasmic endonucleases such as TREX in migrating CGNs in vivo, we would expect to observe substantial cell death, which we hardly see in postmitotic CGNs in the ML and IGL (ED Fig. 5a, 6c). Therefore, even if rupture occurs, we think it likely to be infrequent. We have revised Discussion to provide more comprehensive view of NE rupture during confined migration and clarify the limitation of this study (lines 615-622). We would like to further investigate the relationship between the NE rupture and nuclear and genome integrity through more detailed analyses in future studies.

Referee #4

Thank you for giving us the opportunity to submit a revised manuscript. We sincerely appreciate positive and encouraging comments by the referee. Your review has provided many helpful suggestions, which have greatly improved our paper. In response to these comments, as well as those of the other Referees, we have added several new analyses and experiments. Major revisions are as follows:

1) More live imaging analyses have been performed for quantitative assessment of dynamics of 53BP1 foci formation and nuclear envelope rupture during confined migration in vivo and in vitro. We have reinforced the evidence that migrating neurons generate DSBs independent of nuclear envelope rupture and nuclear entry of cytoplasmic nucleases, mechanisms that have been demonstrated in multiple cancer cells.

2) With additional Top2cc assays and transwell assays, we have strengthened the evidence that confined migration induces the accumulation of stalled TOP2-DNA cleavage complexes (TOP2ccs), which are removed by proteasomal degradation and subsequently religated through the NHEJ pathway.

3) With high resolution imaging of the nuclear membrane and additional transwell assays, we have reinforced the demonstration that nuclear migration through confined spaces induces DSB formation rather than delaying or hindering the repair of DSBs that are already formed in CGNs with or without confined migration.

4) The END-seq analysis has been replaced with new data comparing the patterns after confined and non-confined migration without etoposide treatment with statistical analyses.

We hope that these as well as our point-by-point responses below will answer any uncertainties and be to the satisfaction of the Referee.

So in summary I find this to be an interesting and very well done study. I find it convincing that DNA breaks are induced in migrating neurons, apparently by nuclear deformation as the neurons migrate close spaces. On the other hand, the functional impact of these DNA breaks appears to be modest and so their greater significance is less clear. The breaks appear to be repaired accurately, and so that even in the presence of defective DSB repair (Lig4 conditional mutant) there are few defects at the cellular or organismic role.

Response: We sincerely appreciate positive and encouraging comments by the Referee.

Major Point 1: Adding western blot images will help in understanding how consistent the

levels of gH2AX detected across different experimental conditions and setups (in vivo vs. transwell) are. Assessing reproducibility ensures the reliability of gH2AX as a marker for DSBs (for example, Fig. 2b shown in this study: https://www.researchgate.net/figure/Radiation-induced-DNA-damage-A-DNA-double-strand-breaks-were-visualized-with_fig1_328227387).

Response: According to the Referee's suggestion, we have performed western blotting for gH2AX using transwell cultures. Consistent with the measurement of gH2AX foci by immunostaining, we observed transient increase in gH2AX signals after confined migration through 3 μm pores but not 8 μm pores (new ED Fig.3b). We were not able to repeat western blotting for all sample sets, because we need to collect 10-fold more cells per sample to perform western blotting. It is more challenging to compare gH2AX signals in specific cell types in vivo by western blotting, as brain lysates inevitably include many different cell types. Counting gH2AX foci has been shown to be effective method for quantitative comparison (PMID 24009179 and 12236816) and has been utilized in relevant studies (e.g., Nadar et al., Cell 2021, 34551315; Irianto et al., Curr Biol 2017, 27989676; Xia et al., JCB 2018, 31239284). We hope the Referee would agree with our view.

Major Point 2: It would be helpful to provide some sort of quantification of the videos of 53BP1-mNeonGreen in migrating cells, rather than just show a few examples.

Response: To address the important concerns raised by this and other referees, we have repeated live imaging of 53BP1 foci in cultured CGNs with improved resolution. We have measured the cell passage rate and DSB occurrence rate by pore diameter. New imaging data and quantitative assessment have been added as new Fig.1f, 2f and ND Fig. 1g. We have also repeated live imaging of 53BP1 foci in brain slices (both cerebrum and cerebellum) for more quantitative assessment. Because excessively high expression of 53BP1 increases background noise, while too low expression makes foci undetectable, we track foci only in cells that happen to express 53BP1 at an appropriate level. Although this allows us to identify the cells in which foci are formed in tissue, we must note that quantifying the proportion of cells that express 53BP1 at suitable levels *and* undergo comparable migration, with or without foci formation, is inherently less accurate than doing so in vitro. New imaging data and quantitation have been added as new ED Fig. 1e, 1f, 2c, 2d.

Major Point 3: They examine long-term behavioral effects of persistent DSBs in the Lig4

ND1-Cre mice, and see only modest effects, albeit likely referable to the cerebellum, which is where most of the unrepaired DSBs are found in these mice. They attribute these effects to effects of unrepaired DSBs. Given LIG4's known functions (primarily in NHEJ repair), it is reasonable to attribute these behavioral disorders to some defect in DSB repair. However, we don't really know from these experiments whether the non-repaired DSBs are caused by the developmental mechanism of neuronal migration. It is also possible, perhaps even to my mind more likely, that the progressive, age-related phenotypes reflect some unrelated formation of DSBs in neurons through their lifetime (for example, in the DSBs that are claimed to occur in relationship to gene expression). It would seem to be plausible that this might occur at a level sufficiently low as not to be captured by the gH2AX staining that they perform at later developmental stages. So that while the phenotypes likely relate to unrepaired DSBs, I am not convinced they relate strictly to unrepaired DSBs that originate during neuronal migration, which is the focus of the argument.

Response: We thank the Referee for pointing out important issues. We made two Lig4 cKO mouse lines with NeuroD1-Cre and Gabra6-Cre drivers. Early-onset, robust accumulation of DSBs observed from P8 in NeuroD1-Cre mice is likely caused by migration-induced DSBs, whereas the gradual accumulation seen after 3 months in both NeuroD1-Cre mice and Gabra6-Cre mice is likely due to other stressors, such as transcription (Fig. 3d, e). Motor deficits that were only seen in NeuroD1-Cre mice at 3 months, but not in Gabra6-Cre mice of the same age, are probably caused by the abundant sustained DSBs that were induced by migration. However, since both cKO lines develop similar motor deficits at 12 mo, it is conceivable that the absolute level of DSBs in CGNs rather than the specific effects of migration-induced DSBs determines the onset. END-seq analysis has revealed that the DSBs generated specifically by confined migration are excluded from active promoters, while they are enriched in heterochromatin (LADs and non LADs) and retrotransposon LINEs. As the Referee rightly suggested, these DSBs are unlikely to exert direct effects on gene expression and function; however, their accumulation may influence cellular states in a non-site-specific manner, leading to modest gene expression changes and behavioral defects. We have revised Discussion to clarify these points.

Minor Point 1: Fig. 1b,c shows DNA damage in P4 itself, but it is mentioned that the authors first observed DNA Damage at P6?

Response: Thank you for pointing out that the original writing was misleading. In Fig.1a, we

showed an enlarged view of the cerebellar cortex at P6 when the layer structures, including the outer and inner EGL, ML and IGL, are most clear. We observed gH2AX foci in the emerging IGL at earlier stages (Line 110-113, Fig. 1b,c). We have replaced the word 'first observed' with 'first studied' in Line 100, which referred to the first experiment, to avoid any confusion.

Minor Point 2: Line 875: A word is missing. I think it should read “Once they had a firm [grasp], then they were slowly....”

Response: We thank the Referee for pointing out the error. The sentence has been corrected as suggested.

Referee #1

Thank you for giving us the opportunity to submit a revised manuscript. We really appreciate your thorough and thoughtful consideration of our manuscript. In response to your comments, we have added several new analyses and revised the manuscript. We hope that the revisions and our point-by-point responses adequately address your concerns.

Point 1: In the longer term, it remains unclear how the early onset of DSB accumulation in migrating CGNs contributes to elevated DSB levels later (up to 12 Mo) and whether the initial DSB accumulation in migrating neurons caused the defects observed in animals. This might also explain why it appears difficult to find a title that captures the study's overall functional significance.

Response: We thank the Referee for pointing out important issues. We made two Lig4 cKO mouse lines with NeuroD1-Cre and Gabra6-Cre drivers. Early-onset, robust accumulation of DSBs observed from P8 in NeuroD1-Cre mice is likely caused by migration-induced DSBs, whereas the gradual accumulation seen after 3 months in both NeuroD1-Cre mice and Gabra6-Cre mice is likely due to other stressors, such as transcription (Fig. 3d, e). Motor deficits at 3 mo that were only seen in NeuroD1-Cre mice, but not in Gabra6-Cre mice of the same age, are probably caused by the abundant sustained DSBs that were induced by migration (Fig. 5d). However, since both cKO lines develop similar motor deficits at 12 mo, it is conceivable that the absolute level of DSBs in CGNs rather than the specific effects of migration-induced DSBs determines the onset of the defects. END-seq analysis has revealed that the DSBs generated by confined migration are excluded from active promoters, while they are enriched in heterochromatin and retrotransposons (Fig. 4b, c, ED Fig. 4e, f). These DSBs are unlikely to exert direct effects on gene expression and function; however, their accumulation may influence cellular states in a non-site-specific manner, leading to modest gene expression changes (Fig. 4) and behavioral defects (Fig. 5) at later stages.

The functional significance of DSBs associated with confined migration in cancer and immune cells has been widely discussed, but at present remains largely speculative. It is intriguing that neuronal DSBs appear to have a limited impact on neuronal function and survival, even when unrepaired. We discuss the possibility that this may be related to our END-seq findings, which suggest that these DSB sites are located in relatively safe regions of the genome, thereby protecting DNA from lethal damage, such as that caused by contaminated cytoplasmic DNases in cancer and immune cells; we have revised the title to

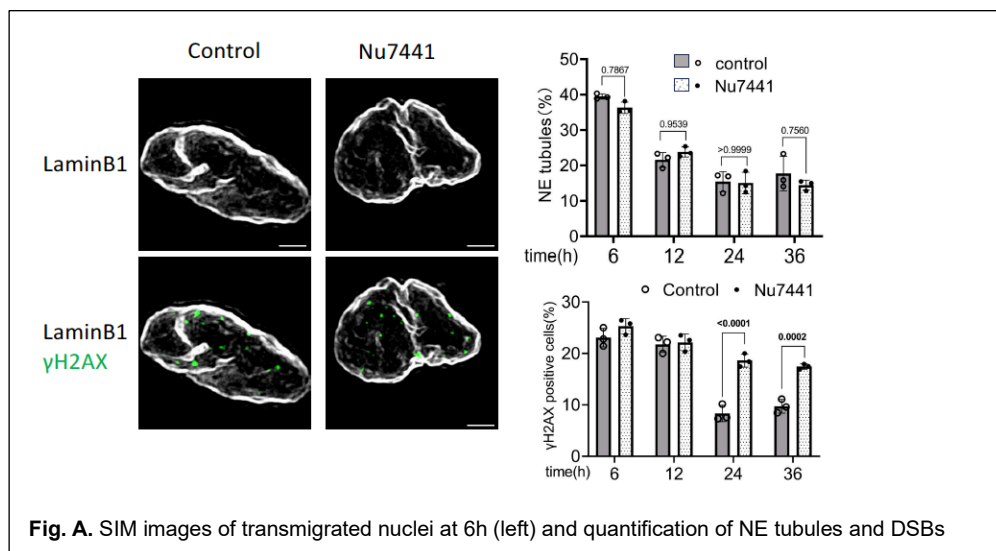
reflect this interpretation. In the Discussion, we also mention the potential relevance to genomic mosaicism, but further studies will be required to address these issues.

Point 2: In the shorter term, the authors present new data showing that the % of cells positive for NE tubules/invaginations increases following confined migration, phenocopying 50 uM ETO treatment in their system and suggesting that this mechanotransduction-based DSB repair mechanism remains functional/activated and thus not disrupted in constricted/migrating neurons. The authors' conclusion here is logical and plausible.

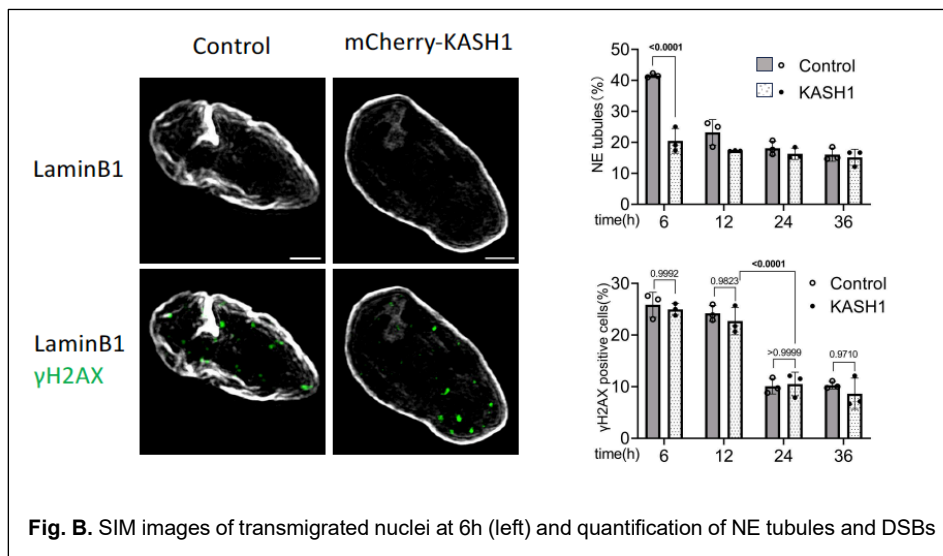
However, the authors also show that treatment with nocodazole prevents tubule formation without inducing detectable increases in the % of DSB-positive cells at 12 h or 24 h. It is also claimed that ATM inhibition, which can disrupt NE tubules, did not increase DSB levels. The nocodazole results are suggestive, but it is difficult to assess the effects of such a microtubule-disrupting drug, especially in neurons, which are highly dependent on microtubules and motors for the transport of critical cargo throughout the cell body. In addition, the cited studies show that NE tubules depend especially on DNA-PKcs and ATM and contribute mainly to NHEJ-dependent repair in mitotically dividing cells. However, DNA repair processes in postmitotic neurons are generally more dependent on DNA-PKcs than ATM.

Therefore, it is likely that DNA-PKcs inhibition disrupts the NE tubules induced by confined migration in neurons. This should be addressed experimentally by quantifying both the % of cells with NE tubules and possibly the surface area of the nuclear envelope. It would also be informative to disrupt in CGNs (or possibly in a surrogate and more tractable neuronal system) the LINC complex or other factor(s) that are reported to be important for tubules/invaginations and DSB-NE interactions in mitotically dividing cells before measuring tubules/invaginations and DSB levels using 53BP1 and γH2AX (% of cells, foci numbers, and more intermediate timepoints). Such mechanistic clarifications could strengthen the coherence of the authors' model, in which confined migration phenocopies TOP2 inhibition, leading to TOP2cc and activating NE tubules/invaginations-dependent NHEJ repair, thereby facilitating repair without NE rupture. Moreover, this could explain why the END-seq data revealed that confined migration yielded the largest increases in absolute DSB peaks at heterochromatic LADs, thereby potentially establishing a stronger connection between Fig. 4 and the remainder of the manuscript. Alternatively, the authors may present other observations to fill the gaps and establish mechanistic coherence.

Response: As you suggested, the hypothesis that DDR kinases activated at the NE contribute to the repair of TOP2-mediated DSBs via NE tubules is compelling and warrants further investigation. In the original version, we showed that inhibition of DNA-PK by Nu7441 impairs DSB repair after confined migration. To test whether the impaired DSB repair is due to defective NE tubule formation, we examined NE invagination in CGNs transmigrated in the presence of Nu7441. However, NE invagination and its resolution was not affected by inhibition of DNA-PK (attached Fig.A and new ED Fig. 5c). As calculating surface area requires acquisition of fine z-serial images of entire cell depth followed by complex analyses, we quantified positive cells, as in the reference study (PMID 38632359). Cells with NE invagination can be clearly defined by confocal and SIM imaging as shown.



In the first round of revision, we showed that the inhibition of NE invagination by nocodazole treatment did not affect DSB repair after confined migration. If nocodazole had inhibited the repair response, off-target/indirect effects would be a concern; however, because the response was normal, there would be little basis to suspect such secondary effects. In the revised version, we further examined the impact of inhibiting NE invagination using a truncated form of Nesprin-1 (KASH1), which disrupts Nesprin-1/2-mediated tethering of MTs to the NE (Wu et al., 2018: PMID 29519888). KASH1 overexpression significantly inhibited NE invagination. Although the rate of transwell migration was decreased, the percentage of DSB formation after transwell migration was comparable to control CGNs (attached Fig.B and new ED Fig.3i and 3k).



Our results therefore suggest that DSBs induced by confined migration can be repaired by NHEJ factors in the nucleoplasm, even in the absence of NE tubules. We observed the formation of NE tubules in CGNs that regained a normal aspect ratio after transwell migration in culture (ED Fig. 3i). In contrast, CGNs migrating in tissue or through repetitive microchannels form and repair DSBs within highly deformed nuclei, where the NE tubule formation may be hindered. We therefore propose that migrating neurons may employ a repair mechanism independent of NE tubules. However, the potential involvement of NE tubules in DSB repair cannot be entirely excluded. We have revised the relevant parts of Results and Discussion accordingly (lines 162-163; 246-249; 385-387).

The regulatory mechanism of TOP2 targeting is yet to be clarified, but topological changes in chromatin by bending or torsion are thought to affect the steric accessibility and activity of TOP2. Large nuclear deformation during confined migration may lead to acute strain accumulation in multiple genome regions, where TOP2 binds and alleviates excessive tensile and torsional stress on chromatin during confined migration. TOP2a breaks during replication have been shown to enrich in heterochromatin and repetitive sequences including LTRs and LINEs, supporting the idea that these regions could be native TOP2 targets for decatenation or resolving topological stress (Amoiridis et al., Nat Comm 2024, 38977669)(Discussion lines 388-431).

Point 3: Related to Fig. 4: First, the text section related to Fig. 4 and its Extended Data is of potential interest, but still appears to be out of place. I suggest significantly shortening this text section, moving 1-2 small panels to another main figure, and moving the remainder of

Fig. 4 to the Extended Data. Second, the statement regarding Fig. 4b, that confined migration shifts DSBs from promoters toward heterochromatin at LADs and non-LADs, does not appear to be statistically incorrect but is somewhat misleading. In terms of the largest increase following confined migration, it appears to be primarily at heterochromatic LADs. The text should be revised to clarify this point. Third, the conclusion at lines 549 to 552 is not convincingly supported and should be removed. Overall, this section stands out as being relatively weak compared to the remainder of the study. It might even be worth considering removing it, unless the heterochromatic LADs phenotype becomes relevant and can be better integrated into the paper's main mechanism and message.

Response: Thank you for your suggestion to delete some of the data shown in Fig. 4. However, we believe that these data are critical for characterizing DSBs generated in neurons during normal development. We demonstrate that neurons generate DSBs in heterochromatin and retrotransposons via a mechanism independent of NE rupture, which may contribute to protecting genome from lethal damage during long-distance migration. These mechanisms might have enabled the expansion of the mammalian brain volume during evolution. We consider this to represent important and informative evidence.

Regarding the second point, as you indicated, the largest increase in DSBs was observed in heterochromatic LADs, and we have revised the text accordingly (lines 298-300).

Regarding the third point, we found significant overlap between unregulated genes in the Lig4 cKO cerebellum and stage 1 cortical neurons in the CK-p25 mouse model of neurodegeneration. Although the neurons of CK-p25 mice exhibit a senescence-like state, according to the paper by Dileep et al., we agree that linking the Lig4 mouse phenotype to the senescence-like state was inappropriate. We have revised the text to more clearly indicate the results: 'there was a significant overlap between the upregulated gene cluster in Lig4^{ND1-Cre} and those upregulated in cortical neurons bearing γ H2AX foci in the early phase of the CKp25 mouse model of neurodegeneration, suggesting a link to DNA damage in neurodegenerative conditions'.

We have changed the reference from which the RNA-seq data were originally provided.

Point 4: This revised title still does not clearly convey the functional significance of the authors' observations.

Response: We consider this study to first identify transient DSBs in migrating neurons and to elucidate their underlying molecular mechanisms; however, their functional significance

remains to be identified. We have revised the title to 'Confined migration induces nonlethal DNA damage in developing neurons' to convey that the DSBs have limited adverse impacts on neuronal survival and function.

All other minor points have been accommodated in the revised manuscript.