Cortical distribution of GABAergic interneurons is determined by migration time and brain size.

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AUTHORS: Pietro Fazzari, Niall Mortimer, Odessa Yabut, Daniel Vogt, and Ramon Pla

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which will involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Fazzari et al investigates the origins of the asymmetric distribution of various cortical interneurons (CIN) using a series of assays determines CIN from different regions and

different times of embryonic development differentially reside in different cortical regions. They first demonstrate a differential distribution of the CIN along the rostro-caudal axis during development.
The authors nicely show that late born (E15) interneurons are preferentially enriched in the cortical areas closer to their respective sites of origin (e.g., MGE-derived interneurons in the somatosensory cortex, and CGE-derived interneurons in the visual cortex). Using in vitro and in vivo transplantation experiments, they Demonstrate that the cortical dispersion of the CINs depends on the developmental stage of the host brain, which constrains the total possible time that can be used for the interneurons to complete migration, and which dictates the size of the field (size of the brain) for them to navigate through. The authors conclude that the cortical interneurons do not show rostro-caudal directionality for their intracortical migration, but rather physical constraints imposed by migration time, speed and cortex volume, contribute to their differential cortical distribution.

Comments for the author

This reviewer found many of the experiments to be well done and many of the results to be of considerable interest. Nonetheless, aspects of the manuscript were considered incomplete or the conclusions drawn not entirely supported by the data, additional studies would seem appropriate. Among the major concerns:

1. The authors need to carefully consider the conclusions they have drawn from the results of the experiments, some of the interpretations seem overstated. For example, they need to provide direct evidence (e.g., cell death marker staining), if they want to state 'Programmed cell death does not influence preferential localization of interneuron subtypes in ...' (line 283). To state that he number of cells is normal and thus there could not be increased cell death is not accurate. It remains possible that proliferation could have been influenced with a similar reduction or abnormality in cell death.
This possibility can certainly not be excluded from the presented data yet it is neither tested or discussed. Another example revolves around the statement that "These findings provide evidence that CIN subtypes are different depending on their birthdate" (line 323-324). However, they do not evaluate interneuron subtype, rather they nicely demonstrate that different location of CINs are dependent on their birthdate. These are but two examples of data not completely supporting the conclusions drawn.

2. In supplementary fig 3 the authors argue that brain is responsible for the differences, that is not necessarily the case. This experiment only shows that E15 born cells traveled further than E12 born cells. Line 361-362 is an overstatement for supplementary fig 3 data. This needs to be replaced with a more accurate statement. Although brain size could be one of the factors affecting CIN distribution the authors cannot rule out the qualitative difference of the brain (E12.5 vs E15.5) as a possible reason why E12.5 born cells cannot travel as E15 born cells; for example some factors that allow dispersion at E12.5 may no longer be present at E15.5.

3. In the results section entitled 'Late born MGE-derive cells are preferentially located in the somatosensory cortex' they look at the anterior-posterior distribution of CIN but not the laminar distribution, this would similarly be valuable to understand in this set of experiments.

4. Some of the results don't quite make sense. For example, in figure 6 it appears the A-P distance traveled are similar in transplants to all ages if you look at the graphs. The differences observed are relatively small and it is unclear if they first statistically tested the whole populations before looking at individual % of the populations. This needs to be re-examined. It is possible other conclusions could be drawn.

5. Doing live migration studies would be extremely informative. They state the cells at E15.5 migrate faster, but in fact they only look at starting and ending locations to evaluate speed. It would certainly be far more informative to conduct live imaging studies, as many labs routinely do for migration studies to determine if it is the result of cells turning more regularly, reversing directions or taking other non-linear routes and thus migrate a shorter distance than cells that might take a more linear route. This possibility is left open and in fact the CIN from E12.5 and

E15.5 might migrate the same speed and still have different distances traveled due to a possibility like this.

Minor comments:
1. Line 365: wrong call out for figures? (no such info in Fig 7A-B).
2. Figure 4I: age info missing (assuming E15)
3. line 402: further explanation is required for a complete understanding.
4. There are numerous typographical and grammatical errors, careful proof reading will be necessary.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, the authors have carried out a series of experiments aimed at identifying the drivers behind the unequal distribution of MGE and CGE cortical interneurons across different cortical areas and in different layers. Their conclusion, as the title says, is that the distribution is dependent on migration time and brain size.

Comments for the author

I agree with their observation that there is unequal distribution of SOME interneurons in SOME layers. This is shown if figures 1-4 (but see comment below). However, I find that all further experiments aimed at testing different possibilities are fundamentally deficient: GABAergic neuron subtype identity is likely to be a key determinant of final distribution. None of the experimental approaches used here account for subtype identity: e.g. by taking 'MGE' cells at E12 and E15 and transplanting them, inevitably, the authors are talking different populations of cells. For example, CGE cells are born later than MGE cells and migrate in all directions, including through the MGE to reach their destination. Therefore, the E15 'MGE' transplants contain more CGE cells than the E12 'MGE' transplants. It is therefore impossible to claim that migration time and brain size are the sole determinants of interneuron spread using the tools in the current manuscript. The authors would need to factor in the subtype identity. At the very least, they should distinguish between MGE and CGE populations in their transplantation experiments using genetic tools.

Other issues with the manuscript:
In figures 1-3 and suppl figure 1, the authors claim that there is unequal distribution of interneurons especially in upper layers. However, the data they show are very selective and perhaps we would not arrive to the same conclusion if the data had been comprehensively presented: for example, what about SST distribution in layers at P14? What about total number distribution of SST, PV and VIP at P30 in different cortical areas? The authors need to show this data.

In figure 5 the authors transplant E12 to E12 MGE and E15 to E15 MGE and conclude that late-born cells spread faster than early-born ones. My question here is how did they measure the speed? If it is by measuring the rostro-caudal distance covered within a certain period of time then that is a bit misleading.
It is possible that the cortical plate, which is more prominent in the late cortex, is less permissive to migration and interneurons instead migrate in a smaller area away from the CP and as a result travel further. Therefore, the distance travelled in one direction does not take into account the multi-directional dispersion factor. A better experiment would be to do the transplantations E12 to E12 and E15 to E12. This will allow them to quantify the speed of migration in the same environment. However, as explained above, by taking MGE transplants at different times, they are taking donor tissue that consists of different populations of cortical interneurons or other subpallial neurons. Hence, the identity of interneurons may be the prime factor, something that is not tested at all by the crude transplantations used throughout the manuscript (Fig 5, Fig 6, Fig 7 and suppl Fig 3).

Reviewer 3

Advance summary and potential significance to field

The aim of the present manuscript is to study the mechanisms that control the dispersion of GABAergic interneurons (CIN) through the cortex during brain development, together with a quantification of the final distribution of cell subtypes across cortical areas (motor, somatosensory and visual) and layers in the adult mouse.
The authors claim that there is no directionality in CIN migrating through the developing cortex. Instead, CIN final location is determined by the time cells spent migrating and the length of the path they traverse. Although this would be an interesting finding, their experimental evidence is not as strong as needed to reach such a conclusion. For that reason, the authors are encouraged to revise their data and go deep in this direction to collect a more solid dataset. I suggest to start the manuscript with the mechanistic set of experiments (figures 6 and 7 of this manuscript plus new data) and leave to the end the quantification because most of this data is already known.

Comments for the author

Major issues:

-Much more data and analysis are required to support the main hypothesis of the manuscript: ?the physical constrains imposed by time, interneuron speed and brain size may contribute to the differential distribution of cortical, interneurons along the rostro-caudal axis? (cited from the abstract). Tackled in figures 6 and 7.

-In the first paragraph of the Results, the authors explain that ?whether CINs distribute equally along the R-C axis or may have different distributions in distinct microcircuits has not been explored?. Figures 1 to 3 show their results in this regard. However, this question has been already tackled by several publications (Xu et al 2010, Van Brededore et al 1990, Gochar and Burkhalter 1997, Nery et al 2002, Keller et al 2019, Ouellet and de Villers-Sidani 2014, and others).

-In the same line, half of the conclusion of figure 2 stated in lines 263-265 is described in Nery et al 2002.

-In figure 1 the authors show the total number of CIN subtypes in the three cortical areas. In figure 2, they show the same data but layer-wise. There is a problem with this quantification because in all areas the total number of cells of each subtype is lower than the sum of cells from layer 2/3, 4 and 5/6.

-With their in vivo transplantation of E12+E15 MGE cells into E15 embryos, they showed that E15 interneurons prefer to migrate towards caudal levels. These results are contradictory with their BrdU experiments in which they showed a preference of E15 MGE interneurons to populate the SS cortex. How do the authors explain this? Also, Figure 6 would benefit of an image of the transplanted cells at P14.

-Makes no sense to show data from VIP+ and PV+, and the data for SST+ in two separate figures (figure2 and supplementary figure 1). Actually, the authors need to use the same data twice: 1) the bars for layer 2/3 in panel J of figure 2, 2) in figure suppl 1 panel H. Moreover, all layer for SST+ should be shown.

-The authors concluded that the lower density of Nkx2 cells at P30 compared to earlier stages is due to programmed cell death and brain enlargement. Although these conclusions result quite logical (and have been partly published) there are no experiments that supports them in the manuscript.

-As the authors explain in the manuscript, the BrdU experiments of figure 4 do not add any new information to what is already published or to what have shown in the previous figures of the manuscript.

Minor:

-Consistency in nomenclature: layer 2/3 or II/III, and other similar cases.

First revision

Author response to reviewers' comments

Point-by-point rebuttal to Reviewer's comments
To the Editor

We are happy to present here the revised version of our manuscript **"Cortical distribution of GABAergic interneurons is determined by migration time and brain size."** by Pietro Fazzari et al.

The response of the Reviewers to our initial version was very positive and constructive. The Reviewers recognized the novelty and the interest of our study. The Reviewers raised relatively minor concerns and asked for a few specific experiments that we added to the manuscript in the amended version.

We are convinced that the updated version of the manuscript together with the accompanying point-to-point rebuttal letter responds properly and extensively to the Reviewers.

The major amendments of the current version of the manuscript concern novel experiments that further support our model and a reformatting of the figure that improved the readability of the manuscript. Specifically, i) we extended the biochemical characterization of the rostrocaudal-distribution of INs (Figure X); ii) we performed live imaging experiments that showed that the differential anterior-posterior distribution of E12 and E15 MGE (Fig X); iii) we generated an *in silico* mathematical model based on the experimental data on the expansion of the cortex and the migration speed of the interneurons. This model shows how cortical expansion impacts on the rostrocaudal distribution of interneurons. We also added control experiments that were suggested by the reviewers for instance to verify the purity of our explants (MGE vs CGE cells; Supplemental Figure x) or the apoptotic cells loss of MGE interneurons (Supplemental Figure x).

Different comments from the reviewers, in particular Reviewer 3, seemed to derive from some misinterpretation of the experiments that we presented. These feedback was very useful and constructive for us since it prompted us to reformat and improve the organization of the figures. This was particularly relevant for the *in vivo* co-transplantation experiments of E12 and E15 cells into host of different ages. The analysis of this experiments entailed a complex stereological quantification of cell migration. This figure confused some of the Reviewers. We are convinced that the current presentation is very effective and clear. It shows that the rostrocaudal distribution of the E12 and E15 interneurons is primarily determined by the particular characteristics of the host brains.

In conclusion, we believe that the updated version of this manuscript together with the extensive rebuttal letter respond to the comments of the reviewers in an adequate and complete manner. We are convinced that it will be a very interesting for the reader of "Development" and in general for all the neurobiologist since it provides novel insights into the mechanisms that regulate the formation of cortical circuits.

We thank the in advance Editor for her kind attention.

To the Reviewers

To facilitate the reading of this response, the comments of the reviewers are quoted in light blue, text from our manuscript is in green.

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript by Fazzari et al investigates the origins of the asymmetric distribution of various cortical interneurons (CIN) using a series of assays determines CIN from different regions and different times of embryonic development differentially reside in different cortical regions. They first demonstrate a differential distribution of the CIN along the rostro-caudal axis during development. The authors nicely show that late born (E15) interneurons are preferentially enriched in the cortical areas closer to their respective sites of origin (e.g., MGE-derived interneurons in the somatosensory cortex, and CGE-derived interneurons in the visual cortex). Using in vitro and in vivo transplantation experiments, they Demonstrate that the cortical dispersion of the CINs depends on the developmental stage of the host brain, which constrains the total possible time that can be used for the interneurons to complete migration, and which dictates the size of the field (size of the brain) for them to navigate through. The authors conclude that the cortical interneurons do not show rostro- caudal directionality for their intracortical migration, but rather physical constraints imposed by migration time, speed and cortex volume, contribute to their differential cortical distribution.

We thank Reviewer 1 for appreciating the originality of our work and its relevance for the field.

Reviewer 1 Comments for the Author:
This reviewer found many of the experiments to be well done and many of the results to be of considerable interest. Nonetheless, aspects of the manuscript were considered incomplete or the conclusions drawn not entirely supported by the data, additional studies would seem appropriate.

We are glad that Reviewer 1 found many experiments in our work well done and interesting. We also appreciate his/her constructive feedback on the fact that some conclusions where not entirely supported by data. In fact, we found that the revision from Reviewer 1 was very careful and accurate. In the revised version of the manuscript that we resubmit added new experiments and revised all the point raised by Reviewer 1. We believe that the current version of the manuscripts fully matches the issues of Reviewer 1. We provide here a point-by-point response.

Among the major concerns:

1. The authors need to carefully consider the conclusions they have drawn from the results of the experiments, some of the interpretations seem overstated. For example, they need to provide direct evidence (e.g., cell death marker staining), if they want to state 'Programmed cell death does not influence preferential localization of interneuron subtypes in ...' (line 283). To state that the number of cells is normal and thus there could not be increased cell death is not accurate. It remains possible that proliferation could have been influenced with a similar reduction or abnormality in cell death. This possibility can certainly not be excluded from the presented data yet it is neither tested or discussed. Another example revolves around the statement that "These findings provide evidence that CIN subtypes are different depending on their birthdate" (line 323-324). However, they do not evaluate interneuron subtype, rather they nicely demonstrate that different location of CINs are dependent on their birthdate. These are but two examples of data not completely supporting the conclusions drawn.

We thank the Reviewer 1 for the constructive feedback. We fully agree with his/her comments and we amended the manuscript accordingly. (Line 318)

With regard to "Programmed cell death", we performed Caspase 3 staining at P7, the peak of apoptosis for cortical interneurons. This experiment failed to reveal any difference in the apoptosis in MGE interneurons in the Motor, SS and Visual cortex. Notably, we performed genetic labeling with Nkx2.1-Cre to focus specifically in the specific cell population derived from MGE. This

experiment support the idea the apoptotic process is not strongly affecting the distribution of interneurons at P7 (this experiments is now presented as Supplemental Figure 2). In addition, the experiments of E12 and E15 MGE cells co-transplanted in an E15 host and analyzed at P0 (Supplemental Figure 3) show that the enrichment of the MGE interneurons in SS cortex can be observed before the onset of apoptosis. Importantly, E12 and E15 cells showed a similar rostrocaudal pattern when co-transplanted at E15. Nonetheless, we did not perform an extensive time course of apoptosis at different stages and we only use activated Caspase 3 as an apoptotic marker. Therefore, we totally agree with Reviewer 1 that our conclusion should not be overstated and we modified the text to take in consideration these limitations. Specifically, **Programmed cell death of MGE-derived interneurons does not alter their distribution across the cortex.** Sentence has been removed.

Regarding the second example provided by the reviewer, we totally agree and we amended the text accordingly. Specifically, Old sentence: These findings provide evidence that CIN subtypes are different depending on their birthdate.

New sentence: These findings provide evidence that location of MGE-derived CINs is dependent on their birthdate. (Line 352-353)

2. In supplementary fig 3 the authors argue that brain is responsible for the differences that is not necessarily the case. This experiment only shows that E15 born cells traveled further than E12 born cells. Line 361-362 is an overstatement for supplementary fig 3 data. This needs to be replaced with a more accurate statement. Although brain size could be one of the factors affecting CIN distribution, the authors cannot rule out the qualitative difference of the brain (E12.5 vs E15.5) as a possible reason why E12.5 born cells cannot travel as E15 born cells; for example some factors that allow dispersion at E12.5 may no longer be present at E15.5.

This point raised from the Reviewer is similar to the previous. Again, we thank the Reviewer and we fully agree with his/her comment. In fact, this comment applies to both co-transplantation experiments (Figure 7 and Supplemental Figure 3). The difference in size is quite remarkable between E12 and E15 (almost double). In addition, in the revised version we present a mathematical based on the experimental data of Cortical expansion and migration speed of the interneurons (maximal spreading distance over time). This model indicate that given the limited speed of the INs, the expansion of the cortex may contribute to the differential rostrocaudal distribution of MGE INs born at different stages. Nevertheless, we cannot exclude that other factors may contribute to the differential dispersion (e.g. guidance cues or the density of the extracellular matrix). The text was amended to include these considerations and avoid overstatements.

Embryonic cortical expansion may affect rostrocaudal distribution of CIN

(Line 414)

3. In the results section entitled 'Late born MGE-derive cells are preferentially located in the somatosensory cortex' they look at the anterior-posterior distribution of CIN but not the laminar distribution, this would similarly be valuable to understand in this set of experiments.

We thank the reviewer for this suggestion. Indeed, our data and previously literature show that E12.5 INs tend to accumulate in lower layers while E15.5 are enriched in upper layers. It should be noted that in the context of Figure 2-3 the analysis we used the distribution by layers to infer the birth date of the cells. This approximation is commonly used. However, this approach is not entirely accurate since birth dating experiments with BrdU or similar methods (again from our lab and others e.g. (Fairen et al., 1986; Pla et al., 2006) showed that not all the neurons in one layer are born at the same stage while INs born at a specific stage are enriched in one layer but may be found in other layers as well. In the experiment mentioned by Reviewer 1 (Figure 4) the birth dating was done precisely with BrdU injection. Since the aim of this experiment is to investigate precisely the rostrocaudal distribution of INs born at different stages we considered that laminar distribution was not crucial in this figure. Following the suggestion of the reviewer we quantified

the laminar distribution of INs for sake of completeness. This data are mentioned in the manuscript at page Figure legend 4

4. Some of the results don't quite make sense. For example, in figure 6 it appears the A-P distance traveled are similar in transplants to all ages if you look at the graphs. The differences observed are relatively small and it is unclear if they first statistically tested the whole populations before looking at individual % of the populations. This needs to be re-examined. It is possible other conclusions could be drawn.

We thank the Reviewer for this constructive comment. We recognize that this kind of graph and presentation is quite complicated to understand if you are not used to this kind of stereological analysis. Also this figure presented a lot of information that was not easy to read. We understand that in saying "The differences observed are relatively small" the Reviewer is probably referring to the area in which 100% of the cell are included (lightest colors in the graphs). The 100% area may look similar because in all cases some outlier cell managed to reach the rostrocaudal extremities of the cortex. However, all the other fractions where very different. For instance, the area that encloses the 80th percentile of the cells is ~45% bigger when cells are co- transplanted in E12.5 host as compared to E15.5 host co-transplants (i.e. AB versus EF). That means that cells spread at further distance when co-transplanted at E12.5. The difference is even bigger for smaller percentiles. The host dependent difference are observed for both E12 and E15 INs. However, the reviewer is right in saying that the distribution of INs from different ages co-transplanted in a host of a specific age spread similarly, (i.e. A vs B and E vs F). In other words, both E12 and E15 INs spread over a wide area when co- transplanted in an E12 host. Conversely, both E12 and E15 INs spread over a smaller area when co- transplanted in an E15 host. This is consistent with our hypothesis that the rostrocaudal distribution of MGE interneurons born at early or late stages (namely E12 and E15) is not intrinsically determined but it is primarily dependent on the characteristic of the cortex at the different stages e.g. brain size.

To address this issue we reformatted the figure as follows: we prepared a new figure (now Figure 7) that presents a "simplified" analysis of the rostrocaudal distribution showing only the anterior-posterior axis. The graph shows that the early and late born INs clearly spread over a wider area when co-transplanted in E12.5 host as compared to the co-transplants in a E15.5 host (Figure 7E). In the graph Figure 7F we plot the area occupied by the 80th percentile of the cells in mm^2. The graph in shows the ratio between the area occupied at E12 over E15 (host age) for both early and late born interneurons. We believe that the current figure conveys effectively the message of this experiment. For sake of completeness, the full stereological analysis was maintained and move to Supplemental Figure 4. The complete description of the stereological analysis and statistics is provided at material and methods **section Statistics analysis** in which we explain that the total distribution is statistically analyzed by the Kolmogorov-Smirnov test before to doing it by percentages.

We believe that this amendment should satisfy the Reviewer's concerns. We thank again the Reviewer for helping us improving the manuscript.

5. Doing live migration studies would be extremely informative. They state the cells at E15.5 migrate faster, but in fact they only look at starting and ending locations to evaluate speed. It would certainly be far more informative to conduct live imaging studies, as many labs routinely do for migration studies to determine if it is the result of cells turning more regularly, reversing directions or taking other non-linear routes and thus migrate a shorter distance than cells that might take a more linear route. This possibility is left open and in fact the CIN from E12.5 and E15.5 might migrate the same speed and still have different distances traveled due to a possibility like this.

We agree with the Reviewer and we thank him/her for the suggestion. We performed the experiment of live imaging suggested by the reviewer in MGE explants cultured in Methylcellulose. In addition, we also performed experiments in explants culture on Matrigel to test the relevance of the extracellular matrix on the CINs migration. These experiments are now presented as Figure 6A-E. The results are in line with the conclusion drawn from the analysis of the migration on flattened cortices from Figure 5. Early and late born INs migrate at similar speed. If something, E15.5 cells

appeared to spread a bit more.

It is worth reminding here that we were prompted to determine INs "speed" by the observation that E15.5 MGE INs are enriched in SS cortex and do not spread as much as E12.5 INs to the frontal and caudal regions of the cortex. This reduction in the rostro-caudal spreading of E15 INs as compared to E12 INs could be attributed to a reduction in the migration speed of E15 cells. However, all the experiments that we performed *in vitro* and *in vivo* seem to indicate that actually E15 INs spread a slightly more than E12.5 cells. Altogether these experiments seem to suggest that a decrease in speed in unlikely the cause of the reduced spreading of E15 cells in the A-P axis.

Incidentally, in all this experiments we use a very simplistic definition of speed: distance covered/time spent to cover the distance. We totally agree with the reviewer that this observation could be due to "cells turning more regularly, reversing directions or taking other non-linear routes" or even pausing in certain moments. In our opinion this different cellular modes are not so critical in the context of this study. Nonetheless, we agree with the reviewer that it would be interesting to investigate this issue. Henceforth, following the suggestion of the Reviewer 1 we tried to determine directionality and persistence of migrating interneurons in live imaging in methylcellulose (two-dimension) (in Matrigel and in flat cortices live imaging is more complicated because of the focusing over different planes in three-dimension). As mentioned above, this experiment shown in Figure Supplementary movies and text line (403): Analysis of these movies revealed that the trajectory of the E12.5 cells moving out the explant appeared less linear as compared to E15.5 born cells. In our experimental settings, trajectories of E12.5 seemed more meandering and displayed longer halts between nucleokinesis than E15.5 born cells.

Minor comments:

1. Line 365: wrong call out for figures? (no such info in Fig 7A-B).

Thanks for pointing this out, the error was corrected

2. Figure 4I: age info missing (assuming E15)

For sake of completeness, Figure 4I shows the density of all YFP cells: i.e. all the cells labeled in Nkx2.1-Cre :: Rosa-YFP. It is not a specific age.

3. line 402: further explanation is required for a complete understanding.

We thank the reviewer for this feedback, the text of the manuscript was revised and extended according to the Reviewer's indications.

4. There are numerous typographical and grammatical errors, careful proof reading will be necessary.

We apologize for the errors. We did our best to correct them in the current version of the manuscript.

- Reviewer 2 Advance Summary and Potential Significance to Field:

In this manuscript, the authors have carried out a series of experiments aimed at identifying the drivers behind the unequal distribution of MGE and CGE cortical interneurons across different cortical areas and in different layers. Their conclusion, as the title says, is that the distribution is dependent on migration time and brain size.

We thank the Reviewer 2 for appreciating the novelty of our work and for the constructive feedback. We present here a point-by-point rebuttal to the concerns of the Reviewer. We are convinced that the revised version of the manuscript addressees all the concerns of Reviewer 2.

Reviewer 2 Comments for the Author:

I agree with their observation that there is unequal distribution of SOME interneurons in SOME layers. This is shown if figures 1-4 (but see comment below). However, I find that all further

experiments aimed at testing different possibilities are fundamentally deficient: GABAergic neuron subtype identity is likely to be a key determinant of final distribution. None of the experimental approaches used here account for subtype identity: e.g. by taking 'MGE' cells at E12 and E15 and transplanting them, inevitably, the authors are talking different populations of cells. For example, CGE cells are born later than MGE cells and migrate in all directions, including through the MGE to reach their destination. Therefore, the E15 'MGE' transplants contain more CGE cells than the E12 'MGE' transplants. It is therefore impossible to claim that migration time and brain size are the sole determinants of interneuron spread using the tools in the current manuscript. The authors would need to factor in the subtype identity. At the very least, they should distinguish between MGE and CGE populations in their transplantation experiments using genetic tools.

We thank the Reviewer for this constructive comment. We agree with the reviewer that subtype identity may possibly have an impact on the distribution of Interneurons. In fact, in Figure 1, 2 and Supplemental Figure 1 we use biochemical markers as a first approach to distinguish different subtypes. We understand that for subtype identity here the Reviewer mainly means MGE vs CGE. The Reviewer is concerned that E12 or E15 MGE explants mays contain slightly different cell populations and contents of cells derived from the CGE. In fact, the approach of taking explants from MGE or CGE at different is an established practice in the field (Flames et al., 2004; Lopez-Bendito et al., 2008; Sanchez-Alcaniz et al., 2011). We consider the concern of the Reviewer is very reasonable. It may actually apply to most if not all of the above mentioned literature. To follow up on the comments of the Reviewer we performed immune-labeling of our MGE and CGE explant with 5HT3, an established marker for the CGE interneurons using the Medial Migratory Stream (MMS) which passes through the lateral part of the MGE in their migration to the amygdala. This experiment is shown at Supplementary Figure 5. This experiment show that MGE explants at E15.5 a highly pure (around 90% of MGE cells) with a very small contamination from CGE cells in our experimental conditions. We believe this is because in explant experiments we used mainly the tissue closer to the ventricular zone (VZ). Line 382-383 and Supplemental Fig legend 3

On this regard, we believe that the concerns of the reviewer are at least in part addressed by the genetic labeling of MGE CINs by Nkx2.1-Cre: in this experiments we exclusively check MGE interneurons. Moreover, the heterochronic experiments in Figure 7 (in the revised manuscript) are very informative on this regard.
There we co-transplant E12 and E15 CINs in hosts of different ages. Even assuming for sake of argument that a minimal CGE contamination may bias the results, the CGE contamination would be the same when we transplants the cells in host at E12 or E15. In fact, these co-transplantation experiments of early and late born interneurons clearly show that the migration pattern in rostro-caudal axis primarily depend on age of the host rather than on the MGE explant themselves. Indeed, E12 and E15 MGE cells migrate in a fairly similar way when co-transplanted together in a host of a specific age. Incidentally, a similar argument would apply also for subtype identity between different MGE cells: even assuming that E12 and E15 contain slightly different proportions of MGE subtypes (we believe it is very possibly the case), this different subtype proportion does not seem to affect massively the rostro-caudal migration pattern. Even more in vivo experiments of CGE transplants on the cortical VZ show that different subtypes of CGE-derived cells are somehow able to reach their final location, pallial or subpallial (amygdala) even although were ectopically grafted (Touzot et al., 2016). Therefore we believe that in our transplants where cells were grafted in the same location from where we took out, CGE-derived cells wouldn't have any trouble to find the amygdala (a region out of the scope of our analysis). Again, we do not exclude that different subtypes may display for whatever reason slightly different migration patterns. However, the age of the host appear much more relevant for the anteroposterior distribution in the experimental paradigms that we used in this study.

We reformatted Figure 7 to present these results more clearly. The complete stereological analysis is now presented as Supplemental Figure 4.

We thank the Reviewer for suggesting this smart control experiments on the presence of CGE in our MGE explants that is normally overlooked by the literature. This experiment gives further strength to our results. We believe that this control experiment and the amendments that we made in the current version of the manuscript respond satisfactorily to the Reviewer's concerns.

Other issues with the manuscript:
In figures 1-3 and suppl figure 1, the authors claim that there is unequal distribution of interneurons especially in upper layers. However, the data they show are very selective and perhaps we would not arrive to the same conclusion if the data had been comprehensively presented: for example, what about SST distribution in layers at P14? What about total number distribution of SST, PV and VIP at P30 in different cortical areas? The authors need to show this data.

We thank the Reviewer for this feedback that prompted us to performed additional analysis to present the manuscript more coherently.

Following the suggestion of the reviewer we now show the distribution of PV, SST and VIP interneurons in upper layers and in the total cortex both at P14 (Figure 1-2) and at P30 (Supplemental Figure 1). We believe that this time course analysis of the expression of this molecular markers is quite interesting. The expression of both PV and SST increases in the early postnatal weeks (see for instance Ouellet and de Villers-Sidani, 2014 or Davila et al., J of Comparative Neurology, 2005;). Consistently, the total number PV+ and SST+ CINs and the apparent distribution of PV+ and SST+ is slightly different at P14 and at P30. The PV cells showed a clear enrichment in the upper layers of the SS cortex at both P14 and at P30. The SST+ cells showed an obvious enrichment in the upper layers at P30 but not at P14. Notably, the density of SST+ cells in layer 2/3 increased between P14 and P30. We speculate that this likely indicates an increase in SST expression in layer 2/3 between P14 and P30. Overall, this observation is not particularly surprising since it is in line with previous studies that showed a change in the expression of INs markers in the first weeks of the postnatal development or depending of neuronal activity. Nonetheless, we are thankful to the reviewer for this suggestion. Showing this time course analysis more extensively gives even more relevance and importance to the following Figure 3. In fact, using genetic labeling combined with birth dating with BrdU represents an important improvement over the use "putative" molecular markers. This approach allowed us to label unambiguously all the MGE cells and to compare directly early vs late born MGE interneurons using an unbiased and stable YFP labeling. This was crucial to establish whether INs born at different time points show different rostrocaudal distribution patterns.

In figure 5 the authors transplant E12 to E12 MGE and E15 to E15 MGE and conclude that late-born cells spread faster than early-born ones. My question here is how did they measure the speed? If it is by measuring the rostro-caudal distance covered within a certain period of time then that is a bit misleading.
It is possible that the cortical plate, which is more prominent in the late cortex, is less permissive to migration and interneurons instead migrate in a smaller area away from the CP and as a result travel further. Therefore, the distance travelled in one direction does not take into account the multi- directional dispersion factor. A better experiment would be to do the transplantations E12 to E12 and E15 to E12. This will allow them to quantify the speed of migration in the same environment. However, as explained above, by taking MGE transplants at different times, they are taking donor tissue that consists of different populations of cortical interneurons or other subpallial neurons. Hence, the identity of interneurons may be the prime factor, something that is not tested at all by the crude transplantations used throughout the manuscript (Fig 5, Fig 6, Fig 7 and suppl Fig 3).

We thank the reviewer for giving us a chance to clarify this point that is quite important. First, the speed in this experiment was measured by literally dividing the "distance covered within a certain period of time" as correctly understood by the reviewer. We think that it is worth reminding here that the goal of this experiment was to test the hypothesis that the reduction in the rostrocaudal spreading of E15 INs was due to a decrease in the distance covered by E15 CINs. With regard to the caveat and "permissiveness" of the cortex to migration we totally agree with the reviewer. Henceforth, in order to look specifically at the "intrinsic" migration speed of E12 vs E15 without the bias of the substrate (i.e. the cortex) we performed *in vitro* experiments to evaluate the migration of INs in the Methylcellulose or in Matrigel (to test the relevance of the Extra-cellular matrix). These experiments are now in Figure 6. In both condition, E15 INs migrated to a similar

speed as E12 cells, just a bit faster. Altogether, these experiments do not indicate that the reduced spreading of E15 INs observe in Figure 4 (*in vivo* genetic labelling) may be due to a reduced velocity. These results are further confirmed by Fig7 and Suplementary fig.5 in vivo experiments. Altogether, these experiments did not reveal major differences between E12 and E15 MGE interneurons that could explain the reduction in rostrocaudal spreading observed in E15 INs *in vivo* in Figure 4. We agree with the Reviewer that this does exclude that there might be some difference in the E12 and 15 populations. This possibility is debated in the discussion Line 555-557. Nonetheless, co-transplantation experiments of E12 and E15 cells showed that the age of the host causes a major difference in the anterior-posterior spreading.

In conclusion, we thank again the reviewer for this constructive feedback that contributed to improve the manuscript. We are convinced that the current version fully responds to the Reviewer's issues.

Reviewer 3
Advance Summary and Potential Significance to Field:

The aim of the present manuscript is to study the mechanisms that control the dispersion of GABAergic interneurons (CIN) through the cortex during brain development, together with a quantification of the final distribution of cell subtypes across cortical areas (motor, somatosensory and visual) and layers in the adult mouse.
The authors claim that there is no directionality in CIN migrating through the developing cortex. Instead, CIN final location is determined by the time cells spent migrating and the length of the path they traverse. Although this would be an interesting finding, their experimental evidence is not as strong as needed to reach such a conclusion. For that reason, the authors are encouraged to revise their data and go deep in this direction to collect a more solid dataset. I suggest to start the manuscript with the mechanistic set of experiments (figures 6 and 7 of this manuscript plus new data) and leave to the end the quantification because most of this data is already known.

We thank the Reviewer for the kind appreciation of the originality of our study. We are also grateful for considering our study an "interesting finding". While considering our work interesting and original, the Reviewer expresses doubt on the robustness of our data that provide novel insight on the migration of interneurons in the anterior-posterior axis of the cortex. This constructive feddback of the Reviewer 3 was very useful since it prompted us to perform new experiments and improve the presentation of our data. In particular, we made clearer the originality of our study in the context of the previous literature. In addition, we completed our dataset in a more comprehensive way and provided novel experiments that further support our model.

We are convinced the revised version of this manuscript matches the Requirements of Reviewer 3. Specific point-by-point replies and amendments are presented below.

Reviewer 3 Comments for the Author:
Major issues:

-Much more data and analysis are required to support the main hypothesis of the manuscript: ?the physical constrains imposed by time, interneuron speed and brain size may contribute to the differential distribution of cortical, interneurons along the rostro-caudal axis? (cited from the abstract). Tackled in figures 6 and 7.

We understand that the Reviewer 3 perceives that we failed to clarify the model that we propose and to present the appropriate supporting evidence to this model (specifically in figure 6 and 7). We agree with the Reviewer 3 that the mechanistic model that we propose for the migration of interneurons along the rostro- caudal axis is not immediately intuitive. Moreover, in the previous version of the manuscript, Figure 6 and 7 were not very easy to "read". In particular, Figure 6 presented a lot of data that may not be straight forward to grasp if you are not familiar with this kind of stereological analysis. We therefore thank the Reviewer 3 for this criticism. In the revised version of the manuscript we present novel experimental evidence, we did improve the

presentation of the data and we present a mathematical model whereby we test in silico our working hypothesis. In this mathematical model we consider the rostrocaudal spreading of interneurons from their site of origin taking into account the concomitant expansion of the cortex while the neurons are migrating. Our experiments showed that interneuron from MGE at E12.5 or E15.5 migrated at a similar speed in a flat mounted cortex (20 um/hour at E12.5; 25 um/hour at E15.5). However, the cortex almost double its size from E12.5 to E15.5 (Fig. 7) and keeps expanding postnatally (supplementary fig. 2). Therefore, E15.5 interneurons migrate in a cortex that is much wider as compared to E12.5. Our mathematical model predicts that this cortical expansion would limit the rostrocaudal spreading of late born cortical interneurons as compared to early born.

The transplantation experiments in figure 6 and 7 are aimed at testing *in vivo* this prediction. These figures 6 and 7 were revised to simplify the understanding of the experiments. In the current version, we present the profiles of E12 and E15 interneurons into hosts at different ages (Fig7). These plots clearly show that early and late born interneurons migrated very similarly when we cotransplanted them in the MGE on the same host mouse. Thereby, the age of the host drastically changed the spreading of interneurons over the anterior posterior axis. The spreading of interneurons transplanted in a host at E15.5 was much wider than the spreading that we observed at transplanting the interneurons at E12.5. For instance, the area delimiting the 80th percentile of cells increased by ~45% (see Figure 7 and sup figure 3).

In other words, E15.5 interneurons migrated at the same distance as early born E12.5 interneurons when co- transplanted hetero-chronically to an E12.5 host. Conversely, early born E12.5 interneurons spread similarly to late born E15.5 interneuron when co-transplanted to an E15.5 host.

Altogether, we believe that this set of experiments demonstrates that the differential distribution pattern of early and late born interneurons is not intrinsically determined but rather depends on the characteristic of the cortex at E12.5 or E15.5. The size of the cortex is arguably the most apparent difference between early and late developmental stages. Based on our mathematical prediction and experimental evidence, we propose that the dramatic cortical expansion that occur during development may contribute at least in part to limited spreading of late born interneurons as compared to the early born cells.

-In the first paragraph of the Results, the authors explain that? whether CINs distribute equally along the R-C axis or may have different distributions in distinct microcircuits has not been explored?. Figures 1 to 3 show their results in this regard. However, this question has been already tackled by several publications (Xu et al 2010, Van Brededore et al 1990, Gochar and Burkhalter 1997, Nery et al 2002, Keller et al 2019, Ouellet and de Villers-Sidani 2014, and others).

We thank the Reviewer 3 for giving us the opportunity to further highlight to originality of our work in the context of the previous literature. Of course, we are familiar with the literature mentioned by Reviewer 3 and indeed we already quoted the studies that are more relevant in the previous version of the manuscript.

In our study we investigate the rostrocaudal distribution of different interneurons subtypes from different embryonic origin and developmental stages. To this aim, we scrutinized concurrently and systematically the distribution if different INs at multiple rostrocaudal levels and layers. Notably, we took advantage of both molecular markers and genetic labeling to trace the localization of Interneurons from different sites of origin in the ganglionic eminences and developmental stages. Most of the studies quoted above either focus of specific areas and/or fail to combine molecular marker with birth dating and genetic labeling.

We first performed this analysis using different molecular markers. The use of markers has obvious limitations due to the intrinsic variability related to the use of different antibodies. More importantly, the approach of using molecular markers falls short to the mayor caveat that the expression of these markers may change during development and in different experimental conditions. This variations would obviously alter the quantification of the total number and

distribution of the neurons. For instance, the interesting study mentioned by Reviewer 3 from Ouellet and de Villers-Sidani 2014 shows a dramatic change in the expression of different markers as PV, Somatostatin and NPY from early postnatal stages until aging (incidentally, the study focus very specifically in rat primary auditory cortex (A1) only). Analogous studies reached similar conclusions (e.g. Bu et al, 2003, https://doi.org/10.1016/S0014-4886(03)00094-3; Pugliese et al., 2004; DOI:10.1002/jnr.20223). Another example is provided by the work of the lab of Pico Caroni (Nature, 2013; doi: 10.1038/nature12866) that showed that different behavioral paradigms regulate the expression of Parvalbumin in interneurons. Another pivotal study from the lab of Prof Greenberg (Mardinly et al., Nature, 2016, doi:10.1038/nature17187) showed that sensory experience strongly regulates the expression of GAD67, PV SST and VIP in interneurons (see figure below. The authors investigate gene expression control mice and in darked-hosed animals upon visual stimulation).

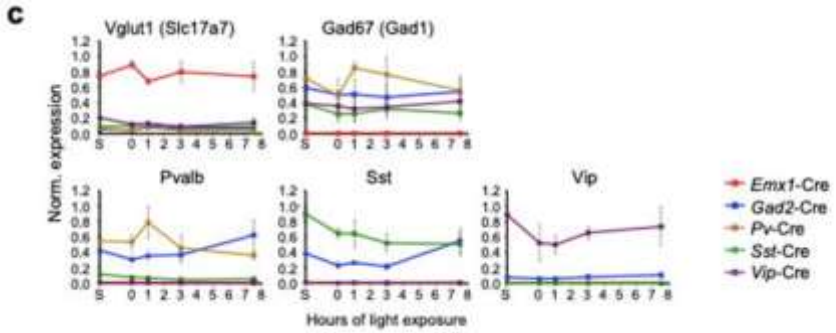

Figure from taken from Mardinly et al., Nature, 2016. Extended Data Figure 1. Validation of the sensory stimulation protocol and the RiboTag-based cell-type-specific purification of mRNA. The authors observed a significant change of the mRNA expression of interneuron markers in upon sensory stimulation.

While a big part of the literature tend to ignore this bias for "pragmatic" reasons, most proteins used as putative interneuron markers do have important functions such as calcium binding (e.g. PV, CR, CB) or signaling functions (e.g. NPY or VIP).

In this context, the use genetic labeling of MGE neurons combined with BrdU birth dating and transplantation *in vivo* provide a sharp improvement over the previous studies (Figure 4, 6 and 7). This kind of technologies allow an unambiguous labeling that is obviously superior to the traditional use of putative biochemical markers. To the best of our knowledge, this is the first study that takes advantage of this combined approaches to determine the rostrocaudal distribution of cortical interneurons from different developmental stages and origins.

The Reviewer 3 mentioned above that "this question has been already tackled by several publications". Very respectfully, we do not agree with this observation and we provide here a point-to-point discussion on the manuscript mentioned by Reviewer 3 in relation to our study:

- Xu et al 2010. **We quoted this study**.

We are well acquainted with the work of the lab of Ed Callaway who is a great scientist that we sincerely admire. In addition, among the studies mentioned by the Reviewer we believe it is the only one that actually investigates the rostrocaudal distribution of different interneurons. Notably, the data are overall consistent with our observations. Nonetheless, we believe that this manuscript displays differences and limitations compared to our study that prompted us to improve upon this work and to perform our own analysis. This manuscript is aimed at characterizing the distribution of chemically distinct classes of interneurons. Henceforth, the authors do not use any kind of genetic labeling to identify the site of origin of the interneurons. We already discussed above the limitations of this approach based on immune-labeling to distinguish subpopulation of interneurons. Moreover, they do not perform birth dating to unambiguously distinguish late and early born interneurons.

Other differences are: Xu et al., worked on C57BL/6 mice while we used a different strain CD1; they worked of frontal cortex while we analyzed the motor cortex; they only present high magnification pictures (so we do not know exactly where the quantification was performed); finally, the age of the mice is not specified (the authors simply mentioned adult mice). As explained in the above mentioned reference *Ouellet and de Villers-Sidani 2014*, the age of the mice can be very relevant in this context. In our study, we analyzed different postnatal stages and we investigated the neuronal distribution with multiple techniques, including a sofisticated combination homo- and hetero- chronic cell transplants *in vivo*. In conclusion, this study from Xu et al., is very interesting but it had a different focus from our study and different technical limitations in comparison to ours. For these reasons, we are convinced that our work provides a very significant advance in comparison to the study of Xu in the context of the understanding the cellular mechanisms of rostrocaudal distribution of cortical interneurons during development.

- Van Bredeodore et al 1990. This study does not investigate the rostro-caudal distribution of interneurons. In addition, it was performed in monkeys and it only focuses on PV and Calbindin expression. In sum, it is not really relevant for our study.

- Gochar and Burkhalter 1997. This study is performed in rats. It only focuses on a specific visual area (on area 17). They do not investigate VIP interneurons. Again, since it does not evaluate the rostrocaudal distribution of interneuron we believe that this study is not relevant for our work.

- Nery et al. 2002. This is a very interesting work from the lab of Gord Fishell, a recognized reference in the field. Of course, **we already quoted this paper in the first version of this manuscript**. The authors found that CGE is distinct from MGE and LGE. Specifically, Nery et al., show CGE cells migrate to different regions of the brain such as nucleus accumbens, striatum and globus pallidus and in the cortex. However, the rostrocaudal migration of different interneurons is not at all the focus of the study. The only reference that we could find in Nery's manuscript on the A-P localization of CGE neurons was at page 1281: *"CGE cells migrated to the cortex, especially at more caudal levels, where they differentiated into neurons that almost exclusively populated layer 5 (Figs. 5a-i and 6h)*". In the figure legend (Fig. 5) these cells are described as "stellate neurons". We are not entirely sure what the authors mean with "stellate neurons" since to the best of our knowledge stellate neurons are normally found in layer 4 and are excitatory. The authors do not use any molecular marker to further define these cells. Regardless of the specific identity of these cells, the authors do not present any quantification. Very respectfully, overall the analysis of rostro-caudal migration is much more superficial than in our study. We do not mean to disregard by any mean the great quality of this study: we simply want to highlight that the rostrocaudal distribution is not the focus of Nery's work and was not studied in depth.

Indeed, with regards to the cortex, the experimental paradigm and the analysis performed by Nery is substantially different from our work. Nery and colleagues performed only homotopic-homochronic transplantation of interneurons born at E13.5. They do not investigate different developmental stages with heterochronic experiments; they do not co-transplant simultaneously cells E12.5 and E15.5 neurons to directly test different migration patterns; they do not use molecular markers to evaluate rostrocaudal distribution; they do not perform genetic labeling; they do not perform birth dating experiments.

In conclusion, the study of Nery is very interesting but it is neither designed nor aimed at investigating specifically rostrocaudal migration. Notably, Nery concludes the Discussion of the manuscript stating as future goal: *"It will be interesting to determine if transplants obtained from different time points of development generate cells of different migratory and cellular fates" (page 1285)*. Exactly one of the issues that we investigate in of our work.

- Keller et al. 2019. This work is very nice descriptive study of the interneurons in the rat somatosensory cortex at P14. The authors do not investigate at all rostrocaudal distribution.

- Ouellet and de Villers-Sidani 2014. We thank the Reviewer for pointing out this very interesting study that we did not mention is the previous version of the manuscript (we first quote it in the revised version at line 283). The authors very specifically focus on the rat primary auditory cortex. They do not investigate at all the rostrocaudal distribution of interneurons. Nonetheless, this work

is very interesting because it provides an extensive longitudinal characterization at different ages of the expression of different proteins usually taken as interneuron markers. The authors observed some changes in the expression of IN markers that may be explained by cells loss associated with early postnatal stages and late aging. However, Ouellet intriguingly observed that the expression curve was different from marker to marker. Notably, the number of positive cells for some mayor markers such as PV, Somatostatin and NPY actually increased in the first postnatal months. NPY expression increased even further with aging. In sum, this interesting work shows a fairly dynamic evolution of the expression pattern for this "putative" markers during life span. These observations further strengthen our experimental approaches of genetic labeling, birth dating and heterochronic transplantations *in vivo*.

In conclusion, we are convinced that our study provides a novel and original perspective on the rostrocaudal migration of cortical interneurons at different time points.

-In the same line, half of the conclusion of figure 2 stated in lines 263-265 is described in Nery et al 2002.

Please see the previous point for the discussion on Nery's work.

-In figure 1 the authors show the total number of CIN subtypes in the three cortical areas. In figure 2, they show the same data but layer-wise. There is a problem with this quantification because in all areas the total number of cells of each subtype is lower than the sum of cells from layer 2/3, 4 and 5/6.

The quantification is expressed in cell density both in Figure 1 and 2. All graphs are labeled cells/mm^2. We indicated that we expressed cell density also in the text of the results section and in the Method section.
Logically, the density calculated in the entire cortex is "*lower than the sum*" of the density calculated in layers 2/3, 4 and 5/6.

-With their in vivo transplantation of E12+E15 MGE cells into E15 embryos, they showed that E15 interneurons prefer to migrate towards caudal levels. These results are contradictory with their BrdU experiments in which they showed a preference of E15 MGE interneurons to populate the SS cortex. How do the authors explain this? Also, Figure 6 would benefit of an image of the transplanted cells at P14.

We thank the Reviewer for prompting us to clarify this issue. In Supplemental Figure 3C we show that both E12 and E15 cells accumulates in the SS cortex. What we show in Supplemental Figure 3D is the ratio between E12 and E15 in SS and Visual cortex. Therefore, this data at P0 are perfectly in line with the previous experiments *in vitro* and *in vivo*. The age of the host at the time of transplantation is the main determinant of cell distribution: when co-transplanted in an E15 host both E12 and E15 INs accumulated in the SS cortex. The fact that the ratio between E15 and E12 is higher in the visual cortex simply confirms our in vitro experiments that E15 cells are a bit faster than E12 cells. In the revised version of the manuscript we present the distribution of cells in SS and Visual cortex. We believe that this way the data are easier to understand and we thank again the Reviewer for the constructive input.

-Makes no sense to show data from VIP+ and PV+, and the data for SST+ in two separate figures (figure2 and supplementary figure 1). Actually, the authors need to use the same data twice: 1) the bars for layer 2/3 in panel J of figure 2, 2) in figure suppl 1 panel H. Moreover, all layer for SST+ should be shown.

We understand that the Reviewer may have overlooked that Figure 2 and Supplemental Figure 1 show 2 different developmental stages, P14 and P30 respectively. Henceforth, the data are not used twice.

We also point out that in the revised version of the manuscript the quantification of SST

interneurons is also presented Figure 2 (P14) while Supplemental Figure 1 now includes also VIP interneurons. It is important to notice that the apparent density of SST+ cells in layer 2/3 seems to change between P14 and P30. This data are in line with the above mentioned work of Oullet that shows a strong increase of SST expression in the early postnatal stages (first weeks and months). Understanding the regulation of the expression SST peptide hormone and its biology is out of the scope of this current study. Nonetheless, this observation nicely exemplifies the kind of caveats and limitations of using this "putative" cell makers. It further stresses the importance of genetic labeling as the most effective and robust approach to overcome this limitations in the context of the current study.

-The authors concluded that the lower density of Nkx2 cells at P30 compared to earlier stages is due to programmed cell death and brain enlargement. Although these conclusions result quite logical (and have been partly published) there are no experiments that supports them in the manuscript.

We thank the Reviewer for this constructive comment. In response to the Reviewer we performed an analysis of brain expansion and cell death. This data are presented in Supplemental Figure 2 of the revised version of the manuscript. Briefly, the cortex significantly increased in size between P7 and P30 (Supp Fig 2 H).

Moreover, we found as expected cell death of GFP positive interneurons at P7 using Caspase-3 as an apoptotic marker. Notably, apoptosis equally affected rostral and caudal levels. Line (327-329)

-As the authors explain in the manuscript, the BrdU experiments of figure 4 do not add any new information to what is already published or to what have shown in the previous figures of the manuscript.

We are not sure what the Reviewer specifically refers to in saying "As the authors explain in the manuscript the BrdU experiments of figure 4 do not add any new information". Respectfully, we did not find in the manuscript a paragraph where we said anything similar to this. Nonetheless, we thank the Reviewer for this comment because it prompts us to highlight the importance of this experiment. Figure 4 is indeed crucial in the context of our study. Our goal here was to determine whether Interneurons originated from the MGE at different time points showed different migration patterns. To accomplish this aim we needed 1) an unambiguous labeling of MGE derived interneurons and 2) a precise birth dating of the interneurons. With unambiguous marker we mean: i) **only** the MGE interneurons express this marker; ii) **all** the MGE interneurons express it; iii) the expression of the marker must be **stable overtime** and independent of the activity or development of the cells in order to ensure a reliable quantification of the interneurons. None of the molecular markers available would fulfill these requirements. Therefore the genetic labelling experiment was crucial for our goal.

Regarding the birth dating, we speculate that possibly the reviewer meant that since the birth date of the neurons may be inferred from the position in the layers, the BrdU is redundant. Very respectfully, we would argue the assuming the birth date of the interneurons from the position in the layers is a rather crude method. Of course, the majority of early born INs are located lower layers while late born INs are found in upper layers. However, this approximation is rather imprecise. On one side, not all the INs in one layer are born at the same time. On the other side, BrdU and other birth dating methods clearly show that interneurons born at a specific age accumulate in one layer but can be found also in other layers. For instance, in Figure 4 we show that some E15 interneurons can be found in layer 4 and some even in layer 5/6 (see figure legend 4) (70% in layers 2/3, 20% in layer 4 and 10% in layers 5/6, across brain areas) and (93% in layers 5/6, 5% in layer 4 and 2% in layers 2/3, across brain areas). This is expected and consistent with previous studies. Henceforth, BrdU labeling is not redundant but is arguably the most accurate method that we could employ to investigate the localization of MGE interneurons born at different time points.

In conclusion, we thank again the Reviewer for helping us to give more importance to this experiment which is much cleaner and refined in comparison to the standard approaches used in

Figure 1-3. The text in the manuscript was also amended to explain better to the reader how this approach significantly improved over the caveats of the methodology in Fig 1-3 (see line 284-306).

Minor:

-Consistency in nomenclature: layer 2/3 or II/III, and other similar cases.

We thank the Reviewer for this feedback. We did our best in the current version to revise and amend these errors.

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Second decision letter

MS ID#: DEVELOP/2019/185033

MS TITLE: Cortical distribution of GABAergic interneurons is determined by migration time and brain size.

AUTHORS: Pietro Fazzari, Niall Mortimer, Odessa Yabut, Daniel Vogt, and Ramon Pla

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that you address th every minor remaiing comments of the referees. In particular, please do remove from your manuscript the concept of cell-cell repulsion a possible mechanism for the dispersion of CIN you observe. Because tere is no direct demonstration that this is the mechanism, it is best to leave it out. Also attend to the remaining points by Rev 2. These final edits will not require further experiments, I believe. Please send us a point-by-point response indicating where you are able to address concerns raised .

Reviewer 1

Advance summary and potential significance to field

Cortical interneurons (CIN) are derived from two primary progenitor locations, the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE). It is know that different subpopulations of CINs are derived from each region and they differentially populate different cortical regions. What was unclear is if over time the CIN derived from each location change over

time and populate different cortical regions or cortical lamina. Using a combination of genetics and transplantation experiments they have addressed these questions and have provided some novel insights into neocortical development. Namely, that early born CIN migrate more broadly across the brain and populate multiple lamina whereas later born CIN remain more localized, despite migrating as fast or faster. The authors suggest using a mathematical model, that this is the result of changes in the brain size, a novel concept and supported by their data.

Comments for the author

This revised manuscript is significantly improved over the previous manuscript.
The have directly addressed the concerns raised with additional experiments and rewriting of the manuscript. Two remaining issues.
1. The manuscript continues to suffer from grammatical errors and will benefit from careful editing. I started to write them down, unfortunately too many.
2. They introduce the concept of cell-cell repulsion, as observed for Cajal Reitzius cells, in the introduction. They then discuss this as a possible mechanism for the dispersion of CIN they observe. However, this mechanism is not tested in any way in this manuscript. I think other mechanisms of dispersion certainly exist and they are not discussed. It would be best if they directly tested this in their studies, short of doing so I feel this should be removed entirely from the manuscript or listed as one of many possible mechanisms in the discussion.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Fazzari et al interrogates the unequal distribution of cortical interneurons along the rostro-caudal axis and the possible mechanism that would explain these variations. They show that, while MGE-derived interneurons born at E15 resided preferentially in the somatosensory cortex, CGE-derived cells are enriched in caudal cortical areas. In both cases, the final destination is a cortical area close to their respective sites of origin. In vivo and in vitro assays show that the differential dispersion of cortical interneurons depends, not on a directional migration, but on the developmental stage of the brain, which confers physical constrains to cell navigation.

Comments for the author

The authors have made a considerable effort to streamline the manuscript and to add data. I really appreciate the comprehensive reply and amendments to address my concern regarding the originality of their manuscript. Their complete explanation and especially the new experimental evidence clarify the focus of the work. I still think that part of the evidence was already known, yet fragmentated in different publications. Then, I reckon the present manuscript as the first attempt to assess in deep the rostro- caudal distribution of different interneurons subtypes from different embryonic origin and developmental stages. What I tried to state was not that there are other publications tackling exactly the same research question, but that some of the results could be found or inferred from previous ones. In any case, this manuscript is relevant because they try to put different pieces together and, importantly, adding new perspectives. Thus, I think that the current version provides enough evidence to support their working hypothesis, a valid and original hypothesis.

I apologize for my mistake regarding the quantification of cell density in figures 1 and 2.

Minor points:

- Adding time-lapse data was a great decision but the quality of the movies presented should be improved and their analysis is not shown in the manuscript. The explants of interest could be centered. Also, more frames could be added as they record overnight. And if one point they want to raise with the movies is that e12.5 cells follow a more tortuous pathways and saltatory migration, the author should present a close- up of a small group of cells or just one cell that illustrates movement behavior at e12 and e15. This could support their statement (lines 414-417)

about how erratic and smooth is the migratory pathway at different stages, however, authors are encouraged to perform a numeric quantification if they really want to make a point of this.

- In line 339, when they say “cortical expansion”, I think they refer to an expansion of the cortical thickness. As in other parts of the manuscript the authors use “cortical expansion” to describe the developmental increase in size of the cortex, they may consider to change the line accordingly.

- They call Fig S5 instead of Fig S3 (line 393) when describing the presence of 5HT3aR+ cells.

- In line 401, Fig 5E should be replaced by Fig 5J.

Second revision

Author response to reviewers' comments

Point-by-point rebuttal to Reviewer’s comments

To the Reviewers

Reviewer 1 Advance Summary and Potential Significance to Field:
Cortical interneurons (CIN) are derived from two primary progenitor locations, the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE). It is know that different subpopulations of CINs are derived from each region and they differentially populate different cortical regions. What was unclear is if over time the CIN derived from each location change over time and populate different cortical regions or cortical lamina. Using a combination of genetics and transplantation experiments they have addressed these questions and have provided some novel insights into neocortical development. Namely, that early born CIN migrate more broadly across the brain and populate multiple lamina whereas later born CIN remain more localized, despite migrating as fast or faster. The authors suggest, using a mathematical model, that this is the result of changes in the brain size, a novel concept and supported by their data.

We thank Reviewer 1 for appreciating the novelty of using a mathematical model supported by our cell transplant experiments.

Reviewer 1 Comments for the Author:
This revised manuscript is significantly improved over the previous manuscript. The have directly addressed the concerns raised with additional experiments and rewriting of the manuscript. Two remaining issues.
1. The manuscript continues to suffer from grammatical errors and will benefit from careful editing. I started to write them down, unfortunately too many.
2. They introduce the concept of cell-cell repulsion, as observed for Cajal Reitzius cells, in the introduction. They then discuss this as a possible mechanism for the dispersion of CIN they observe. However, this mechanism is not tested in any way in this manuscript. I think other mechanisms of dispersion certainly exist and they are not discussed. It would be best if they directly tested this in their studies, short of doing so I feel this should be removed entirely from the manuscript or listed as one of many possible mechanisms in the discussion.

We are glad that Reviewer 1 found the new paper has improved compared to the previous one due to the new experiments done and the rewriting. In the revised version of the manuscript we took carefully in consideration his comments.

1. We believe that the current version of the manuscript is grammatically correct for the final text was revised carefully by the native English speaker coauthors of the manuscript. In this respect we put our best effort to make sure that we corrected all the grammatical errors.

2. We have revised the document and removed any reference to the cell-cell repulsion as CIN

dispersion from the Introduction and the results. We only mention this type of migration as a possibility to explain the R-C CIN migration in the discussion. We believe that this amendment should satisfy the Reviewer's concerns. We thank again the Reviewer for helping us improving the manuscript.

Reviewer 3
The authors have made a considerable effort to streamline the manuscript and to add data. I really appreciate the comprehensive reply and amendments to address my concern regarding the originality of their manuscript. Their complete explanation and especially the new experimental evidence clarify the focus of the work. I still think that part of the evidence was already known, yet fragmentated in different publications. Then, I reckon the present manuscript as the first attempt to assess in deep the rostrocaudal distribution of different interneurons subtypes from different embryonic origin and developmental stages. What I tried to state was not that there are other publications tackling exactly the same research question, but that some of the results could be found or inferred from previous ones. In any case, this manuscript is relevant because they try to put different pieces together and, importantly, adding new perspectives. Thus, I think that the current version provides enough evidence to support their working hypothesis, a valid and original hypothesis.

We thank the Reviewer for the kind appreciation in the effort we made to improve the manuscript. We are also grateful for considering our study the first one to put all the pieces of the puzzle together and give a general idea of the intracortical migration by different CIN subtypes. We agree with the reviewer that in recent years several authors have partially addressed different aspects of how CINs settle in the cortex, but no one has provided a mechanism to explain it yet. This is why we consider the topic so important and we thing this manuscript can shed light on the CIN arrangement in the cortex.

Reviewer 3 Comments for the Author:
Minor points:
- Adding time-lapse data was a great decision but the quality of the movies presented should be improved and their analysis is not shown in the manuscript. The explants of interest could be centered. Also, more frames could be added as they record overnight. And if one point they want to raise with the movies is that e12.5 cells follow a more tortuous pathways and saltatory migration, the author should present a closeup of a small group of cells or just one cell that illustrates movement behavior at e12 and e15. This could support their statement (lines 414-417) about how erratic and smooth is the migratory pathway at different stages, however, authors are encouraged to perform a numeric quantification if they really want to make a point of this.

We thank the reviewer for this feedback, and in order to support our statement we added a new figure (Supplementary Fig.6). There, we quantify the migratory straightness (from 0 to 1) in both CIN populations. In addition as reviewer suggest, we show a close-up of some cells migrating at different time-points.

- In line 339, when they say "cortical expansion", I think they refer to an expansion of the cortical thickness. As in other parts of the manuscript the authors use "cortical expansion" to describe the developmental increase in size of the cortex, they may consider to change the line accordingly.

We agree with the reviewer and we did our best to amend the mistakes in this new version.

- They call Fig S5 instead of Fig S3 (line 393) when describing the presence of 5HT3aR+ cells.

Thanks for pointing this out, the error was corrected.

- In line 401, Fig 5E should be replaced by Fig 5J.
Thanks for pointing this out, the error was corrected.

Third decision letter

MS ID#: DEVELOP/2019/185033

MS TITLE: Cortical distribution of GABAergic interneurons is determined by migration time and brain size.

AUTHORS: Pietro Fazzari, Niall Mortimer, Odessa Yabut, Daniel Vogt, and Ramon Pla
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.