Notch signaling regulates neural stem cell quiescence entry and exit in *Drosophila*

Chhavi Sood, Virginia T. Justis, Susan E. Doyle and Sarah E. Siegrist
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Original submission decision letter

MS ID#: DEVELOP/2020/198242

MS TITLE: Neural stem cell quiescence is controlled by daughter cell mediated Notch activation in Drosophila

AUTHORS: Chhavi Sood, Virginia T Justis, Susan Doyle, and Sarah Siegrist
ARTICLE TYPE: Research Report

Dear Dr. Siegrist,

I apologise for the delay. 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 from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. They point to a number of interesting observations but feel that some results require further clarification. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential importance of this work. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

Reviewer 1

Advance Summary and Potential Significance to Field

In this manuscript, the authors investigate the function of Notch signaling in the regulation of neuroblast quiescence at the end of embryogenesis in Drosophila.
For this purpose, they rely on various reporter lines and antibodies to investigate the expression of the Notch ligand Delta, and Notch signaling activation. They also use RNAi transgenes to generate

knockdown conditions in neuroblasts. Using these types of assay, they evaluate when neuroblasts enter or leave quiescence. Their data lead to the conclusion that Delta expression in neuroblasts and their progeny that are successively generated during embryogenesis leads to a dynamic regulation of Notch signaling in NBs that is important to trigger quiescence towards the end of embryonic stages.
This manuscript therefore provides a lineage intrinsic mechanism that contributes to scheduling neural stem cell quiescence during development.

Comments for the author

I liked very much the idea of a dynamic regulation of Notch activity in NBs driven by opposing temporal gradients of Delta cis-inhibition and trans-activation. In this model, decreasing loads of Delta in dividing NBs reduce cis-inhibition. The concomitant increasing trans-activation by Delta from the undifferentiated progeny promotes Notch activity. As Notch signaling ultimately promotes NB quiescence, trans-activation progressively stops as progeny stop being generated and differentiate Ultimately, this allows for down-regulation of Notch signaling in quiescent NBs, and later reactivation of NBs during early larval stages upon feeding.

However, all RNAi experiments aiming at down-regulating Notch signaling in embryonic NBs led to very mild phenotypes with apparently only a few NBs delaying or escaping quiescence whatever the genetic background (Figures 2, 3). It is therefore hard to extrapolate a general role for Notch signaling based on these very mild phenotypes, and I have to say that I am not fully convinced by the data here.

What about using mutants instead of RNAi in embryonic neuroblasts?

- e.g. Kuz e29-4 that had already been used in NBs for abrogating Notch signaling (Bivik et al 2016). Maybe the phenotype would be more convincing…

- If the hypothesis is correct, blocking NB division during embryogenesis should prevent trans-activation of Notch signaling in NBs and also prevent entry in quiescence. This could be investigated using a string mutant to block cell cycle progression or a pebble mutant to block cytokinesis. In the latter mutant, neuroblasts should still cycle at the end of embryogenesis and not activate Notch signaling. These mutants have been extensively used by the Doe lab.

- In your model, maintenance of Notch signaling in quiescent NBs should prevent cell cycle re-entry. Can you test this using NICD?

References to Figure 4 is wrong throughout the paragraph p8… :(Figure 4: Make sure Figure legend matches with Figure…

Minor comments or non-essential experiments:
The term “non-MB NBs” is confusing and complicate the reading. Why don’t you call them CB NBs (central brain) as opposed to MB NBs. I think it would make the manuscript easier to read.

P8: add Reference to “(REF)”

Figure 3B-H: please indicate the GAL4 driver used to mis-expressed RNAi transgenes.

The presented model of opposing temporal gradiants of cis-inhibition in NBs and trans-activation by NB progeny is attractive. It would have benefited from some kind of numerical simulation in order to better visualize how it can provide a mechanism for the dynamic regulation of Notch signaling in dividing neuroblasts.

What happens in castor mutant embryos in which NBs do not enter quiescence. Do they exhibit strong or reduced Notch signaling activation?

Would you also see trans-activation in context of NB amplification such as upon mis-expression of constitutively activated aPKC or loss of prospero. Could one expect cis-inhibition in NBs to overcome trans-activation?

Reviewer 2

Advance Summary and Potential Significance to Field

How stem cells enter and exit quiescence is an important question. The authors here have focused on the role of Notch in this regulation, investigating pathway activity and function at the transitions in Drosophila neural stem cells (NBs). The results are interesting but confusing.

1. Notch activity increases after the NBs exit quiescence, when the NBs are reactivated in a nutrition dependent manner.

2. Notch activity is needed for quiescence

3. Notch activity is very low in quiescent NBs

4. Perturbing Notch prevents NBs entering quiescence.

Taken at face value the results seem contradictory. Quite possibly Notch role changes at different stages but this is not overtly discussed. Likewise point 2 (Notch is required for quiescence) is more likely a manifestation from perturbing the entry into quiescence (point 4). The way the results are presented does not really recognize this not join the dots together very well.

The authors formulate a model involving cis-inhibition and trans-activation that is not very well substantiated by their data and is highly speculative. It is not easy to tease apart these different actions, and the experiments, as reported, do not do so. It is also unclear how Notch is promoting quiescence, given that it's levels decline in the quiescent NBs. There are a number of targets that could be investigated. They also need to rule out that this is a consequence of a change in NB temporal fates.

In summary there are some interesting observations and the data have been well quantified for the most part. But because the findings are complicated it leaves many gaps in the story. For example there is no real insight into how Notch might be promoting quiescence at the end of embryogenesis and, if so, what the relevance the subsequent downregulation has

Comments for the author

The paper would need substantial additional work to justify the title and the conclusions reached.

1.More mechanistic insight is needed and a more fine-scaled dissection of when Notch activity is required to drive quiesecnce and whether its essential that it is shut off. What would happen with ectopic NICD? Would that drive quiescence (as their model predicts) or block it (because it later needs to be down-regulated)? It remains plausible that the regulation is indirect and is bought about by a failure of the NBs to transition to the right stage (e.g. by a switch in temporal factors).

2. How is Notch regulating quiescence. There are several studies showing it regulates cell cycle genes like cyclin E and decapo also that these are negatively regulated by the E(spl) targets of Notch (e.g. papers from Stefan Thor; Bivik et al, 2016). Some of these would be obvious targets to investigate.

3. The model re cis-inhibition is speculative and the results with Dl RNAi are contradictory/confusing. What would be the prediction if they used drivers to ablate Dl in the progeny only? Can they use a more fine-scaled temporal approach to deplete Delta at different stages? Using KuzDN that will only affect trans signaling and not cis-inhibition may be another approach.

4. In far too many cases conclusions are reached without substantiation. For example, there is a correlation between NBs that divide and those that retain E(spl)my and Delta. But this does not prove that reduced Dl is required for quiescence nor does it prove that the division of the GMCs is the cause of the reduced Dl even though it is a nice model

5. The expression of E(spl)my-GFP in larval NBs as they enter and exit quiescence has already been described along with experiments demonstrating that Dl signaling from the GMC is important for Notch activity. This work should be properly cited (Zacharioudaki et al., 2012).
6. Can they explain why they have effects from Dl RNAi on PCNA but not on EdU in Figure 2?

7. The authors state: “Delta is negatively regulated by the Notch target gene, Hes-1”. There is not Hes1 in Drosophila and the effects of Notch activity in Dl regulation are not well characterized in this system. Levels may be higher from this Gal4 but not for the reason stated. This comment should be removed as it is misleading.

8. They test a range of RNAi to investigate possible regulatory relatuionships between Notch and other inputs. The logic for these is unclear (aren't there more direct ways to perturb specific cell cycle steps?) In addition the fact that an RNAi does not have an effect may be due to poor knock down, it is hard to make firm conclusions from negative results unless the effects on the cell cycle are tested directly in that context.

9. The section claiming the levels of nuclear NICD and of Delta change to explain an increase in signaling is interesting but further validation is needed (e.g. negative controls). Very few groups have properly quantified nuclear NICD as it is almost undetectable. Given that they use the MIMC line for Delta, it would be advisable to reproduce with the Delta antibody (available from public sources).

Original submission

First decision letter

MS ID#: DEVELOP/2021/200275

MS TITLE: Notch signaling regulates neural stem cell quiescence entry and exit in Drosophila

AUTHORS: Chhavi Sood, Virginia T Justis, Susan E Doyle, and Sarah E Siegrist

I am extremely sorry for the very long delay before being able to comeback to you. 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 the remaining referees' comments can be satisfactorily addressed. Please attend to the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

Shows Notch activity promotes neural stem cell quiescence.

Comments for the author

In my opinion, the previous reviewers comments have been addressed and I have only minor comments on this revised manuscript.

Minor comments for authors:
The sentence in the abstract "... neuroblast Notch activity incrases inducing neuroblast cell cycle" does not make sense to me; please revise.
page 5 the term Notch-deltaECD is used without it being defined. Please explain the genotype at first use. Is it the same as Notch-ICD?
Everywhere it says "data not shown" should be converted into a supplemental figure or deleted, depending on importance to the conclusions.
Title on page 8 should be "...in late stage embryonic CB NBs" (not "staged").

Reviewer 2

Advance summary and potential significance to field

This study addresses how stem cells enter and exit from quiescence, focusing on the developing Drosophila central brain neuroblasts (NBs). They find some evidence for that the Notch pathway acts both during NB entry into and exit from quiescence. Regarding entry into quiescence, a number of previous studies have addressed this issue during Drosophila embryogenesis, in both the VNC and brain and found evidence for the involvement of Notch in this decision, as well as cas and dacapo. Regarding exit from quiescence, the involvement of balanced Notch pathway is novel, but this issue is not rigorously addressed, and is in fact the weaker part of the study. The study provides limited novel insight into entry into/exit from quiescence, other than unsurprisingly linking Notch to these decisions also in the central brain NBs. It furthermore feels premature and is fraught with experimental issues.

Comments for the author

1) Regarding the Notch pathway, in the embryonic CNS Notch is also engaged in the control of how many NBs are formed, in addition to NB entry into quiescence, and perhaps exit from quiescence. Resolving these different Notch phenotypes in the brain, without using restricted and selective NB/lineage Gal4 lines/markers is a challenge.

2) Using worGAL4 they express the UAS-RNAi transgenes during an extended period of neurogenesis, from late stage 8 and onward. In fact, previous studies indicate that wor itself is already expressed in the ectoderm prior to NB delamination. Against this backdrop, their claims that they are specifically studying the late embryonic quiescence decision are difficult to embrace. How do they know at what stage they inhibit the Notch pathway? For instance, in Fig 1G the quantification of proliferating NBs shows one extra NB in one whole brain lobe (out of an estimated 106 NBs, not taking the 8 Type II NBs into consideration). But minor effect could represent an extra NB formed during the NB delamination process (which plays out into stage 11), and not an NB prevented from entering quiescence. The same issue applies to the kuz mutants. NB counts in the RNAi and kuz mutants would help address this, although restricted NB markers would be even better.

3) Figure 1: Show Dpn staining in separate panels. There are many more labelled cells than just the four MB NBs, presumably GMCs generated by the NBs during the 2-hour EdU pulse. But this is not commented upon in the Results.

4) Figure 2: Again, please show Dpn only panels.

5) Figure 4F-J: Add control immune panels and data to graphs and show quantifications.

6) Figure 5D-F: How long was the EdU pulse in these experiments?

7) Figure 6: How can they claim to address exit out of quiescence in larval NBs using a Gal4 driver that commences in stage 8 embryos and onward (worGAL4)?
What do these brains look like at stage 17? Maybe the NBs never enter into quiescence in the embryo. Or are they using an inducible system? They list tub-Gal80[ts] in Table S1, but in Table S2 it is listed that tub-Gal80[ts] is used in Figure 3F-I, not for Figure 6. On that note, regarding Figure 3, the use of tub-Gal80[ts] is not mentioned in the Results of Figure legend, nor is the stage of heat-shock described.

Reviewer 3

Advance summary and potential significance to field

In this paper Sood, Siegrist et al show that Notch-Delta signaling between Drosophila larval neuroblasts (NBs) and their progeny ganglion mother cells (GMCs) regulates NB quiescence. This is an expected mechanism that has already been demonstrated in other stem and progenitor cell lineages in flies (adult midgut stem cells, ovarian follicle cells), and it's similar to the lateral inhibition signaling that occurs in embryonic NB specification. However it seems it has not yet been demonstrated to regulate NB quiescence at the embryonic/larval stage, and therefore this study has significant value. The paper is clear and the results, as far as they go, support the conclusions well. It's a bit difficult to read because of at the acronyms and stage designations and other experimental details scattered throughout the text, but is nevertheless concise and accurate. Although the conclusions are meaningful and valuable, the paper raises some questions it fails to answer, and there are several obvious important experimental tests that seem straightforward to do but are nevertheless missing. I think the paper is appropriate for Development, but it would be advisable for the authors to perform a few more experimental tests, as noted below.

Comments for the author

1. (Most important) The paper concludes that strong Delta-Notch signaling between the GMC and the NB promotes quiescence. Although Notch and Numb are tested genetically, Delta is only implicated by expression. The functional importance of Delta needs to be tested using mutations or RNAi's targeting Delta.

2. In Figure 3, it would be good to confirm the FUCCI results with EdU incorporation data.

3. Please add the data from Fig S2D,E to main Fig 2 (NB numbers data).

4. In the introduction, it is said that trbl regulates insulin signaling. But trbl also regulates the stability of stg.
Is this function important here?

5. The text in the introduction needs a few more commas between clauses, to be easy to understand.

6. On page 6, the authors make the conclusion that cell cycle exit suppresses Notch activity. The result is clear, but the mechanism underlying this effect is mysterious and in fact the effect is not expected. Can the authors propose a mechanism to explain this interesting effect of cell cycle progression on Notch activity?

7. Throughout the paper, the authors state that this "regulates" that without specifying whether the regulation is positive or negative. This is confusing.
Please use "suppress" and "promote" or "positively regulates" and "negatively regulates" instead, for clarity.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

The sentence in the abstract "... neuroblast Notch activity incrases inducing neuroblast cell cycle" does not make sense to me; please revise.
The sentence has been changed.

page 5 the term Notch-deltaECD is used without it being defined. Please explain the genotype at first use. Is it the same as Notch-ICD? It is not the same as NotchICD, but it is reported to function in the same manner. We provide a reference for further information.

Everywhere it says "data not shown" should be converted into a supplemental figure or deleted, depending on importance to the conclusions. Thank you. We have corrected this by either including the data or removing the statement. Data that has been added can now be found in S1 G-J and S4 I,J.

Title on page 8 should be "...in late stage embryonic CB NBs" (not "staged").
Thank you. We have corrected this.

Reviewer 2 Comments for the Author:
1)Regarding the Notch pathway, in the embryonic CNS Notch is also engaged in the control of how many NBs are formed, in addition to NB entry into quiescence, and perhaps exit from quiescence. Resolving these different Notch phenotypes in the brain, without using restricted and selective NB/lineage Gal4 lines/markers is a challenge. Correct and this is the reason why mutant analysis of most Notch pathway components is not possible. Notch mutants have early defects starting with neuroblast specification and delamination. Therefore, we use *UAS-RNAi* transgenes targeted against Notch pathway components in conjunction with *worGal4* to restrict GAL4 expression to neuroblasts after specification and delamination.

2)Using worGAL4 they express the UAS-RNAi transgenes during an extended period of neurogenesis, from late stage 8 and onward. In fact, previous studies indicate that wor itself is already expressed in the ectoderm prior to NB delamination. Against this backdrop, their claims that they are specifically studying the late embryonic quiescence decision are difficult to embrace. How do they know at what stage they inhibit the Notch pathway?
Because we do not observe a neurogenic phenotype in *worGAL4,UAS-NotchRNAi* animals, we know that Notch levels are being reduced after neuroblast specification/delamination. We have never detected worGAL4 expression or *worniu* transcript in the ectoderm. We have only ever visualized expression in neuroblasts after delamination, consistent with previous reports (Ashraf et al., 1999). In fact, this was one of the motivations in making *worGAL4* trangenic animals to begin with (Albertson, 2003).

For instance, in Fig 1G, the quantification of proliferating NBs shows one extra NB in one whole brain lobe (out of an estimated 106 NBs, not taking the 8 Type II NBs into consideration). But minor effect could represent an extra NB formed during the NB delamination process (which plays out into stage 11), and not an NB prevented from entering quiescence. The same issue applies to the kuz mutants. NB counts in the RNAi and kuz mutants would help address this, although **restricted NB markers** would be even better.
We quantified the number of Dpn positive CB NBs (which include Type IIs) in *Notch RNAi* animals (Fig 2I)and as requested in *kuz* mutants as well. We found no differences compared to controls.

3)Figure 1: Show Dpn staining in separate panels. There are many more labelled cells than just the four MB NBs, presumably GMCs generated by the NBs during the 2-hour EdU pulse. But this is not commented upon in the Results. We have included grayscale images of the Dpn channel alone as requested. Yes, MB NBs generate EDU positive progeny during the EdU pulse, which is stated in the text.

"At this time, the MB and VL NBs are larger than quiescent CB NBs and are actively dividing based on expression of the S-phase indicator *pcna:GFP*, incorporation of the thymidine analogue EdU, and their generation of EdU positive progeny".

4)Figure 2: Again, please show Dpn only panels. We have included grayscale images of the Dpn channel alone as requested.

5)Figure 4F-J: Add control immune panels and data to graphs and show quantifications. We are not sure what is being requested here. Do you mean no primary or secondary antibody controls or something else? Panel H and I show cas mutants alone. At this time, controls have only EdU positive MB+VL NBs (5 per brain hemisphere). This has been shown in previous figures.

6)Figure 5D-F: How long was the EdU pulse in these experiments? For D, E: animals were fed EdU for 12 hours and F for one hour.

7)Figure 6: How can they claim to address exit out of quiescence in larval NBs using a Gal4 driver that commences in stage 8 embryos and onward (worGAL4)? This is addressed above. What do these brains look like at stage 17? Maybe the NBs never enter into quiescence in the embryo. We are using *numbRNAi* as a tool to elevate Notch activity in quiescent NBs. NBs with elevated levels of Notch activity fail to reactivate in response to dietary nutrients. If NBs never enter quiescence in the embryo, then we would expect that they would still be proliferating at this time. We do not see this.

Or are they using an inducible system? They list tub-Gal80[ts] in Table S1, but in Table S2 it is listed that tub-Gal80[ts] is used in Figure 3F-I, not for Figure 6. On that note, regarding Figure 3, the use of tub-Gal80[ts] is not mentioned in the Results of Figure legend, nor is the stage of heat-shock described. Genotypes of all panels are listed in supplementary table 2. In Fig. 3, animals were raised at 29˚C.

Reviewer 3 Comments for the Author:

1. (Most important) The paper concludes that strong Delta-Notch signaling between the GMC and the NB promotes quiescence. Although Notch and Numb are tested genetically, Delta is only implicated by expression. The functional importance of Delta needs to be tested using mutations or RNAi's targeting Delta. Unfortunately, we do not have the means of knocking down Delta in GMCs and Delta mutants have a strong neurogenic phenotype which makes them unsuitable for this analysis.

2. In Figure 3, it would be good to confirm the FUCCI results with EdU incorporation data. Correct, we have not EdU treated our fucci animals. However, at this time (0h ALH), we expect only 1-3 EdU positive CB NBs (minus MB+VL NBs) in *Notch RNAi* based on our results in Fig. 1. This number roughly corresponds with number of RFP positive cells shown in the histogram (pink Fig. 3D).

3. Please add the data from Fig S2D,E to main Fig 2 (NB numbers data). We have made the change as suggested.

4. In the introduction, it is said that trbl regulates insulin signaling. But trbl also regulates the stability of stg.
Is this function important here? Great question. We have included your point in the intro.

5. The text in the introduction needs a few more commas between clauses, to be easy to understand. Thank you. We have added commas.

6. On page 6, the authors make the conclusion that cell cycle exit suppresses Notch activity. The result is clear, but the mechanism underlying this effect is mysterious and in fact the effect is not expected. Can the authors propose a mechanism to explain this interesting effect of cell cycle progression on Notch activity?

Newborn GMCs express high levels of Delta, but over time Delta levels decrease in the lineage. We think that Delta levels decline as a result of GMC division. GMCs divide soon after their birth giving rise to neurons or glia.

Towards the end of embryogenesis, NBs stop dividing and stop producing newborn Delta-expressing GMCs. As a consequence, Notch activity becomes attenuated. The newborn GMCs that were once the source for Notch pathway transactivation in NBs, continue onward with their developmental program to divide and and make neurons/glia. Thus because NBs stop dividing, they stop producing their own ligand expressing daughters.

Images of Delta GFP expression can be found in S4B,D.

7. Throughout the paper, the authors state that this "regulates" that without specifying whether the regulation is positive or negative. This is confusing. Please use "suppress" and "promote" or "positively regulates" and "negatively regulates" instead, for clarity.
Thank you. We have changed the text as suggested.

Second decision letter

MS ID#: DEVELOP/2021/200275

MS TITLE: Notch signaling regulates neural stem cell quiescence entry and exit in Drosophila

AUTHORS: Chhavi Sood, Virginia T Justis, Susan E Doyle, and Sarah E Siegrist
ARTICLE TYPE: Research Article

I looked at the response to reviewers and in light of this I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.