Physical interactions between Gsx2 and Ascl1 balance progenitor expansion versus neurogenesis in the mouse lateral ganglionic eminence

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MS TITLE: Physical interactions between Gsx2 and Ascl1 regulate the balance between progenitor expansion and neurogenesis in the mouse lateral ganglionic eminence

AUTHORS: Kaushik Roychoudhury, Joseph Salomone, Shenyue Qin, Masato Nakafuku, Brian Gebelein, and Kenneth Campbell

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

As you will see, the three referees are positive and express great interest in your work, but they also have 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 may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their 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 Roychoudhury et al investigates how the co-expression of Gsx2 and Ascl1 in the germinal layers of ventral telencephalon may fine tune the balance between progenitor expansion and differentiation. Previous work from the same group has shown Gsx2 to maintain neural progenitor identity in the lateral ganglionic eminence (LGE) of ventral telencephalon, despite inducing Ascl1 expression. Maintenance of Gsx2 expression allows for further expansion of the lineage, raising the question of how the differentiation promoted by Ascl1 is delayed. In this manuscript the authors found that Gsx2 can physically interact with Ascl1, thereby repressing its pro-differentiation activity. They start by characterizing the co-expression of each factor in the LGE, and how this correlates with the rate of neurogenesis observed in different LGE domains at distinct developmental stages. Following on results from previous published studies using misexpression of Ascl1 or Gsx2 in the dorsal telencephalon, they use a mouse genetic model to show that co-expression of Gsx2 inhibits differentiation promoted by Ascl1. This observation is followed by a series of experiments aiming at identifying the molecular basis for this inhibition. The authors conclude that Gsx2 directly interacts with Ascl1, hindering dimerization of Ascl1 and consequently DNA binding. Although some of results presented are based on in vitro assays, such as yeast 2-hybrid and 3-hybrid system, these are backed up by other approaches, in particular a proximal ligation assay performed in embryonic brain sections.
This is a scientifically sound study that addresses a very relevant question, namely how is the Ascl1 activity differentially regulated at distinct stages of the neuronal lineage. In particular, it provides an additional mechanism to explain how Ascl1 expression can be maintained in neural progenitors, without triggering neuronal differentiation. On the weak side, the authors do not provide experimental evidence to explain how the mechanism proposed can be compatible with the activation of a sub-set of Ascl1 target genes in Gsx2 expressing progenitors. This important point is nevertheless properly discussed.

Comments for the author

The quality of the work presented could be further improved if the authors addressed the following points:
1) In Figure 2, can the authors better characterize the ectopic expression of Ascl1 and Gsx2 at cellular resolution? In particular, it would be important to show co-expression of both transcription factors occurring in individual cells.
2) The transcriptional assay showing Gsx2 inhibiting Ascl1 function is quite compelling, however it was performed solely with a portion of the achaete gene promoter, in transfected Drosophila S2 cells. Given the importance of this assay in showing Gsx2 interfering with Ascl1 function at a target gene level, it would be important to have similar data with an Ascl1 target gene, in transfected mammalian cells. This should not be difficult to obtain, given the information available in the literature on Ascl1 target genes. The high levels of expression used should overcome the confounding effects originated from endogenous expression of Ascl1 and/or Gsx2.
3) The differences observed in the yeast two-hybrid assay between Gsx2 and Gsx1 are potentially very interesting, considering previous results showing that misexpression of Gsx1 results in increased neurogenesis. I suggest the authors co-express also Gsx1 with Ascl1 in the transcriptional assay. Although Gsx1 is not the focus of this work, information along this line will provide a more solid understanding of Gsx2 function in the gene regulatory network it operates.
4) The ability to coimmunoprecipitate Ascl1with Gsx2 using protein extracts from ventral telencephalon is a very relevant result, which requires further controls. These should include negative control using tissue expressing one factor but not the other. Also, input chromatin and molecular weight markers should be present in the figure.

Reviewer 2

Advance summary and potential significance to field

The authors provide a set of clear and molecular biology convincing data supporting the notion that Gsx2 and Ascl1 physically interact and that this interaction attenuates homo- and hetero-

dimerization, DNA binding, and thus likely transactivation of target genes, by Ascl1. These molecular data are supported by findings that Gsx2 and Ascl1 also very probably bind directly in vivo. The major conclusion is that this physical interaction is important for ensuirng that cells expressing both factors remain in a progenitor state until Gsx2 is downregulated allowing Ascl1 to drive differentiation. Overall the results are interesting, provide insight into the fine regulation of neural progenitor cell transitions and add a very interesting element to the merging literature on the intricate regulation of proneural proteins during neurogenesis.

Comments for the author

I have three specific concerns. The first concern is general and relates to the fact that the manuscript does not provide clear insight into the biology of progenitors as they experience a change in the balance between Gsx2 and Ascl1 expression. Experiments either in vivo or in culture titrating the levels of either protein by shRNA while observing markers of state transitions such as cell cycle exit or induction of Ascl1 target genes would add to the manuscript. The second concern is experimental and relates to the use of the non-DNA binding mutant form of Gsx2 in the EMSA experiments. While I understand the logic, this remains a mutant form and could have unexpected differences with the wild type protein. Confirming the observations of competitive binding with the wild type protein would help make the arguments more convincing. The third concern is minor and relates to what happens to the Gsx2/Ascl1 heterodimer in progenitors. Is it fully nuclear, or is it at least partly cytoplasmic? If it is nuclear, is not possible that this is an active transcriptional complex that basically favors expression of progenitor genes while preventing expression of genes associated with differentiation?

Reviewer 3

Advance summary and potential significance to field

Important question in neural development that is articulated nicely in the abstract and the introduction that addresses how 2 transcription factors, Gsx2 and Ascl1 interact molecularly to explain the roles attributed to them phenotypically during ventral telencephalon development. The authors carefully describe the expression of these factors, their protein-protein interaction, and the consequences of their interaction on binding DNA and activating transcription. Gsx2 and Ascl1 co-expression define a population of LGE intermediate progenitors. Misexpression of Gsx2 and Ascl1 in dorsal telencephalon progenitors severely limits Ascl1-driven neurogenesis. Gsx2:Ascl1 and Ascl1:Tcf3 (E-protein) are distinct complexes and Gsx2:Ascl1 interactions predominate in LGE ventricular zone cells (maintaining progenitors) and Ascl1:Tcf3 interactions characterize SVZ progenitors (differentiating cells). The Gsx2:Ascl1 complex does not bind the E-box motif. The experiments are high quality, nicely controlled, convincing and add substantially to understanding how these key factors are functioning to control neural progenitor/neural differentiation decisions. Importantly, very few interacting factors for these essential regulatory factors have been identified. The authors provide an intriguing model supported by their findings for Gsx2:Ascl1 interactions supporting progenitor expansion while Ascl1:Tcf3 interactions promote cell cycle exit and subsequent neurogenesis. This is an important finding in neural development.

Comments for the author

I only have minor comments pointing out typos and a suggestion.

Minor:
1. The dashed box in Fig. 2A is not correctly marking the inset. It is not needed.
2. Typo: Ascl1 was misspelled as Asc1 in first paragraph of results.
3. I suggest adding a supplemental figure associated with Fig. 4 showing the amino acid sequence of Ascl1 with the numbering so that it is easily known what residues are deleted in the series of deletion constructs.

First revision

Author response to reviewers' comments

Reviewer 1:
1)In Figure 2, can the authors better characterize the ectopic expression of Ascl1 and Gsx2 at cellular resolution? In particular, it would be important to show co-expression of both transcription factors occurring in individual cells.
We have improved the quality of the images in Fig. 3 (previously Fig. 2) and provided high power insets to show the ectopic co-expression of Gsx2 and Ascl1 in the dorsal telencephalon VZ with cellular resolution, in panels C and D.

2)The transcriptional assay showing Gsx2 inhibiting Ascl1 function is quite compelling, however it was performed solely with a portion of the achaete gene promoter, in transfected Drosophila S2 cells. Given the importance of this assay in showing Gsx2 interfering with Ascl1 function at a target gene level, it would be important to have similar data with an Ascl1 target gene, in transfected mammalian cells. This should not be difficult to obtain, given the information available in the literature on Ascl1 target genes. The high levels of expression used should overcome the confounding effects originated from endogenous expression of Ascl1 and/or Gsx2.
We have now performed a similar luciferase assay in mammalian mK4 cells using a multimerized (6X) E2 site and found that Ascl1 activated gene expression (i.e. luciferase activity) was efficiently reduced by both wild type Gsx2 and Gsx2N253A, similar to what was observed in the Drosophila S2 cells. This new data has been incorporated into Fig. 4G. While we acknowledge that this may not fully address the reviewer's concern regarding actual Ascl1 target genes, it does show that the interference of Ascl1 activated gene expression can occur in both Drosophila and mammalian cells using a tool (multimerized E-box sequences) that has been extensively utilized in prior studies to characterize the transcriptional properties of bHLH proteins.
To further understand how Gsx2 affects Ascl1 target gene expression in LGE progenitors, we have performed single cell (sc)RNA-seq experiments on E12.5 mouse ventral telencephalon to examine transcriptional differences between progenitors expressing Gsx2, Ascl1 or both. Our scRNA-seq studies found that the Ascl1+-only expressing LGE progenitors showed enrichment for neuronal genes including Ascl1 targets Tubb3 and Gad2, while the LGE progenitors expressing either Gsx2+-only or Gsx2+Ascl1+ showed enrichment only for genes typical of LGE progenitors. While correlative, this data supports a model where Gsx2 must be downregulated in LGE progenitors in order for Ascl1 to promote neurogenesis. The scRNA-seq results have been described in the results on pages 7-8 and in Fig. 2, Table 1 and Tables S1, S2.

3)The differences observed in the yeast two-hybrid assay between Gsx2 and Gsx1 are potentially very interesting, considering previous results showing that misexpression of Gsx1 results in increased neurogenesis. I suggest the authors co-express also Gsx1 with Ascl1 in the transcriptional assay. Although Gsx1 is not the focus of this work, information along this line will provide a more solid understanding of Gsx2 function in the gene regulatory network it operates.
We agree with the reviewer that the differential roles of Gsx1 and Gsx2 as well as their potential interactions with Ascl1 are very interesting. However, after considering the reviewers comment, we feel it is important to do a more thorough analysis of the potential relationship between Gsx1 and Ascl1. In addition to the luciferase assays, we plan to do co-IP's and PLA analysis. Unfortunately, due to time constraints and space limitations in this current manuscript, this will have to be a part of a future publication. Thus, we have removed the section showing the yeast 2-hybrid test between Gsx1 and Ascl1 (previous Fig. 4D) as well as the paragraph in the Discussion where the difference between Gsx1 and Gsx2 in neurogenesis is discussed.

4) The ability to coimmunoprecipitate Ascl1with Gsx2 using protein extracts from ventral telencephalon is a very relevant result, which requires further controls. These should include negative control using tissue expressing one factor but not the other. Also, input chromatin and molecular weight markers should be present in the figure.
We have redone the co-IP assay and included input lanes and molecular weight markers to replace panel D in the new Fig. 5 (previously Fig. 4E). Unfortunately, there are no brain regions that express Gsx2, but not Ascl1, so it is difficult to do the negative control suggested by the reviewer. We would like to point out, however, that the results from the co-IP assay (Fig. 5D) have been confirmed and extended by our PLA analysis (Fig. 8A,B). Importantly, PLA was performed using

brain sections from Gsx2 knockouts (Fig. 8C) as well as from Gsx2 and/or Ascl1 misexpressing animals (Fig. 8D-F) to demonstrate the specificity of the molecular interaction between Gsx2 and Ascl1 in vivo. Especially pertinent to the reviewer's comment, we found that when Ascl1 is misexpressed throughout the entire telencephalon, Gsx2 remains in the ventral telencephalon (Fig. 3B) and accordingly, PLA signal was only observed in the LGE (Fig. 8E).

Reviewer 2:
The first concern is general and relates to the fact that the manuscript does not provide clear insight into the biology of progenitors as they experience a change in the balance between Gsx2 and Ascl1 expression. Experiments either in vivo or in culture titrating the levels of either protein by shRNA while observing markers of state transitions such as cell cycle exit or induction of Ascl1 target genes would add to the manuscript.
The reviewer raises an important point. Since the co-expression of Gsx2 and Ascl1 is transient and occurs only in a subset of progenitors in the LGE, we believe it would be difficult for us to perform the experiments suggested above. Alternatively, we performed scRNA-seq experiments on mouse E12.5 ventral telencephalon and analyzed gene enrichment profiles in subsets of LGE progenitors expressing either Gsx2 alone, Ascl1 alone, or Gsx2 and Ascl1. Our results show that the Ascl1+ only LGE progenitors show enrichment for neuronal genes such as Tubb3 and Gad2, while Gsx2+ only progenitors express genes enriched in radial glia, i.e. apical progenitors (e.g. Glast and Fabp7) and Gsx2+Ascl1+ double positive LGE progenitors showed genes enriched in SVZ progenitors (e.g. Dlx1). While these results are only correlative, they do support a model where Gsx2 maintains progenitor status and when it is downregulated Ascl1 promotes neurogenesis. This data had been added to the first section of the results on pages 7-8 and in Fig. 2, Table 1 and Tables S1 and S2.

The second concern is experimental and relates to the use of the non-DNA binding mutant form of Gsx2 in the EMSA experiments. While I understand the logic, this remains a mutant form and could have unexpected differences with the wild type protein. Confirming the observations of competitive binding with the wild type protein would help make the arguments more convincing.
We have now performed the competitive EMSA experiments using wild type Gsx2 and show similar results interfering with Ascl1 homo- and heterodimer binding in new Fig. S4.

The third concern is minor and relates to what happens to the Gsx2/Ascl1 heterodimer in progenitors. Is it fully nuclear, or is it at least partly cytoplasmic? If it is nuclear, is not possible that this is an active transcriptional complex that basically favors expression of progenitor genes while preventing expression of genes associated with differentiation?
We have now performed confocal imaging on the PLA results (Fig. 8B) and the majority of the PLA signal overlaps with DAPI+, suggesting that most of the Gsx2:Ascl1 complex is in the nucleus. The reviewer raises an interesting point that this association may also serve as a transcriptional complex which favors progenitor (but not neuronal) gene expression. While we don't have any data to address this more clearly, we have discussed this possibility briefly on page 20.

Reviewer 3:
1.The dashed box in Fig. 2A is not correctly marking the inset. It is not needed
We have revised Fig. 2, which is now Fig. 3, by removing the dashed box and previous insets. As suggested by Reviewer 1, we added high power insets in Fig. 3C and D to show the ectopic co-expression of Gsx2 and Ascl1 in the VZ of the dorsal telencephalon.

2.Typo: Ascl1 was misspelled as Asc1 in first paragraph of results.
We have corrected the misspelling of Ascl1 in the beginning of the results (as well as few other typos we found).

3.I suggest adding a supplemental figure associated with Fig. 4 showing the amino acid sequence of Ascl1 with the numbering so that it is easily known what residues are deleted in the series of deletion constructs.
We added a new supplementary figure (Fig. S2) that shows the amino acid sequence of the bHLH and details the AAs deleted in each of the tested constructs that correspond with the numbers listed in Fig. 5 (previous Fig. 4).

Second decision letter

MS ID#: DEVELOP/2019/185348

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ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

The manuscript by Roychoudhury et al investigates how the co-expression of Gsx2 and Ascl1 in the germinal layers of ventral telencephalon may fine tune the balance between progenitor expansion and differentiation. Previous work from the same group has shown Gsx2 to maintain neural progenitor identity in the lateral ganglionic eminence (LGE) of ventral telencephalon, despite inducing Ascl1 expression. Maintenance of Gsx2 expression allows for further expansion of the lineage, raising the question of how the differentiation promoted by Ascl1 is delayed. In this manuscript the authors found that Gsx2 can physically interact with Ascl1, thereby repressing its pro-differentiation activity. They start by characterizing the co-expression of each factor in the LGE, and how this correlates with the rate of neurogenesis observed in different LGE domains at distinct developmental stages. Following on results from previous published studies using misexpression of Ascl1 or Gsx2 in the dorsal telencephalon, they use a mouse genetic model to show that co-expression of Gsx2 inhibits differentiation promoted by Ascl1. This observation is followed by a series of experiments aiming at identifying the molecular basis for this inhibition. The authors conclude that Gsx2 directly interacts with Ascl1, hindering dimerization of Ascl1 and consequently DNA binding. Although some of results presented are based on in vitro assays, such as yeast 2-hybrid and 3-hybrid system, these are backed up by other approaches, in particular a proximal ligation assay performed in embryonic brain sections.

Comments for the author

Having read the revised version of the manuscript by Roychoudhury et al, I found the authors to have addressed in a reasonable way (either experimentally or by discussing them) all the points of concern that I raised previously. Most notably, new scRNAseq data from ventral telencephalon cells were added to the manuscript. Although of a descriptive nature, this evidence provides a solid support to the model provided by the authors describing the interactions between Gsx2 and Ascl1 along the neuronal lineage.

Reviewer 2

Advance summary and potential significance to field

The authors provide a set of clear and molecular biology convincing data supporting the notion that Gsx2 and Ascl1 physically interact and that this interaction attenuates homo- and hetero-dimerization, DNA binding, and thus likely transactivation of target genes, by Ascl1. These molecular data are supported by findings that Gsx2 and Ascl1 also very probably bind directly in vivo. The major conclusion is that this physical interaction is important for ensuirng that cells expressing both factors remain in a progenitor state until Gsx2 is downregulated allowing Ascl1 to drive differentiation. Overall the results are interesting, provide insight into the fine regulation of

neural progenitor cell transitions and add a very interesting element to the merging literature on the intricate regulation of proneural proteins during neurogenesis.

Comments for the author

I have no further comments and believe the manuscript can be accepted.