Dynamic profiling and functional interpretation of histone lysine crotonylation and lactylation during neural development

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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 BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees 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. In particular, they criticise the frequent overinterpretation of the data, with statements on the regulatory role of the Kcr and Kla modifications in chromatin organisation and transcription regulation, when only correlations are observed. Reviewer 3 also requests that you validate some of the result obtained in P19 cells in a more physiological model of embryonic progenitors, and recommends that you assess more directly the regulatory functions of the Kcr and Kla modifications by silencing some of their known writers or erasers . 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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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.

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

In their manuscript Dai et al. characterize a series of novel histone lysine acylations in the context of the telencephalic development. Their work provides an interesting and novel dataset that sets the stage for future exploration of their functional significance. One major issue throughout the manuscript is the authors ascribe regulatory relationships with data that merely shows descriptive associations. Without additional experimental evidence these regulatory mechanisms invoked throughout the paper cannot be substantiated. Even the title suggesting that 'Kcr and Kla are novel regulators of neuronal fate during neural development' is not supported by any of the current experimental evidence provided throughout manuscript.
The paper can also do with more example of genes across all the comparison of chipseq datasets and particularly when discussing GO terms/functional enrichment

Comments for the author

Major points:
Figure 1: immunostaining shows significant expression of marks outside of radial glia/germinal zones. Most of the paper focuses mainly on this expression and does not comment on the in vivo expression in neurons that inevitably drives identified peaks in the chipseq samples, particularly in e16.5 timepoint. They should bin Kcr and Kla positive cells across the cortex to illustrate their enrichment across the progenitors zones intermediate zone and cortical plate or alternatively radial glia, IPCs and neurons by co-staining with markers.
This in situ analysis would be a more compelling illustration of histone acylation dynamics than the nestin and Tuj1 co-staining done in P19 cells. There is a visually interesting reduction of expression in different zones for different marks.
There are major concerns with the western blots shown in Figure 1B (and Figures 4 and 5). The histone H3 control intensity compared to the H3Kcr and Kla is very weak. When the authors measure each mark compared to the total histone H3, it appears the relative amouts of H3Kcr and Kla is greater than the total amount of H3. Howerver in Figure 4, the H3 intensity is quite strong and the relative amounts of Kcr and Kla marks are all less than the total H3. The while the intensity of H3 is consistent in the individual figures, it is a concern that the antibody used for these assays may not be a reliable internal control.
Figure 2: Well done characterization with associations but conclusions regarding "regulation" is unsubstantiated.
Figure 3: The authors select two timepoints of e.13.5 and e16.5 to examine PTM dynamics during neurogenesis. They show a change of expression correlated with differential histone PTMs- however conclude a regulation on neurogenesis. Because this is bulk, changes in the expression correlated with changes in PTMs could be because of the growing pool of neurons in the chipseq samples in the alternate timepoint.
Figure 3G,H: Using GO analysis as shown in panels 3G&H the authors conclude that upregulated genes with enriched H3Kcr or K3K18la are involved in neuronal differentiation. However, this conclusion is not supported by the GO terms that are shown. These are mainly genes involved in neuron function and does not elude to differentiation.
Figure 4: This is a good control experiment. The authors don't see a reduction in acetylation marks using the two inhibitors compared to the paper they cite (Martin et al. 2021). which suggests a potential differential inhibitor mechanism in inhibiting transcription as Martin et al used actinomycin D in mESCs, whereas the authors used triptolide and flavopiridol. I don't know which would be most appropriate for inhibiting transcription, however it would be interesting to see if they can recapture the loss of Kac marks (as shown in Martin et al) from actinomycin D and whether the novel PTMs behave similarly or not.
Figure 5: Interesting effect of pan-HDACs on PTM expression. Suggests the involvement of HDACs in the accumulation of the novel PTMs in the developing telencephalon. However, they present no evidence that these HDACs are specifically lysine delactylases except for an association. They also

choose to focus the effect of simultaneous HDAC knockdown on lactyl marks but not crotonyl marks despite there being evidence of an effect with crotonyl marks in the inhibitor experiments. It would be interesting to see crotonyl dynamic changes as well.
Figure 6: Data was clustered into 3 distinct clusters based on expression, with cluster 1 being focused on. It would be nice to see all 3 clusters presented together instead of removing c2 and c3 into the supplement.
Providing individual or small groups of genes as exemplars of these 3 clusters would help bolster their conclusions would also be helpful as the gene ontology here and in the supplementary clusters are not informative.

Minor Points:
The paper could greatly benefit from editing for English language vocabulary and grammar.
A lot of supplementary data is extensively discussed in the results section, it may be worthwhile to reevaluate the data that is included in the main figures versus the supplementary data.

Reviewer 2

Advance summary and potential significance to field

Dai et al. show novel histone lysine acylations like crotonylation (Kcr) and lactylations (Kla) exist and cooperatively regulate the neuronal fate during mouse development. They show that Kcr and Kla are undergoing global changes during the development by profiling their genome-wide occupancy.
Furthermore, they show the genes that contain three modifications (Kac, Kcr and Kla) acquire more chromatin accessibility and transcription compared to the genes with single modifications and drives the cell fate changes. They show that HDAC1-3 as a novel eraser of H3K18la and inhibition of them preactivated the neuronal transcriptional programs by promoting histone lysine acylations. While the genome-wide distribution of this modification and its role in transcriptional regulation has been provided in development and disease, the insight in the central nervous system is less characterized and understood. Therefore, the scope of study is in time and has great potential of receiving a broad interest from the community.

Comments for the author

1. In Fig.1 germinal layer markings are missing (VZ, SVZ,CP). Cells from the VZ regions are progenitor cells and SVZ region contains mostly differentiating cell populations and CP contains the differentiated functional neurons. In Fig.1A level of H3K18cr is most enriched in the cortical plate regions it would be better if authors could differentiate/quantify the enrichment level of these modifications across the layers.
2. In Fig1.C and Fig.S1E authors quantified the levels of these modifications during P19 cells differentiation. Similar quantification should be done at cellular levels in in vivo experiments to show the dynamics of these modifications across the various cell types during cortical development (VZ/SVZ/CP).
3. In line number 121 authors have written H3K9ac, H3K9cr and H3K18la were mainly located in promotor regions. But from their Fig.2A H3K18al was most enriched in intron and intergenic regions.
4. Authors should extend their ChIP-seq data analysis. example: motif enrichment analysis and what are the transcription factors that target these motifs and co-occurrence of these modifications with super-enhancers. This extended analysis will provide additional knowledge to the scientific community.
5. In Fig. 2D authors only showed gene ontology terms for H3K9cr and H3K18la and in the end claim H3K9ac, H3K9cr and H3K18la are involved in different pathways. gene ontology terms for H3K9ac should be provided.
Fig2D. Number of genes contributed to biological functions are missing.
6. Line number 148 they mentioned that they, systemically analyzed genome-wide changes in H3K9ac, H3K9cr, and H3K18la at early (E13.5) and late (E16.5) stages of embryonic neurogenesis and referred (Fig.S5) which is RNA-seq data. If they profiled genome-wide changes in H3K9ac, H3K9cr, and H3K18la they should refer the ChIP-seq data.

7. In Fig 4. they showed Pol ll inhibition resulted in transcription reduction by measuring the levels of Pol ll CTD phosphorylation. instead, it would have been better to include some additional experiments to show the transcription reduction. like, puromycin incorporation assays.
In Fig.4C the western blot signals look weak. And control H3 signals are unequal based on their given image. They should provide better higher exposed images. All the western blot images should be labeled with proper molecular weight markers.
8. Fig 5. All the western blot images should be labeled with proper molecular weight markers.
9. Fig.5G-H. they showed knockdown of HDAC 1-3 increased the level of H3K18la levels in P19 but they didn't mention what happens to the H3K9cr and H3K18cr levels in P19 cells in the same knockdown conditions.
10. Authors inhibited the HDAC1-3 activity by MS-275 in differentiating P19 cells in vitro and claims inhibition of HDAC1-3 pre-activated the neuronal transcriptional programs through multiple histone acylation.
However, this needs to be proved in in vivo experiments.
11. Fig.7 should be explained properly in the legends.
12. In line number 250: histone Kcr an Kla should be Kcr and Kla
13. In line number 269: authors wrote that Both CBP and H3K9/14ac are already bind the promoter of Tuj1. Histone modifications will not bind to the promotor regions the correct term for this is enrichment. Authors should write H3K9/14ac are already enriched at the promoter of Tuj1 and bound by CBP at E13.
14. Line number 282: switch of "actyl-spray" should be acetyl-spray? And this term confusing more. Should give proper explanation or meaning of the term.
15. Line number 289: at cell level should be at cellular level.
16. Lin number 66: though should be through.
17. The authors also seem to conclude that Kcr is closely associated with highly expressed genes, when the results show a much more mixed picture of activation and repression.
18. Lastly, many figures and figure legends lack the details needed to interpret the results presented. There are also several typos in the figure labels. This makes it difficult to properly review the manuscript.

Reviewer 3

Advance summary and potential significance to field

There is little on the dynamics of these epigenetic marks during neurogenesis, so the study is of potential significance to the field, at least at the phenomenological level.

Comments for the author

The study by Dai et al focuses on two histone lysine modifications, Kcr and Kla. The authors set out to investigate their dynamics in the context of neuronal differentiation by utilizing mostly in vitro models. They also report a clear effect of histone deacetylase inhibitors on these modifications, correlating with changes in transcription at many developmentally regulated loci.
This is a potentially interesting study utilizing a descriptive approach in assessing distribution and dynamics of the two marks during neurogenesis. The part on HDACi is also interesting and provides some evidence for an potential link between their action and Kcr and Kla distribution. I have a number of points that are listed below:

Main points
- Use of the P19EC line is convenient, but it somewhat limits the impact of the study. A degree of confirmation in another more physiological model is required, e.g. in embryonic NSCs, given that they retain developmental timing in vitro (neuronal differentiation first, followed by astroglial differentiation). Alternatively, human foetal NSCs are also available via several repositories.
- In Figure 2 and 3, Authors tend to make claims that aren't supported by evidence of causal interactions. Such claims need to watered down, such as "cooperatively regulate chromatin and gene expression…" or the title related to Fig 3/S3 "…promote neural differentiation by transcriptome remodeling". No such data is there to back this up. Only correlations are shown.

- Experiments in Figure 4 should also be attempted in differentiation conditions, ie what happens if I block transcription in postmitotic/differentiating cells?
- The HDACi data are quite interesting. However, one could argue that some of the effects on transcription would be independent from Kla and Kcr modifications. One could think of a way to directly inhibit or enhance these modifications. Given the growing literature on writers, erasers and readers at least for Kcr, authors should address whether knockdown of some of these players affect these modifications, other chromatin marks and transcription. Some attempts need to be made at least in in-vitro settings. Since RNAi is already working in their hands, I don't see why this would not be feasible in a normal revision turnaround.

Overall, I believe this study contains important insights, but suffers from data overemphasizing, debatable choice of in-vitro models and lack of direct evidence for a role of writers or erasers of these marks, especially Kcr in differentiation.

First revision

Author response to reviewers' comments

Responses to the comments

We appreciate the editors and all anonymous reviewers for your in-depth comments, suggestions, and corrections, which have greatly improved the manuscript. We have addressed all your comments and revised the manuscript. The point-by-point responses (in blue) to each comment (in italic) are shown below, and all of the major revisions are highlighted in red in the revised manuscript.

Point-by-Point Responses to Reviewers Comments
The comments from the reviewers are in italic and our responses are as plain text.

Comments made by Reviewer #1

Reviewer 1 Advance summary and potential significance to field
1. In their manuscript Dai et al. characterize a series of novel histone lysine acylations in the context of the telencephalic development. Their work provides an interesting and novel dataset that sets the stage for future exploration of their functional significance.
Response: We really appreciate the Reviewer#1 for careful review and positive evaluation on our manuscript.

2. One major issue throughout the manuscript is the authors ascribe regulatory relationships with data that merely shows descriptive associations. Without additional experimental evidence these regulatory mechanisms invoked throughout the paper cannot be substantiated. Even the title suggesting that 'Kcr and Kla are novel regulators of neuronal fate during neural development' is not supported by any of the current experimental evidence provided throughout manuscript.
Response: Many thanks for this critical comment. At present, specific agents to stimulate or block Kcr and Kla are still a great challenge in the field, which in turn hinder the exploration of regulatory mechanism of these relatively novel modifications. We have discussed the limitation of our present study (Lines 332–340) and tuned down all the claims about regulatory relationships and mechanisms in the revised manuscript (Lines 15–17, 27–29, 95–96, 148–150, 152, 176–179, 180–183, 262, and 264–265). In addition, we modified the title from "Histone Kcr and Kla are novel regulators of neuronal fate during neural development" to "Dynamic profiling and functional interpretation of histone Kcr and Kla during neural development" (Lines 1–2). We believe that our dataset will be helpful for understanding the functional significance of histone Kcr and Kla during neural development.

3. The paper can also do with more example of genes across all the comparison of chipseq datasets and particularly when discussing GO terms/functional enrichment.
Response: We appreciate this instructive comment. We have provided representative genes for the comparison of chipseq datasets (Fig. 6A, S3B, S8A, and S8B) and for the GO terms/functional

enrichment analyses in the revised manuscript (Fig. 2D, 3G, 3H, 6D, S4B, S5D, S6, S8C, S8D, S9E, S9F, S12G–I, and S13A).

Reviewer 1 Comments for the author
Major points:
1. Figure 1: immunostaining shows significant expression of marks outside of radial glia/germinal zones. Most of the paper focuses mainly on this expression and does not comment on the in vivo expression in neurons that inevitably drives identified peaks in the chipseq samples, particularly in e16.5 timepoint. They should bin Kcr and Kla positive cells across the cortex to illustrate their enrichment across the progenitors zones, intermediate zone and cortical plate or alternatively radial glia, IPCs and neurons by co-staining with markers. This in situ analysis would be a more compelling illustration of histone acylation dynamics than the nestin and Tuj1 co-staining done in P19 cells. There is a visually interesting reduction of expression in different zones for different marks.
Response: Many thanks for these constructive comments. We have quantified the enrichment levels of H3K9cr, H3K18cr, H3K14la, and H3K18la across the VZ/SVZ, IZ, and CP layers across the cortex (Fig. 1B; Fig. S1A), and provided our new data clearly illustrated that increased H3K9cr and H3K18la levels in the cortical plate regions than that of the ventricular/subventricular zone in our revised manuscript (Lines 104–109).

2. There are major concerns with the western blots shown in Figure 1B (and Figures 4 and 5). The histone H3 control intensity compared to the H3Kcr and Kla is very weak. When the authors measure each mark compared to the total histone H3, it appears the relative amouts of H3Kcr and Kla is greater than the total amount of H3. However in Figure 4, the H3 intensity is quite strong and the relative amounts of Kcr and Kla marks are all less than the total H3. The while the intensity of H3 is consistent in the individual figures, it is a concern that the antibody used for these assays may not be a reliable internal control.
Response: Many thanks for these critical comments. We have updated these blots with higher exposed images to ensure that the intensity between histone marks and H3 loading control is comparable (Fig. 1D, 4C, 4D, 5A, 5C, and 5G). Although we displayed one H3 loading control blot at the bottom of the individual figures, we quantified the relative histone marks levels to their respective H3 blots (not shown) by stripping antibodies of histone marks firstly and then incubating the antibody of histone H3.

3. Figure 2: Well done characterization with associations but conclusions regarding “regulation” is unsubstantiated.
Response: Many thanks for this correction. We have modified the conclusions regarding “regulation” to “correlation” (Lines 149–150).

4. Figure 3: The authors select two timepoints of e.13.5 and e16.5 to examine PTM dynamics during neurogenesis. They show a change of expression correlated with differential histone PTMs- however conclude a regulation on neurogenesis. Because this is bulk, changes in the expression correlated with changes in PTMs could be because of the growing pool of neurons in the chipseq samples in the alternate timepoint.
Response: We totally agree with the reviewer’s perspective. We have changed the statement from “regulation on neurogenesis” to “involvement in neurogenesis” in the revised manuscript (Lines 180–183).

5. Figure 3G,H: Using GO analysis as shown in panels 3G & H the authors conclude that upregulated genes with enriched H3Kcr or K3K18la are involved in neuronal differentiation. However, this conclusion is not supported by the GO terms that are shown. These are mainly genes involved in neuron function and does not elude to differentiation.
Response: Thanks a lot for this critical comment. We performed KEGG pathways analysis in Fig. 3 G and H, but there were no any terms related to “neuron differentiation” in the KEGG pathway databases. However, our GO analysis indicated that upregulated genes with increased H3K9cr or H3K18la enrichment were involved in positive regulation of neuron differentiation (Fig. S10), and we have provided these new data in our revised manuscript (Lines 176–179).

6. Figure 4: This is a good control experiment. The authors don’t see a reduction in acetylation marks using the two inhibitors compared to the paper they cite (Martin et al. 2021). which suggests a potential differential inhibitor mechanism in inhibiting transcription as Martin et al used actinomycin D in mESCs, whereas the authors used triptolide and flavopiridol. I don’t know which

would be most appropriate for inhibiting transcription, however it would be interesting to see if they can recapture the loss of Kac marks (as shown in Martin et al) from actinomycin D and whether the novel PTMs behave similarly or not.
Response: We appreciate the reviewer's constructive suggestions. Actinomycin D intercalates into DNA for blocking the progression of RNA polymerases. Triptolide inhibits TFIIH and thereby prevents transcription initiation, whereas flavopiridol is a PTEF-b inhibitor that blocks release from promoter-proximal pausing (Bensaude, 2011; Vaid et all., 2020). According to the comments, we have detected the dynamic changes of histone acylations under the treatment of actinomycin D, our new results indicated that H3K9ac, H3K9cr, and H3K18la are drastically reduced after 60 min treatment of 25 μg /mL actinomycin D (Response Fig. 1A), which is consistent with the findings of a previous study (Martin et al,2021). However, the amount of extracted nuclear histones from the same number of cells have doubled in our study (Response Fig. 1B), which suggesting that actinomycin D may influence metabolism of histones, besides inhibiting the transcription (possible reason for reduced histone acylations due to the dilution effect of histone modifications). Moreover, both triptolide and flavopiridol treatments significantly inhibited the transcription in our study, as evidenced by the following data: (1) The loss of RNA polymerase II (RNAP2) serine 5 CTD phosphorylation (S5p, the mark for transcription initiation) and RNAP2 serine 2 CTD phosphorylation (S2p, the mark for transcription elongation) (Fig. 4A); (2) The decrease of nascent transcripts from Actb and Gapdh genes (Fig. 4B). Meanwhile, we did not observe obvious changes in histone Kac, Kcr, and Kla in proliferating NSPCs and postmitotic/differentiating neurons (Fig. 4C, D), which were consistent with the results of a recent study in which no global changes of histone Kac were observed in drosophila S2 cell safter triptolide and flavopiridol treatments (Vaid et all., 2020).

Response Fig 1. Actinomycin D treatment decreases histone acylations in P19 EC cells.
A. Images showing changes of H3K9ac, H3K18ac, H3K9cr, and H3K18la after DMSO and Actinomycin D (25 μg/mL) for 1h.
B. Quantitative analysis of changes of histones content under DMSO or actinomycin D treatment. Data are presented as mean ± SEM of three independent biological replicates, n = 3. Unpaired two-tailed Student's t test was used to analyze statistical significance; NS: no significance, **P < 0.01.

7. Figure 5: Interesting effect of pan-HDACs on PTM expression. Suggests the involvement of HDACs in the accumulation of the novel PTMs in the developing telencephalon. However, they present no evidence that these HDACs are specifically lysine delactylases except for an association. They also choose to focus the effect of simultaneous HDAC knockdown on lactyl marks but not crotonyl marks despite there being evidence of an effect with crotonyl marks in the inhibitor experiments. It would be interesting to see crotonyl dynamic changes as well.
Response: Many thanks for this constructive comment. When we prepared the manuscript, a preprint study by Olsen and colleagues reported the histone lysine delactylation activity of HDAC1-3 in vitro (Moreno-Yruela et all., 2021). Our data in the present study support the specificity of HDACs as histone delactylases both in vitro and in vivo together with their results. According to the reviewers' valuable suggestions, we have examined the dynamic changes of H3K9ac, H3K9cr, and H3K18cr after Hdac1–3 knockdown, and found that H3K9ac and H3K9cr levels increased but H3K18cr level did not change after Hdac1–3 knockdown (Fig. S11A). We provided these new data in our revised manuscript (Lines 216–219).

8. Figure 6: Data was clustered into 3 distinct clusters based on expression, with cluster 1 being focused on. It would be nice to see all 3 clusters presented together instead of removing c2 and c3 into the supplement. Providing individual or small groups of genes as exemplars of these 3 clusters would help bolster their conclusions would also be helpful as the gene ontology here and in the supplementary clusters are not informative.
Response: Thanks for this constructive suggestion. Following the reviewer's advice, we have put all 3 clusters together and provided representative genes for Fig. 6D in our revised manuscript.

Minor Points:
The paper could greatly benefit from editing for English language vocabulary and grammar.
Response: The revised manuscript has been carefully proofread by a native English speaker.

A lot of supplementary data is extensively discussed in the results section, it may be worthwhile to reevaluate the data that is included in the main figures versus the supplementary data.

Response: We greatly appreciate this constructive comment. We have incorporated some supplementary data in the main figures to improve the presentation of our data (Fig. 3A and B, Lines 167 and 170; Fig. 6D, Lines 246 and 248).

Reviewer 2 Advance summary and potential significance to field
Dai et al. show novel histone lysine acylations like crotonylation (Kcr) and lactylations (Kla) exist and cooperatively regulate the neuronal fate during mouse development. They show that Kcr and Kla are undergoing global changes during the development by profiling their genome-wide occupancy. Furthermore, they show the genes that contain three modifications (Kac, Kcr and Kla) acquire more chromatin accessibility and transcription compared to the genes with single modifications and drives the cell fate changes. They show that HDAC1-3 as a novel eraser of H3K18la and inhibition of them preactivated the neuronal transcriptional programs by promoting histone lysine acylations. While the genome-wide distribution of this modification and its role in transcriptional regulation has been provided in development and disease, the insight in the central nervous system is less characterized and understood. Therefore, the scope of study is in time and has great potential of receiving a broad interest from the community.
Response: We appreciate Reviewer#2's positive evaluation and constructive comments.

Reviewer 2 Comments for the author
1.In Fig.1 germinal layer markings are missing (VZ, SVZ,CP). Cells from the VZ regions are progenitor cells and SVZ region contains mostly differentiating cell populations and CP contains the differentiated functional neurons. In Fig.1A level of H3K18cr is most enriched in the cortical plate regions it would be better if authors could differentiate/quantify the enrichment level of these modifications across the layers.
Response: Thanks a lot for the instructive comments. We have added the geminal layer markings in Fig. 1A, and quantified the enrichment levels of H3K9cr, H3K18cr, H3K14la, and H3K18la across the layers (Fig. 1B; Fig. S1A), and provided these new data in our revised manuscript (Lines 104–109).

2.In Fig1.C and Fig.S1E authors quantified the levels of these modifications during P19 cells differentiation. Similar quantification should be done at cellular levels in in vivo experiments to show the dynamics of these modifications across the various cell types during cortical development (VZ/SVZ/CP).
Response: Many thanks for this excellent suggestion. We have quantified the dynamics of H3K9cr, H3K18cr, H3K14la, and H3K18la between neural stem/progenitor cells (VZ/SVZ) and neurons (CP) (Fig. 1A, B; Fig. S1A). We have provided these new data in our revised manuscript (Lines 104–109).

3.In line number 121 authors have written H3K9ac, H3K9cr and H3K18la were mainly located in promotor regions. But from their Fig.2A H3K18al was most enriched in intron and intergenic regions.
Response: Many thanks for this comment. We have corrected it from "First, H3K9ac, H3K9cr, and H3K18la were mainly located in promoter regions (Fig. 2A)" to "First, H3K9ac and H3K9cr were mainly located in promoter regions, while H3K18la was most enriched in intron and intergenic regions (Fig. 2A)" in the revised manuscript (Lines 125–127).

4.Authors should extend their ChIP-seq data analysis. example: motif enrichment analysis and what are the transcription factors that target these motifs and co-occurrence of these modifications with super-enhancers. This extended analysis will provide additional knowledge to the scientific community.
Response: We appreciate these instructive comments. We performed motif enrichment analysis of H3K9ac, H3K9cr, and H3K18la peaks and provided new data in our revised manuscript (Fig. S4C–E, Lines 144–147). H3K27ac is the widely accepted histone mark to identify super-enhancers. Sadly, we do not have performed anti-H3K27ac ChIP assay in the present study. Hopefully, we will explore the co-occurrence of these modifications with super-enhancers in the future. We have discussed this future outlook for co-occurrence of these modifications with super-enhancers in the revised manuscript (Lines 332–335).

5.In Fig. 2D authors only showed gene ontology terms for H3K9cr and H3K18la and in the end claim H3K9ac, H3K9cr and H3K18la are involved in different pathways. gene ontology terms for H3K9ac should be provided.
Response: We apologize for not providing the gene ontology terms for H3K9ac in the original manuscript. We have them in Fig. S4B in the revised manuscript (Lines 140–141).

Fig2D. Number of genes contributed to biological functions are missing.
Response: We have provided the number of genes contributed to biological functions in Fig. 2D and Fig. S4B.

6.Line number 148 they mentioned that they, systemically analyzed genome-wide changes in H3K9ac, H3K9cr, and H3K18la at early (E13.5) and late (E16.5) stages of embryonic neurogenesis and referred (Fig.S5) which is RNA- seq data. If they profiled genome-wide changes in H3K9ac, H3K9cr, and H3K18la they should refer the ChIP-seq data.
Response: Many thanks for this correction. We have corrected it from "Thus, we systemically analyzed genome-wide changes in H3K9ac, H3K9cr, and H3K18la at early (E13.5) and late (E16.5) stages of embryonic neurogenesis (Fig. S5)" to "Thus, we systemically analyzed genome-wide changes in H3K9ac, H3K9cr, and H3K18la at early (E13.5) and late (E16.5) stages of embryonic neurogenesis by ChIP-seq (Fig. S3A), and RNA-seq (Fig. S6)." in the revised manuscript (Lines 156–158).

7.In Fig 4. they showed Pol ll inhibition resulted in transcription reduction by measuring the levels of Pol ll CTD phosphorylation. instead, it would have been better to include some additional experiments to show the transcription reduction. like, puromycin incorporation assays.
Response: Thanks a lot for this constructive comment. We have directly detected the transcription reduction effects after Pol ll inhibition by quantifying the changes of nascent transcripts from Actb and Gapdh genes (two house-keeping genes), and found that both triptolide and flavopiridol treatment significantly inhibited the transcription of Actb and Gapdh genes. We have included these new data in our revised manuscript (Fig. 4B, Lines 192–194).

In Fig.4C the western blot signals look weak. And control H3 signals are unequal based on their given image. They should provide better higher exposed images. All the western blot images should be labeled with proper molecular weight markers.
Response: We have updated these blots to ensure higher exposed images in Fig. 4C, and labeled all the western blot images with proper molecular weight markers in the revised manuscript (Fig. 4A, 4C, and 4D).

8.Fig 5. All the western blot images should be labeled with proper molecular weight markers.
Response: We have labeled all the western blot images with proper molecular weight markers in Fig. 5 in the revised manuscript (Fig. 5A, 5C, 5D, and 5G).

9.Fig.5G-H. they showed knockdown of HDAC 1-3 increased the level of H3K18la levels in P19 but they didn't mention what happens to the H3K9cr and H3K18cr levels in P19 cells in the same knockdown conditions.
Response: Actually, we measured the levels of H3K9ac, H3K9cr, and H3K18cr levels after knockdown of HDAC 1–3, and found that knockdown of HDAC1–3 increased H3K9ac and H3K9cr levels, but not H3K18cr levels in P19 EC cells. We have provided these data in our revised manuscript (Fig. S11A, Lines 216–219).

10.Authors inhibited the HDAC1-3 activity by MS-275 in differentiating P19 cells in vitro and claims inhibition of HDAC1-3 pre-activated the neuronal transcriptional programs through multiple histone acylation. However, this needs to be proved in in vivo experiments.
Response: This is an excellent suggestion. In the present study, we observed that the sizes of embryonic brains in the MS-275 treatment group were significantly reduced compared with that in the DMSO control group (data not shown). Consistent with our observation, Tang and colleagues also reported that the brain and cerebral cortex size in particular were dramatically reduced in HDAC1/2 cKO mice compared to wild-type controls (Tang et all., 2019). As ChIP-seq and RNA-seq assays of the telencephalon with mixed cell types and severe developmental defects may not reflect the direct effects of HDAC1-3 inhibition or deletion, we did not examine the changes of neuronal transcriptional programs through multiple histone acylation in vivo. To further validate the claim, we have detected the dynamics in histone Kac and Kcr after MS-275 treatment in cultured NSPCs that were isolated from E13.5 mouse forebrain, and validated that gene with increased histone Kac or Kcr enrichment after MS-275 treatment were mainly involved in neuronal differentiation and maturation. These results were highly consistent with our conclusions drawn from P19 EC cells-derived NSPCs (Fig. 6). We have provided these new data in our revised

manuscript (Fig. S14, Lines 248–252) and discussed this limitation of our present study in the revised manuscript (Lines 337–340).

11.Fig.7 should be explained properly in the legends.
Response: We have provided a more detailed legend for Fig.7 in the revised manuscript (Lines 884–890).

12.In line number 250: histone Kcr an Kla should be Kcr and Kla.
Response: We have corrected this error in the revised manuscript (Line 267).

13.In line number 269: authors wrote that Both CBP and H3K9/14ac are already bind the promoter of Tuj1. Histone modifications will not bind to the promotor regions the correct term for this is enrichment. Authors should write, H3K9/14ac are already enriched at the promoter of Tuj1 and bound by CBP at E13.
Response: Many thanks for this correction. We have fixed it in the revised manuscript (Lines 286–287).

14.Line number 282: switch of “actyl-spray” should be acetyl-spray? And this term confusing more. Should give proper explanation or meaning of the term.
Response: We have revised the sentence from “we propose the existence of a switch of “actyl-spray” targeting regulatory elements of genes regulating neural fate decisions” to “we propose the existence of a switch for histone acylations at regulatory elements of genes regulating neural fate decisions” (Lines 298–299).

15.Line number 289: at cell level should be at cellular level.
Response: We have corrected it in the revised manuscript (Line 305).

16.Lin number 66: though should be through.
Response: We have corrected it in the revised manuscript (Line 67).

17. The authors also seem to conclude that Kcr is closely associated with highly expressed genes, when the results show a much more mixed picture of activation and repression.
Response: Many thanks for pointing this confusing point out. Instead of assigning one-to-one mapping between binding sites and genes (commonly used peak annotation method), BETA models the influence of a binding site on the expression of a gene with a monotonically decreasing function that is based on the distance between the binding site and transcription start site (Wang et all., 2013). The results in Fig. 3 and Fig. S12 suggested that the increase in histone Kcr and Kla enrichment was mainly associated with up-regulated genes (a smaller p-value of Upregulate than that of downregulated genes), indicating that increased histone Kcr and Kla might act as a gene activator rather than a repressor during neural development. We have rewritten the text in the revised manuscript (Lines 229–233).

18. Lastly, many figures and figure legends lack the details needed to interpret the results presented. There are also several typos in the figure labels. This makes it difficult to properly review the manuscript.
Response: We apologize for not providing detailed figure legends in the original manuscript. We have made detailed interpretation for the results in figure legends and corrected typos for all figures in our revised manuscript (Fig. 2C, 2D, 3G, 3H, 6C, and 6D, Lines 869–871, 884–890).
Reviewer 3 Advance summary and potential significance to field
There is little on the dynamics of these epigenetic marks during neurogenesis, so the study is of potential significance to the field, at least at the phenomenological level.

Reviewer 3 Comments for the author
The study by Dai et al focuses on two histone lysine modifications, Kcr and Kla. The authors set out to investigate their dynamics in the context of neuronal differentiation by utilizing mostly in vitro models. They also report a clear effect of histone deacetylase inhibitors on these modifications, correlating with changes in transcription at many developmentally regulated loci. This is a potentially interesting study utilizing a descriptive approach in assessing distribution and dynamics of the two marks during neurogenesis. The part on HDACi is also interesting and provides some

evidence for an potential link between their action and Kcr and Kla distribution. I have a number of points that are listed below:
Response: We thank the reviewer for the encouraging and constructive comments and suggestions for improving our manuscript.

Main points
-Use of the P19EC line is convenient, but it somewhat limits the impact of the study. A degree of confirmation in another more physiological model is required, e.g. in embryonic NSCs, given that they retain developmental timing in vitro (neuronal differentiation first, followed by astroglial differentiation). Alternatively, human foetal NSCs are also available via several repositories.
Response: We appreciate this critical comment. In our recent publication (Dai et all., 2021), we treated cultured NSPCs isolated from E13.5 mouse forebrains with 1 μM MS-275 for 24 h (the same conditions used in the present study) and performed anti-histone Kac and Kcr ChIP-seq assays to detect their dynamic changes. We found that NSPCs pre-treated with MS-275 were prone to differentiate into neurons. Through in-depth analysis of these public datasets (GEO databases, GSE124540), we found that genes with increased histone Kac or Kcr enrichment after MS-275 treatment were mainly involved in neuronal differentiation and maturation (Fig. S14, Lines 242–245) These results were highly consistent with our conclusions drawn from P19 EC cells-derived NSPCs (Fig. 6)
We tried several new batches of H3K18la antibodies but failed to pass the quality control for ChIP experiments of MS-275 treated embryonic NSPCs. So, we hope that further detailed analysis in the dynamics of these modifications in more physiological model (such as human foetal NSCs) will be included in a future publication. We have discussed this limitation of our present study in the revised manuscript (Lines 330–332).

-In Figure 2 and 3, Authors tend to make claims that aren't supported by evidence of causal interactions. Such claims need to watered down, such as "cooperatively regulate chromatin and gene expression…" or the title related to Fig 3/S3 "…promote neural differentiation by transcriptome remodeling". No such data is there to back this up. Only correlations are shown.
Response: We appreciate this critical comment. Please see above response to the second point of Advance summary and potential significance to fieldcomment made by Reviewer #1.

-Experiments in Figure 4 should also be attempted in differentiation conditions, ie what happens if I block transcription in postmitotic/differentiating cells?
Response: Many thanks for this excellent suggestion. We have detected the dynamics of H3K9cr, H3K18cr, H3K14la, and H3K18la levels after transcription inhibition in postmitotic/differentiating cells (P19 EC cells-derived neurons at Day8), and found that there were no significant changes of these modifications. We have provided these new data in our revised manuscript (Fig. 4D, Lines 191–193).

-The HDACi data are quite interesting. However, one could argue that some of the effects on transcription would be independent from Kla and Kcr modifications. One could think of a way to directly inhibit or enhance these modifications. Given the growing literature on writers, erasers and readers at least for Kcr, authors should address whether knockdown of some of these players affect these modifications, other chromatin marks and transcription. Some attempts need to be made at least in in-vitro settings. Since RNAi is already working in their hands, I don't see why this would not be feasible in a normal revision turnaround.
Response: We appreciate these instructive comments. Following the reviewer's advice, we have detected the changes of H3K9ac, H3K9cr, H3K18cr, H3K9me3, H3K4me3 as well as transcription after Hdac1-3 knockdown, and found that HDAC1-3 mainly influenced histone acylations (H3K9ac, H3K9cr, and H3K18la) but not histone methylation (H3K9me3 and H3K4me3) (Fig. S11A and B). In addition, transition elongation (S5p levels) but not transition initiation (S2p levels) was enhanced after Hdac1-3 knockdown, which was consistent with the previous findings that the pan HDACs inhibitor TSA promoted the release of promoter-proximal paused RNAP2 by histone Kac (Vaid et all., 2020). We have provided these new data in our revised manuscript (Lines 211–214).

Overall, I believe this study contains important insights, but suffers from data overemphasizing, debatable choice of in-vitro models and lack of direct evidence for a role of writers or erasers of these marks, especially Kcr in differentiation.

Response: We greatly appreciate the reviewer's insightful comments that have significantly helped us to improve the manuscript. We hope that our revised manuscript will satisfy the comments of the reviewer.

Reference

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Second decision letter

MS ID#: DEVELOP/2021/200049

MS TITLE: Dynamic profiling and functional interpretation of histone lysine crotonylation and lactylation during neural development

AUTHORS: Shang-Kun Dai, Pei-Pei Liu, Xiao Li, Lin-Fei Jiao, Zhao-Qian Teng and Chang-Mei Liu

My apologies for the long time it has taken us to receive the referees' reporting your manuscript. I have now received the reports of two of the referees who reviewed the first version of your manuscript and I 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 maintain their interest in your work, but Referee 3 still has significant criticisms and recommends a substantial further revision of your manuscript before we can consider publication. I have consulted with a member of our Advisory Board, and we both agree with Referee 3 that additional experiments are needed to link the H3K18la or H3K9cr modifications with target gene transcription. Referee 3 recommends RNAi-mediated knockdown of writers, readers or erasers in cell culture. The Advisory Board members also suggests adding excess amounts of lactic acid or crotonic acid to culture medium so as to modulate local H3K18la and H3K9cr levels. If you are able to revise the manuscript along the lines suggested, 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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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.

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 2

Advance summary and potential significance to field

The authors have satisfactorily addressed all of my concerns. I recommend publication.

Comments for the author

The authors should have their manuscript proofread once carefully for English/grammar prior to publication.

Reviewer 3

Advance summary and potential significance to field

The Authors have improved their manuscript by addressing many of the comments I had raised. However, a key point remains unanswered, ie to connect these modifications to chromatin and transcription and how this may influence neurogenesis. The Authors mention "functional" in the title, but little functional insights have been provided.

Comments for the author

What the Authors failed to provide is evidence supporting the proposed link between these histone modifications, chromatin and neurogenesis. As highlighted in my review, given the growing literature on writers, erasers and readers at least for Kcr, authors should address whether knockdown of some of these players affect these modifications, other chromatin marks and transcription. Some attempts need to be made at least in in-vitro settings. Since RNAi is already working in their hands, I don’t see why this would not be feasible in a normal revision turnaround. I do not believe this point has been addressed, and I am convinced some evidence should be provided.

Second revision

Author response to reviewers' comments

Responses to Editorial comments

We appreciate the editors for your in-depth comments, suggestions, and corrections, which have greatly improved the manuscript. We have addressed all your comments and revised the manuscript. The point-by-point responses (in blue) to each comment (in italic) are shown below, and all of the major revisions are highlighted in red in the revised manuscript.

As you will see, the referees maintain their interest in your work, but Referee 3 still has significant criticisms and recommends a substantial further revision of your manuscript before we can consider publication. I have consulted with a member of our Advisory Board, and we both agree with Referee 3 that additional experiments are needed to link the H3K18la or H3K9cr modifications with target gene transcription. Referee 3 recommends RNAi-mediated knockdown of writers, readers or erasers in cell culture. The Advisory Board members also suggests adding excess amounts of lactic acid or crotonic acid to culture medium so as to modulate local H3K18la and H3K9cr levels.

Response: We have performed additional experiments to knockdown the writer (GCN5) and eraser (HDAC1-3) for H3K9cr in cell culture, and linked H3K9cr with transcription in the revised manuscript (Lines 217–225, Fig. S11).
In addition, we have added 10 mM crotonate to cultured NSPCs to modulate H3K9cr levels (Response Fig. 1) and found that crotonate-treated NSPCs differentiated into more TUJ1+ neurons than those in the control group (Fig. S15C and D). In our recent publication (Dai et all., 2021), we have also proved that enhanced histone Kcr levels induced by crotonate treatment promotes gene expression by regulating chromatin accessibility and RNAP2 recruitment.
Response Fig 1 Crotonate modulates H3K9cr levels in cultured NSPCs.

Point-by-Point Responses to Reviewers Comments
The comments from the reviewers are in italic and our responses are as plain text.

Comments made by Reviewer #2

Reviewer 2 Advance summary and potential significance to field
The authors have satisfactorily addressed all of my concerns. I recommend publication.
Response: We appreciate Reviewer #2's tremendous efforts to support and improve our work.

Reviewer 2 Comments for the author
The authors should have their manuscript proofread once carefully for English/grammar prior to publication.
Response: Thanks a lot for this suggestion. We have proofread the manuscript with a friend who is a native English speaker and corrected grammar errors in the revised manuscript.

Comments made by Reviewer #3

Reviewer 3 Advance summary and potential significance to field
The Authors have improved their manuscript by addressing many of the comments I had raised. However, a key point remains unanswered, ie to connect these modifications to chromatin and transcription and how this may influence neurogenesis. The Authors mention "functional" in the title, but little functional insights have been provided.
Response: We appreciate Reviewer #3's careful review and positive comments on our revised paper. We have performed several new experiments and provided functional insights in the revised manuscript by following Reviewer #3's instructive comments.

Reviewer 3 Comments for the author
What the Authors failed to provide is evidence supporting the proposed link between these histone modifications, chromatin and neurogenesis. As highlighted in my review, given the growing literature on writers, erasers and readers at least for Kcr, authors should address whether knockdown of some of these players affect these modifications, other chromatin marks and transcription. Some attempts need to be made at least in in-vitro settings. Since RNAi is already working in their hands, I don't see why this would not be feasible in a normal revision turnaround. I do not believe this point has been addressed, and I am convinced some evidence should be provided.
Response: We deeply appreciate these instructive comments. Following Reviewer #3's advice, we examined whether knockdown of the writer (GCN5) and eraser (HDAC1-3) for H3K9cr affect other chromatin marks, such as histone methylation, and transcription. There were significantly reduced H3K9ac, H3K9cr, and transcription elongation (S2p) levels after knockdown of GCN5, while obviously increased H3K9ac, H3K9cr, and transcription elongation (S2p) levels after knockdown of HDAC1–3 (Fig. S11). However, there were no evident changes in histone methylations, such as H3K4me3, H3K9me3, and H3K27me3, and in transcription initiation (S5p) levels (Fig. S11). These new data suggested that knockdown of writer or eraser for H3K9cr might predominately affect H3K9ac and H3K9cr levels, but not other chromatin marks (active mark: H3K4me3, and repressive mark: H3K9me3 or H3K27me3).
HDACs inhibition by TSA promotes the release of promoter-proximal paused RNAP2 (thus increased transcription elongation) via enhancing histone Kac (Vaid et all., 2020), while knockdown of GCN5 increases RNAP2 pausing (thus reduced transcription elongation) because of the loss of global H3K9ac (Gates et all., 2017). Consistent with previous findings, we observed reduced transcription elongation levels (S2p levels) (Fig. S11C) as well as a sharp decline in H3K9ac and H3K9cr levels

after knockdown of GCN5 (Fig. S11A). Meanwhile, we also observed enhanced transcription elongation levels (S2p levels) (Fig. S11D) as well as an increase in H3K9ac and H3K9cr levels after knockdown of HDAC1–3 (Fig. S11B). These results suggested dynamic changes in H3K9cr linked with transcription elongation to regulate gene expression.
To examine whether H3K9cr and H3K18la are functionally correlated with neurogenesis, we treated cultured NSPCs from E13.5 mouse forebrain with MS-275, and found that MS-275-treated NSPCs displayed increased levels of H3K9ac, H3K9cr, and H3K18la (Fig. S15B), and that they differentiated into more TUJ1+ neurons than those in the control group (Fig. S15C and D). These new results were highly consistent with the changes in histone Kac and Kcr (Fig. S14) as well as the transcriptional programs (Fig. S15A).
Taken together, we believe that changes in H3K9cr levels affect transcription elongation to regulate gene expression (Fig. S11). Functionally, enhanced H3K9cr levels by MS-275 treatment pre-activates neuronal transcriptional programs in P19 ECCs-derived NSPCs (Fig. 6) and promotes neuronal differentiation in cultured NSPCs isolated from E13.5 mouse forebrain (Fig. S14, S15). We have added these new data in the revised manuscript (Lines 217-224, Lines 254-259, Fig. S11, S15).

Third decision letter

MS ID#: DEVELOP/2021/200049

MS TITLE: Dynamic profiling and functional interpretation of histone lysine crotonylation and lactylation during neural development

AUTHORS: Shang-Kun Dai, Pei-Pei Liu, Xiao Li, Lin-Fei Jiao, Zhao-Qian Teng and Chang-Mei Liu
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.