**Peer Review File**

**Manuscript Title: A complete temporal transcription factor series in the fly visual system**

## Reviewer Comments & Author Rebuttals

**Reviewer Reports on the Initial Version:**

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript by Konstantinides et al. aims to identify the suite of temporal transcription factors that generate the diverse neurons in the Drosophila optic lobe. This work builds on an earlier publication by Li et al. (Nature [2013] 498, 456-462) that identified the transcription factors Hth, Ey, Slp, D and Tll as temporal transcription factors (tTFs) in the optic lobe. Here the authors use single cell RNA sequencing to identify new candidate tTFs. They tested the genetic interactions between these candidate factors and the previously identified tTFs, showing that three of the factors appear to be new tTFs, while others modulate the temporal series. They also used scRNAseq to follow the differentiation of one of the neuronal subtypes, showing that cell cycle genes and DNA replication genes are expressed first, followed by genes regulating translation, dendrite development, axon guidance and finally neuronal function. Interestingly, there appeared to be a considerable delay between transcription and translation of neurotransmitter-related genes, although the mechanism for this was not explored. Finally, they showed a similar sequence of expression of neuronal differentiation genes in human cortical neurons, deduced from single nuclear RNA-seq data.

The authors generated nearly 50,000 transcriptomes from cells in the larval brain. These were arrayed in a developmental trajectory based on known markers expressed in the neuroepithelium, neuroblasts, ganglion mother cells, neurons and glia. When the previously identified tTFs (Hth, Ey, Slp, D and Tll) were arrayed on this map, a gap was evident between Hth and Ey, suggesting that there might be one or more new tTFs to be discovered. Seven candidate transcription factors with restricted temporal expression were identified in the scRNAseq dataset (Opa, Erm, Hbn, Scro, BarH1, Oaz, Esg). Of these Esg was excluded as a candidate (it was not expressed in all neuroblasts) as was Oaz (there were no antisera to confirm temporal expression) leaving five potential tTFs.

The authors emphasize that restricted temporal expression of a transcription factor is not sufficient to label it as temporal factor, it also has to progress the temporal series. To do this, temporal transcription factors activate expression of the next tTF in the series and repress expression of the previous tTF. The authors used clonal analysis or RNAi to remove or knock down each candidate tTF (Opa, Erm, Hbn, Scro, BarH1) and then assayed expression of the prior and subsequent tTFs in the series. From their results they proposed the genetic interactions between tTFs shown in Figure 3A.

According to the authors' definition of tTFs, not all of the factors in Figure 3A are temporal transcription factors. The authors state that Hth is not a tTF; it is not required for progression of the

series as Ey is still expressed in Hth mutants. Although the authors place Hth upstream of Erm, there is no evidence that Hth activates Erm expression. Opa does not appear to be a tTF as it is not absolutely required for Ey expression (Figure 3C). In addition, Ey is proposed to repress Opa, but the two genes appear to be co-expressed (Figure 3E; double labeling in color would be helpful here). Similarly, what is the evidence that Bar-H1 is a temporal factor; does Bar-H1 repress D? On the evidence presented, only Erm, Hbn and Scro can be called tTFs. In Figure 3A it would be useful to clearly distinguish between the previously published temporal tTFs and the candidate factors identified in this work and to label the factors that actually progress the temporal series, as shown for previous tTFs.

Next, the authors investigated whether the tTFs regulate neuronal identity, by first searching in the scRNAseq data for neurons that maintain expression of the tTFs. This section is confusing in places. What is meant by "Moreover, although Erm is expressed in neurons that also inherited Ey from the neuroblast, and D is co-expressed with Hbn in neurons that come from the Hbn temporal window (arrows), this represents a different function of these genes."? It could also be made clearer which results are from previous papers, for example "the Tll window is known to give rise to glial cells" is not referenced. Similarly, "We evaluated the assignments of the neurons to specific temporal windows by looking at neurons whose temporal window is already known" is not referenced. The authors should discuss how their results overlap or extend those published by Li et al. (Nature [2013] 498, 456-462; summarized in Figure 6) that previously showed the neurons derived from the Hth, Ey, Slp, D and Tll temporal windows and whether Notch was on or off. In Figure 4C, Hbn and BarH1 are both in white; how were they distinguished from each other?

In the next section of the manuscript, the progression of neuronal differentiation was followed in one neuronal subtype, Mi1, by merging the larval scRNAseq with the authors' previously published pupal scRNAseq data to generate a neuronal differentiation trajectory. While the timing of expression of these genes is of interest, their order of expression is unsurprising: cell cycle genes and DNA replication genes first, followed by genes regulating translation, dendrite development, axon guidance and neuronal function. All neurons appear to follow the same trajectory. Interestingly, neurotransmitter-related genes were transcribed in larval brains but not translated, although the mechanism of this post transcriptional regulation was not investigated. Similar experiments were performed on human cortical neurons and their differentiation trajectory, based on Gene Ontology, was similar to that in Drosophila with the exception of genes regulating translation. Finally, the authors searched for expression of the Drosophila tTFs in published scRNA seq data from mouse cortical radial glia at E12-E15 and for orthologs of mouse tTFs in the optic lobe but found no conservation other what had previously been shown for Pax6 and Foxg1.

In summary, the authors identified three new, bona fide, temporal transcription factors, Erm, Hbn and Scro in the Drosophila optic lobe and described the genetic interactions between these factors, the known tTFs (Hth, Ey, Slp, D and Tll), and transcription factors that influence the temporal series without progressing it (Opa and Bar-H1). The authors state, incorrectly, that "several tTFs directly control the progression by activating the next tTF and repressing the previous one…" None of the interactions they show can be called direct without some evidence of direct binding to the regulated gene, for example by ChIP. The authors show that expression of most tTFs is maintained in neurons and that Hbn is required for neuronal expression of Traffic-Jam and Otd, while Opa is required for

TfAP-2 expression. A neuronal differentiation trajectory was generated and shown to be similar in human cortical neurons. Drosophila differentiation genes were expressed much earlier in development than expected and at least some of the genes appeared to be regulated post-transcriptionally. The experiments were well designed and presented although the methods used are now fairly routine (scRNAseq and generation of pseudotime trajectories using Monocle (Nat Biotechnol. 2014 Apr;32(4):381-386).

Referee #2 (Remarks to the Author):

In this study, Konstantinides and colleagues used single cell RNA sequencing data of the developing Drosophila optic lobe to answer fundamental questions about neuronal temporal specification and neuronal differentiation. The presence of many repeated, serially developing lineages permits profiling neural development with exquisite temporal resolution. Analysis of medulla neural stem cells (NB) both using UMAP and cell trajectory building (Monocle) allowed the authors to discover what appears to be a comprehensive series of temporal transcription factors (tTF). With these tTF, the authors were able to predict a series of 12 TF codes that could be expressed by medulla NBs in consecutive order. Together with spatial patterning cues and binary Notch signaling, this newly discovered discover tTF series could account for the specification of 120 of the 200 discovered medulla neuron types. Further, they used the UMAP data of NBs, intermediate daughter cells and differentiated neurons (with types annotated) to predict the birth order of known medulla neuron types.

In sum, this work has exceeded previous high-profile papers on the use of single cell sequencing technologies in genome-wide molecular genetic studies. It greatly expands our knowledge of neuronal temporal fating mechanisms and lays a comprehensive framework for seeing how the genome encodes each neuron type in the complex nervous system.

General concern:

Given the wealth of new info, it is understandable that some analyses scratched the surface of many things and detailed mechanisms were not thoroughly investigated. However, one could have done more in-depth analysis and presentation of the data to show the respective neuronal outputs for the predicted 12 temporal windows in the NB. The authors predicted birth order of medulla neurons (this was in a supplemental table that was absent from the pdf), but it was not clear how this birth order related to the tTFs and the 12 temporal windows.

Main comments on the scientific findings

1. Identification of newly identified tTFs (as well as old tTFs)
a. BarH2 antibody staining and genetic interactions were not shown. Is BarH2 protein and regulation identical to BarH1?
b. Oaz-Gal4 pattern may be discernible with a labile reporter.
c. According to the RNASeq data in Figure S3A and in the UMAP data of the NBs (Figure 1F), Hth appears to have a 2nd expression window in NBs. Is there a later role for a second wave of Hth?

2. Is the current list of tTFs comprehensive?
a. Have the gaps truly been filled between Hth and Ey? Can Hth, Opa and Ey antibody stains be done together with a counter stain (Dpn, Ase or even Dapi) to be able to visualize all of the cells?
b. Hbn nested within Ey. Are there Ey-only subwindows both preceding and following Hbn?

3. Neuronal output…
a. Do the 12 temp windows translate to predicted neuronal types? This is not clear from Table S1 which does not use the same tTF temporal windows.
b. Some windows (particularly in the neuronal data) seem broader than others. Does this make sense? Are the broader windows broken down into smaller windows?
c. Is it known approximately how many divisions each NB undergoes? 200 types >>> divide by 2 notch status =100 >>> divide by 5 spatial domains = 20. Could the tTF windows make up 20 different NB TF codes?

4. Progression of tTF hub (are the hubs really hubs?)

Please indicate more clearly which interactions have/have not been tested. (For example, the Hth RNAi experiments were not included the results section or main figure). The diagram showing genetic interaction of tTFs would benefit from adding information about which genes were tested but did not show interactions (that could also be in a separate but similar diagram).

Please include the N values of the data presented.

a. Interaction between Opa and Erm: According to Fig 3C, Opa mutant had normal Erm expression, however antibody staining was not very good, are there additional experiments where erm antibody works better? Does Erm inhibit Opa?
b. In Fig3C, Opa mutant clone had altered Ey expression (expressed at edge of clone). Could this result come from mechanisms other than a temporal delay? Have you tested Vsx-Gal4 driven Opa RNAi?
c. Ey was previously shown to activate Slp, right? Why is that missing from the diagram in main figure now?
d. Loss of Hbn was not tested for Sco
e. Slp mutant not tested for Opa (2nd wave)
f. unclear whether Opa mutant clone's effect on Slp is via Ey as the figure suggests
g. No interaction was tested between Opa and D
h. No interaction was tested between Opa and BarH1

Minor points on text/data presentation/figures

In all figures with UMAP data, having the absence of gene expression be labeled as a dark color (gray/black) makes it difficult to see the nearby relevant gene expression. Can it be changed to a lighter color?

Fig 1A: Please explain what the different colors are supposed to represent (are these tTFs?). In neurons, what does filled vs outlined neurons mean for a particular temporal color?

Fig1B:
• This UMAP clearly includes the later introduced P15 pupal RNAseq data set. Does the shape of the UMAP/interpretation of the data change if only the L3 data is included?
• The way GMCs are labeled, it appears only the right side of the "hourglass" are GMCs while the other half are neurons. Same goes for the labeling of the Neuroblasts, not clear where the cells transition from NE to NBs based on the labeling.
• The "hourglasses" could be highlighted in some way.
• The red dashed box (referring to Fig3F) is confusing, as it is a similar color as Ase—making it seem like all cells within are GMCs
• What does dark grey color represent? Absence of marker genes?
Curious cell types:
• I noticed a couple of populations of Ase/Elav double positive cells that are not clustered with the GMCs: larval Mi1 and to the immediate left of the Pm1/2 cells. Are these GMCs that are more "differentiated" towards their neuron types?
• I'm also intrigued by the three-pronged structure which contains NE, NB and GMC, and glial markers. Are these glioblasts or NBs at a late, glial stage?

Fig 1C: This is clearly the same data as 1B (represented with different labels) but that is not clearly stated. In the legend, please state the stage of the pupal data.

Figure 1D: Figure legend does not clearly state what is being shown (UMAP of larval and pupal OLs). It is not written like a figure legend, rather like the results section.

Figure 1E: The colors having dark gray being lack of expression makes it difficult to see and interpret the data. It is very hard to tell the difference between dark magenta, dark blue and dark green.

Figure 1F: Which cells are GMCs and which NBs? It is unclear how to interpret the different shades of colors that aren't quite the colors given. What does brighter or darker color mean? There is no "Blank" row of NBs but row of dark gray (please explain what gray means—NBs not expressing known tTFs). It would be helpful to have an arrow demonstrating the temporal direction of the tTFs.

Figure 2A: All 14 potential tTFs are not on this graph. Please add BarH2 and Slp2.

Figure 2B: It would be helpful to have the same orientation of the OLs in B and B'

Figure 2C: In the figure legend, please explain what data was used to create this model. Is it based on RNAseq data, immunostaining, or a combination? Is it known approximately how many division each NB undergoes?

Figure 2D: Do both D and D' show both waves of Opa? It would be helpful to have a counterstain (Dpn, Ase or Dapi) to see the cells that are not positive for either antibody?

Figure 4 A-B: Please label the neuronal populations per figure 1C.

Figure 5C: what stages are contained in this UMAP?

Referee #3 (Remarks to the Author):

In this manuscript, Konstantinides et al. reported a comprehensive identification of the temporal transcription factors (tTFs) in neuroblasts that specify most Drosophila medulla neurons in the optic lobe using single-cell mRNA sequencing. Notably, the tTFs remain expressed in differentiating neurons and regulate neuronal identity. The authors also examined the early steps of neuronal differentiation and found that the terminal differentiation genes are present as transcripts, but not proteins, in immature neurons days before being used in functioning neurons. Finally, the authors also suggested that some of the mechanisms are conserved in humans. Overall, this is an interesting study and provides new insights into the temporal control of lineage progression and neuronal diversity.

Main comments:

1. Based on scRNAseq, the authors identified eight new candidate TFs such as Opa, Erm, Esg, Hbn, as temporal TFs based on the previously known tTF framework. Given the limitation of 10x genomics in sequencing depth, the dataset that authors used shows a median of 1343 genes per cell (200 genes cutoff). Should a dataset with more gene depth be used, would the authors expect to identify more tTFs?

2. The authors took advantage of the complex genetic interaction data to build the tTF network that controls the temporal progression of neuroblast lineages. While the genetic interaction data is powerful and informative, it does not provide any direct knowledge on gene regulation (e.g., transcriptional activation or suppression). Could the authors obtain any evidence on direct gene regulation between the tTFs, especially those newly identified ones?

3. Given that the tTFs often remain expressed in neurons (in addition to neuroblasts) and regulate neuronal identity, how would the authors know that the phenotype of mutants is due to their functions in neuroblasts or neurons? How would tTF network act in postmitotic neurons?

4. In the human scRNA-Seq dataset from GW19 cortical plate, the authors reported a trajectory from Pax6+ radial glia progenitors (RGPs) to Eomes+ intermediate progenitors (IPs) and Neurod2+ postmitotic neurons. Did the authors use the entire cortex, as the cortical plate often refers to the mantle region without the proliferative zone?

5. The authors checked the expression of Drosophila tTFs in mouse cortical radial glia and found no obvious conservation of a common temporal series of TF between flies and mice. Since the previous studies have suggested some Drosophila tTFs orthologs in the developing mouse retina, could the authors compare fly optic lobe and mouse/primate retina (instead of the cortex)?

Minor comments:

1. In the abstract “Moreover, we show that tTFs not only….” This sentence is incomplete.

2. Fig 1B: Could the authors clearly label the lineage trajectories for lobula plate neurons and lobula neurons?

Fig 1E: It appears that Vsx1+ cells are present in two separate clusters. How does this relate to the spatial or temporal regulation of the lineages?

3. Fig S3 (line 1328), the known tTFs should be in purple and the new tTFs should be in green as shown in the Figure S3A.

4. There are numerous typos/errors in the Methods (e.g., 25C). The authors should carefully review and revise.

Referee #4 (Remarks to the Author):

Konstantinides et al., “A comprehensive series of temporal transcription factors in the fly visual system.” The starting point of this study is single cell sequencing of the Drosophila optic lobe performed at the third larval instar stage (L3) (Figure 1). This dataset complements other RNAseq by the same lab performed at other time points(Ozel, et al. 2020). From this dataset authors identify a series of transcription factors that are dynamically expressed in neuroblasts (Figure 2). Some of these factors have been previously identified to be temporal transcription factors in the optic lobe (Li et al., 2013), and some had not been previously reported to have dynamic temporal expression. Using loss-of-function experiments, the authors try to examine the regulatory relationships among these factors (Figure 3). Then the authors examine the expression and role of these factors in intermediates (GMCs) and neurons. They document expression patterns and using loss-of-function manipulations examine the regulation of marker gene expression as a stand in for examining neuronal diversity (Figure 4). The authors then continue to mine their dataset and transition to asking a new question about how neurons differentiate. Unexpectedly they show that some transcripts (e.g., for neurotransmitters) are expressed early in development, but not translated into protein until much later. They show a similar phenomenon occurs in Human cortical neurons at least at the mRNA level (Figure 5). This is responsive to A) Summary of key results. B) Originality and significance; C) Data & methodology.

First, I want to commend the authors on an overall well-written manuscript and beautiful figures, and also providing so much RNAseq data, which will likely be a resource the field can mine for years to come. The statistical methods are appropriate. This is responsive to: C) Data & methodology, D) Statistics, and H) Clarity and context.

Overall, it seems like this manuscript presents two separate stories each of which are under developed; and that these stories were stuck together in one large paper. I do not find them

complementary. E) Conclusions, H) Clarity and context.

The first story is about comprehensive description of temporal transcription factors in the medulla. This story is not that novel, temporal transcription factors were discovered 20 years ago in the Drosophila nerve cord (Isshiki and Doe, 2001). And it has been known for nearly a decade that there are temporal transcription factors in Drosophila medulla neuroblasts and that the complete set of temporal transcription factors had not been identified (Li et al., 2013). This is responsive to B) Originality and significance.

In reading the text, it is not particularly well-justified why the authors were looking for additional temporal transcription factors. The findings reported here are interesting, but do little to change the framework of how I think about temporal transcription factors. Instead the text gives the impression that the authors had a lot of RNAseq data and needed to come up with a story. This is responsive to B) Originality and significance. H) Clarity and Context.

The claims the authors make about the factors they identify being temporal transcription factors may be true. But currently, their claims are overstatements and do not meet the standard of evidence in the field. Specifically, they rely on two types of data of 1) gene expression patterns in wild type tissue and 2) loss of function manipulation. While these are a good starting point, the authors do not include gain of function experiments. I find this some what perplexing because in previous reports from this lab, the lab has met this standard (see Li et al., 2013). This is responsive to E) Conclusions and F) Suggested improvements.

The most exciting parts of the temporal transcription factor story were some of the novel and more complex interactions among the temporal transcription factors. For example, the idea that some temporal transcription factors acted as hubs was interesting. Hub has meaning in bioinformatics as a node in a network that gets multiple inputs and or has multiple outputs. The thing that is interesting about the hub concept is that it implies some time of computation is taking place at that node in the network. In my opinion, the authors have an opportunity and the technical means to determine what computations are being performed by the hubs and to relate this to the biology of the system. That line of experimentation would greatly improve the story and likely raise the novelty and impact. Additionally, this comment also applies to their other discoveries of more non-traditional temporal transcription factors. This is responsive to F) Suggested improvements.

The second story the authors present is about the steps neurons take on their path to differentiation. I find this story very interesting, but very underdeveloped. I would recommend removing this story line from the manuscript, and using it as a starting point for a different study that uses experiments to delve into some of their claims. For example, the authors claim, in lines 493-4 “…we show that the differentiation process of Drosophila optic lobe neurons is fixed and independent of neuronal identity.” I think this is an interesting idea. But I do not agree that they show this. They raise the possibility that this could occur, but there is no experimental support, just gene and protein expression. This is responsive to B) Originality and significance, E) Conclusions, F) Suggested improvements.

**Author Rebuttals to Initial Comments:**

Referees' comments:

Referee #1:

The manuscript by Konstantinides et al. aims to identify the suite of temporal transcription factors that generate the diverse neurons in the Drosophila optic lobe. This work builds on an earlier publication by Li et al. (Nature [2013] 498, 456-462) that identified the transcription factors Hth, Ey, Slp, D and Tll as temporal transcription factors (tTFs) in the optic lobe. Here the authors use single cell RNA sequencing to identify new candidate tTFs. They tested the genetic interactions between these candidate factors and the previously identified tTFs, showing that three of the factors appear to be new tTFs, while others modulate the temporal series. They also used scRNAseq to follow the differentiation of one of the neuronal subtypes, showing that cell cycle genes and DNA replication genes are expressed first, followed by genes regulating translation, dendrite development, axon guidance and finally neuronal function. Interestingly, there appeared to be a considerable delay between transcription and translation of neurotransmitter-related genes, although the mechanism for this was not explored. Finally, they showed a similar sequence of expression of neuronal differentiation genes in human cortical neurons, deduced from single nuclear RNA-seq data.

The authors generated nearly 50,000 transcriptomes from cells in the larval brain. These were arrayed in a developmental trajectory based on known markers expressed in the neuroepithelium, neuroblasts, ganglion mother cells, neurons and glia. When the previously identified tTFs (Hth, Ey, Slp, D and Tll) were arrayed on this map, a gap was evident between Hth and Ey, suggesting that there might be one or more new tTFs to be discovered. Seven candidate transcription factors with restricted temporal expression were identified in the scRNAseq dataset (Opa, Erm, Hbn, Scro, BarH1, Oaz, Esg). Of these Esg was excluded as a candidate (it was not expressed in all neuroblasts) as was Oaz (there were no antisera to confirm temporal expression) leaving five potential tTFs.

The authors emphasize that restricted temporal expression of a transcription factor is not sufficient to label it as temporal factor, it also has to progress the temporal series. To do this, temporal transcription factors activate expression of the next tTF in the series and repress expression of the previous tTF. The authors used clonal analysis or RNAi to remove or knock down each candidate tTF (Opa, Erm, Hbn, Scro, BarH1) and then assayed expression of the prior and subsequent tTFs in the series. From their results they proposed the genetic interactions between tTFs shown in Figure 3A.

We thank the reviewer for the very thorough review of our manuscript and for the deep understanding of the message. The kind words about the experimental design and methodology are also appreciated.

According to the authors' definition of tTFs, not all of the factors in Figure 3A are temporal transcription factors. The authors state that Hth is not a tTF; it is not required for progression of the series as Ey is still expressed in Hth mutants. Although the authors place Hth upstream of Erm, there is no evidence that Hth activates Erm expression.

Hth was positioned on the left side of Erm because Erm inhibits Hth. Moreover, Hth is expressed prior to Erm, which justifies its position in the schematic. We explain this in the Figure legend. We tested whether Hth activates Erm expression by overexpressing Hth in a restricted region of the OPC, but we did not see any effect on Erm expression (Extended Data Figure 6B).

Opa does not appear to be a tTF as it is not absolutely required for Ey expression (Figure 3C):

As the reviewer correctly states, Opa is not required for the expression of Ey *per se*, but it is required for the correct timing of Ey expression. Since we are discussing temporal patterning, correct timing is at the core of the mechanism, therefore we think that it is a *bona fide* tTF. Moreover, new data included in the revised manuscript show that Opa regulates the expression of Oaz (Figure 3D). However, in light of the reviewer's comment, we introduced a sentence in the discussion to highlight this distinction in function. The fact that different tTFs have different functions in the series is one of the exciting findings of our study.

In addition, Ey is proposed to repress Opa, but the two genes appear to be co-expressed (Figure 3E; double labeling in color would be helpful here)

We apologize for the confusion, which stems from the fact that Opa is expressed in two waves. Opa's first wave of expression is repressed by Ey (Figure 3G), while its second wave largely overlaps with it (Extended Data Figure 4A). We added an extra figure showing this (Extended Data Figure 4A), and also state this fact in the main text.

Similarly, what is the evidence that Bar-H1 is a temporal factor; does Bar-H1 repress D?

We have now added experiments that show that Bar-H1 is a *bona fide* tTF: It activates Tll (Figure 3R) but does not repress D (Extended Data Figure 5Q), with which it partially overlaps (Figure 2K).

On the evidence presented, only Erm, Hbn and Scro can be called tTFs. In Figure 3A it would be useful to clearly distinguish between the previously published temporal tTFs and the candidate factors identified in this work and to label the factors that actually progress the temporal series, as shown for previous tTFs.

Based on new data, we have revised the Figure. All new tTFs have a role in controlling the progression of the temporal series, except for Oaz. We have also indicated the previously published tTFs in magenta and new ones in green.

Next, the authors investigated whether the tTFs regulate neuronal identity, by first searching in the scRNAseq data for neurons that maintain expression of the tTFs. This section is confusing in places. What is meant by "Moreover, although Erm is expressed in neurons that also inherited Ey from the neuroblast, and D is co-expressed with Hbn in neurons that come from the Hbn temporal window (arrows), this represents a different function of these genes."?

We know that some of the tTFs, such as Erm and D, are expressed in neurons that were not born during the Erm or D neuroblast temporal windows. In other words, neuronal expression of a tTF is not always inherited from the neuroblast but can be activated independently. We rephrased this sentence to make it clearer: *"Erm, D, and Scro were expressed widely in both early- and late-born neurons, hence, they are not only maintained in neurons coming from*

*neuroblasts of their respective temporal windows, but they are also activated independently in different neurons after neuronal birth (Figure 4A').”*

It could also be made clearer which results are from previous papers, for example “the Tll window is known to give rise to glial cells” is not referenced. Similarly, “We evaluated the assignments of the neurons to specific temporal windows by looking at neurons whose temporal window is already known” is not referenced.

We apologize for these omissions. We have added references for both statements and were careful throughout the text to reference previous data.

The authors should discuss how their results overlap or extend those published by Li et al. (Nature [2013] 498, 456-462; summarized in Figure 6) that previously showed the neurons derived from the Hth, Ey, Slp, D and Tll temporal windows and whether Notch was on or off.

We have added a very extensive new section in the Results describing the generation of neuronal diversity (“**Temporal window of origin and birth order of medulla neurons**”). We used multiple different neuronal markers (i.e. concentric genes), some of which were used by Li et al., 2013 and Hasegawa et al., 2011 (Hth, Bsh, Run, Vvl and Toy), while many others were new (Kn, Fd59A, Hbn, Otd, Sox102F, etc) to identify the temporal window of origin and birth order of all neuronal types (clusters) in the medulla. This is presented in the very extensive Table S1, which represents a great resource that will be useful to anyone interested in the specification of medulla neurons.

In Figure 4C, Hbn and BarH1 are both in white; how were they distinguished from each other?

This Figure was meant as an illustration for the inheritance of the temporal factors in newly born neurons. Since Hbn and BarH1 antibodies were both raised in rabbit, they are shown in the same channel and they could only be distinguished based on their position in the tissue. We included Figures in the Supplementary material (Extended Data Figure 4G) to show that Hbn is indeed expressed in neurons born earlier than BarH1 by using an anti-BarH1 antibody from rat that we recently obtained.

In the next section of the manuscript, the progression of neuronal differentiation was followed in one neuronal subtype, Mi1, by merging the larval scRNAseq with the authors' previously published pupal scRNAseq data to generate a neuronal differentiation trajectory. While the timing of expression of these genes is of interest, their order of expression is unsurprising: cell cycle genes and DNA replication genes first, followed by genes regulating translation, dendrite development, axon guidance and neuronal function. All neurons appear to follow the same trajectory. Interestingly, neurotransmitter-related genes were transcribed in larval brains but not translated, although the mechanism of this post transcriptional regulation was not investigated. Similar experiments were performed on human cortical neurons and their differentiation trajectory, based on Gene Ontology, was similar to that in Drosophila with the exception of genes regulating translation. Finally, the authors searched for expression of the Drosophila tTFs in published scRNA seq data from mouse cortical radial glia at E12-E15 and for orthologs of mouse tTFs in the optic lobe but found no conservation other what had previously been shown for Pax6 and Foxg1.

In summary, the authors identified three new, bona fide, temporal transcription factors, Erm, Hbn and Scro in the Drosophila optic lobe and described the genetic interactions between these factors, the known tTFs (Hth, Ey, Slp, D and Tll), and transcription factors that influence the temporal series without progressing it (Opa and Bar-H1).

We hope that the new data convince the reviewer that not only Erm, Hbn and Scro, but also Opa and BarH1, are *bona fide* tTFs.

The authors state, incorrectly, that "several tTFs directly control the progression by activating the next tTF and repressing the previous one…" None of the interactions they show can be called direct without some evidence of direct binding to the regulated gene, for example by ChIP.

We agree with the reviewer that we cannot claim that the regulations are direct. Interestingly, a recent preprint from Andrea Brand's lab (Tang *et al.*, Figure 5J biorxiv doi: https://doi.org/10.1101/2021.06.07.447332,) showed direct binding of Ey on the *hbn* enhancer in the optic lobe. Moreover, based on the speed of the progression of the series (the whole series lasts less than 20 hours), we predict that most interactions must be direct. We are now mentioning these points in the Discussion but we no longer mention direct interactions in the text.

The authors show that expression of most tTFs is maintained in neurons and that Hbn is required for neuronal expression of Traffic-Jam and Otd, while Opa is required for TfAP-2 expression.

A neuronal differentiation trajectory was generated and shown to be similar in human cortical neurons. Drosophila differentiation genes were expressed much earlier in development than expected and at least some of the genes appeared to be regulated post-transcriptionally. The experiments were well designed and presented although the methods used are now fairly routine (scRNAseq and generation of pseudotime trajectories using Monocle (Nat Biotechnol. 2014 Apr;32(4):381-386).

We again want to thank the reviewer for their kind words and constructive comments. We, of course, agree that we take advantage of widely used methods in the field (such as MARCM clones, scRNAseq, and trajectory inference), which, however, continue to be state of the art methods and amazingly powerful to address our questions.

**Referee #2:**

In this study, Konstantinides and colleagues used single cell RNA sequencing data of the developing Drosophila optic lobe to answer fundamental questions about neuronal temporal specification and neuronal differentiation. The presence of many repeated, serially developing lineages permits profiling neural development with exquisite temporal resolution. Analysis of medulla neural stem cells (NB) both using UMAP and cell trajectory building (Monocle) allowed the authors to discover what appears to be a comprehensive series of temporal transcription factors (tTF). With these tTF, the authors were able to predict a series of 12 TF codes that

could be expressed by medulla NBs in consecutive order. Together with spatial patterning cues and binary Notch signaling, this newly discovered discover tTF series could account for the specification of 120 of the 200 discovered medulla neuron types. Further, they used the UMAP data of NBs, intermediate daughter cells and differentiated neurons (with types annotated) to predict the birth order of known medulla neuron types.
In sum, this work has exceeded previous high-profile papers on the use of single cell sequencing technologies in genome-wide molecular genetic studies. It greatly expands our knowledge of neuronal temporal fating mechanisms and lays a comprehensive framework for seeing how the genome encodes each neuron type in the complex nervous system.

We thank reviewer #2 for the very useful comments and very kind words but also for the in-depth evaluation and understanding of our work. These comments were very important for improving the paper further.

General concern:
Given the wealth of new info, it is understandable that some analyses scratched the surface of many things and detailed mechanisms were not thoroughly investigated. However, one could have done more in-depth analysis and presentation of the data to show the respective neuronal outputs for the predicted 12 temporal windows in the NB. The authors predicted birth order of medulla neurons (this was in a supplemental table that was absent from the pdf), but it was not clear how this birth order related to the tTFs and the 12 temporal windows.

We thank the reviewer for this comment that prompted us to do a very detailed analysis of neuronal output of the different temporal windows, which has improved the paper significantly. Moreover, we apologize for a confusing Table S1. We have now added an extra section ("**Temporal window of origin and birth order of medulla neurons**"), a Figure (Figure 5) and three Extended Data Figures (Extended Data Figures 7, 8, and 9) where we describe how neuroblast temporal windows and Notch activity correlate with neuronal TFs (concentric genes) expression. By looking at the relative expression of these neuronal genes in the optic lobes, we were able to identify the birth order and predict the temporal window of origin of the 105 neuronal types in the medulla. The expression of different concentric genes as well as detailed explanations as to how each neuronal type is assigned to a specific temporal window are now presented in Figure 5 and Table S1 and will be a useful and detailed resource for anyone working on medulla neuron specification.

Main comments on the scientific findings
1. Identification of newly identified tTFs (as well as old tTFs)

a. BarH2 antibody staining and genetic interactions were not shown. Is BarH2 protein and regulation identical to BarH1?

In the absence of an antibody specific to BarH2, we used HCR RNA-FISH to probe the expression of BarH2 mRNA. BarH2 is expressed in the same neuroblasts as BarH1, both by FISH and in the scRNASeq dataset. These data are now presented in Extended Data Figure 4F. Given their similarity in gene expression and proximity of their genomic loci, we believe that BarH1 and BarH2 are regulated in an identical manner.

b. Oaz-Gal4 pattern may be discernible with a labile reporter.

We performed HCR RNA-FISH to probe the expression of Oaz, which is expressed in a temporal manner, as was suggested by the single-cell RNA sequencing data (Extended Data Figure 4F). At the same time, we managed to make the Oaz antibody work by modifying our fixation protocol: the immunostaining shows that the protein is indeed temporally expressed, starting from the Opa window and ceasing before the expression of D, in agreement with the scRNAseq data (Figure 2E-F and Extended Data Figure 3A). We also performed genetic interaction experiments with Oaz and found that it is activated by Opa (Figure 3D) and inhibited by Slp (Figure 3O) but does not regulate the expression of the other tTFs (Extended Data Figure 5F-J).

c. According to the RNASeq data in Figure S3A and in the UMAP data of the NBs (Figure 1F), Hth appears to have a 2nd expression window in NBs. Is there a later role for a second wave of Hth?

We thank the reviewer for this careful observation. We had noticed this second expression window. However, this later window of mRNA expression does not correspond to protein expression (based on antibody staining: Extended Data Figure 4A), suggesting that the *hth* mRNA is not translated (Extended Data Figure 4A). Hence, in the absence of protein, this is not relevant to the progression of the series. We have added a sentence explaining this in the manuscript.

2. Is the current list of tTFs comprehensive?

a. Have the gaps truly been filled between Hth and Ey? Can Hth, Opa and Ey antibody stains be done together with a counter stain (Dpn, Ase or even Dapi) to be able to visualize all of the cells?

We performed this experiment: staining against Hth, Opa, Ey and Armadillo (to label all cells), shows that there are no gaps between these tTFs. This experiment is now described in Extended Data Figure 4A.

b. Hbn nested within Ey. Are there Ey-only subwindows both preceding and following Hbn?

There are indeed Ey-positive and Hbn-negative neuroblasts before the Hbn temporal window showing that Hbn is nested within Ey. These data are now shown in Extended Data Figure 4C.

3. Neuronal output…

a. Do the 12 temp windows translate to predicted neuronal types? This is not clear from Table S1 which does not use the same tTF temporal windows.

We apologize for the previous presentation of Table S1, where neuronal clusters were not ordered by temporal window of origin. As explained in response to reviewer #1, the comments

of both reviewers prompted us to do an extremely detailed analysis of neuronal output of the different temporal windows that has improved the paper significantly. We have now added an extra section ("**Temporal window of origin and birth order of medulla neurons**"), a Figure (Figure 5) and three Extended Data Figures (Extended Data Figures 7, 8 and 9) where we describe how neuroblast temporal windows and Notch activity correlate with neuronal concentric gene expression and hence neuronal output. We were able to identify the birth order and predict the temporal window of origin of the 105 neuronal types in the medulla. We labeled in different colors the distinct temporal windows in Figure 5B and Table S1, which should now be a great resource for anyone working on medulla neuron specification.

b. Some windows (particularly in the neuronal data) seem broader than others. Does this make sense? Are the broader windows broken down into smaller windows?

Some tTFs, such as Ey and Scro, are expressed during more than one neuroblast division, making their temporal windows broader. Moreover, some temporal windows can be subdivided by the overlaps between tTFs expression in neuroblasts, *e.g.* Hbn+Ey and Hbn+Opa, Slp1+D, D+BarH1, etc.

c. Is it known approximately how many divisions each NB undergoes?

This is something we are very interested in. Based on our preliminary *ex vivo* observations, we believe NBs undergo ~15-16 divisions, but this is not yet unambiguously demonstrated and, thus, cannot be published.

200 types >>> divide by 2 notch status =100 >>> divide by 5 spatial domains = 20. Could the tTF windows make up 20 different NB TF codes?

The 200 types include all optic lobe neurons, as well as a few central brain neurons that come from neuroblasts that are close to the optic lobe. Our work describes the specification of the ~120 neuronal types (clusters) in the medulla:

120 neuronal types divided by 2 notch statuses = 60 >>> divided by 6 spatial domains (Optix, Vsx and Dpp spatial domains, both dorsal and ventral) = 10 neuroblast TF codes. Given that the last NB temporal window (Tll) generates glia, the 11 tTF windows that we describe agree very well to the predicted number of NBs codes necessary to generate 120 different neuronal types. We have added a sentence about this in the Discussion.

4. Progression of tTF hub (are the hubs really hubs?)

In light of new data that challenge the role of Ey as a hub (please see Figure 3A), and because of a confusion with the term "hub" (see comment from Reviewer 4 regarding the bioinformatic use of the term), we have removed this term from the manuscript.

Please indicate more clearly which interactions have/have not been tested. (For example, the Hth RNAi experiments were not included the results section or main figure). The diagram showing genetic interaction of tTFs would benefit from adding information about which genes were tested but did not show interactions (that could also be in a separate but similar diagram).

We have added this diagram in Extended Data Figure 5A.

Please include the N values of the data presented.

We apologize for this important omission; we have now included N values in Figures 2-6, and Extended Data Figures 3-8.

a. Interaction between Opa and Erm: According to Fig 3C, Opa mutant had normal Erm expression, however antibody staining was not very good, are there additional experiments where erm antibody works better? Does Erm inhibit Opa?

We apologize for the quality of the Figure that intended to show the effect of Opa on Ey. We have added Extended Data Figure 5D showing that, in Opa mutant clones, Erm is not affected. We also performed *erm* mutant clones and we now show that Erm represses Opa (Figure 3E).

b. In Fig3C, Opa mutant clone had altered Ey expression (expressed at edge of clone). Could this result come from mechanisms other than a temporal delay? Have you tested Vsx-Gal4 driven Opa RNAi?

We tried Opa RNAi, but unfortunately, all the lines that we tested were not functional, and did not decrease significantly Opa protein.

c. Ey was previously shown to activate Slp, right? Why is that missing from the diagram in main figure now?

In light of the new data, we know that Ey activates Hbn and Hbn activates Slp, hence Ey (genetically) activates Slp indirectly, which is why we removed the arrow from Ey to Slp. The genetic arrow in the previous paper was correct.

d. Loss of Hbn was not tested for Scro

We apologize for this important omission. We tested this interaction: Hbn activates Scro and this is now shown in Figure 3J.

e. Slp mutant not tested for Opa (2nd wave)

We have also added this experiment: Slp inhibits the second wave of Opa (Figure 3N)

f. unclear whether Opa mutant clone's effect on Slp is via Ey as the figure suggests Based on the expression pattern of Opa, the most parsimonious explanation is that Opa regulates the timing of Ey and, hence, Slp. However, the reviewer is correct and Slp could be regulated independently by the second wave of Opa. This is almost impossible to disentangle, as we would have to inhibit only the second wave of Opa without affecting the first. Given that neuroblasts go through the temporal series as a wave, we cannot use Gal80$^{ts}$ approaches. To avoid any confusion and misinterpretation, we removed this result from the paper.

g. No interaction was tested between Opa and D

We generated *D* mutant clones and showed that D does not inhibit Opa (Extended Data Figure 5L). In *opa* mutant clones, and as described above, we cannot disentangle the effect of the second Opa window from the first.

h. No interaction was tested between Opa and BarH1

We performed RNAi against BarH1: BarH1 does not affect Opa expression (Extended Data Figure 5O).

Minor points on text/data presentation/figures

In all figures with UMAP data, having the absence of gene expression be labeled as a dark color (gray/black) makes it difficult to see the nearby relevant gene expression. Can it be changed to a lighter color?

We agree with the reviewer, but these plots are very difficult to manipulate. In several instances, we have lightened the color that indicates absence of gene expression. However, this also lightens the colored dots, and the figure appears washed out. For this reason, we now present individual UMAP plots, whenever possible (e.g. Figure 4A-A'') to make sure that relevant gene expression is more visible.

Fig 1A: Please explain what the different colors are supposed to represent (are these tTFs?). In neurons, what does filled vs outlined neurons mean for a particular temporal color?

We have explained this in the Figure legend: The different colors represent the different tTFs, while filled *vs*. outlined neurons represents the Notch status of the neuron ($Notch^{ON}$ *vs.* $Notch^{OFF}$).

Fig1B:
• This UMAP clearly includes the later introduced P15 pupal RNAseq data set. Does the shape of the UMAP/interpretation of the data change if only the L3 data is included?

The UMAP plot indeed changes shape when including only the L3 data. However, the interpretation of the data does not change. The progression from NE to NB to GMC is still visible, and, of course, every following step that involves subsetting neuroblasts or neuronal clusters is exactly the same (Figure below for L3 data only).

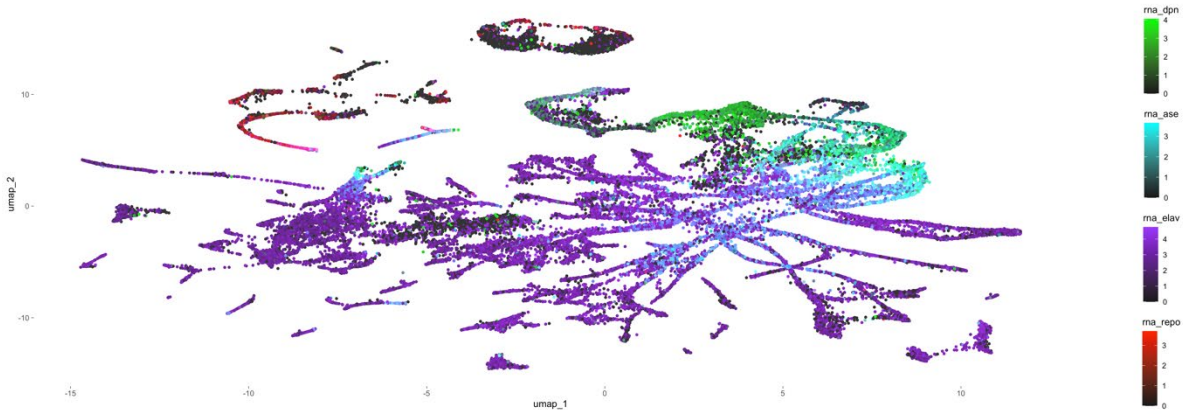


**Figure legend:** This is the equivalent of Figure 1B when including only larval cells. Although the shape changes, the progression from NE to NB (green) to GMCs (cyan) to neurons (purple) is retained. The glial cells cluster outside of the neuronal trajectories (red).

We decided to only use a single UMAP representation for simplicity. We explicitly state this in the Figure legend.

• The way GMCs are labeled, it appears only the right side of the “hourglass” are GMCs while the other half are neurons. Same goes for the labeling of the Neuroblasts, not clear where the cells transition from NE to NBs based on the labeling.

We improved the labeling to show that GMCs are on both sides of the “hourglass”. However, we do not know exactly where the transitions happen. Transcriptionally, the transition from NE to NB and from GMC to neuron is continuous and not an abrupt process (as also illustrated by the UMAP). Therefore, we do not have very good markers that clearly label GMCs vs neurons and NE vs NBs.

• The “hourglasses” could be highlighted in some way.

We now show the hourglasses with red arrows.

• The red dashed box (referring to Fig3F) is confusing, as it is a similar color as Ase—making it seem like all cells within are GMCs

We changed the color of the dashed box to black.

• What does dark grey color represent? Absence of marker genes?

Yes, we now state this in the Figure legend.

Curious cell types:

• I noticed a couple of populations of Ase/Elav double positive cells that are not clustered with the GMCs: larval Mi1 and to the immediate left of the Pm1/2 cells. Are these GMCs that are more “differentiated” towards their neuron types?

In fact, there are GMCs (or, at least, cells with Ase transcripts) at the base of every branch. They are more visible at the base of the Mi1 cells, because Mi1 cells are separate from the other medulla neurons (it is unclear why). The ones that are to the immediate left of the Pm1/2

cells are the central brain GMCs, which are also separate. The same is true for the GMCs of T4/T5 cells that can also be easily separated.

• I'm also intrigued by the three-pronged structure which contains NE, NB and GMC, and glial markers. Are these glioblasts or NBs at a late, glial stage?

This is a great observation. This structure represents a special population of neuroglioblasts that we have shown to give rise to both lamina wide-field neurons and epithelial and marginal glia (Chen *et al.*, Cell Reports 2016).

Fig 1C: This is clearly the same data as 1B (represented with different labels) but that is not clearly stated. In the legend, please state the stage of the pupal data.

Thank you for noticing this. We corrected this.

Figure 1D: Figure legend does not clearly state what is being shown (UMAP of larval and pupal OLs). It is not written like a figure legend, rather like the results section.

We fixed this.

Figure 1E: The colors having dark gray being lack of expression makes it difficult to see and interpret the data. It is very hard to tell the difference between dark magenta, dark blue and dark green.

We apologize for this, but changing the background color of "absence of expression" changes also the background color of the other dots, not alleviating the problem. We agree that the difference between dark gray and dark blue is not very clear, but when observed carefully, it can be deciphered (please see part of the Figure below).

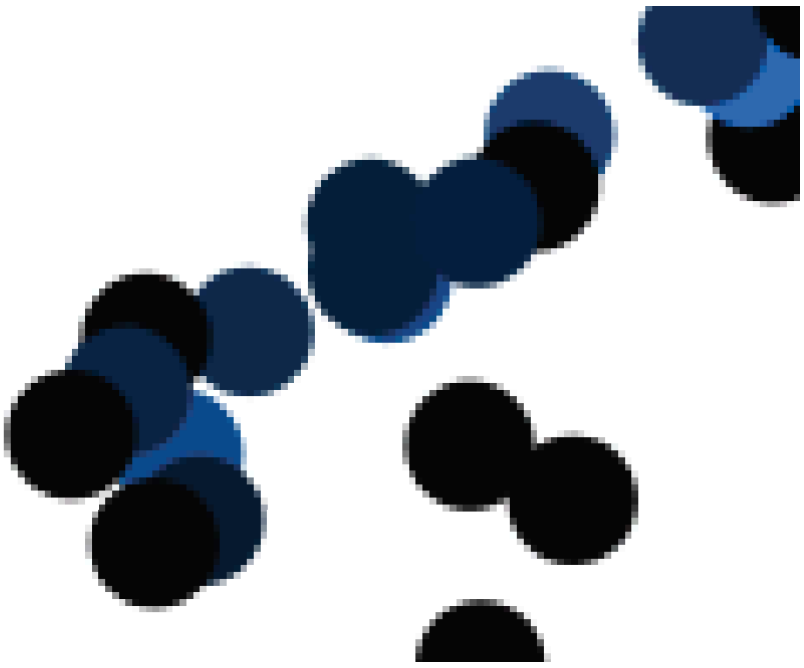

Figure 1F: Which cells are GMCs and which NBs? It is unclear how to interpret the different shades of colors that aren't quite the colors given. What does brighter or darker color mean? There is no "Blank" row of NBs but row of dark gray (please explain what gray means—NBs not expressing known tTFs). It would be helpful to have an arrow demonstrating the temporal direction of the tTFs.

The rightmost cells are NBs and the leftmost are GMCs. This is now indicated in the Figure. Brighter color means more expressed, while darker color means less expressed. Indeed black/gray means "no expression". These are now specified in the Figure legend. We also added an arrow showing the temporal direction of the tTFs.

Figure 2A: All 14 potential tTFs are not on this graph. Please add BarH2 and Slp2. Done.

Figure 2B: It would be helpful to have the same orientation of the OLs in B and B'

Done.

Figure 2C: In the figure legend, please explain what data was used to create this model. Is it based on RNAseq data, immunostaining, or a combination?

This schematic is now shown in Figure 5A and it is based on immunostainings. This is explained in the Figure legend.

Is it known approximately how many division each NB undergoes?

Reviewer #1 asked the same question: The number of NB divisions is something of significant interest to us. Based on our preliminary *ex vivo* observations, we believe NBs undergo ~15-16 divisions, but this is not yet unambiguously demonstrated and thus, cannot be published.

Figure 2D: Do both D and D' show both waves of Opa? It would be helpful to have a counterstain (Dpn, Ase or Dapi) to see the cells that are not positive for either antibody?

We have added a staining of Opa with Arm in Extended Data Figure 4A to show that the two waves of Opa do not overlap. Moreover, we improved the Opa staining in Figure 2D, where the two waves can be clearly seen.

Figure 4 A-B: Please label the neuronal populations per figure 1C.

We modified this Figure to make clearer where each tTF is being expressed. The neuronal populations that arise from each temporal window are shown in Figure 5B.

Figure 5C: what stages are contained in this UMAP?

This is the same UMAP as in Figure 1. We now mention the L3 and P15 stages in the Figure legend.

**Referee #3:**

In this manuscript, Konstantinides et al. reported a comprehensive identification of the temporal transcription factors (tTFs) in neuroblasts that specify most Drosophila medulla neurons in the optic lobe using single-cell mRNA sequencing. Notably, the tTFs remain expressed in differentiating neurons and regulate neuronal identity. The authors also examined the early steps of neuronal differentiation and found that the terminal differentiation genes are present as transcripts, but not proteins, in immature neurons days before being used in functioning neurons. Finally, the authors also suggested that some of the mechanisms are conserved in humans. Overall, this is an interesting study and provides new insights into the temporal control of lineage progression and neuronal diversity.

We thank the reviewer for their kind words and for the helpful review.

Main comments:

1. Based on scRNAseq, the authors identified eight new candidate TFs such as Opa, Erm, Esg, Hbn, as temporal TFs based on the previously known tTF framework. Given the limitation of 10x genomics in sequencing depth, the dataset that authors used shows a median of 1343 genes per cell (200 genes cutoff). Should a dataset with more gene depth be used, would the authors expect to identify more tTFs?

This is indeed a valid concern for all scRNA-Seq studies. While we cannot exclude the presence of lowly expressed tTFs, we believe that this is not the case for several reasons:

- First, we did identify TFs that appeared to be temporally expressed at low levels (Extended Data Figure 3). However, when we assayed the expression of several of them with antibodies or reporters, they were not validated as tTFs. We have added Extended Data Figures 3C-D to illustrate this. This means that we were able to capture “noisy” expression; any meaningful expression should be higher than this and should have been detected.

- Second, we managed to identify all previously known tTFs that had been identified with very sensitive antibody stainings.

- Third, while the previous temporal series had some clear gaps, with NBs that did not express any known tTFs (*e.g.* between Hth and Ey and between D and Tll), this temporal series covers all neuroblasts (Figure 2, Figure 5A, Extended Data Figure 4A and E) and can explain the progression of the series through the described genetic interactions (Figure 3A).

-Fourth, these 10 temporal windows (since the Tll temporal window only generates glia) can explain the generation of the entire neuronal diversity generated in the medulla (105 clusters) together with spatial patterning (6 spatial domains) and binary Notch decision. 10x6x2=120 (see also response to reviewer #1)

For the above reasons, we believe that the temporal series is complete. We now mention this in the Discussion section.

2. The authors took advantage of the complex genetic interaction data to build the tTF network that controls the temporal progression of neuroblast lineages. While the genetic interaction data is powerful and informative, it does not provide any direct knowledge on gene regulation (e.g., transcriptional activation or suppression). Could the authors obtain any evidence on direct gene regulation between the tTFs, especially those newly identified ones?

This is indeed an important point. As mentioned earlier in response to a comment from Reviewer 1, a recent preprint from Andrea Brand's lab (Tang *et al.*, biorxiv doi: https://doi.org/10.1101/2021.06.07.447332, Figure 5J) showed direct binding of Ey to the Hbn enhancer in the optic lobe. Moreover, based on the speed of the progression of the series (the whole series lasts less than 20 hours), we predict that most interactions are direct. However, obtaining such evidence would require very extensive experiments using Cut&Run or nanoDam that goes beyond the scope of this paper. This is something that we will pursue in the future. We mention this in the Discussion.

3. Given that the tTFs often remain expressed in neurons (in addition to neuroblasts) and regulate neuronal identity, how would the authors know that the phenotype of mutants is due to their functions in neuroblasts or neurons?

This is a very interesting question, in which the lab is actively working. However, in the current work, we were interested in the function of tTFs in neuroblasts that are responsible for the progression of the series and the specification of neurons born during each temporal window. tTFs relay temporal identity to neurons by being inherited in newly born neurons and by regulating downstream effector TFs (such as concentric genes). When we generate MARCM mutant clones in a given tTF in NBs, we are eliminating that tTF both in NBs and in neurons (since they are inherited). Hence, we used concentric genes as a readout of neuronal tTFs effectors that we could analyze. Experimentally, we could disentangle the function of a given tTF in neuronal specification vs. neuronal differentiation by driving a given tTF RNAi specifically in neurons using Elav-Gal4, although this might not be expressed early enough to achieve our goal, as in many cases (such as Opa or Slp1) the protein endures even in the absence of transcript. However, for the present work, we were mainly interested in the role of tTFs in NBs and how their absence affected neuronal specification (the presence or absence of specific neuronal progeny).

How would tTF network act in postmitotic neurons?

This is also a very interesting question as not all tTFs remain expressed in mature postmitotic neurons. However, the "temporal identity" is locked in because tTFs are inherited in newly born neurons (see Figure 4B) and activate relay TFs. We believe that the temporal series does not progress in neurons because the differentiation program somehow overrides the progression of the series, which might also require cell division.

4. In the human scRNA-Seq dataset from GW19 cortical plate, the authors reported a trajectory from Pax6+ radial glia progenitors (RGPs) to Eomes+ intermediate progenitors (IPs) and Neurod2+ postmitotic neurons. Did the authors use the entire cortex, as the cortical plate often refers to the mantle region without the proliferative zone?

We indeed collected the cortical plate and did not include the germinal matrix (proliferation zone). However, at GW19, they are very close together and, as the germinal matrix is very expansile, some of the outer subventricular zone was likely included. We have added plots (Extended Data Figure 10D) showing that our trajectory includes both outer (HOPX-positive) and ventricular (FBXO32-positive) radial glia.

5. The authors checked the expression of Drosophila tTFs in mouse cortical radial glia and found no obvious conservation of a common temporal series of TF between flies and mice. Since the previous studies have suggested some Drosophila tTFs orthologs in the developing mouse retina, could the authors compare fly optic lobe and mouse/primate retina (instead of the cortex)?

It is indeed notable that several of the fly VNC tTFs are expressed in the developing mouse retina. The reviewer was absolutely right that it was important to perform this analysis that is now presented in Extended Data Figure 11C-D. In it, we identify the VNC tTFs, Pou2f1/Pou2f2 (Pdm) and Casz1 (Castor) as being temporally expressed, as was previously shown (Mattar *et al.*, Neuron 2015; Javed *et al.*, Development 2020). Ikzf1 (Hb) was not identified to be expressed temporally, but this is probably due to low levels as previously described (Shiau F *et al.*, Dev Biol 2021). However, optic lobes tTFs are not temporally expressed in the retina. The only optic lobe tTFs that are expressed are the orthologs of Hth (Meis2), Opa (Zic5), and D (Sox12), which are restricted to embryonic stage 12, while the ortholog of Ey (Pax6) is expressed constitutively. Interestingly, Nr2e1 is mainly expressed when retinal progenitor cells that generate Müller glia, similar to its fly optic lobe ortholog, Tll, that generates glial cells. These observations are described in the manuscript and Extended Data Figure 11C-D.

Minor comments:

1. In the abstract “Moreover, we show that tTFs not only….” This sentence is incomplete.
We apologize for that. We fixed it.

2. Fig 1B: Could the authors clearly label the lineage trajectories for lobula plate neurons and lobula neurons?

We added arrows on the figure to show lobula plate neurons emerging from the IPC. Most lobula neurons are generated by central brain NBs and are not indicated here.

Fig 1E: It appears that Vsx1+ cells are present in two separate clusters. How does this relate to the spatial or temporal regulation of the lineages?

The two separate clusters of Vsx1 cells likely correspond to the ventral and dorsal sides of the neuroepithelium that are marked by Hedgehog, which is expressed at earlier larval stages in the ventral neuroepithelium but is not maintained at L3. However, *hh* is not expressed at this stage, therefore we don’t include this speculation in the paper.

3. Fig S3 (line 1328), the known tTFs should be in purple and the new tTFs should be in green as shown in the Figure S3A.

Fixed.

4. There are numerous typos/errors in the Methods (e.g., 25C). The authors should carefully review and revise.

Fixed.

**Referee #4:**

The starting point of this study is single cell sequencing of the Drosophila optic lobe performed at the third larval instar stage (L3) (Figure 1). This dataset complements other RNAseq by the same lab performed at other time points(Ozel, et al. 2020). From this dataset authors identify a series of transcription factors that are dynamically expressed in neuroblasts (Figure 2). Some of these factors have been previously identified to be temporal transcription factors in the optic lobe (Li et al., 2013), and some had not been previously reported to have dynamic temporal expression. Using loss-of-function experiments, the authors try to examine the regulatory relationships among these factors (Figure 3). Then the authors examine the expression and role of these factors in intermediates (GMCs) and neurons. They document expression patterns and using loss-of-function manipulations examine the regulation of marker gene expression as a stand in for examining neuronal diversity (Figure 4). The authors then continue to mine their dataset and transition to asking a new question about how neurons differentiate. Unexpectedly they show that some transcripts (e.g., for neurotransmitters) are expressed early in development, but not translated into protein until much later. They show a similar phenomenon occurs in Human cortical neurons at least at the mRNA level (Figure 5). This is responsive to A) Summary of key results. B) Originality and significance; C) Data & methodology.

First, I want to commend the authors on an overall well-written manuscript and beautiful figures, and also providing so much RNAseq data, which will likely be a resource the field can mine for years to come. The statistical methods are appropriate. This is responsive to: C) Data & methodology, D) Statistics, and H) Clarity and context.

We thank the reviewer for these kind comments.

Overall, it seems like this manuscript presents two separate stories each of which are under developed; and that these stories were stuck together in one large paper. I do not find them complementary. E) Conclusions, H) Clarity and context.

Accumulating evidence in the field (eg. Telley et al, 2019, Jain et al, biorxiv 2021 https://doi.org/10.1101/2020.09.18.304410 and others) support the idea that neuronal development is separated into two (not always distinct) stages, a) Specification, that is driven by the spatiotemporal identity of the progenitors and largely instructs neuronal identity and b) Differentiation that represents the implementations of neuronal identity. Both stages are

essential for a neuron to acquire its final identity. We therefore find it is important to study both contexts together in order to understand how neuronal diversity is generated.

However, we agree with the reviewer that the presentation of the results was not optimal, which might have led to the interpretation of “sticking” two stories together. For this reason, we have added a new large section on neuronal specification (“Temporal window of origin and birth order of medulla neurons”), a Figure (Figure 5), three Extended Data Figures (Extended Data Figures 7, 8 and 9), and significantly updated Table S1 to show how neurons interpret their temporal identity by expressing “concentric genes” in order to realize their identity. With the addition of these data, we hope that the reviewer will appreciate the importance of connecting these two (specification and differentiation) developmental stages.

The first story is about comprehensive description of temporal transcription factors in the medulla. This story is not that novel, temporal transcription factors were discovered 20 years ago in the Drosophila nerve cord (Isshiki and Doe, 2001). And it has been known for nearly a decade that there are temporal transcription factors in Drosophila medulla neuroblasts and that the complete set of temporal transcription factors had not been identified (Li et al., 2013). This is responsive to B) Originality and significance.

By no means did we imply that temporal transcription factors are a novel idea. We tried to be very careful citing the earliest papers that described temporal patterning in flies starting with Brody and Odenwald (2000) and the beautiful work of Chris Doe’s lab. However, we believe that our manuscript is novel in several respects:

- While the idea of temporal patterning has been known for 20 years, its complexity, as well as the fine mechanisms for the progression of the series have not been fully investigated. We think that this is of the utmost importance to understand the generation of neuronal diversity and for future studies that address its development and evolution.

- While a temporal series of TFs is partly known in several neuronal structures, we do not know how this leads to the generation of neuronal diversity and, in particular, the neuronal types that arise from each temporal window. While there has been very significant work in studying the neuronal output of temporal windows in the VNC, we believe that this is the first time this has been achieved for any neuronal structure to this extent.

- Moreover, we compare temporally expressed factors in flies and vertebrates, providing insights into the evolution of temporal patterning in both systems.

- Finally, in this study, we present an experimental and analytical roadmap for future studies to use single-cell sequencing to study temporal patterning and its link to neuronal diversity in different species.

In reading the text, it is not particularly well-justified why the authors were looking for additional temporal transcription factors. The findings reported here are interesting, but do little to change the framework of how I think about temporal transcription factors. Instead the text gives the impression that the authors had a lot of RNAseq data and needed to come up with a story. This is responsive to B) Originality and significance. H) Clarity and Context.

We respectfully disagree with the reviewer. Our lab has worked extensively over the last 10 years to identify the temporal series of tTFs in the optic lobes and its effects on neuronal diversity. Having the complete temporal series was always of great importance for our lab and for the field. We knew since 2013 that we had not identified all tTFs since rows of neuroblasts did not express any of the known tTFs; it was also unclear how different tTFs were activated or repressed. Finally, it was clear that five tTFs were not sufficient to explain the generation of the entire neuronal diversity. To identify the missing tTFs, we initially generated a large number of antibodies against different transcription factors, but unfortunately, we did not identify additional tTFs. Therefore, when single-cell mRNA sequencing became available, it gave us the amazing possibility to identify the complete series with higher resolution. For this reason, our initial goal in using scRNAseq was exactly to identify these tTFs as this was the question we absolutely wanted to address.

Finally, while our 2013 paper described a linear progression of the series, we have now uncovered a higher complexity underlying this progression. Therefore, these data have changed how we think of temporal transcription factors and uncover their different roles in different aspects of the temporal cascade.

Therefore, knowing these tTFs in NBs allowed us to link temporal patterning with specific neuronal outputs and these data open new avenues of research to understand with more detail specification mechanisms (both temporal and spatial) of specific neuronal types.

The claims the authors make about the factors they identify being temporal transcription factors may be true. But currently, their claims are overstatements and do not meet the standard of evidence in the field. Specifically, they rely on two types of data of 1) gene expression patterns in wild type tissue and 2) loss of function manipulation. While these are a good starting point, the authors do not include gain of function experiments. I find this some what perplexing because in previous reports from this lab, the lab has met this standard (see Li et al., 2013). This is responsive to E) Conclusions and F) Suggested improvements.

We favored loss of function experiments that are usually considered as much stronger tests of interactions. However, following the reviewer's advice, we have now performed gain-of-function experiments for most tTFs that are described in Extended Data Figure 6.

Hth misexpression in NBs didn't affect Erm or Opa expression (Extended Data Figure 6A-B), in agreement with previous experiments where Erm and Opa expression were not affected upon Hth knock-down (Extended Data Figure 5B-C, respectively). As shown in Extended Data Figure 6D, Ey misexpression activated Hbn, in agreement with previous experiments (see Figure 3H). However, Scro expression was not present in the NBs where Ey was misexpressed. Furthermore, misexpression of Hbn in NBs did not activate the next tTF, Slp, earlier than its temporal window, probably due to the low level of Hbn protein produced that we observe (we have reasons to believe that high levels of Hbn are detrimental to development) (Extended Data Figure 6E arrowhead). The same was the case when we misexpressed Scro, as D was not activated earlier (Extended Data Figure 6F, arrowhead). When D was misexpressed early, Scro expression was repressed, uncovering a new interaction between these tTFs (Extended Data Figure 6G). Moreover, D misexpression early repressed Bar-H1, which is not consistent with previous results where B-H1 expression was lost in D mutant clones (see Figure 3Q). Since misexpression experiments often do not

recapitulate native protein levels and they are not expressed at the proper time, the interactions uncovered using mutant clones in NBs are likely to be correct. Finally, Tll misexpression suppressed the expression of D, in agreement with previous data indicating that Tll was sufficient but not necessary to inhibit D (Li et al., 2013). Moreover, Tll misexpression inhibited B-H1 expression, although this effect could be indirect, through D inhibition (Extended Data Figure 6H). In summary, most (but not all) of the misexpression results support loss-of-function.

The most exciting parts of the temporal transcription factor story were some of the novel and more complex interactions among the temporal transcription factors. For example, the idea that some temporal transcription factors acted as hubs was interesting. Hub has meaning in bioinformatics as a node in a network that gets multiple inputs and or has multiple outputs. The thing that is interesting about the hub concept is that it implies some time of computation is taking place at that node in the network. In my opinion, the authors have an opportunity and the technical means to determine what computations are being performed by the hubs and to relate this to the biology of the system. That line of experimentation would greatly improve the story and likely raise the novelty and impact. Additionally, this comment also applies to their other discoveries of more non-traditional temporal transcription factors. This is responsive to F) Suggested improvements.

We agree with the reviewer that the non-traditional roles played by some of the tTFs are very exciting, and we are planning to study them further. However, as mentioned earlier, this greatly exceeds the scope of this paper that already presents a huge amount of data. Moreover, in response to a comment from reviewer #2, and in light of new data that challenge the role of Ey as a hub (please see Figure 3A), we have removed the term hub from the manuscript.

The second story the authors present is about the steps neurons take on their path to differentiation. I find this story very interesting, but very underdeveloped. I would recommend removing this story line from the manuscript, and using it as a starting point for a different study that uses experiments to delve into some of their claims. For example, the authors claim, in lines 493-4 "…we show that the differentiation process of Drosophila optic lobe neurons is fixed and independent of neuronal identity." I think this is an interesting idea. But I do not agree that they show this. They raise the possibility that this could occur, but there is no experimental support, just gene and protein expression. This is responsive to B) Originality and significance, E) Conclusions, F) Suggested improvements.

As stated earlier, there are accumulating data that support the idea that specification and differentiation are two important components of neuronal identity (eg. Telley *et al*, 2019). The fact that all neurons, independent of neurotransmitter identity, express neurotransmitter-related genes at the same stage of differentiation is a very interesting observation that we think is very important to publish. This is also true for synaptic genes, ion channels etc. Hence, we strongly believe that we have shown unambiguously, in multiple neuron types, that "the differentiation process is fixed and independent of neuronal specification". However, we have toned it down to address the reviewer's concerns.

Interestingly, a recent preprint from the Homem lab has shown that this early transcription of neurotransmitter-related genes is also true in the central brain (Marques *et al.*, biorxiv doi:

https://doi.org/10.1101/2021.06.22.449317), validating our findings. We have added a sentence in the Discussion mentioning this paper.

**Reviewer Reports on the First Revision:**

Referees' comments:

Referee #2 (Remarks to the Author):

Konstantinides et al., have done a very thorough job revising the manuscript and addressing reviewers' concerns. The new section "Temporal window of origin and birth order of medulla neurons" greatly improves the paper and elevates its impact to the community.

Minor comments on where the text could be improved:

Lines 46-50: please simplify sentence, it is very long with many parts

Lines 116-117: "where each [neuroblast] expresses at least one of the tTFs"
Should this mean when each of the tTF stages is represented within the neurogenic region? As written, is suggests only that each NB expresses one (or more) tTF. It does not suggest all tTF stages are represented.

Lines 187-188: I suggest removing "and tTFs in the neuroblasts," as the tTFs are not discussed in this paragraph.

Line 202: "based on their age" should be based on neuroblast age.

Lines 217-219: "This might be observed because genes involved in the epithelium-to-neuroblast transition in the first case, or in the terminal asymmetric division of GMCs in the second case, are obscuring more specific identity features."
This sentence is unclear as written. Does this imply that the genes expressed in EtoN or GtoN transition make sequencing the temporal genes more difficult (technical issue), or the transitions decrease the abundance of temporal genes?

Line 225: I suggest changing "any tTFs" to "any known tTFs".

Line 249: Please clarify that "They" refers to the candidate tTFs, not all tTFs.

Line 259-261: "Escargot (Esg) was expressed within the Ey temporal window, albeit in a salt-and-pepper manner, indicating that it is likely not a bona fide tTF as it was not expressed in all neuroblasts."
After this statement, you may want to say that you will not explore Esg further.

Line 267: "BarH2 transcript was expressed in the same neuroblasts as BarH1"
Here, you may want to clarify that while you couldn't examine BarH2 (lack of antibody), its transcript was expressed in same NBs as BarH1, suggesting a shared expression pattern.

Line 269-271: "Therefore, we could confirm that the predicted medulla tTF proteins (and BarH2

mRNA) are expressed temporally in the developing optic lobe, defining new temporal windows as the neuroblasts progress through divisions."
I suggest removing BarH2 mRNA from this sentence. I also recommend concluding which of the candidate tTFs (i.e. not Esg) you are proceeding with as presumptive tTFs for genetic interaction testing.

Lines 286-305: This single paragraph may be easier to read if broken into 2.
This section lack discussion about Oaz. While new genetic interaction studies were done (Extended Data Figure 5F-J), they are not mentioned in this section.

Line 298: "Opa activates the expression of Oaz" is a conclusion rather than a description of the data.

Line 307: For clarity, please change "We had shown" to "We had previously shown"

Line 317: "Slp then inhibits Ey28, Hbn, Opa, and Oaz"
1) "then" is confusing as you were just talking about Hbn inhibiting Erm.
2) Slp inhibiting Ey is not show in Figure 3—does it do so via Hbn?

Line 327: "At the same time" is confusing as you are talking about temporal transitions—the same time as what?

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Referee #3 (Remarks to the Author):

The authors have addressed my previous concerns. I support the publication.

Referee #4 (Remarks to the Author):

Reviewer 4 here.

Thank you for your thoughtful revision.

I think that you have addressed my many of my major concerns.

1. The manuscript seemed dis-jointed.
Addition of the "concentric" neuron expression data and correlating it with the TTF windows provides a nice bridge now.

2. The TTF experiments lacked Gain-of-function validation.
Thank you for including this.

3. Superficial treatment of the complex TTF interactions.
I can see that this is beyond the scope of the work, and with the addition of the neuronal expression data there is no room for this in the current version of the story. I am looking forward to learning about these more complex interactions in the future.

4. Adding the human data did nothing to enhance the story in my opinion.
This is still my opinion. But, it is fine to leave this in.