Dr. John Rubenstein  
1550 4th Street  
San Francisco 94158  
UNITED STATES  
  
  
Aug 27, 2019  
  
RE: CELL-REPORTS-D-19-02779  
"LiCl Treatment Rescues Cortical Neuronal Spine Maturation and Synaptogenesis in Tbr1 Mutants"  
  
Dear Dr. Rubenstein,  
  
Thank you for submission of your paper to Cell Reports. I have appended the comments that reviewers made on your paper, which I hope you will find useful and constructive. As you will see, there is a split in opinion between the two reviewers. They both agree the area and the question are interesting, but Reviewer 1 has concerns about the advance over the related Neuron paper from 2018, and also feels that stronger data are required to support the conclusions. After discussion with the editorial team, we remain interested in the paper, but agreed that Reviewer 1's substantial critiques would need a strong response for the paper to go forward here.  
  
It is difficult to say whether it would be appropriate for us to consider a revised paper without knowing whether you can address the further experiments that the reviewers require. If you are interested in pursuing further consideration of this work at Cell Reports, you would need to first submit a point-by-point response that indicates how you intend to address the reviewer concerns and outlines how any corresponding results and/or edits would be incorporated into a revised manuscript. Once I receive this document I would assess the plan for revision, perhaps in consultation with the reviewers. Based on these considerations, we would either reactivate the manuscript file to enable resubmission or recommend submission elsewhere. Please note that we take into account the published literature up until the date of our final editorial decision.  
  
Yours sincerely,  
  
Natalie Cain, Ph.D.  
Associate Editor, Cell Reports  
  
**Reviewers' comments:**  
  
Reviewer #1: This study by Fazel Darbanti et al. addresses postnatal Tbr1 molecular regulations and synaptic function in L5 mPFC neurons. In addition, the authors use LiCl in an attempt to rescue the phenotypes observed upon deletion of Tbr1 in L5 neurons.  
This study is closely related to Fazel Darbanti et al., Neuron 2018, which focuses on Tbr1 function in L6 neurons. The experimental layout and figures essentially overlap between the two studies, which somewhat reduces the novelty of the current manuscript.  
I have several major concerns on this manuscript, since in critical sections, data quality and display fail to support the authors very bold claims. Only very few illustrations of the original data are provided (e.g. photomicrographs) and, when present, they are of low quality. How analyses were conducted is insufficiently reported, including with regard to number of experiments/ animals/ neurons/ synapses analyzed.  
The authors' claim that LiCl i.p. injection at P60 rescues synapse density and axon arborization within 24h seems dramatically overblown and is hard to understand: how could LiCl induce the expression of embryonic/early postnatal molecular programs and allows the growth of axon in mature brains within 24h. This observation is only sparsely documented (and yet highlighted in the title and extensively in the discussion) and should be much further experimentally addressed to become convincing.  
  
Point-by-point comments:  
  
1. Tbr1 regulates neurogenesis and synaptogenesis in layer 5 pyramidal neurons of neonatal medial prefrontal cortex. Regarding the "clear separation" between Tbr1WT and Tbr1L5: could this be due to batch effect, since 1 library was performed by condition (no replicates)? How were unhealthy cells defined? A supplementary figure with quality controls on single cells would help, with raw data and thresholds chosen for filtering. Thirty percent of mitochondrial gene content seems high for this young age. Feature plots showing expression of classical L5 markers should be presented.

We completely agree with the reviewer that batch effects can be an issue with single cell RNA-seq data. However, to avoid this major issue, our single cell RNA-seq experiments for all three genotypes was performed simultaneously and run on the same 10X chip to mitigate any batch effect. TSNE displays cell-types from different library preparations clustering together, showing cell-type is a stronger predictor than library preparation. The separation between Tbr1WT and Tbr1L5 is only seen in the neuronal population. Further, this replicates across other single-cell datasets. The PCA shown here incudes data from a pilot experiment not included in the manuscript due to lower coverage and lack of Tbr1 L5 het. Again, cell-types from different library preparations and different chips cluster together.

We will happily add this additional supplementary figure to show quality controls on single cells, with raw data and thresholds chosen for filtering.

Low quality cells were defined by more than 30% mitochondrial genes or less than 3000 reads per cell. Both thresholds are based on the distribution for all cells illustrated above. The thirty percent mitochondrial gene content was chosen to be lenient given the increasing literature supporting mitochondrial dysfunction in ASD. We further validated that percent mitochondrial gene content accounts for less than 1% of the total variance.

Atypical neuronal cells were defined by having low expression levels of Neurod6 and Nrgn (two excitatory neuronal markers) and high levels of housekeeping genes. We actually think the cells could be \_\_\_\_ but do not currently have the evidence to conclusively support that idea, so we choose to remove them.

After removing low quality and atypical cells, we used more than 7,000 neuronal cells for differential expression analysis. DID WE EVER SAY UNHEALTHY IN MANUSCRIPT?

Additionally, we will happily show feature plots for the expression of classical L5 markers in our data sets.

DEX: Different color codes should be used for z-scores and ontologies. Cell body ontology is not meaningful in the context of this study. Top genes should be highlighted with either feature plots using the tSNE or violin expression plots to facilitate interpretation.

We used the same coloring scheme between the heatmap and GO-terms to make it easier for the reader to follow what proportion of the dysregulated genes were associated with those GO terms. We can add text to better explain the color codes are representing the same information or we will change the coloring scheme. We include “cell body” ontology because it was one of the top ontology terms using the uniqueness score in REVIGO and we do not know if cell body isn’t meaningful in this context. We can re-evaluate and clarify how we select ontology terms. We will add violin expression plots below for the top genes in our scRNA-seq data.

"ISH to validate the expression of several DEX genes": How were the genes chosen (top DEX genes, interesting function?)? Expression plots between Tbr1WT and Tbr1L5 for the genes presented in Fig2 should be presented.

The candidate genes for ISH experiments were in the top DEX genes Calm2 (log2FC=1.65), Mgst3 (log2FC=0.97), and Kif1a (log2FC=-2.20). [and of interest based on \_\_\_]. Wnt7b ISH was added to confirm that WNT-signaling is impaired in layer 5 as well due to Tbr1 LoF. Our scRNA-seq data showed that Wnt7b levels are reduced in Tbr1L5 null, however, it doesn’t reach statistical significance. We will add violin expression plots for these genes to further clarity.