Divergent evolution of developmental timing in the neocortex revealed by marsupial and eutherian transcriptomes

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MS TITLE: Divergent evolution of developmental timing in the neocortex revealed by marsupial and eutherian transcriptomes

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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

Reviewer 1

Advance summary and potential significance to field

This pioneer study by Kozulin and colleagues presents the first transcriptomic analysis of the developing cortex of the fat-tailed dunnart, a mouse-sized marsupial with interest in evolutionary neurobiology. This is a real tour de force given the very limited existing genomic resources on this

species. Most importantly, they cleverly exploit this new dataset by comparing it with mouse transcriptomes at equivalent developmental stages, reaching the fascinating conclusion that cortical development progress is heterochronous between these two species, which future work may find is extensive to eutherians versus metatherians at large. By performing a variety of comparative analyses between their bulk transcriptome of timed developing dunnart cortex versus mouse, they find that the transcriptome changes very little in dunnart as compared to mouse along a comparable developmental period, in reference to the progression of cortical neurogenesis. Their convincing analyses and interpretations lead to the solid conclusion that this difference involves a precocious maturation of the dunnart cortical transcriptome as compared to mouse, but then a delayed or much protracted further maturation. These results not only further support the emerging concept that cortical evolution essentially involved changes in the gene regulatory landscape, rather than the emergence of novel, species-specific genes, but also that temporal regulation of gene expression may have been part of this evolution.

I find this study fascinating and seminal, both at the technological level (as a goldmine resource) and conceptual level. There are only a handful of issues that should be addressed before this manuscript is ready for publication, which otherwise should be fast-tracked.

Comments for the author

There is no clear information on whether the bulk mouse transcriptomes at early and late cortical development were experimentally obtained by the authors, or from public datasets. If the former, this is not described in Methods nor there are accession numbers to the datasets. If the latter, there is no clear information on the source of the dataset/s used.
In page 7, lines174-177: “In contrast, genes that were up-regulated during ECD (relatively down-regulated during LCD) did not show a notable change in GO enrichment between developmental periods in either species with respect to the aforementioned developmental processes.” - This is strange, as one might expect a reciprocal change. How is this interpreted?
Page 8, lines 200-206: “Interestingly, the transcripts with a higher expression ranking in dunnarts relative to mice during ECD (Table S7A) were enriched for GO terms related to advanced stages of neural maturation, such as neuron differentiation, neuronal projection development, ion transport and synaptic signalling. In contrast, transcripts with a higher expression ranking in mice relative to dunnarts during ECD (Table S7B) were enriched for GO terms that negatively regulate processes related to mitosis and cell cycle (Fig. 3B; Table S6B).” - This seems contradictory, as positive regulation of neurogenesis and neuron differentiation should go along with negative regulation of cell cycle not the opposite... In fact, how is it that in ECD higher in mouse they find greater negative reg of forebrain neuron differentiation, but positive reg of cell differentiation? In early cortical development in mouse, neuroepithelial cells and early radial glia cells undergo fast self-amplification, so regulation of mitotic cell cycle should be importantly represented.

Reviewer 2

Advance summary and potential significance to field

The authors present an interesting comparative study between the mouse and the marsupial S. Carssicaudata (Dunnart). This paper is a novel characterization that provides information on patterns of neocortical development and gene expression that could be relevant to models of neocortical evolution.

Comments for the author

The authors present an interesting comparative study between the mouse and the marsupial S. Carssicaudata (Dunnart). The authors find a number of genes have an interesting heterochrony in expression, and suggest a general pattern of precocious and extended development in the Dunnart. The figures are of high quality and the display of results and their analysis well-executed. This paper while purely a characterization with no functional test, would be of interest to those interested in developmental patterns of neocortex in different species.
I have one crtique and suggestion that could be easily added to significantly improve the manuscript and usefulness to readers. Specifically, all micrographs provided are relatively high

magnification. Because Necortex can have significant regional differences in gene expression and laminar dimensions, it would be important to add some lower magnification images of coronal sections, so the reader can appreciate differences or similarities in regional patterns of markers in the two species.

Reviewer 3

Advance summary and potential significance to field

In this study the authors compared neocortical transcriptome of marsupial fat-tailed dunnart and those of eutherian mice, at equivalent developmental stages. The authors identified unique features of neocortical transcriptome in marsupials and concluded that neocortical developmental programs of marsupials exhibit precocious and protracted compared with those of eutherians. This is the first comprehensive transcriptome analysis of developmental marsupial neocortex that provided several valuable information relevant to development and evolution of the mammalian neocortex. Experimental design and results are convincing; however, there are several ambiguous points in their conclusions that need to be substantially revised.

Comments for the author

1. The authors identified equivalent developmental stages (ECD and LCD) of dunnart and mice based on pulse-chase birthdate analysis of deep and superficial layer neurons. This approach is unique and appropriate, but still significant information is absent, which are 1) when neocortical neurogenesis starts, 2) how long ECD and LCD are lasting (i.e., the duration of L5/6 and L2/3 neuron production), and 3) when neurogenesis finishes in the developing dunnart neocortex. These lines of evidence are critical to evaluate heterochronic changes in neocortical programs in dunnart occurs at specific timing or not.

2. The conclusion of “protracted” neuronal maturation program is unclear. In figure4, the authors demonstrated the dominance of progenitor type gene expression (ranking) in dunnart at ECD, while neuron-type gene expression is conversely dominant in mice at LCD, but it is ambiguous what kind of programs are “protracted” in later stages of dunnart neocortex. The authors focused on NeuroD1 and Tbr1, and these markers are relevant to label differentiated neurons and layer-specific neurons, but not appropriate to evaluate specific developmental timing or stage.

3. Similarly, the thickness of Sox2 or NeuroD1-positive areas in the neocortex (Figure4) demonstrates relative ratios of progenitors and neurons at given time points but does not provide evidence for precocious or protraction of neurogenesis. Since ECD and LCD were determined as “equivalent stages” between two species, the terms “precocious” or “protraction” are contradictory to the criteria. The data taken from two time points are snapshots, thus needs to show the histological data of other developmental stages in order to argue the dynamics of progenitor and neuronal pools and species differences.

4. It is necessary to validate the antibodies used in this study recognize appropriate antigens in each species by western blotting. In addition, significant differences of expression patterns of Lhx2 must be confirmed by in situ hybridization.

5. Details of statistics and p-values must be described in Method and figure legends.

First revision

Author response to reviewers' comments

Thank you to the reviewers for their excellent suggestions to this manuscript. We found the reviews extremely helpful and the suggestions have greatly improved the article. We provide a point-by-point response to each of the reviewers' comments below.

Many thanks for your consideration,
Laura Fenlon

Reviewer 1 Comment 1 (R1.1) There is no clear information on whether the bulk mouse transcriptomes at early and late cortical development were experimentally obtained by the authors, or from public datasets. If the former, this is not described in Methods nor there are accession numbers to the datasets. If the latter, there is no clear information on the source of the dataset/s used.

Author response (AR): We have now amended the supplementary methods (beginning line 32) to make this information clearer, which reads:

"The P12 dunnart data was compared against three existing age-matched (Early Cortical Development, ECD) wildtype C57BL/6 mouse RNA-seq datasets of dorsal cortex at E12 from the publicly available NCBI Sequence Read Archive (SRR1509162, SRR1509163, SRR1509164). Similarly, the P20 dunnart data was compared against four existing age-matched (Late Cortical Development, LCD) wildtype C57BL/6 mouse neocortical datasets at E16 from the publicly available NCBI Gene Expression Omnibus (SRR5755669, SRR5755670, SRR5755671, SRR5755672) (Bunt et al., 2017)."

R1.2 In page 7, lines174-177: "In contrast, genes that were up-regulated during ECD (relatively down-regulated during LCD) did not show a notable change in GO enrichment between developmental periods in either species with respect to the aforementioned developmental processes." - This is strange, as one might expect a reciprocal change. How is this interpreted?

AR: We agree that this is an interesting phenomenon, and one possible explanation may be a broadly additive trend of gene expression during development. In other words, as there is a progressively increasing diversity of cell types later in development, entirely new suites of genes may be expressed, while those that were highly expressed in early cortical development remain relatively stable, as progenitor populations still remain. We have added this possible interpretation into the manuscript beginning line 183, which now reads:

"This trend may reflect a relative stability of early progenitor populations (and thereby their gene expression) across these two stages of cortical development, with the majority of interstage differences arising in mice driven by the transcriptomes of later-born populations (such as mature projection neurons) in LCD."

R1.3 Page 8, lines 200-206: "Interestingly, the transcripts with a higher expression ranking in dunnarts relative to mice during ECD (Table S7A) were enriched for GO terms related to advanced stages of neural maturation, such as neuron differentiation, neuronal projection development, ion transport and synaptic signalling. In contrast, transcripts with a higher expression ranking in mice relative to dunnarts during ECD (Table S7B) were enriched for GO terms that negatively regulate processes related to mitosis and cell cycle (Fig. 3B; Table S6B)." - This seems contradictory, as positive regulation of neurogenesis and neuron differentiation should go along with negative regulation of cell cycle, not the opposite... In fact, how is it that in ECD higher in mouse they find greater negative reg of forebrain neuron differentiation, but positive reg of cell differentiation? In early cortical development in mouse, neuroepithelial cells and early radial glia cells undergo fast self-amplification, so regulation of mitotic cell cycle should be importantly represented.

AR: We thank the reviewer for pointing this out, and use this opportunity to discuss an important point in more detail. We now provide an interesting potential mechanism (and supporting

references), that may reconcile this seeming contradiction, with the following statement beginning line 216:

"It has previously been reported that differing lengths of various phases of the cell cycle are related to differing developmental outcomes in the cerebral cortex. For example, a shorter S-phase is linked to differentiation (Arai et al., 2011). It is therefore possible that the relative enrichment of GO terms for negative regulation of mitotic cell cycle and cell cycle phase transition in mouse are functionally linked with the downregulation of differentiation via a mechanism such as lengthening S-phase and thereby inhibiting differentiation."

R2.1 I have one crtique and suggestion that could be easily added to significantly improve the manuscript and usefulness to readers. Specifically, all micrographs provided are relatively high magnification. Because Necortex can have significant regional differences in gene expression and laminar dimensions, it would be important to add some lower magnification images of coronal sections, so the reader can appreciate differences or similarities in regional patterns of markers in the two species.

AR: We agree that lower magnification images would add to this finding, and have now included these for each of the progenitor and neuronal markers at both early and late cortical development stages for both species in a new supplementary figure (Fig. S2), and have added reference to this new figure in the main text.

R3.1 The authors identified equivalent developmental stages (ECD and LCD) of dunnart and mice based on pulse-chase birthdate analysis of deep and superficial layer neurons. This approach is unique and appropriate, but still significant information is absent, which are 1) when neocortical neurogenesis starts, 2) how long ECD and LCD are lasting (i.e., the duration of L5/6 and L2/3 neuron production), and 3) when neurogenesis finishes in the developing dunnart neocortex. These lines of evidence are critical to evaluate heterochronic changes in neocortical programs in dunnart occurs at specific timing or not.

AR: We have previously published a detailed comparative EdU timecourse and staging system in fat-tailed dunnart, that we did not reference explicitly. We have now amended the manuscript to explicitly reference this and have included the requested information in text, beginning line 90, which reads:

"We have previously shown with birth-dating data that cortical neurogenesis is initiated and terminated at equivalent stages between species (S19-S25) and while each stage within this period spans one day in mouse, it spans approximately three days in dunnart (Paolino et al., 2020)."

R3.2 & 3.3 The conclusion of "protracted" neuronal maturation program is unclear. In figure4, the authors demonstrated the dominance of progenitor type gene expression (ranking) in dunnart at ECD, while neuron-type gene expression is conversely dominant in mice at LCD, but it is ambiguous what kind of programs are "protracted" in later stages of dunnart neocortex. The authors focused on NeuroD1 and Tbr1, and these markers are relevant to label differentiated neurons and layer-specific neurons, but not appropriate to evaluate specific developmental timing or stage… Similarly, the thickness of Sox2 or NeuroD1-positive areas in the neocortex (Figure4) demonstrates relative ratios of progenitors and neurons at given time points but does not provide evidence for precocious or protraction of neurogenesis. Since ECD and LCD were determined as "equivalent stages" between two species, the terms "precocious" or "protraction" are contradictory to the criteria. The data taken from two time points are snapshots, thus needs to show the histological data of other developmental stages in order to argue the dynamics of progenitor and neuronal pools and species differences.

AR: We agree that the above two points might have stemmed from a confusion around the terms "protracted" and "precocious" throughout the manuscript. We have addressed this by clarifying the benchmark that we are using by which we consider development to be protracted and precocious, that is, the timing of birth of equivalent cortical layers at early cortical development (deep layers) and late cortical development (upper layers). We have now clarified this each time these terms are used throughout the manuscript, including the abstract beginning line 29, which now reads:

"Enrichment analyses revealed more mature gene networks in marsupials at the early stage, which reverted at the later stage, suggesting a more precocious but protracted neuronal maturation program relative to birth timing of cortical layers."

We have also further clarified that these stages were chosen as benchmarks due to matched timing of neurogenesis of equivalent populations, as well as overall physiological development in the results beginning line 103, which now reads:

"We then used these ages as comparable benchmark periods of development for all subsequent collections and transcriptomic analyses, due to the equivalency of overall development according to our staging system, as well as the match in timing of birth for equivalent cortical layers (see Materials and Methods). We refer to these stages as early cortical development (ECD) and late cortical development (LCD) hereafter."

Finally, we have now amended the manuscript to explicitly reference histological data that spans multiple age points using a marker of postmitotic neurons occupying the cortical plate of both species that we have previously published to contextualise the snapshots that we included in this paper. This section starting on line 298 now reads:

"We have previously shown, using a marker for post-mitotic cortical neurons, that the expansion of the cortical plate in both mouse and dunnart is a broadly additive process across multiple stages of development (Paolino et al., 2020). We therefore interpret this data as two timepoints along a relatively linear continuum of cortical plate growth in both species."

R3.4 It is necessary to validate the antibodies used in this study recognize appropriate antigens in each species by western blotting. In addition, significant differences of expression patterns of Lhx2 must be confirmed by in situ hybridization.

AR: We respectfully disagree that antibody validation by western blotting is required to support the conclusions of this manuscript. Antibody staining is very commonly published without western blot validation across novel species, and indeed antibodies against many of these proteins have previously been published in developing marsupials, showing similar localisations to our results (Puzzolo and Mallamaci 2010; Tbr1, Pax6). Given that the immunohistochemical labellings that we show in this manuscript are clearly cell-type specific, in keeping with the location of known populations in the mouse, and corroborate the trends shown by the RNA-seq data for several markers, we do not consider that further western blot validation is necessary to support the conclusions of the paper. We also think that the inclusion of multiple markers and their corroboration with each other is sufficiently convincing compared to single markers alone. In addition, we have now included lower magnification images of the antibodies used in figure 4 in new supplementary figure S2, which we think adds to the evidence of cell-type specificity and conservation between species for these markers.

We also consider that we have already shown differences between levels of expression of Lhx2 at the mRNA level in our RNA-seq results, and that an in situ hybridisation is not necessary to support this. As the final immunohistochemical labelling of Lhx2 is an interesting validation of one of the candidates found in the RNA-seq comparison, and we do not make any definitive claims about its differing function or expression at the mRNA versus protein levels, we do not think that an in situ hybridisation is necessary to support our broader conclusions.

R3.5 Details of statistics and p-values must be described in Method and figure legends.

AR: Further details of statistics and p-values have been added to the supplementary methods (lines 94-96; 145-147) as well as Figure legends 2, 3 and 4.

Second decision letter

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AUTHORS: Peter Kozulin, Rodrigo Suarez, Qiongyi Zhao, Annalisa Paolino, Linda J Richards, and Laura R Fenlon
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

This pioneer study by Kozulin and colleagues presents the first transcriptomic analysis of the developing cortex of the fat-tailed dunnart, a real tour de force given the very limited existing genomic resources on this species. Most importantly, they cleverly exploit this new dataset by comparing it with mouse transcriptomes at equivalent developmental stages, reaching the fascinating conclusion that cortical development progress is heterochronous between these two species, which future work may find is extensive to eutherians versus metatherians at large. By performing a variety of comparative analyses between their bulk transcriptome of timed developing dunnart cortex versus mouse, they find that the transcriptome changes very little in dunnart as compared to mouse along a comparable developmental period, in reference to the progression of cortical neurogenesis. Their convincing analyses and interpretations lead to the solid conclusion that this difference involves a precocious maturation of the dunnart cortical transcriptome as compared to mouse, but then a delayed or much protracted further maturation. These results not only further support the emerging concept that cortical evolution essentially involved changes in the gene regulatory landscape, rather than the emergence of novel, species-specific genes, but also that temporal regulation of gene expression may have been part of this evolution. I find this study fascinating and seminal, both at the technological level (as a goldmine resource) and conceptual level.

Comments for the author

The authors have satisfactorily addressed all my previous concerns, and thus I now fully endorse publication of this fantastic paper.

Reviewer 2

Advance summary and potential significance to field

This is an interesting, and novel, comparison of neocortical development between two species not compared previously.

Comments for the author

The authors addressed my one suggestion.

Reviewer 3

Advance summary and potential significance to field

The authors responded adequately to the points.

Comments for the author

No further revisions are required for publication.