DYRK1A overexpression enhances STAT activity and astrogliogenesis in a Down Syndrome mouse model

Nobuhiro Kurabayashi, Minh Dang Nguyen and Kamon Sanada

Corresponding author: Kamon Sanada, The University of Tokyo

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Esther Schnapp

1st Editorial Decision 27 March 2015

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, the referees agree that the findings are potentially interesting and of relevance for understanding the molecular changes occurring in Down Syndrome. However, all of the referees also pinpoint different aspects of the study that are weak and require significant strengthening. We think that all referee concerns are very valid and can be addressed in a straightforward manner, which we therefore invite you to do. Referee 1 remarks that it should be distinguished whether DYRK1 has a stage-specific effect on neurons and astrocytes, or whether it promotes astrogliogenesis by inhibiting neurogenesis. Referee 2 notes that it needs to be examined whether DYRK1 promotes normal astrogenesis or whether it causes astrogliosis, which is an interesting aspect in the context of the syndrome. Referee 3 finally points out that the effect of DYRK1 on STAT is not convincing and needs to be strengthened and supported by additional data. Another important point this referee raises is whether DYRK1 has a role in astrogenesis during normal cortical development.

As I said, we think that all of these concerns are valid and relevant and we therefore would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript.
will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the issue further.

I very much appreciate that you include all statistical information in the figure legends, and that you use scale bars in all microscope images, which is very laudable as, unfortunately, rare. I just have one question: Are the data in figures 5D and 6B based on a single experiment? Please add this information to the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

In the manuscript by Kurabayashi et al "DYRK1A overexpression enhances STAT activity and astrogliogenesis in a Down's Syndrome mouse model" the authors use in vitro and in vivo electroporation approaches to show increased differentiation of late (E17) cortical progenitors towards astrocytic fates in the Ts1Cje mouse model of Down's Syndrome, and phenocopy this by overexpression of DYRK1A, a kinase encoded in the trisomic region. The authors had previously argued that DYRK1A overexpression in neurogenic progenitors (E11-13), delays neuronal differentiation thus altering laminar fate (Kurabayashi, Gen Dev 2013). In this study they suggest that once progenitors have become competent to generate glia (~E16 in cortex), overexpression of this gene promotes astrogliogenesis.

This paper is in the context of evidence that DS brains have fewer neurons and more glia. Some have suggested astrogenesis at the expense of neurogenesis (Lu, Plos1 2011), others show that DS iPSC astrocytes have non-cell autonomous effects on neighboring neurons, and express higher levels of "mature" or reactive astrocyte markers such as S100 and GFAP (Chenn, Nat Comm 2014.)

In this context, it is important to understand at a mechanistic level how DS associated genes affect the production of neurons and glia. The paper is carefully written and most data are well supported. In combination with their previous study and controls in the current study (kinase dead versions of Dyrk1a for example), they make a strong case that this kinase is a major component of the progenitor phenotype they are studying in Ts1Cje mice. They also make a strong case for the increased expression of some astroglial mature markers when this gene is electroporated during the gliogenic period.

The primary weakness of this study is that it is conceptually very dependent on the results of their earlier paper, and neurogenesis/gliogenesis are really two sides of the same coin. However, their earlier paper (Genes Dev 2013) electroporated E11-13 but did not look past E16, when gliogenesis is just beginning, and did not examine any glial markers. Conversely, this paper electroporates during a gliogenic period and does not really look at neurons in a detailed way. It looks at the expression of two fairly late stage astrocyte markers, but does not examine any earlier markers of glial competence (Aldh1l1, Glast, FGFR3, etc.) It is difficult to know if we are seeing stage specific effects of the same gene (as the authors argue), or whether this gene can both delay neuronal differentiation and simultaneously increase eventual astroglial competence at both stages.

Specific points (chronological)

P6: the authors states that cortical progenitor cells were cultured at "clonal density" which is not accurate. Cells appear to be cultured at very high density, and clonality is based on sparse efficiency of GFP expression. It would be helpful to clarify the actual clonality of the assay (how many GFP positive colonies per well?)
P7-At this very early stage (4DIV), it is not surprising that 75-85% of colonies express neither neuronal or glial markers. It would be interesting to monitor the multipotent colonies which will develop over the next 4-14 DIV, measuring the appearance of neuronal and glial markers, or changes in the timing of glial appearance.

P7, Figure 2a: Why would the authors speculate that GFP labeled cells with astrocytic appearance are all in the lower cortical plate? With enough time, astrocytes migrate and proliferate locally in all layers of the cortex (Ge et al, Nature 2012), so this apparent migratory defect could suggest a premature exit from multipotency towards a glial fate.

Fig 2D, Fig 3E- Why is percent of NeuN+ cells shown at P30 and not at P5? Surely if this is a reduction in neurogenesis any defect in the numbers of neurons should be evident at both ages?

P9, top - The electroporation at E13 seems like the most important data in the paper, and the only data linking this study to their previous, highly complementary paper. Why is this data only described and not shown, and most importantly, when was the analysis performed? If it was done at E16, the negative result could simply mean that the authors did not wait long enough to see the increase in astroglial markers they have chosen, which are not expressed until much later.

Referee #2:

In the research manuscript entitled "DYRK1A overexpression enhances STAT activity and astrogliogenesis in a Down's syndrome mouse model” Kurabayashi et al. investigates the link between DYRK1A dosage and astrocyte development with relevance to Down's syndrome. Since Down's syndrome is a neurodevelopmental disorder, the mechanistic roles of these over expressed genes in affecting neurogenesis vs gliogenesis may be key to uncovering the causes of mental impairment, thus this topic is of high importance. Previously, the authors reported that overexpression of DYRK1A and DSCR1 together altered the timing of neurogenesis. Here, they use similar techniques but examine in detail the link between DYRK1A kinase activity and STAT phosphorylation.

This manuscript reports the key finding that increased DYRK1A generates an overabundance of astrocytes through STAT activation, which is of high significance to both the field of gliogenesis and broadly to Down's syndrome research. The evidence provided (measuring astrocyte percentages after overexpression and knockdown of DYRK1A, as well as the impact on STAT) supports their conclusions.

In general, the manuscript is written clearly and provides sufficient experimental details and references. There is only one major issue that should be addressed before this reviewer recommends this paper for acceptance.

Issue 1. The only major unaddressed issue in this study is whether DYRK1A is pushing progenitors to generate normal astrocytes through the canonical STAT developmental pathway, or whether the increase of markers GFAP, S100B, and STAT are a result of increased reactive astrocytes. The decrease of neurons supports the former model, though according to some of the image examples the astrocytes processes appear more complex, which is a marker of astrogliosis. Increased STAT3 is usually observed in reactive astrocytes in injury models and thus it would be important to know whether DYRK1A is inducing a reactive response in the Down's syndrome model. The authors should address this by comparing one or more measures of reactivity in the DYRK1A overexpressing astrocytes to controls. For example, by measuring either GFAP intensity, astrocyte morphology (i.e. scholl analysis), neighboring microglial activation, presence of inflammatory cytokines, oxidative stress, or increased proteoglycan deposition.

Issue 2. Minor issue. It is interesting that the DYRK1A astrocytes are generated in a lower region of the cortical plate compared to controls. Is this a defect of migration or due to their earlier generation during the inside-out development of the cortex? Please elaborate on this topic in the discussion.
Referee #3:

In this paper the authors seek to understand the molecular basis for increased astrocyte production associated with Down Syndrome. Using a mouse model of DS, Ts1Cje, they find that there is increased production of astrocytes during cortical development. These results nicely correlate with observations from other groups, associating increased astrocyte production with DS. Next, they turn their attention to DYRK1A, as it has been shown to influence progenitor proliferation and is also duplicated in DS and in the Ts1Cje mouse. Overexpression of DYRK promotes astrocyte differentiation, whereas its knockdown in Ts1Cje mice, attenuates the increased astrocyte generation. To uncover the mechanistic basis for these roles during astrocyte differentiation, they find that the Ts1Cje mouse has increased phosphorylation of STAT, which appears to be the result of increased DYRK.

In general, the first part of this paper is very well executed and convincing. The links between DS, astrocytes, and DYRK1 are very interesting and make this part of the paper worth reading. The problems in this paper lie in the mechanistic studies. The links to STAT activity and phosphorylation are extremely modest.

1) Should show expression of DYRK in astrocytes during cortical development in both the Ts1 mouse, as well as WT controls. Would like to see the correlation and co-expression of DYRK with GFAP during normal and Ts1 cortical development.

2) In the knockdown experiments in figure 2, they need to show reduced DYRK expression. I agree that there is a difference in astrocyte production, just need confirmation of DYRK knockdown.

3) In figure 4E it seems that astrocyte production in the Ts1 mouse is more sensitive to DYRK knockdown than WT. Any reason why the Ts1 mouse is more sensitive. This also raises the question of whether DYRK1 is required in normal astrocytes, or whether its function is simply related to DS. This should be addressed.

4) In general, the effects on STAT3 are very, very modest. Not sure I believe there is a real effect. If indeed, DYRK1 is influencing astrocyte differentiation through STAT signaling, then the authors should try a STAT-dependent, astrocyte production assay. Simply adding LIF to astrocyte cultures will provoke STAT dependent, astrocyte differentiation. In this paradigm, they could manipulate DYRK expression and test whether it influenced astrocyte production. This might provide a functional link between STAT and DYRK. While this experiment may show a functional link, the general phenomenon of DYRK influencing STAT phosphorylation would still remain nebulous.

5) The methods of quantifying STAT in figure 6 are not acceptable. Also in 6E, the Cherry and GFP are BOTH elevated in the DYRK overexpression making it very difficult to interpret the results of this experiment.

6) An explanation of the STAT reporter is needed. Nowhere in the paper is there a reference or explanation for GFIL-GFP.

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**Referee #1:**

Point 1:

The referee stated “…, this paper electroporates during a gliogenic period and does not really look at neurons in a detailed way. It looks at the expression of two fairly late stage astrocyte markers, but does not examine any earlier markers of glial competence (Aldh1l1, Glast, FGFR3, etc.) It is difficult to know if we are seeing stage specific effects of the same gene (as the authors argue), or whether this gene can both delay neuronal differentiation and simultaneously increase eventual astroglial competence at both stages.”
Our previous study shows that overexpression of DYRK1A and DSCR1 during the neurogenic phase delays neuronal differentiation of progenitor cells (Kurabayashi and Sanada, 2013). The present study reveals that DYRK1A overexpression at E16 or later (at the beginning of the gliogenic phase) promotes astrocytic differentiation. Based on these combined data, we propose that increased dosage of DYRK1A impairs neurogenesis during early stages of corticogenesis, as well as astrogliogenesis during later stages of corticogenesis (astrogliogenic phase).

As pointed out by the referee, because the two studies were performed at different stages of corticogenesis, it is possible that DYRK1A overexpression affects simultaneously the neurogenic and astrogliogenic machinery of progenitor cells. If the effects were simultaneous, this will implicate increased dosage of DYRK1A as a neurogenic-to-astrogliogenic fate switch during corticogenesis. One theoretical way to evaluate this possibility is to use earlier astroglial lineage markers. Unfortunately, ALdh1l1, Glast and FGFR3 are not only expressed in astrocytes, but also in radial glial progenitor cells (Foo and Dougherty (2013) Glia 61: 1533; Fukuchi-Shimogori and Grove (2003) Nat. Neurosci. 6: 825; Malatesta et al. (2003) Neuron 37: 751). The absence of true astrocyte-specific marker for detection of early gliogenic stages makes this experiment uninterpretable.

To explore the possibility that DYRK1A overexpression simultaneously affects neurogenic and astrogliogenic machinery, we measured phosphorylation of STAT3 at Ser727, STAT activity and astrogial differentiation. In the initial version of the manuscript, we found that DYRK1A overexpression promotes STAT3 Ser727 phosphorylation, STAT activity and astrogial differentiation of E16 or later stage progenitor cells. Our recent data now demonstrate that at E13-14, DYRK1A overexpression promotes STAT3 Ser727 phosphorylation but does not alter STAT-dependent transcription (activity) and astrogial differentiation of progenitor cells (Supplemental Figure S7). Thus, we reason that DYRK1A modifies the astrogliogenic machinery of progenitors at both E13 and E16. It is well known that signaling events downstream and / or upstream of STAT are suppressed during the neurogenic phase (Martynoga et al., 2012; Miller and Gauthier, 2007; Okano and Temple, 2009). Therefore, it is not surprising that STAT-dependent transcription and astrogial differentiation of progenitor cells are suppressed in earlier stages of corticogenesis (such as at E13-14) despite increased dosage of DYRK1A and STAT Ser727 phosphorylation.

In summary, supported by our Genes & Development publication in 2013, the present study strengthens the notion that overexpression of DYRK1A impairs neuronal fate acquisition and advances the idea that increased dosage of DYRK1A modifies the astrogliogenic machinery of neural progenitors in DS.

Point 2:

"P6: the authors states that cortical progenitor cells were cultured at "clonal density" which is not accurate. Cells appear to be cultured at very high density, and clonality is based on sparse efficiency of GFP expression. It would be helpful to clarify the actual clonality of the assay (how many GFP positive colonies per well?) "

We apologize that our original statement was inaccurate and misleading. The experimental details are now included in the Materials and Methods section, and the text has been revised accordingly. Briefly, E16 brains were first electroporated with GFP-expressing plasmid. The neocortical cells containing GFP-labeled cells were then immediately prepared and diluted with neocortical cells derived from the non-electroporated littermates. Cells were plated at a density of 0.2 x 10^6 cells on sterile coverslips precoated with poly-D-lysine and laminin overnight in wells of 24-well plates. GFP expression was used to confirm that the GFP-labeled cells were well separated from each other so that clones could be unambiguously identified. In our plating conditions, 20-60 clones per well were observed. More than 100 clones were analyzed for each condition.

Point 3:

"It would be interesting to monitor the multipotent colonies which will develop over the next 4-14 DIV, measuring the appearance of neuronal and glial markers, or changes in the timing of glial appearance."

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As suggested by the referee, we have performed clonal analysis at DIV4, DIV8 and DIV12. As shown below, we have found an increase in the percentage of astroglial clones over time in cultures. On the other hand, the percentage of neuronal clones was unchanged. Also, mixed clones containing both Tuj1-positive and GFAP-positive cells were not detected. Importantly, DYRK1A-introduced progenitors gave rise to significantly more astroglial clones at all time points when compared to control progenitors. Thus, in our experimental conditions, generation of astroglial clones is accelerated without affecting the production of neuronal clones. As the data at 4 DIV are clearly representative of the effect of DYRK1A overexpression on astrocytic differentiation of progenitor cells, we have only included these data in the revised manuscript.

**Figure for the Referee**

![Graph showing astroglial and neuronal clones](image)

**Point 4:**
"P7, Figure 2a: Why would the authors speculate that GFP labeled cells with astrocytic appearance are all in the lower cortical plate? With enough time, astrocytes migrate and proliferate locally in all layers of the cortex (Ge et al, Nature 2012), so this apparent migratory defect could suggest a premature exit from multipotency towards a glial fate."

In Fig 2A, we electroporated GFP-expressing plasmid into E17 neocortices, harvested the brains at P5, and examined the identity of the GFP-labeled cells by immunostaining. As indicated by the referee, a relatively large population of GFP-labeled cells with astrocyte appearance was located in the lower part of the cortical plate. Ge et al. have shown that more than 90% of astrocytes derived from GFP-electroporated P0-P2 progenitor cells reside in lower part of the cortical plate at P16-20 and that astrocyte in the upper part of the cortical plate expand their population through local proliferation thereafter. As the proliferation rate of astrocytes is quite low during the first postnatal week (Bandeira et al. (2009) *PNAS* 106: 14108), we reason that in P5 neocortex, the majority of GFP-labeled astrocytes are in lower cortical plate. We have now cited Ge et al. and described the similarities between our findings and their data (page 7).

**Point 5:**
"Fig 2D, Fig 3E- Why is percent of NeuN+ cells shown at P30 and not at P5? Surely if this is a reduction in neurogenesis any defect in the numbers of neurons should be evident at both ages?"

As immature neurons are less or not positive for NeuN (Mullen et al. (1992) *Development* 116: 201; Sarnat et al. (1998) *Brain Dev.* 20: 88), we could not measure the percentage of NeuN-positive cells among the GFP-labeled cells at P5. To overcome the issue, we have stained the GFP-labeled cells at P5 with an antibody against Cux1, a marker for layer 2-4 neurons in the neocortex. As neurons...
generated at later stages of corticogenesis are destined to layers 2-4, one would expect that almost all GFP-labeled cortical plate cells generated at E17 are positive for Cux1 or GFAP at P5. As anticipated, the GFP-labeled cells in the cortical plate of control brains were 87% Cux1-positive and 12% GFAP-positive. Importantly, in Ts1Cje mice, a significantly smaller fraction (67%) of GFP-labeled cells in the cortical plate was positive for Cux1 and a remarkably larger population (28%) was positive for GFAP. Finally, in DYRK1A-introduced neocortices, we observed a significant decrease in the proportion of cells positive for Cux1. In sum, these results indicate increased astrogliogenesis, with a corresponding reduction of neurogenesis, in both P5 and P30 Ts1Cje mice and DYRK1A-overexpressing neocortices. These data are now included in new Fig 2D, E and Fig3E, F, and cited in page 7 and pages 9-10 of the manuscript, respectively.

Point 6:
“P9, top - The electroporation at E13 seems like the most important data in the paper, and the only data linking this study to their previous, highly complementary paper. Why is this data only described and not shown, and most importantly, when was the analysis performed? If it was done at E16, the negative result could simply mean that the authors did not wait long enough to see the increase in astroglial markers they have chosen, which are not expressed until much later.”

We apologize for the lack of information provided for the clonal analysis using E13 progenitors. The experiment was done at DIV4 (E17), a period in culture in which an increase in GFAP-positive clones was clearly observed in the case of E16 progenitors. We have now included the data in Supplemental Figure S7, and revised the text and Materials and Methods section accordingly.

We also understand that experiments using E13 embryos are very important for a better understanding of the function of DYRK1A in astrogliogenesis. As described in point #1, we have analyzed STAT3 Ser727 phosphorylation, STAT-dependent transcription (activity) and astrogial differentiation at E13. We found that Ser727 phosphorylation of STAT3 was enhanced upon DYRK1A overexpression in E13 progenitor cells. Importantly, STAT-dependent transcription in E13-14 progenitor cells and astrogliogenesis were not significantly increased upon DYRK1A overexpression at that stage. This result is consistent with our idea that DYRK1A overexpression does not instruct astrocytic differentiation of progenitor cells at earlier stages of corticogenesis. These data can be found in supplemental figure S7.

Referee #2:

Point 1. The referee stated “The only major unaddressed issue in this study is whether DYRK1A is pushing progenitors to generate normal astrocytes through the canonical STAT developmental pathway, or whether the increase of markers GFAP, S100B, and STAT are a result of increased reactive astrocytes. The decrease of neurons supports the former model, though according to some of the image examples the astrocytes processes appear more complex, which is a marker of astrogliosis. Increased STAT3 is usually observed in reactive astrocytes in injury models and thus it would be important to know whether DYRK1A is inducing a reactive response in the Down’s syndrome model. The authors should address this by comparing one or more measures of reactivity in the DYRK1A overexpressing astrocytes to controls. For example, by measuring either GFAP intensity, astrocyte morphology (i.e. scholl analysis), neighboring microglial activation, presence of inflammatory cytokines, oxidative stress, or increased proteoglycan deposition.”

The referee is concerned that an increase in GFAP positive cells and STAT phosphorylation / activity upon DYRK1A overexpression is reflective of increased astrogliosis rather than enhanced astrogliogenesis. To address this issue, we have examined GFAP intensity of GFP-labeled astrocytes, astrocytic morphology (by Sholl analysis of GFAP-positive glial process) and activation of microglia in the vicinity GFP-labeled astrocytes in DYRK1A-introduced brains. Briefly, P30 neocortices electroporated at E17 were immunostained with antibodies against GFAP and MAC2 (a marker for activated microglial cells). GFAP immunofluorescence intensity of GFP-labeled astrocytes in DYRK1A-introduced brains was similar to that of control astrocytes. Further, Sholl analysis of GFAP-positive glial processes demonstrated that astrocytic morphology was not
significantly altered following DYRK1A overexpression. Finally, almost no MAC2-positive cells were observed in both control and DYRK1A-overexpressed brains. Taken together, these data indicate that DYRK1A increased dosage does not induce astrogliosis but rather promotes astrogliogenesis (see Supplementary Fig S3 and pages 10-11).

Point 2:
The referee stated “Minor issue. It is interesting that the DYRK1A astrocytes are generated in a lower region of the cortical plate compared to controls. Is this a defect of migration or due to their earlier generation during the inside-out development of the cortex? Please elaborate on this topic in the discussion.”

This is in reference to the original Fig 3F that showed the distribution of GFP-labeled cells in P30 brains electroporated at E17. To confirm the differential distribution of GFP-labeled astrocytes between control and DYRK1A-introduced brains, we divided the cerebral wall into bins (with bin 1 being at the ventricle surface and bin 10 at the pial surface) and counted the number of GFP-positive astrocytes in each bin. As shown below, our analysis revealed that the position of astrocytes is not significantly lower in the DYRK1A-introduced brains versus control brains. In the original figure, we did not align properly the position of the ventricular surface of control, DYRK1A (WT) and DYRK1A (KD) brain images and this may have given the referee the impression of differential distribution of astrocytes. This mistake has been corrected in the new Fig. 3G.

Referee #3:

Point 1:
The referee stated “Should show expression of DYRK in astrocytes during cortical development in both the Ts1 mouse, as well as WT controls. Would like to see the correlation and co-expression of DYRK with GFAP during normal and Ts1 cortical development.”

First, we examined expression of DYRK1A in astrocytes in cultures. Briefly, progenitor cells prepared from E16 neocortices of Ts1Cje/euploid were stimulated with CNTF, and differentiated astrocytes were immunostained with antibodies against DYRK1A and GFAP. As shown below, in both Ts1Cje and euploid cultures DYRK1A immunofluorescent signals were detected in almost all
GFAP-positive cells. In addition, as expected, immunofluorescence intensity of DYRK1A in Ts1Cje astrocytes was ~1.4-fold higher than that in euploid cells. Interestingly, as detected with western blotting, DYRK1A expression in wild-type neocortex gradually decreases over time (from E13 to adult) whereas GFAP expression increases (see below). Thus, no correlation exists between DYRK1A expression and GFAP expression during corticogenesis.

Having previously showed that DYRK1A is expressed in progenitors and enriched in the ventricular zone of E14 neocortex (Kurabayashi and Sanada, 2013), DYRK1A is likely to be expressed predominantly in progenitor cells. This would explain the decreased levels of DYRK1A observed during brain maturation. Since the role of DYRK1A in progenitor cells constitutes the centerpiece of our manuscript, the data concerning DYRK1A expression in astrocytes, although interesting and worth pursuing in another study, do not enhance our understanding of the role of DYRK1A in progenitors. For this particular reason, we have not included them in the revised manuscript but could integrate them if the referee feels strong about it. That DYRK1A is expressed in neurons and astrocytes is now mentioned in page 8.

Point 2:
The referee stated “In the knockdown experiment in figure 2, they need to show reduced DYRK expression. I agree that there is a difference in astrocyte production, just need confirmation of DYRK knockdown.”

Confirmation of DYRK1A knockdown in progenitor cells was shown in the original Supplementary Fig S2 (now Supplementary Figure S4).

Point 3:
The referee stated “In figure 4E it seems that astrocyte production in the Ts1 mouse is more sensitive to DYRK knockdown than WT. Any reason why the Ts1 mouse is more sensitive. This also raises the question of whether DYRK1 is required in normal astrocytes, or whether its function is simply related to DS. This should be addressed.”

As pointed out by the Reviewer, DYRK1A knockdown had no or less effect on astrocytic differentiation of euploid progenitors when compared to that in Ts1Cje progenitors. Furthermore, we found that knockdown of DYRK1A in euploid progenitors has no significant effect on STAT3 Ser727 phosphorylation (0.99 ± 0.01-fold in control sh versus 0.98 ± 0.01-fold in DYRK1A sh#1, P = 0.76 versus control by a two-tailed Welch’s t-test). Thus, in normal progenitor cells DYRK1A
appears to not contribute to their astrocytic differentiation. In contrast, increased dosage of DYRK1A in DS progenitors impacts their astrocytic differentiation.

Point 4:
The referee stated “In general, the effects on STAT3 are very, very modest. Not sure I believe there is a real effect. If indeed, DYRK1 is influencing astrocyte differentiation through STAT signaling, then the authors should try a STAT-dependent, astrocyte production assay. Simply adding LIF to astrocyte cultures will provoke STAT dependent, astrocyte differentiation. In this paradigm, they could manipulate DYRK expression and test whether it influenced astrocyte production. This might provide a functional link between STAT and DYRK. While this experiment may show a functional link, the general phenomenon of DYRK influencing STAT phosphorylation would still remain nebulous.”

We have listened to the referee’s suggestion and performed in vitro clonal analysis in the presence of CNTF to induce STAT-dependent astrocyte differentiation. In this experiment, neocortical progenitors were prepared from E16 brains immediately after electroporation, cultured, and stimulated with CNTF at 3DIV. The cellular composition of the GFP-labeled clones was then examined at 5DIV by immunostaining. We found that DYRK1A overexpression significantly increased the percentage of astroglial clones (42.9 ± 2.9% in control versus 67.7 ± 4.5% in DYRK1A, P < 0.01 by a two-tailed Student’s t-test; see Supplementary Fig S2 and page 9). These results are consistent with our model that DYRK1A overexpression enhances astrocytic differentiation of progenitors through STAT-dependent pathway.

In brains of DS patients and mouse models, DYRK1A expression is increased by ~1.5-fold. To mimic DS condition, we reduced the concentration of plasmids electroporated. This allowed us to achieve modest expression of ~1.5 fold the levels of DYRK1A in wild-type brains or to decrease the expression of DYRK1A by ~0.6-fold in Ts1Cje progenitors. Upon DYRK1A knockdown, astrocytic differentiation and Ser727 phosphorylation of STAT3 in Ts1Cje progenitors were reduced by ~30 to 50%. Moreover, astrocytic differentiation and Ser727 phosphorylation of STAT3 in wild-type DYRK1A-overexpressing progenitors were increased by 1.5 to 3-fold. Thus, modest variations in DYRK1A levels are consistent with the modest effects on STAT3 phosphorylation and astroglial differentiation.

Points 5 and 6:
The referee stated “The methods of quantifying STAT in figure 6 are not acceptable. Also in 6E, the Cherry and GFP are BOTH elevated in the DYRK overexpression making it very difficult to interpret the results of this experiment.”

The referee stated “An explanation of the STAT reporter is needed. Nowhere in the paper is there a reference or explanation for GF1L-GFP.”

We guessed the Reviewer refers to Fig. 5 (not Fig. 6) that examines the effect of DYRK1A overexpression on STAT activity.

In brief, E16 embryos were electroporated with the CAG-mCherry plasmid, either GF1L-dGFP or GF1L-SBSPM-dGFP plasmids, and various plasmids as indicated. The embryos were harvested 24 hours later. The brain cryosections (20 µm) were immunostained with antibodies against Pax6 and GFP. Images of GFP and mCherry in the individual sections were obtained under identical parameters with a 63x objective (Plan-Apochromat; Zeiss) on a Zeiss LSM 5 confocal microscope. Mean fluorescence intensity of mCherry and GFP in the soma of individual cells expressing mCherry was measured using Zeiss Zen2010 Software. In addition, the average background fluorescence intensity was measured in individual sections using areas with no electroporated-cells present and was subtracted from the mCherry and GFP intensity measurements. Cells with saturated signal were not used for the analysis. The ratio of fluorescence intensity of GFP to that of mCherry in individual cells was calculated and defined as STAT activity levels. Note that in the original manuscript, we mentioned “…we utilized a reporter construct that drives expression of destabilized
GFP (dGFP) under a control of the 2.5-kb GFAP promoter (GF1L) containing a STAT binding motif (Nakashima et al., 1999).”

Many studies have used similar method to monitor the activity of other transcription factors in cortical progenitors such as β-catenin activity (e.g. Woodhead et al. (2006) JNS, 26; 12620; Munji et al. (2011) JNS, 31: 1676). In our study, we used a plasmid expressing dGFP under the control of GF1L (2.5-kb GFAP promotor containing a STAT-binding motif). To confirm the validity of the above methods, we have now provided additional data showing that mean immunofluorescence intensities of mCherry among the individual GFP-labeled progenitors counted was not significantly different between control and DYRK1A overexpressing condition (control, 75.1 ± 15.5; DYRK1A (WT), 64.0 ± 16.2 in arbitrary unit). On the other hand, mean immunofluorescence intensity of dGFP was increased upon DYRK1A overexpression (control, 29.2 ± 8.2; DYRK1A (WT), 122.7 ± 24.9 in arbitrary unit). These data are now included in the main text (page 13). Also, we have replaced the Fig 5E and F with better images in which mCherry expression is almost equivalent to control and are, we hope, more convincing to the referee.

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees.

As you will see, all referees overall are supportive of publication of the study by EMBO reports now. However, referee 3 points out that DYRK expression in astrocytes through perinatal development into adulthood should be analyzed directly by IHC or ISH. I am not sure how much time this will take you, and whether you have sections from different time-points already in the lab. It is our journal policy that manuscripts must be accepted at the latest 6 months after a first decision was made, which was at the end of March for you. Your paper therefore needs to be accepted latest in September, which means that we need to receive the revised, final version some time in August. Does this work for you? We can discuss this issue further if you foresee any problems.

EMBO reports now integrates supplementary information in the main text file "inline". Supplementary figures, now called expanded view, expand online when they are clicked. At the moment, we can offer this for 5 supplementary figures. Can you please label the supplementary figures expanded view EV1, 2, etc. and upload each EV figure as a separate file? Please include the figure legends for EV figures at the end of the main manuscript text file. Some of your supplementary figures are very small. I suggest that you combine them with other supplementary or main figures to reach a total of 5 expanded view figures. Please let me know if you think that this cannot be done. You can find more information about expanded view figures on our website at: http://embor.embopress.org/authorguide#expandedview

I also noticed that the legends for figure 5B and SF3 do not explain the *, that the legends for SF1B, SF3 and SF4 do not specify from how many independent experiments the data is derived, and that the legend for SF7A does not define the scale bar size. Please add this missing information.

I look forward to seeing a final, revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

In this revision of the manuscript, the authors clarify some of the issues raised in the first round of review, and help to strengthen evidence of a pro-astrogenic effect of Drk1a.

However, there is clearly an increase in gliogenesis at the expense of neuronal production. Future studies (perhaps beyond the scope of the current manuscript) should analyze <i>multipotent</i> clones (not just clones of glial restricted or neuron restricted progenitors) with either improved in
vitro techniques or in vivo analysis of clones electroporated at E13 and followed through the gliogenic phase.

Referee #2:

The authors have satisfactorily responded to all of my concerns and appear to address issues from other reviewers. The revised manuscript appears suitable for publication pending report from other referees.

Referee #3:

The authors did an adequate job of responding to my comments. However, I totally disagree with their response to "Point 1". I contend that it is essential to show that DYRK is expressed in astrocytes in the developing cortex. The in vitro and Western data are not sufficient evidence for this. Moreover, they argue that DYRK expression is decreased in adulthood, based on Western blotting, and therefore it is not in astrocytes, etc. There are no adult timepoints mentioned and no intermediate timepoints between P5 and adulthood shown. Also, this analysis is based on Western blotting, which is a fundamentally flawed approach for assessing cell lineage expression.

Thus, I feel that the authors need to show the dynamics of DYRK expression in astrocytes over the course of perinatal development and into adulthood, using immunohistochemistry (or in situ) on sections from mouse cortex at various developmental, perinatal, and adult timepoints.

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2nd Revision - authors' response 10 August 2015

Referee #1:

“In this revision of the manuscript, the authors clarify some of the issues raised in the first round of review, and help to strengthen evidence of a pro-astrogenic effect of Drk1a. However, there is clearly an increase in gliogenesis at the expense of neuronal production. Future studies (perhaps beyond the scope of the current manuscript) should analyze multipotent clones (not just clones of glial restricted or neuron restricted progenitors) with either improved in vitro techniques or in vivo analysis of clones electroporated at E13 and followed through the gliogenic phase.”

In the present study, we examined roles of DYRK1A at later stages of corticogenesis, with a particular focus on astrocytic differentiation. We understand that the experiments suggested by the Referee are important to elucidate DYRK1A’s actions throughout corticogenesis. However, we also agree with the Referee that these experiments are well beyond the scope of the present study.

Referee #3:

The referee stated “I feel that the authors need to show the dynamics of DYRK expression in astrocytes over the course of perinatal development and into adulthood, using immunohistochemistry (or in situ) on sections from mouse cortex at various developmental, perinatal, and adult timepoints.”

According to the Referee’s suggestion, we have performed immunohistochemistry on E14, P7, P14 neocortices and adult brains. At E14, DYRK1A immunoreactivity was detected in BLBP-positive neural progenitor cells as well as MAP2-positive neurons located in the cortical plate. These results are totally consistent with the previous study from Hämmerle et al. (2008) showing the same results. In P7 and P14 neocortices, almost no DYRK1A immunoreactivity was observed in neurons and GFAP-positive astrocytes. On the other hand, in the adult neocortex, DYRK1A immunoreactivity was detected in neurons, but not in astrocytes. Based on the study by Marti et al. (2003) and our data showing detectable expression of DYRK1A in cultured astrocytes, we suggest that DYRK1A is
expressed at low levels in this cell type. We have included these results in Appendix figure S1, and revised the manuscript as follows:

“As assessed by immunohistochemistry, DYRK1A immunoreactivities were detected in neural progenitor cells and immature neurons of the embryonic neocortex, as well as mature neurons of the adult brain (Appendix Fig. S1), consistent with previous studies [35, 40]. DYRK1A expression in astrocytes is also reported [41].”