Endocannabinoid signalling in stem cells and cerebral organoids drives differentiation to deep layer projection neurons via CB_1 receptors

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MS TITLE: Endocannabinoid signalling in stem cells and cerebral organoids drives differentiation to deep layer projection neurons via CB1 receptors

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

As you will see, all the referees express interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they request a rescue of the CB1 knockdown phenotype and a more quantitative of this phenotype with cellular resolution analysis. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may 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.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The paper by Paraiso-Lune et al., entitled “Endocannabinoid signalling in stem cells and cerebral organoids drives differentiation to deep layer projection neurons via CB1 receptors” deals with the molecular mechanisms controlling the formation of deep vs upper layer neurons in the developing cerebral cortex. The identification of these mechanisms is of great interest as they are critical to generate a functional cerebral cortex which mediates our intellectual capabilities. Moreover, this study has the potential to bring insights into the devastating effects cannabinoids can have on fetal brain development.

Using an in vitro culture system to generate mouse cortical neurons, the authors first show that activating endocannabinoid signalling by using either THC, the CB1 receptor agonist HU-210 or by inhibiting the degradation of endogenous cannabinoids led to an increased formation of deep layer Ctip2+ neurons and to a concomitant reduction in upper layer Satb2 neurons. In contrast, knocking-down the CB1 receptor had the opposite effect. Analysing the electrophysiological properties of newly formed neurons also showed that activating endocannabinoid signalling promote the acquisition of neuronal activity and maturation. Finally, the authors demonstrate that activating endocannabinoid signalling also induces higher proportions of deep layer neurons in a human organoid model. Taken together, this work provides some interesting conclusions but the authors need to revise this manuscript before publication.

Comments for the author

Major points
The manuscripts lacks novelty. The role of endocannabinoid signalling has extensively been studied in the mouse in vivo using full knock-out and conditional mutants and in vitro slice cultures. Using these approaches, the lab of the corresponding authors and other groups have shown effects of this signalling pathway on neural progenitors (Diaz-Alonso et al., 2015; Ao et al., 2020) and on the formation of deeper vs lower cortical projection neurons (Diaz-Alonso et al., 2012). As such the mouse 2D culture system does not provide novel insights compared to these previous study, in particular as it represents a somehow artificial system compared to the mouse as a model. The only novel aspect appears the use of a human organoid system but this has not been analysed in very much detail with no mechanistic insights. Also, the specificity of CB1 signalling is not tested by using AM-251 inhibition in this system and the authors need to indicate how they did their cell counts.

The mechanisms by which activating endocannabinoid signalling elicits an increase in Ctip2+ neurons and a concomitant reduction in Satb2+ neurons remains largely unclear. Based on their luciferase assays the authors seem to suggest that this pathway controls neuronal fate by controlling the transcriptional regulation of Ctip2 but the changes in promoter activity remain rather modest (less than 1.5 fold) which might not be sufficient to induce a fate change. In fact, there are a number of cells after CB1 knock-down which express very low levels of Satb2 if at all (Supplementary Figure 4). This raises the possibility that these cells still express high levels of Ctip2 and might not even have changed fate. It will be important to quantify Ctip2 and Satb2 expression levels in individual knock-down cells rather than using bulk qRT-PCR. The authors should also consider the possibility that this signalling pathways acts on neural progenitor cells as was described recently by themselves and others (Diaz-Alonso et al., 2015; Ao et al., 2020). They should determine the proportions of radial glial cells, basal progenitors and neurons in their culture

systems. A BrdU cell cycle exit experiment would also help to determine whether there is an increase in neuron formation at the expense of basal progenitor cell formation, i.e. an increase in direct neurogenesis which would explain the phenotype as well.

There are discrepant results between the AM-251 treatment which has no effect and the CB1 gene knock-down which causes an increased number of Satb2+ neurons. The authors do not explain this discrepancy raising doubts about the specificity of the reagents used. To address the specificity of the knock-down reagents, the authors should perform rescue experiments with a CB1 expression construct. The authors should also explain why AM-251 treatment on its own has no effect. Is it because the pathway is not active under normal conditions?

It is unclear which statistical tests were used for individual experiments, hence it is impossible to evaluate the significance of the data. Rather than stating which types of statistical tests were performed in the Methods section in general, each figure legends should contain the relevant statistical test, n numbers and p-values.

Given the broad readership of “Development” the manuscript would greatly benefit from better descriptions of the endocannabinoid signalling pathway, the various enzymes and signalling molecules. A simple cartoon as part of figure 1 would certainly help to better illustrate this pathway to the non-expert reader.

Minor points

p7: it should read “development” rather than “evolution”.

p10: it should read Fig. 4a,b instead of Fig. 5a,b.

The CB1 Western blot in Figure 1C needs to be quantified.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, the authors have investigated endocannabinoid signalling in two in vitro models of neuronal differentiation, the R1 murine embryonic stem cell line, as well as in human iPSCs. In studies of R1 mESCs induced to differentiate into neurons using a protocol with cyclopamine administration, the authors report elevated expression levels of eCB components, including the CB1 recpetor and eCB metabolic enzymes. Immunostaining expeirments show that CB1 co-expression is co-detected with stem cell markers OCT4 and Nestin, as well as markers of neurons (TUJ1 and NeuN), and with CTIP2 (gene symbol is Bcl11b). Pharmacological exposure of neurodifferentiating R1 cells to the cannabinoid receptor agonist THC leads to changes in the expression of CTIP2/Bcl11b, a marker of a subset of cerebral cortex neurons. Electrophysiological recordings of cultured neurons confirm their functional maturation, a feature which is correlated with endocannabinoid gene expression.

In separate experiments, shRNA-mediated knockdown of CB1 expression leads to changes in neurodifferentiation, and the expression of markers of cerebral cortex neurons, including CTIP2/Bcl11b (which identifies deep-layer cortical projection neurons of the mammalian cerebral cortex, and SATB2 (which identifies cortical projection neurons of the mammalian cerebral cortex which lie superficial to Bcl11b immunopositive cells). This is an interesting finding to show that CB1 is necessary for neurodifferentiation and neuronal marker expression. In studies with hiPSC-derived cerebral organoids, the authors show that exposure to agonists THC and HU-210 modulates the expression of markers of cortical neurons CTIP2/BCL11B and SATB2. These findings are consistent with the notion that stimulation of endocannabinoid signalling influences neuronal development.

The advance made in this paper is that the authors demonstrate that CB1 is necessary for neuronal differentiation, and that pharmacological manipulation of eCB signalling influences the expression of markers of cerebral cortex neurons, such as CTIP2/BCL11B and SATB2.

The significance to the field is in the authors' development of robust in vitro assays with mouse and human stem cell lines to investigate the impact of endocannabinoid signalling in neuronal differentiation. This is an important technological advance to define the underlying molecular mechanism for eCB signalling in cerebral cortex development.

Comments for the author

The major issue to this manuscript is that the data provided does not support the authors' claim that modulation of endocannabinoid signalling drives differentiation to deep layer projection neurons via CB1 receptors. In studies of R1 mESCs and hiPSC-derived organoids, the authors have detected changes in the expression of markers of cortical layer neurons including CTIP2 and SATB2. While their findings can be extrapolated to suggest that eCB signal modulation leads to changes in cortical layer neurons in the mouse and human cerebral cortex, there is no such experiment in this study to support this (see major comment 1).

A related issue in the work is the lack of details as to how the authors determined the working concentrations of eCB modulating compounds. This becomes more important when interpreting findings for cultures exposed to combinations of agonist/antagonist (see major comment 2). Further, the authors show that knockdown of CB1 receptor affects neurodifferentiation but it is unclear if this is specific to the loss of CB1 expression (see major comment 3).

What is unique to this work is that the authors show that endocannabinoid expression is relevant to stem cell neurodifferentiation, and is modulated by pharmacological modulation. The authors should consider this when revising their manuscript text.

Major comments:
1. In their title, the authors claim "Endocannabinoid signalling in stem cells and cerebral organoids drives differentiation to deep layer projection neurons via CB1 receptors"
Without an experiment to show that endocannabinoid signalling drives differentiation to deep layer projection neurons via CB1 receptors, the authors cannot make this claim in their title.

2. A combination of the agonist THC and the inverse agonist AM-251 was applied to study eCB signalling in neurodifferentiating R1 mESCs. Given their different binding affinities, can the authors please clarify how how they arrived at the working concentrations? The authors used 100nM of HU-210 and 1microM of AM-251 in experiments.

Incubation with the agonist HU210 led to elevated NeuN and neuronal marker expression, but incubation with the agonist AM-251, did not. Does this mean that THC signalling promotes neurodifferentiation, but blockade with antagonist does not influence neurodifferentiation?

Were combinations of different concentrations of THC and AM-251 trialled and did this affect the results in the study?

3. Studies with a targeting shRNA construct shows that CB1 receptor expression is necessary for neurodifferentiation. However, the authors do not have sufficient data to show if this effect is specific to the loss of CB1, or if it is a non-specific effect. A rescue experiment with a knockdown-resistant CB1 receptor construct could be performed. Alternatively, the authors could treat CB1 knockdown cells with THC or HU-210 to determine if there is a partial rescue of neuronal marker gene expression.

Minor comments:
1. In the INTRODUCTION, the sentence, "Specifically, the cannabinoid receptor agonists THC and HU-210, as well as the monoacylglycerol lipase (MGL) inhibitor JZL-184, increased the generation of deep layer pyramidal neurons, at the expense of upper layer pyramidal neurons, by modulating the CTIP2/SATB2-dependent neurogenic program." is not accurate. The authors report that JZL-184 application on cerebral organoids led to changes in the expression of markers for deep layer pyramidal neurons, and a corresponding decrease in the expression of a marker for upper layer pyramidal neurons. Their findings suggest that eCB signalling via agonist and by CB1 receptor modulation influence cortical neuron layer markers during cerebral cortex development, but such direct evidence is lacking. Furthermore, the authors show that eCB modulation leads to changes in

CTIP2/BCL11B and SATB2, rather than to show that it modulates the “CTIP2/SATB2 neurogenic program”. Please consider revising this statement.

2. In the INTRODUCTION, the sentence, “This cell-autonomous role of eCB signalling contributes to understand the neurobiological impact of embryonic cannabinoid exposure and the associated risk to developing neuropsychiatric alterations and neurodevelopmental disorders.” is an over-reach. The authors have insufficient evidence to claim that the effects of eCB signalling are “cell-autnomous”. The knockdown studies merely show that CB1 signalling is necessary for the expression of layer markers in R1 mESCs at ND stage. For this statement to be true, the authors will have to perform a rescue experiment to show that the changes to marker expression are restored by balancing CB1 receptor expression and/or signalling (related to major comment 3).

3. In RESULTS, the subsection title, “The eCB system is induced during ES cell-derived default neuronal differentiation” is confusing. What do the authors mean by "default neuronal differentiation"?

4. In RESULTS, please clarify this statement, “After differentiation most cells were neurons, as indicated by TUJ1 and NeuN expression and immunofluorescence for vGLUT1 combined with vGAT1, or glutamate and GABA revealed that the majority of differentiated cells were excitatory glutamatergic neurons (Supplementary Fig. 1c).”. In Supp. Fig. 1c, the y-axis is labelled Cells+/Dapi+, which means that there are 70% vGlut+ cells and 18% GABA+ cells?
For the statement “…that the majority of differentiated cells were excitatory glutamatergic neurons” to be true, the authors would have to show proportions of NeuN+/vGlut+, or Tuj1+/vGlut+ cells. If not, this sentence will have to be revised.

5. In the RESULTS, for the authors to be confident of their statement, that “…inhibition of 2AG-degradation promotes deep layer neuronal differentiation by enhancing CB1 receptor activity.”, they should show that the effects of JZL-184 is corrected by a dominant-negative form of CB1, or by blockade of CB1 expression/function using their shRNA.

6. In the DISCUSSION, the statement “ES-derived differentiation of pyramidal neurons, particularly by promoting the generation of deep layer cortical neurons…” is one interpretation. It is possible that CB1 modulation leads to a delay in the specification of cortical neuron markers CTIP2 and SATB2 rather than the promotion of deep layer cortical neurons.

Reviewer 3

Advance summary and potential significance to field

Conceptual advance is marginal

Comments for the author

In the manuscript by Pariso-Luna et al., the authors investigate the role of endocanabinoid signalling in differentiation of deep layer neocortical projection neurons. In order to address this, they use mouse ES cells differentiated in vitro. The main conclusion of the manuscript is that CB1 receptor is required for correct proportions of deep/upper layer neurons. The authors show that eCB system is expressed during neuronal differentiation of mouse ES cells. They also use agonists and antagonists of the endocanabinoid signalling to show that it is required for deep layer production. They show a similar effect in human iPS derived human organoides. Although the work is relatively well done, I am not entirely convinced about the novelty of this manuscript. The authors have already reported in the mouse knock-out model that CB1 Receptor is required for deep layer production as well as to Pax6-Tbr2 transition. I do not really understand why the authors switched to mouse ES cells in vitro system, to basically confirm what they found in vivo several years ago?

One experiment with human iPS cells might partially justify this switch, but this is just one figure out of seven. However, it should be noted, this concern is partially balanced by the fact that the authors show CB1 mediated control of Ctip2 activity depends on Erk pathway, but not on mTORC1

or JNK. I think that this finding would potentially be novel and have more value if it was done in vivo, or with primary cells derived from the knock-out mice.

First revision

Author response to reviewers' comments

POINT BY POINT ANSWER TO REVIEWERS

Reviewer 1 Major points.

R: The manuscripts lacks novelty. The role of endocannabinoid signalling has extensively been studied in the mouse in vivo using full knock-out and conditional mutants and in vitro slice cultures. Using these approaches, the lab of the corresponding authors and other groups have shown effects of this signalling pathway on neural progenitors (Diaz-Alonso et al., 2015; Ao et al., 2020) and on the formation of deeper vs lower cortical projection neurons (Diaz-Alonso et al., 2012). As such the mouse 2D culture system does not provide novel insights compared to these previous study, in particular as it represents a somehow artificial system compared to the mouse as a model. The only novel aspect appears the use of a human organoid system but this has not been analysed in very much detail with no mechanistic insights. Also, the specificity of CB_1 signalling is not tested by using AM-251 inhibition in this system and the authors need to indicate how they did their cell counts.

A: We apologize if the novelty of our study was not appropriately explained in the original manuscript. Hence, we have amended this issue in the revised version. Whereas there have been several studies addressing the impact of CB_1 receptor regulation in prenatal pyramidal development, most of these studies were conducted *in vivo* (Díaz-Alonso et al., 2012, 2015, de Salas-Quiroga et al., 2015). In these studies, the interpretation of the results is always complex, and one cannot define a cell-autonomous or a non-cell- autonomous contribution of CB_1 receptor signalling to the regulation of neuronal differentiation. Hence, one of the objectives of the present study and the use of an alternative approach based on stem cells was to clarify this important aspect. Thanks to the editorial feedback and new experiments performed to improve the study, we have now generated additional pieces of evidence for the specificity of CB_1 receptor signalling manipulation both by pharmacological (JZL-184) treatment and shRNA-mediated CB_1 knockdown. These new findings have been included in the revised manuscript as **new Figures 3, 4 and Figs S2, S3 and S4.** Regarding the cerebral organoid studies, we fully agree with the importance to follow up with this important methodology, and this is planned indeed. Organoid-derived data so far were obtained in collaborative experiments and after the COVID19 crisis it will take still some time to set them up in our facilities, while travelling was not a possibility during these hectic times. We have included in the revised manuscript a more detailed explanation of cell quantification in the organoid experiments (**page 27**).

R: The mechanisms by which activating endocannabinoid signalling elicits an increase in Ctip2+ neurons and a concomitant reduction in Satb2+ neurons remains largely unclear. Based on their luciferase assays the authors seem to suggest that this pathway controls neuronal fate by controlling the transcriptional regulation of Ctip2 but the changes in promoter activity remain rather modest (less than 1.5 fold) which might not be sufficient to induce a fate change. In fact, there are a number of cells after CB_1 knock-down which express very low levels of Satb2 if at all (Supplementary Figure 4). This raises the possibility that these cells still express high levels of Ctip2 and might not even have changed fate. It will be important to quantify Ctip2 and Satb2 expression levels in individual knock-down cells rather than using bulk qRT-PCR. The authors should also consider the possibility that this signalling pathways acts on neural progenitor cells as was described recently by themselves and others (Diaz-Alonso et al., 2015; Ao et al., 2020). They should determine the proportions of radial glial cells, basal progenitors and neurons in their culture systems. A BrdU cell cycle exit experiment would also help to determine whether there is an increase in neuron formation at the expense of basal progenitor cell formation, i.e. an increase in direct neurogenesis which would explain the phenotype as well.

A: We are grateful to the reviewer for raising these relevant methodological notes, interpretation aspects and experimental proposals to improve the relevance of our study. During the revision process we assessed at the cellular level the expression of CTIP2 and SATB2 by immunofluorescence after CB_1 receptor knockdown, and we could confirm our previous qualitative analyses. We did so by quantifying CTIP2- and SATB2-positive cells in shCB_1 and shCtrl transfected GFP^+ cells. Unfortunately, whereas performing single-cell mRNA analyses were not feasible in a reasonable timeline in the context of the COVID19 pandemic, as an alternative approach we also quantified the CTIP2 and SATB2 immunoreactivity area in knockdown and control cells, providing similar results (**Fig. S3C, D**). The reviewer is correct in pointing to the likely existence of differential CB_1 receptor downstream signalling coupling in neural progenitors and postmitotic neurons. CB_1 receptors mediate apical to basal neural progenitor transition in a PI3K/mTORC1-dependent manner (Díaz-Alonso et al., 2015), whereas deep layer corticofugal neuronal differentiation and CTIP2 activity relies on ERK signalling (present study). Hence, we included in the revised Discussion a mention to this important mechanistic aspect that helps to clarify the different cell fate consequences of CB_1 receptor activation at different stages of neuronal differentiation. In addition, the revised manuscript includes a new Discussion paragraph regarding the limitations of our study (**page 16**) and the different mechanism by which CB_1 signalling can regulate neurogenesis (**page 19**).

R: There are discrepant results between the AM-251 treatment which has no effect and the CB_1 gene knock-down which causes an increased number of Satb2+ neurons. The authors do not explain this discrepancy raising doubts about the specificity of the reagents used. To address the specificity of the knock-down reagents, the authors should perform rescue experiments with a CB_1 expression construct to do experimentally. The authors should also explain why AM-251 treatment on its own has no effect. Is it because the pathway is not active under normal conditions?

A: The reviewer has correctly pointed to the differences that exist between the absence of effect of AM- 251 treatment in neuronal differentiation and CB_1 receptor knockdown. In these respect, the new rescue experiments of CB_1 receptor expression (**new Fig. 3 and Fig. S2**), on one hand confirmed the selectivity of the silencing strategy and the involvement of CB_1 receptors in neuronal differentiation. Similarly, these experiments allowed us to unequivocally ascribe a fundamental role of CB_1 receptors as essential mediators of the effect produced by MGL inhibition and increased endocannabinoid levels in neuronal differentiation. Overall, the absence of action of AM-251 alone can be attributed to the intrinsic and context-dependent complexity of action of CB_1 receptor antagonists and inverse agonists (Pertwee, Life Sciences 2005). Pharmacological regulation by CB_1 inverse agonists depend not only of their affinity, but also on the endocannabinoid tone, the constitutively active receptor population, and receptor density. Similarly, in other studies investigating neural cell fate regulation by endocannabinoid signalling, the use of the CB_1 inverse agonist AM251 (Xapelli et al., Plos One 2013; Rodrigues et al., Frontiers Pharmacol.2017) or SR141716 (Aguado et al., Faseb J., 2005; Díaz-Alonso et al., J. Neurosci. 2012; Cerebral Cortex 2015, 2017) prevented agonist-induced actions, but at times was ineffective alone.

R: It is unclear which statistical tests were used for individual experiments, hence it is impossible to evaluate the significance of the data. Rather than stating which types of statistical tests were performed in the Methods section in general, each figure legends should contain the relevant statistical test, n numbers and p-values.

A: We apologize for the lack of detailed information on this regard. We have included a **Table S4** that shows all the statistical information on the data.

R: Given the broad readership of “Development” the manuscript would greatly benefit from better descriptions of the endocannabinoid signalling pathway, the various enzymes and signalling molecules. A simple cartoon as part of figure 1 would certainly help to better illustrate this pathway to the non-expert reader.

A: As suggested, we have included a cartoon panel in **new Fig.1A** of the revised manuscript describing the major endocannabinoid synthesis and degradation enzymes and their functional connection with CB_1 receptors.

Reviewer Minor points: (p7: it should read “development” rather than “evolution”. p10: it should read Fig. 4a,b instead of Fig. 5a,b. The CB_1 Western blot in Figure 1C needs to be quantified.)

A: The misspellings and quantification of CB_1 Western blot have been corrected and included.

Reviewer 2 Major comments:

1. In their title, the authors claim “Endocannabinoid signalling in stem cells and cerebral organoids drives differentiation to deep layer projection neurons via CB_1 receptors” Without an experiment to show that endocannabinoid signalling drives differentiation to deep layer projection neurons via CB_1 receptors, the authors cannot make this claim in their title.

A: We thank the reviewer for raising this important note. During the revision period we performed new experiments to support the involvement of CB_1 receptors in JZL-184-induced actions in neuronal differentiation. Hence, JZL-184 was ineffective in both stable and acute CB_1 receptor-knockdown cells, but active in control cells. In addition, we observed increased endocannabinoids levels (2AG and AEA) as a consequence of MGL inhibition. These results are included in the new **Figure 4 and Fig. S4.**

2. A combination of the agonist THC and the inverse agonist AM-251 was applied to study eCB signalling in neurodifferentiating R1 mESCs. Given their different binding affinities, can the authors please clarify how they arrived at the working concentrations? The authors used 100nM of HU-210 and 1microM of AM- 251 in experiments. Incubation with the agonist HU210 led to elevated NeuN and neuronal marker expression, but incubation with the agonist AM-251, did not. Does this mean that THC signalling promotes neurodifferentiation, but blockade with antagonist does not influence neurodifferentiation? Were combinations of different concentrations of THC and AM-251 trialled, and did this affect the results in the study?

A: All pharmacological regulation studies were conducted after preliminary dose-dependency experiments in different cellular models including R1 cells. Although this was mentioned in the original manuscript, it has been clarified in the revised Methods section. Typically, in 2D stem-cell models, THC and the CB_1 antagonists SR141716 and AM251 above 10 micromolar negatively affect cell survival. Hence, low micromolar range is the optimal dose for THC to stimulate CB_1 signalling without inducing remarkable acute receptor desensitization and hence acting as a functional antagonist (Pertwee and Cascio, Handbook of cannabis, Oxford University Press, 2014). On the other hand, HU-210 is a much more potent CB_1 receptor agonist, and its optimal concentration for *in vitro* studies usually ranges from 50 to 100 nM. Thus, the use of HU-210 at these nM range of concentrations allows to evaluate the specificity of THC actions *via* CB_1 receptors. Along many years of experience, we and others have found and used similar concentrations of agonists and antagonists/inverse agonists to study CB_1 receptor-evoked signalling (*e.g.*, Liu et al., Biochem J, 2000; Galve-Roperh et al., Mol Pharmacol 2002; Aguado et al., J. Biol. Chem. 2007). The absence of effect of AM-251 alone can be attributed to the intrinsic and context-dependent complexity of action of CB_1 receptor antagonists and inverse agonists (*e.g.*, Pertwee, Life Sciences 2005). Pharmacological regulation by CB_1 inverse agonists depends not only of their affinity, but also on the endocannabinoid tone, the constitutively active receptor population, and receptor density. Similarly, in other studies investigating neural cell fate regulation by endocannabinoid signalling, the use of the CB_1 inverse agonists AM251 (*e.g.*, Xapelli et al., Plos One 2013; Rodrigues et al., Frontiers Pharmacol. 2017) or SR141716 (*e.g.*, Aguado et al., Faseb J., 2005; Díaz-Alonso et al., J. Neurosci. 2012; Cerebral Cortex 2015, 2017) prevented agonist-induced actions, but at times was ineffective alone. Hence, the intrinsically different mechanism of CB_1 receptor inhibition by genetic silencing and pharmacological manipulation through CB_1 inverse agonism may explain the differences observed, namely the absence of effect of AM- 251 treatment alone versus the CB_1 receptor knockdown-induced alterations of neuronal differentiation. In these respect, the new rescue experiments of CB_1 receptor expression, on one hand, confirmed the selectivity of the silencing strategy and the involvement of CB_1 receptors in neuronal differentiation. On the other hand, these experiments allowed us to unequivocally ascribe a fundamental role of CB_1 receptors as essential mediators of the effect produced by MGL inhibition -and the concerted increase of endocannabinoid levels- in neuronal differentiation. The revised manuscript includes a new Discussion paragraph regarding the limitations of our study (**page 16**).

3. Studies with a targeting shRNA construct shows that CB_1 receptor expression is necessary for neurodifferentiation. However, the authors do not have sufficient data to show if this effect is specific to the loss of CB_1, or if it is a non-specific effect. A rescue experiment with a knockdown-resistant CB_1 receptor construct could be performed. Alternatively, the authors could treat CB_1 knockdown cells with THC or HU- 210 to determine if there is a partial rescue of neuronal marker gene expression.

A: We thank the reviewer for this important note. We fully agree on the importance of such control. Hence, CB_1 expression rescue experiments have been performed. Results are included in **new Fig. 3** and demonstrate that inhibited deep layer neuronal differentiation in shCB1-ES cells is reversed by re- expression of a hCB1 resistant to shRNA-induced knockdown (immunofluorescence and gene expression analyses). Additional characterization and controls of CB_1 expression rescue are shown in **Fig. S2** including: CB_1 mRNA and protein levels (western blot and immunofluorescence), as well as the functional demonstration that restored CB_1 expression rescues the ability of the CB_1 agonist THC to activate the ERK pathway. In addition, in the revised manuscript we have included new evidence for the involvement of CB_1 receptors in the pro-neurogenic response induced by the MAGL inhibitor JZL-184. Thus, inhibition of MGL was ineffective in promoting deep layer neuronal differentiation in shCB1-ES cells, while it was effective in shControl cells (**New Fig. 4**).

Minor comments:

1. In the INTRODUCTION, the sentence, “Specifically, the cannabinoid receptor agonists THC and HU-10, as well as the monoacylglycerol lipase (MGL) inhibitor JZL-184, increased the generation of deep layer pyramidal neurons, at the expense of upper layer pyramidal neurons, by modulating the CTIP2/SATB2- dependent neurogenic program.” is not accurate. The authors report that JZL-184 application on cerebral organoids led to changes in the expression of markers for deep layer pyramidal neurons, and a corresponding decrease in the expression of a marker for upper layer pyramidal neurons. Their findings suggest that eCB signalling via agonist and by CB_1 receptor modulation influence cortical neuron layer markers during cerebral cortex development, but such direct evidence is lacking. Furthermore, the authors show that eCB modulation leads to changes in CTIP2/BCL11B and SATB2, rather than to show that it modulates the “CTIP2/SATB2 neurogenic program”. Please consider revising this statement.

A: As suggested, we have tempered the interpretation of our findings in the revised version of our study and have eliminated the concept of a “CTIP2/SATB2 neurogenic program”.

2. In the INTRODUCTION, the sentence, “This cell-autonomous role of eCB signalling contributes to understand the neurobiological impact of embryonic cannabinoid exposure and the associated risk to developing neuropsychiatric alterations and neurodevelopmental disorders.” is an over-reach. The authors have insufficient evidence to claim that the effects of eCB signalling are “cell-autnomous”. The knockdown studies merely show that CB_1 signalling is necessary for the expression of layer markers in R1 mESCs at ND stage. For this statement to be true, the authors will have to perform a rescue experiment to show that the changes to marker expression are restored by balancing CB_1 receptor expression and/or signalling (related to major comment 3).

A: We thank the reviewer for this important issue, that, as discussed above, has been addressed by new findings and controls derived from CB_1 expression rescue experiments.

3. In RESULTS, the subsection title, “The eCB system is induced during ES cell-derived default neuronal differentiation” is confusing. What do the authors mean by "default neuronal differentiation"?

A: We have edited the subsection title for “The eCB system is induced during ES cell-derived neuronal differentiation”.

4. In RESULTS, please clarify this statement, “After differentiation most cells were neurons, as indicated by TUJ1 and NeuN expression and immunofluorescence for vGLUT1 combined with vGAT1, or glutamate and GABA, revealed that the majority of differentiated cells were excitatory glutamatergic neurons (Supplementary Fig. 1c).”. In Supp. Fig. 1c, the y-axis is labelled Cells+/Dapi+, which means that there are 70% vGlut+ cells, and 18% GABA+ cells? For the statement

"…that the majority of differentiated cells were excitatory glutamatergic neurons" to be true, the authors would have to show proportions of NeuN+/vGlut+, or Tuj1+/vGlut+ cells. If not, this sentence will have to be revised.

A: As suggested, in the revised manuscript we have included an additional characterization of neuronal differentiation including the quantification of TUJ1+-vGLUT1+ neurons (Results, **page 6** and **Fig. S1D**).

5. In the RESULTS, for the authors to be confident of their statement, that "…inhibition of 2AG-degradation promotes deep layer neuronal differentiation by enhancing CB_1 receptor activity.", they should show that the effects of JZL-184 is corrected by a dominant-negative form of CB_1, or by blockade of CB_1 expression/function using their shRNA.

A: We thank the reviewer for raising the importance of this control. In agreement, we have performed new experiments with JZL-184 in shCB1-ES and shControl cells (**new Fig. 4**) and we could substantiate the data with pharmacological AM-251 antagonism. In addition, we have validated the increase in 2AG and the other major eCB AEA in JZL-184-differentiated neurons (**Fig. S4A**) and performed acute shCB1-mediated receptor knockdown controls (**Fig. S4B, C**).

6. In the DISCUSSION, the statement "ES-derived differentiation of pyramidal neurons, particularly by promoting the generation of deep layer cortical neurons…" is one interpretation. It is possible that CB_1 modulation leads to a delay in the specification of cortical neuron markers CTIP2 and SATB2, rather than the promotion of deep layer cortical neurons.

A: The revised manuscript includes a new paragraph in the Discussion section (**page 19**) regarding the alternative mechanisms by which CB_1 receptor signalling could influence neurogenesis.

Reviewer 3 Comments for the Author:

The authors show that eCB system is expressed during neuronal differentiation of mouse ES cells. They also use agonists and antagonists of the endocanabinoid signalling to show that it is required for deep layer production. They show a similar effect in human iPS derived human organoids. Although the work is relatively well done, I am not entirely convinced about the novelty of this manuscript. The authors have already reported in the mouse knock-out model that CB_1 Receptor is required for deep layer production as well as to Pax6-Tbr2 transition. I do not really understand why the authors switched to mouse ES cells in vitro system, to basically confirm what they found in vivo several years ago? One experiment with human iPS cells might partially justify this switch, but this is just one figure out of seven. However, it should be noted, this concern is partially balanced by the fact that the authors show CB_1 mediated control of Ctip2 activity depends on Erk pathway, but not on mTORC1 or JNK. I think that this finding would potentially be novel and have more value if it was done in vivo, or with primary cells derived from the knock-out mice.

A: We apologize for not making clear the novelty and rationale of our study in the original manuscript. Hence, we have clarified this issue in the revised version of the manuscript. Whereas there have been several studies addressing the impact of CB_1 receptor regulation in prenatal pyramidal development, most of these studies were conducted *in vivo* (Díaz-Alonso et al., 2012, 2015, de Salas-Quiroga et al., 2015; Harkany's group studies and others). In these studies, the interpretation of the results is always complex, and one cannot define a cell-autonomous or a non-cell-autonomous contribution CB_1 receptor signalling to the regulation of neuronal differentiation. Hence, one of the objectives of the present study and the use of an alternative approach based on stem cells was to clarify this important aspect.

In addition, the revised manuscript includes new results, as pointed by other reviewers, regarding the selectivity of CB_1 receptor knockdown experiments by the use of a genetic expression rescue strategy. We have also extended the demonstration of the requirement of CB_1 receptors in JZL-184-induced regulation of neuronal differentiation by new experiments in shCB_1-R1 cells. These new findings have been included in the revised manuscript as **new Figures 3, 4 and Figs S2-S4**. Overall, besides the identification of the ERK signaling pathway as the downstream effector of CB_1 signaling controlling Ctip2/Bcl11b expression, the revised manuscript contributes to the field, with new methodological approaches, demonstrating the cell-autonomous role of CB_1 receptors in pyramidal

neuron differentiation, and the possibility to pharmacologically regulate this process either directly (CB_1 agonists) or indirectly (MGL inhibition).

Whereas we agree that confirming the involvement of ERK pathway in CB_1 receptor mediated control of Ctip2 activity *in vivo* would be relevant, we had to concentrate efforts in the limited time available for reviewing our study in the context of the disruptions caused by the COVID19 pandemic, to unequivocally demonstrate the selectivity of CB_1 receptor manipulation. Inhibition of ERK signalling as a crucial pro- survival and proliferation pathway during embryonic development requires significant experimental validation to avoid toxicity. Hence, addressing the involvement of ERK signalling *in vivo* would overcome the limits of the present study. In any case, we have enriched the Discussion of the revised manuscript including previous evidence that support that ERK signalling is crucial for deep layer V and corticospinal neuronal development *in vivo* (Xing et al., 2016).

Second decision letter

MS ID#: DEVELOP/2020/192161

MS TITLE: Endocannabinoid signalling in stem cells and cerebral organoids drives differentiation to deep layer projection neurons via CB1 receptors

AUTHORS: Juan Paraiso-Luna, Jose Aguareles, Ricardo Martin, Ane C Ayo-Martin, Samuel Simon-Sanchez, Daniel Garcia-Rincon, Carlos Costas-Insua, Elena GarcÃ¤A-Taboada, Adan De Salas-Quiroga, Javier Diaz-Alonso, Isabel Liste, Jose Sanchez-Prieto, Silvia Cappello, Manuel Guzman, And Ismael Galve-Roperh

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

The reviewers’ evaluation is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address their remaining suggestions and comments. 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

N/A as this is a re-review

Comments for the author

While the authors have addressed some of my concerns, a number of open questions still remain.

1) Novelty: I can understand that the authors could not perform further experiments on the human ornganoids. I also start to see from their rebuttal letter what might be novel about this study but this is not made any clearer in the main text. The authors should include a few sentences in their introduction about what are the questions left open from previous analyses that this paper is going to address.

2) Mechanisms underlying the increased formation of deep layer neurons: I appreciate that the authors have quantified Ctip2 and Satb2 expression levels but they have not addressed with any experiments the possibility that CB1 signalling might act at the progenitor level. For this reason, they cannot conclude that CB1 promotes the formation of deep layer neurons thought the regulation of Bcl11b and Satb2 expression in their system. They should remove all sentences making this over-conclusion and discuss the various possibilities that could lead to this phenotype side by side.

3) Statistical tests: This has very much approved but it looks like the authors only provide p-values of the post-hoc tests, p values for the One-way ANOVA are missing.

4) Endocannaboid signalling: I acknowledge that the authors have added a schematic illustrating the CB1 signalling pathway, however, the schematic appears overly complex containing a lot of irrelevant information. Moreover, this schematic is not explained in the text at all making it impossible for a non-expert on endocannaboids to understand this pathway and the different pharmacological reagents used in this study. A couple of sentences at the beginning of the results section would have done a great job. Finally, all abbreviations in the schematic should be explained in the figure legend.

Minor point: The term “evolution” was not amended.

Reviewer 2

Advance summary and potential significance to field

The advance made to this paper is a demonstration of the cell-autonomous impact of CB1 receptor signalling on the development and maturation of cerebral cortical neurons within cerebral organoids. By combining studies of gene disruption (knockdown) and rescue, with an investigation of the pharmacological disruption of CB1 signalling in cerebral organoids, the authors provide a powerful means through which one may analyse the molecular and cellular effects of CB1 function in cerebral cortex development. These findings have potential implications for our understanding of fetal exposure to modulators of CB1 signalling and the production and maturation of neurons in the brain.

The authors have now revised the manuscript to a form which is suitable for publication, pending a few suggestions to very minor edits to the manuscript file (see below). In the revised version of the manuscript, the authors have conducted additional experiments to address the concerns of all reviewers, provided additional clarification of procedures and protocols and deposited their raw data in Dryad, although I could not access the data when I clicked on the weblink.

Comments for the author

Minor comments:
1. For the sentence, "This cell-autonomous role of eCB signalling contributes to understand the neurobiological impact of embryonic cannabinoid exposure and the associated risk to developing neuropsychiatric alterations and neurodevelopmental disorders."

Please consider revising to, "These findings for a cell-autonomous role for eCB signalling contributes to our understanding of the neurobiological impact of embryonic cannabinoid exposure and the associated risk in developing neuropsychiatric alterations and neurodevelopmental disorders."

2. For the sentence, “Considering the available evidences of CB1 receptors role in neuronal differentiation in vivo (Mulder et al., 2008; Díaz-Alonso et al., 2012; de Salas- Quiroga et al., 2015)

and in vitro (present study) we characterized in more detail the impact of CB1 receptor signalling manipulation by direct pharmacological regulation."

Please consider revising to, "Given the evidence for CB1 receptors in neuronal differentiation in vivo (Mulder et al., 2008; Díaz-Alonso et al., 2012; de Salas- Quiroga et al., 2015), we next characterized the impact of CB1 receptor signalling by direct pharmacological manipulation."

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

1) Novelty: I can understand that the authors could not perform further experiments on the human organoids. I also start to see from their rebuttal letter what might be novel about this study but this is not made any clearer in the main text. The authors should include a few sentences in their introduction about what are the questions left open from previous analyses that this paper is going to address.

A: We thank the reviewer for this note that will make clearer to the reader the novelty of our study. Accordingly, we have included a new paragraph in the Introduction (page 5) describing the rationale for this new study.

2) Mechanisms underlying the increased formation of deep layer neurons: I appreciate that the authors have quantified Ctip2 and Satb2 expression levels but they have not addressed with any experiments the possibility that CB1 signalling might act at the progenitor level. For this reason, they cannot conclude that CB1 promotes the formation of deep layer neurons thought the regulation of Bcl11b and Satb2 expression in their system. They should remove all sentences making this over-conclusion and discuss the various possibilities that could lead to this phenotype side by side.

A: In this regard, we would like to point out that, in our previous study by Diaz-Alonso et al. (2012), we demonstrated that CB1 receptor regulation of deep layer neuron differentiation is a postmitotic event. Thus, deep layer V neuronal differentiation was impaired in Nex-CB1-deficient mice similarly to complete CB1- deficient mice. Importantly, neural progenitor proliferation and identity was not affected in Nex-CB1- deficient mice, as it is the case in complete CB1-deficient mice. Hence, we have included a new sentence in the Discussion section (page 19) to make this information evident to the reader and support the interpretation of the data from the present manuscript.

3) Statistical tests: This has very much approved but it looks like the authors only provide p-values of the post-hoc tests, p values for the One-way ANOVA are missing.

A: New Supplementary Fig. 4 has been amended to include the requested p values.

4) Endocannaboid signalling: I acknowledge that the authors have added a schematic illustrating the CB1 signalling pathway, however, the schematic appears overly complex containing a lot of irrelevant information. Moreover, this schematic is not explained in the text at all making it impossible for a non- expert on endocannaboids to understand this pathway and the different pharmacological reagents used in this study. A couple of sentences at the beginning of the results section would have done a great job. Finally, all abbreviations in the schematic should be explained in the figure legend.

A: In agreement, we have made an explanatory reference to the eCB signalling pathway shown in Fig. 1A in the Results section (page 7) and we completed the abbreviations explanations in Figure 1 legend (page 31).

Minor point: The term “evolution” was not amended.

A: We apologize for having missed this issue. It has been modified in the revised manuscript.

Reviewer 2. In the revised version of the manuscript, the authors have conducted additional experiments to address the concerns of all reviewers, provided additional clarification of procedures and protocols, and deposited their raw data in Dryad, although I could not access the data when I clicked on the weblink.

A: Regarding the raw data in Dryad, we uploaded the data and we remain waiting for instructions from the server. Currently, in the present link (https://datadryad.org/stash/share/_ub5ATJzhMOuOO48wr4ffexnN8yYq3rZCSNlFvpqelM) the data can be downloaded but the quality control of the system is still pending

Minor comments: …

A: We thank the reviewer for the suggestions to improve the writing of the mentioned sentences, and accordingly we have incorporated them in the new version of our manuscript.

Third decision letter

MS ID#: DEVELOP/2020/192161

MS TITLE: Endocannabinoid signalling in stem cells and cerebral organoids drives differentiation to deep layer projection neurons via CB1 receptors

AUTHORS: Juan Paraiso-Luna, Jose Aguareles, Ricardo Martin, Ane C Ayo-Martin, Samuel Simon-Sanchez, Daniel Garcia-Rincon, Carlos Costas-Insua, Elena Garcia-Taboada, Adan De Salas-Quiroga, Javier Diaz-Alonso, Isabel Liste, Jose Sanchez-Prieto, Silvia Cappello, Manuel Guzman, And Ismael Galve-Roperh

ARTICLE TYPE: Research Article

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