Δ9-tetrahydrocannabinol inhibits Hedgehog-dependent patterning during development

Hsiao-Fan Lo, Mingi Hong, Henrietta Szutorisz, Yasmin L. Hurd and Robert S. Krauss
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MS TITLE: Δ9-tetrahydrocannabinol inhibits Hedgehog-dependent patterning during development

AUTHORS: Hsiao-Fan Lo, Mingi Hong, Henrietta Szutorisz, Yasmin L. Hurd, and Robert S. Krauss

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

As you will see, the referees express considerable interest in your work, but have some questions and criticisms that require attention before we can consider publication. All three referees make constructive suggestions to improve the presentation of your data and raise points that need your clarification. If you are able to revise the manuscript along the lines suggested, which may involve further experiments or inclusion of additional data, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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 manuscript presents a series of studies examining the developmental impact of prenatal THC exposure on craniofacial development neural patterning and investigating inhibition of Sonic Hedgehog signaling as a mechanism of action. These studies follow publications from other groups demonstrating that endocannabinoids and cannabinoids have the capacity to inhibit Shh signaling and potentially cause craniofacial malformations. The present study benefits from incorporation of a sensitizing genetic factor that reveals THC exposure as a "conditional teratogen", or a chemical that is unlikely to independently cause malformations but could contribute in the presence of other genetic or environmental factors. The manuscript also presents the most meticulous mechanistic analysis of Shh pathway inhibition by THC to-date and makes a compelling case that inhibition occurs independently of cannabinoid receptor 1 as recently suggested. The manuscript is nicely constructed and the data appear of high quality and are described clearly and appropriately. As a whole, this study provides important context to our understanding of developmental impact of THC exposure and new and important insight into the underlying mechanism of action, and would be a valuable contribution to the developmental biology and developmental toxicology fields. I have only minor comments to consider.

Comments for the author

Minor comments:
1. In the third to las sentence in the introduction, the preface of "In contrast" is confusing and should be removed.
2. In the discussion section, the last sentence of the second paragraph beginning "In fact, THC treatment and Shh…." is unclear and should be restated.
3. A recent paper by Shiota et al in BDRA (PMID: 33660946) on the intrauterine fate of embryos with HPE could be added as a reference in the last paragraph of the discussion.

Reviewer 2

Advance summary and potential significance to field

The study presented here demonstrates that the psychoactive cannabinoid THC and related molecule CBD can attenuate Sonic Hedgehog (SHH) signaling through targeting the SHH signal transducing protein Smoothened (SMO). The results clearly show that when combined with a subthreshold deficit in SHH signaling, in this case knockout of the gene encoding a SHH co-receptor Cdon, exposure to THC can induce mild Holoposencephaly (HPE) phenotypes. THC, but not CBD, is found to attenuate SMO trafficking into the primary cilium and reduce the SHH transcriptional response. CBD is also shown to reduce SHH-target gene induction, and despite being related to THC, appears to act through a slightly different mechanism (not determined here). The studies are directly relevant to human health because THC and CBD exposures tested in mouse studies are likely to correlate with what humans could reasonably be exposed to in cases of recreational or medicinal cannabis use.
The work clarifies a misconception in the literature about whether cannabinoid receptors are contributing factors for attenuation of SHH signaling by THC or CBD – they are not. THC is clearly shown to act through SMO. The manuscript is well written, experiments are well designed and executed, and the results are very clearly and presented.

Comments for the author

All of the points raised below are minor and can be addressed with text and/or figure edits.
Cdon +/- mice exposed to THC look to have a very minor phenotypic shift with reduced SHH target gene expression. It does not qualify as HPE, so is not discussed in the results, but I'm wondering if this is a meaningful change or just noise in the phenotypic presentation.

Figure 1A – it would be helpful to indicate the dose of THC used in the figure next to the embryo images to appreciate how it correlates with the dose curve shown in 1B.

Page 9 – perhaps rephrase the sentence "…perturbation of primary cilia, the cellular site of SMO signaling." to "…perturbation of primary cilia, the cellular site of signaling to GLI." because GLI-independent signals can occur from outside the PC.

Page 10 – "CBD may therefore inhibit HH signaling by a mechanism somewhat distinct from either SANT-1-type or cyclopamine-type SMO inhibitors." You might consider discussing some potential alternative mechanisms. The TM core binding site in SMO is very deep with several potential binding postures for small molecules. CBD might bind in a deeper portion of the transmembrane pocket than cyclopamine, so does not displace the bodipy label. Deeper binding may induce different inhibitory conformation shifts that don't alter ciliary trafficking, but still block the active conformation. It could also be that CBD binds to the amino-terminal cysteine rich domain and displace allosteric activators that bind this site. These points could be added to the discussion on page 14.

Figure 5: The method for how bodipy signal intensity is shown is a little unclear.
Is signal intensity shown per field of cells or per a specified number of cells or per field/experiment? The effect is obvious in the images shown, so I don't think any experiments need to be repeated for quantification. The method just needs to be expanded for clarity.

Reviewer 3

Advance summary and potential significance to field

The manuscript describes studies to examine the effect of Δ9-tetrahydrocannabinol (THC) on Hedgehog signalling and on embryonic development in fibroblast cells and in mouse embryos. Given the potential for THC exposure during pregnancy understanding whether and how this may act as a risk factor for birth defects is an important question.

THC has previously been found to repress Shh signalling in a reporter cell line and the authors have confirmed this finding in 3T3 cells and provide evidence that THC acts at the level of SMO as found for other cannabinoids and insight into the mechanism of action in vitro.

Interestingly, the effect of THC did not depend on CBR1, which had been proposed to mediate the effect of cannabinoids on Hh signalling. This receptor also appeared unlikely to be required for THC effects on neural tube patterning – highlighting a need for future studies to address this mechanism.

A novel approach to reveal potential effects of THC during development is the use of Cdon null mice which are sensitised to Hh signalling impairment. This suggests that THC exposure may cause holoprosencephaly, which could be mediated through impaired Hh signalling, as well as ventral neural tube patterning alterations.

Comments for the author

The authors confirm that THC inhibits response to Hh signalling in vitro – likely through action at the level of SMO. These studies are well described and illustrated.

The authors show evidence that CB1R is absent or at very low levels prior to E11.5, does not bind SMO based on lack of co-i.p. and did not alter in vitro THC inhibition of HH response. While these studies are no definitive they suggest that if THC affects HH signalling this not via the different receptor.

My main comments focus on the analysis of THC effects in vivo – (i) evidence that there is a THC-mediated phenotype and (ii) that is mediated via suppression of Hh signaling.
In the mouse studies, there is an apparent additive effect of THC treatment and Cdon loss of function which sensitizes to HPE. I think the conclusions could be better supported by an expanded description of the HPE phenotype and analysis of impaired Hh signalling, in particular:

- An image of affected fetus is shown at E14. Given that the numbers are not large and the phenotype is scored as yes/no for HPE, more information should be given about how this was scored e.g. what were the criteria used for defining HPE, what were the quantitative measures?
- At E10.5 (Fig 1C) and the authors describe impaired Hh signalling on the basis of reduced Gli1 expression in the rostroventral midline. This experiment is based on quantification of WMISH – this is not a quantitative technique so additional evidence is needed to claim this (e.g. qRT-PCR), particularly in view of questions about potential effect of THC on growth/development (see below). Sections of the WMISH would also help reveal the tissue location of described abnormalities.
- I would also suggest that ANOVA would be more appropriate than multiple t-tests for the analysis.
- At E10.5, The THC treated Cdon-/- embryo appears smaller but without a whole embryo image or quantification of stage/size (e.g. defined by somite numbers and crown-rump length), it is not clear whether the THC treatment had an overall retarding effect on growth and development. This would also help with interpretation of the assessment of whether the NT patterning alteration (at E9.5) represents a specific effect of THC.
- It is surprising that Hh signalling read-out in the forebrain was not assessed at earlier stages, especially given that treatment was applied at E7.5 and analysed at E10.5 whereas abnormal Hh dependent patterning of the forebrain (ie, the most relevant domain for HPE) would be detectable earlier. Indeed, embryos were collected at E9.5 for the analysis of spinal cord patterning. For the NT counts the raw data (ie, numbers of sections and numbers of positive/total cells should be included in supplementary).

Additional points
- Stage of treatment differs in Fig 1 and 2 so the stage of treatment should be included with the dose information in the fig legends)
- Fig1B shows the proportion of affected fetuses but it is necessary to go to supplementary material for details (Table S1 – incidentally the column #Cdon +/-, should read -/- I assume). It would be easy to describe numbers of embryos scored as HPE (eg, 4/13 and 4/11 at 10 mg/kg and 15 mg/kg) in the legend.
- It is a strength of the study that the levels of THC in maternal plasma was investigated (Fig S1) – as this is stated as THC and metabolites it would be helpful to include some detail in this Fig legend of what was actually measured in this kit.
- Fig 1. Describe angle and level of section (preferably indicate levels on whole embryo image). Scale bars images are missing in A and C)
- Fig 1 and 2. These include a mixture of stages and it would help the reader to indicate these on the figures (fig 2 currently does not mention the stage in the legend either).
- As part of in vitro studies, the authors compared effects of THC with CBD. The authors discuss the possibility that CBD (which may be more widely consumed than THC) should be tested for conditional teratogenicity – given that they used this extensively in cells these experiments could presumably have been tried?

First revision

Author response to reviewers' comments

We thank the reviewers for their helpful comments about the manuscript. Responses to specific comments follow.

Reviewer 1 Advance Summary and Potential Significance to Field:

The manuscript presents a series of studies examining the developmental impact of prenatal THC exposure on craniofacial development neural patterning and investigating inhibition of Sonic Hedgehog signaling as a mechanism of action. These studies follow publications from other groups demonstrating that endocannabinoids and cannabinoids have the capacity to inhibit Shh signaling and potentially cause craniofacial malformations. The present study benefits from incorporation of a sensitizing genetic factor that reveals THC exposure as a

"conditional teratogen", or a chemical that is unlikely to independently cause malformations but could contribute in the presence of other genetic or environmental factors. The manuscript also presents the most meticulous mechanistic analysis of Shh pathway inhibition by THC to-date and makes a compelling case that inhibition occurs independently of cannabinoid receptor 1 as recently suggested. The manuscript is nicely constructed and the data appear of high quality and are described clearly and appropriately. As a whole, this study provides important context to our understanding of developmental impact of THC exposure and new and important insight into the underlying mechanism of action, and would be a valuable contribution to the developmental biology and developmental toxicology fields. I have only minor comments to consider.

We thank the reviewer for their supportive comments.

Reviewer 1 Comments for the Author:

Minor comments:
1. In the third to las sentence in the introduction, the preface of "In contrast" is confusing and should be removed.

The reviewer refers to a sentence in the Abstract, rather than the Introduction, and we have made this change.

2. In the discussion section, the last sentence of the second paragraph beginning "In fact, THC treatment and Shh…." is unclear and should be restated.

We rephrased this sentence as: "In fact, THC treatment and *Shh* heterozygosity acted similarly in *Cdon*$^{-/-}$ mice, in that each enhanced the effects of *Cdon* mutation on facial midline and VNT patterning, but neither was sufficient to perturb pattering on their own (Tenzen et al., 2006)."

3. A recent paper by Shiota et al in BDRA (PMID: 33660946) on the intrauterine fate of embryos with HPE could be added as a reference in the last paragraph of the discussion.

We have added this reference at the suggested spot.

Reviewer 2 Advance Summary and Potential Significance to Field:

The study presented here demonstrates that the psychoactive cannabinoid THC and related molecule CBD can attenuate Sonic Hedgehog (SHH) signaling through targeting the SHH signal transducing protein Smoothened (SMO). The results clearly show that when combined with a subthreshold deficit in SHH signaling, in this case knockout of the gene encoding a SHH co-receptor Cdon, exposure to THC can induce mild Holoposencephaly (HPE) phenotypes. THC, but not CBD, is found to attenuate SMO trafficking into the primary cilium and reduce the SHH transcriptional response. CBD is also shown to reduce SHH-target gene induction, and despite being related to THC, appears to act through a slightly different mechanism (not determined here). The studies are directly relevant to human health because THC and CBD exposures tested in mouse studies are likely to correlate with what humans could reasonably be exposed to in cases of recreational or medicinal cannabis use. The work clarifies a misconception in the literature about whether cannabinoid receptors are contributing factors for attenuation of SHH signaling by THC or CBD – they are not. THC is clearly shown to act through SMO. The manuscript is well written, experiments are well designed and executed, and the results are very clearly and presented.

We thank the reviewer for their supportive comments.

Reviewer 2 Comments for the Author:

All of the points raised below are minor and can be addressed with text and/or figure edits.

Cdon +/- mice exposed to THC look to have a very minor phenotypic shift with reduced SHH target gene expression. It does not qualify as HPE, so is not discussed in the results, but I'm wondering if this is a meaningful change or just noise in the phenotypic presentation.

We thank the reviewer for this question. We have sometimes seen minor changes in gene expression in *Cdon* heterozygotes (with or without THC exposure), but these are apparently insufficient to result in HPE phenotypes. It is difficult to rigorously discern whether these changes are meaningful or noise (or both). This issue is of potential significance to human HPE, where all mutations are heterozygous, as well as differences between mice and humans, but we feel that exploring this question – even in the text only – is beyond the scope of the current manuscript. We hope the reviewer agrees.

Figure 1A - it would be helpful to indicate the dose of THC used in the figure next to the embryo images to appreciate how it correlates with the dose curve shown in 1B.

We have added this information to Figures 1A and 1C.

Page 9 - perhaps rephrase the sentence "…perturbation of primary cilia, the cellular site of SMO signaling." to "…perturbation of primary cilia, the cellular site of signaling to GLI." because GLI-independent signals can occur from outside the PC.

We have made this change.

Page 10 - "CBD may therefore inhibit HH signaling by a mechanism somewhat distinct from either SANT-1-type or cyclopamine-type SMO inhibitors." You might consider discussing some potential alternative mechanisms. The TM core binding site in SMO is very deep with several potential binding postures for small molecules. CBD might bind in a deeper portion of the transmembrane pocket than cyclopamine, so does not displace the bodipy label. Deeper binding may induce different inhibitory conformation shifts that don't alter ciliary trafficking, but still block the active conformation. It could also be that CBD binds to the amino-terminal cysteine rich domain and displace allosteric activators that bind this site. These points could be added to the discussion on page 14.

We thank the reviewer for this comment and have added the following sentences to the Discussion: "The SMO transmembrane domain has several potential binding modalities for small molecules (Kowatsch et al., 2019; Qi and Li, 2020), and it is possible that CBD binds in a deeper portion of the transmembrane pocket than cyclopamine, and so does not displace it. Such binding may induce a SMO conformation that does not efficiently block ciliary trafficking but inhibits adoption of an active conformation. Alternatively, CBD could bind to the amino-terminal cysteine-rich domain, displacing allosteric regulators (Huang et al., 2018)".

Figure 5: The method for how bodipy signal intensity is shown is a little unclear. Is signal intensity shown per field of cells or per a specified number of cells or per field/experiment? The effect is obvious in the images shown, so I don't think any experiments need to be repeated for quantification. The method just needs to be expanded for clarity.

The cells were imaged on a Leica DM5500 B upright microscope, capturing 10 fields of view from each of three experiments, with the mean intensity from the 10 fields/experiment plotted as data points in Figure 5B. We have added this information to the Methods section.

Reviewer 3 Advance Summary and Potential Significance to Field:

The manuscript describes studies to examine the effect of Δ9-tetrahydrocannabinol (THC) on Hedgehog signalling and on embryonic development in fibroblast cells and in mouse embryos. Given the potential for THC exposure during pregnancy understanding whether and how this may act as a risk factor for birth defects is an important question.

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We thank the reviewer for their supportive comments.

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-An image of affected fetus is shown at E14. Given that the numbers are not large and the phenotype is scored as yes/no for HPE, more information should be given about how this was scored e.g. what were the criteria used for defining HPE, what were the quantitative measures?

Mice were scored as positive for HPE if they displayed a fused upper lip, an unambiguous phenotype without gradation that arises as a consequence of defective craniofacial midline patterning (Hong et al. 2012, in the references). We have added this statement to the second paragraph of the Results.

-At E10.5 (Fig 1C) and the authors describe impaired Hh signalling on the basis of reduced Gli1 expression in the rostroventral midline. This experiment is based on quantification of WMISH - this is not a quantitative technique so additional evidence is needed to claim this (e.g. qRT- PCR), particularly in view of questions about potential effect of THC on growth/development (see below). Sections of the WMISH would also help reveal the tissue location of described abnormalities.

We thank the reviewer for this comment. We have dropped the quantification of the WMISH signal and instead performed the suggested qRT-PCR analysis for several HH pathway target genes (new Figure 1D). The new results are consistent with the WMISH results in Figure 1C and bolster our claim that impaired HH signaling occurs in vivo in response to THC and underlies the phenotype. We have also pointed out more clearly midline alterations seen in *THC-treated* Cdon *mutants (Figure 1A), and quantified the reduced width of the nasal septum cartilage in THC-treated* Cdon$^{-/-}$ *embryos (new Figure S2). Unfortunately, the embryos used for WMISH were discarded, and we are not in a financial position to perform any further more work for this project (please see the comment below about CBD experiments for a fuller explanation). We hope the reviewer will agree*

that the combination of well-documented, mild HPE phenotypes, diminished HH target gene expression seen in qRT-PCR analyses, and VNT patterning defects combine to support our overall conclusion that THC inhibits HH signaling in Cdon *mutant embryos.*

-I would also suggest that ANOVA would be more appropriate than multiple t-tests for the analysis.

We reanalyzed the data in Figures 1D, 2B, 3A, 3B, 4B, 5B, S2, and S3 with ordinary one-way ANOVA, with Tukey's multiple comparison test. All conclusions held up to the new statistical analysis.

-At E10.5, The THC treated Cdon-/- embryo appears smaller but without a whole embryo image or quantification of stage/size (e.g. defined by somite numbers and crown-rump length), it is not clear whether the THC treatment had an overall retarding effect on growth and development. This would also help with interpretation of the assessment of whether the NT patterning alteration (at E9.5) represents a specific effect of THC.

Embryo stages were defined by number of somites. THC did not have obvious effects on embryo size at any stage. We have now added this information to the figure legends and text.

-It is surprising that Hh signalling read-out in the forebrain was not assessed at earlier stages, especially given that treatment was applied at E7.5 and analysed at E10.5 whereas abnormal Hh dependent patterning of the forebrain (ie, the most relevant domain for HPE) would be detectable earlier. Indeed, embryos were collected at E9.5 for the analysis of spinal cord patterning. For the NT counts the raw data (ie, numbers of sections and numbers of positive/total cells should be included in supplementary).

As described above, we have now assessed HH signaling at an earlier stage in the requested qRT-PCR experiments shown in new Figure 1D. For the VNT results we now report the number of sections analyzed, and numbers of FOXA2+, NKX2.2+, OLIG2+ and total NT cells, in the Methods and newly added Supplementary Table 2.

Additional points

-Stage of treatment differs in Fig 1 and 2 so the stage of treatment should be included with the dose information in the fig legends)

We have added this information to the figure legends.

-Fig1B shows the proportion of affected fetuses but it is necessary to go to supplementary material for details (Table S1 - incidentally the column #Cdon +/-, should read -/- I assume). It would be easy to describe numbers of embryos scored as HPE (eg, 4/13 and 4/11 at 10 mg/kg and 15 mg/kg) in the legend.

We have added this information to the figure legend.

-It is a strength of the study that the levels of THC in maternal plasma was investigated (Fig S1) - as this is stated as THC and metabolites it would be helpful to include some detail in this Fig legend of what was actually measured in this kit.

The kit measures total levels of THC (Δ9-THC) plus the THC metabolites (11-hydroxy-THC and 11-nor-9-carboxy-THC). We have added this information to the legend for Figure S1.

-Fig 1. Describe angle and level of section (preferably indicate levels on whole embryo image). Scale bars images are missing in A and C)

We have added a graphic to describe the angle and level of embryo sections in Figure 1A. Scale bars have been added for Figures 1A and 1C.

-Fig 1 and 2. These include a mixture of stages and it would help the reader to indicate these on

the figures (fig 2 currently does not mention the stage in the legend either).

We have added this information to the figure legends.

-As part of in vitro studies, the authors compared effects of THC with CBD. The authors discuss the possibility that CBD (which may be more widely consumed than THC) should be tested for conditional teratogenicity - given that they used this extensively in cells these experiments could presumably have been tried?

We agree with the author that these would be worthwhile studies. Unfortunately, we have not had a chance to perform any in vivo experiments with CBD. While we are eager to know the effects of CBD in vivo, we view such experiments as beyond the scope of the present study as they would require detailed dose-responses tests and multiple types of analysis. It had not been in our initial plans to investigate CBD in this study, and we mainly used it as an in vitro control for THC. Because the effects of THC on SMO translocation to primary cilia were different from those reported for CBD in Khaliullina et al., we felt it was incumbent on us to address whether we could reproduce these earlier results with CBD. Our results with CBD in vitro are similar to those of Khaliullina et al., revealing that THC and CBD act somewhat differently on HH signaling. CBD therefore also became a useful control for our studies with bodipy-cyclopamine.

This study was supported by a two-year award from the NIH. The studies were interrupted by the pandemic, and although we were fortunate to be able to continue salary support during lockdown to those who did the work, this has resulted in the grant funds now being fully expended. We hope to acquire additional support to rigorously study CBD in vivo, but to do this properly will require a new line of support and significant time. We hope the reviewer will understand our current situation.

Second decision letter

MS ID#: DEVELOP/2021/199585

MS TITLE: Δ9-tetrahydrocannabinol inhibits Hedgehog-dependent patterning during development

AUTHORS: Hsiao-Fan Lo, Mingi Hong, Henrietta Szutorisz, Yasmin L. Hurd, and Robert S. Krauss
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

The revised version of the manuscript retains the strengths outlines in my initial review while addressing each of the comments raised.

Comments for the author

The revised version of the manuscript retains the strengths outlines in my initial review while addressing each of the comments raised.

Reviewer 3

Advance summary and potential significance to field

The manuscript describes studies to examine the effect of Δ9-tetrahydrocannabinol (THC) on Hedgehog signalling and on embryonic development in fibroblast cells and in mouse embryos. Given the potential for THC exposure during pregnancy understanding whether and how this may act as a risk factor for birth defects is an important question.

Reviewer 3 Comments for the Author:

The authors have made revisions to the manuscript and I agree with their comments and changes. Congratulations on this interesting study.