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## Miswiring the brain: $\Delta^9$ -tetrahydrocannabinol disrupts cortical development by inducing an SCG10/stathmin-2 degradation pathway

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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.)

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1st Editorial Decision

19 July 2013

Thank you for submitting your manuscript to The EMBO journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the analysis interesting and important. However, they also raise a number of significant concerns that would have to be resolved in order to consider publication here. Both referees 2 and 3 find that the data at present is not strong enough to support that the effects of cannabis treatment on synaptic plasticity and wiring are mediated via altered JNK/ERK activation and SCG10 degradation. This issue would have to be resolved with the inclusion of additional data. The referees suggest a number of ways to address this issue, but there might be other ways to do so as well. Should you be able to address the concerns raised in full, then I would like to invite you to submit a suitably revised version for our consideration. I should remind you that it is our policy to allow one major round of revision and that it is therefore important to resolve the raised concerns at this stage.

I also realize that to address the concerns raised that this can take some additional time beyond the 3 months that we normally allow for revisions. In this case I can extend the revision to 6 months if that is helpful.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:

<http://www.nature.com/emboj/about/process.html>

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

## REFEREE REPORTS

### Referee #1

In this study, the authors have sought to identify proteins involved in neuronal development that could be significantly altered following THC administration in utero. The issue of whether or not marijuana abuse might impair CNS development is a very important one and only seldom it has been approached in such a rigorous, comprehensive and mechanism-oriented manner. The authors are to be commended for the accuracy and in-depth of their investigation, which clearly provides an unprecedented insight on potential targets of pre-natal THC abuse and the way they modify neuronal development and function. I have only three minor, but nevertheless important points that the authors may wish to address in a revised version:

1) The authors suggest that THC is acting as a functional antagonist in this experimental set-up, and indeed they provide both pharmacological and knockout mouse data to substantiate this hypothesis. However, the authors also suggest that THC effect might be due to counteraction of endocannabinoid tone at CB1 whereas the possibility also exists that chronic THC administration causes instead desensitization and decreased expression-targeting of CB1 receptors. Did the authors perform any experiment other than that shown in fig. 1 to distinguish between the two possibilities? Furthermore, what was the overall effect of THC treatment on CB1 mRNA and protein expression?

2) The experiment carried out with cannabis is a very important one as it provides physiopathological relevance to the other experiments. Since street cannabis may contain also high levels of cannabidiol, and this non-CB1-acting cannabinoid might modify CB1-mediated effects of THC via other receptors, it would be important to see whether a 1:1 ratio between CBD and THC (higher than that normally found in contemporary strains of cannabis used for recreational purposes) produces at least some of the same effects as THC alone, or if instead the co-presence of CBD counteracts or reduces the effect of THC. This would also provide important information regarding Sativex, which is now approved in the EU for the treatment of spasticity in multiple sclerosis (although this drug cannot be prescribed to pregnant women);

3) The authors seem to have tested only one dose of THC. It would have been useful to see whether similar effects of THC can also be seen at lower dosages, which are possibly more relevant to the blood/placental concentrations of THC during marijuana smoking.

### Referee #2

The question how fetal drug exposure affects long-term brain structure and function is of high medical importance. The authors have investigated the long-term consequences of fetal cannabis exposure in mice and humans, and they find that altered activation of CB1R during development leads to postnatal changes in the density of CB1R positive boutons in the somatosensory cortex, and altered synaptic density and function in the stratum oriens of CA1 in the hippocampus. A subsequent proteomic approach revealed alterations in a broad spectrum of proteins, in particular a reduction in SCG10. The authors then went on to characterize the role of SCG10 in mediating the effects of altered CB1R activation and found that SCG10 is co-localized with CB1R in the sub-ventricular zone and cortical plate. They show that SCG10 mRNA is reduced in human fetal brain after cannabis exposure, and they performed in vitro experiments with cortical neurons that provide evidence that THC promotes SCG10 degradation via activation of JNK or ERK mediated phosphorylation. The authors also show that a pseudo-phosphorylated SCG10 mutant phenocopies THC effects in reducing neurite length in cultured cortical neurons. In general, these are interesting and important data because they point to the relevance of microtubule dynamics in long-term effects on wiring and synaptic plasticity in the brain. Nevertheless, the paper is not fully convincing in providing proof that the long-term effects of fetal cannabis treatment are mediated via altered

JNK/ERK activation and phosphorylation induced degradation of SCG10.

1. Proof is missing that counteracting reduced SCG10 expression rescues long-term effects of fetal THC treatment on cortical synapses and hippocampal synaptic plasticity. If reduced SCG10 mediates the effect of fetal THC treatment, then increased expression of SCG10, i.e. by transgenic overexpression or by inhibition of JNK, either genetically, i.e. by reducing JNK expression in JNK +/- mice, should counteract the observed effects *in vivo* and lead to normalization of synaptic density in cortex and LTD in Schaffer collaterals. In this context, the study by Jeanneteau et al. *Nature Neuroscience* 13, 1373-9, 2010 should be cited and discussed. MKP1 knockout mice show reduced JNK activation and reduced phosphorylation of Stathmin-1 and SCG10, and these mice could be used to study whether reduced JNK activation counteracts the effects of fetal THC exposure. Admittedly, this is difficult, but such data would greatly enhance the quality of these data. Alternatively, the authors have to dampen the conclusion that altered JNK and SCG10 activity is responsible for the long-term alterations in cortex and hippocampus shown in Fig. 1.

2. Altered JNK activation does not only act on SCG10, but also on other signaling pathways that modulate synaptic plasticity. In a previous paper published in 2005, the authors have shown that altered CB1R function regulates the migration and morphogenesis of developing neurons by transactivating Trk-B. Thus the question arises whether altered Trk-B signaling contributes to the effects shown in Fig. 1 on cortical synaptogenesis and hippocampal LTD in Schaffer collaterals. Is there any reduction of Trk-B phosphorylation in THC treated mouse embryos? This could easily be tested. If there is a difference, then the possibility that altered TrkB function modulates development and function of synapses also needs to be taken into account.

3. It is not clear whether the effects of altered CB1R signaling on corticofugal axon fascicles are mediated through altered function of Stathmin-2. If THC inhibits SCG10 function, then SCG10 suppression or knockout should phenocopy the effects of THC treatment. Such evidence would be necessary to support the strong statement "The identified SCG10 is a novel "master effector" of morphogenetic CB1R signaling..." in the first paragraph of the discussion.

4. The data shown in Fig. 2F-G are not convincing. Resolution is relatively low, and it is not clear whether at what extent SCG10 and CB1R are colocalized in individual axons and growth cones in the cortical plate at E18.5. Fig. 1B and F point to altered synaptic morphology but it is not clear whether this is due to altered microtubule stability in presynaptic terminals, or whether it reflects a combination of pre- and postsynaptic effects. This needs to be worked out by more detailed analysis of SCG10 and CB1R distribution in developing cortex and hippocampus, and analysis whether alterations of microtubules in terms of distribution of acetylated and tyrosinated tubulin in pre- and postsynaptic terminals correlate with altered JNK phosphorylation in the pre- and postsynaptic parts of these synapses.

5. The authors make a strong point that SCG10 is reduced by JNK/ERK mediated phosphorylation and subsequent proteasomal degradation. However, the data shown for human fetal brain in Fig. 3 do not fit into this picture. These data provide some evidence that the mRNA for SCG10 is reduced in the developing brain. It would be much more convincing to show that JNK phosphorylation is enhanced and that SCG10 protein levels are reduced in order to fit these data into the concept of proteasomal degradation of SCG10.

Referee #3

Tortiello and colleagues show in this study that SCG10 levels are decrease in  $\Delta^9$ -tetrahydrocannabinol (THC)-treated mice and human cannabis-exposed fetuses and propose that SCG10 as a novel "master effector" of morphogenic the type 1 cannabinoid receptor (CB1R) signaling. One of the strongest point of the study is the finding that aberrant THC induced CB1R activation leads to JNK1 mediated SCG10 phosphorylation and degradation, thereby revealing a signaling pathway through which THC can exert its effects. Although the finding that JNK1 phosphorylation drives proteasomal degradation of SCG10 is not novel (Shin et al. *PNAS* 2012), it is still a relevant observation. Moreover, there are not many examples in literature in which receptor activation can be linked to altered SCG10 expression.

However, there are major criticisms to this work. The molecular mechanisms of 'life-long circuit modifications' in offspring of THC-treated mice remain largely unclear. In fact, the statement in the title and abstract implying that THC induced 'miswiring' of the brain is regulated through SCG10 degradation is a strong over-claim. This is not at all shown in this study. At best it might be discussed at the end of the manuscript that SCG10 is a factor that might be involved in modifying neuronal wiring. The only data showing that miswiring occurs after fetal THC exposure is the altered density of CB1R boutons in somatosensory cortex and hippocampus. However these data are not convincing and rather confusing. It is not clear whether the altered density is achieved by changes in cell-birth, migration, elongation, branching and/or synapse formation. To map the morphology of specific CB1R expressing cell types in the brain after THC exposure would partly address this issue. Moreover, it is not at all clear whether this effect is caused by altered SCG10 expression. In fact it is very likely that many other factors and cellular processes play a role. For example, THC is known to control Rho signaling and the authors show clear effects of THC on actin cytoskeleton (Figure 5C). In summary, the authors should provide evidence that 1) SCG10 plays a role in the rewiring of the in vivo brain and 2) THC induced altered morphology of CB1R expressing neurons is a direct effect of SCG10 degradation and microtubule alterations.

Other and related major concerns:

1. In figure 1, the authors conclude that THC administration during pregnancy effects normal connectivity and functionality of the cortical circuitry of the offspring. However, there is substantial evidence that THC administration to mice alters their behavior. Therefore the observed effects in the offspring could also be assigned to differences in maternal care. To rule out this possibility extra control experiments are needed, i.e. switch offspring from treated and non-treated mice.
2. Furthermore, the authors show both a decrease and increase in the amount of CB1R positive boutons in the cortex or striatum, respectively. How can this be explained? How do these opposite results relate to SCG10 degradation pathway?
3. From figure 2A,B, the authors claim that THC can act as a functional antagonist for CB1R. This cannot be concluded from these experiments and additional data should be provided.
4. Human fetuses are used to investigate the effect of maternal cannabis use on SCG10 mRNA levels in hippocampus. The results in Figure 3 show a decrease in SCG10 mRNA levels of cannabis-exposed fetuses, however it remains questionable whether this is a specific effect or due to loss of cellular density.
5. It is confusing that THC acts as a CB1R agonist on JNK recruitment and SCG10 degradation but as a functional antagonist during development. This might imply two different mechanisms, suggesting that the altered connectivity might not be due to increased SCG10 degradation.
6. The authors use cultured cortical neurons to investigate the effect of THC application on SCG10 levels and neuronal morphology. Additional knock-down experiments of CB1R and SCG10 could be performed to confirm that CB1R, JNK, SCG10 and microtubule dynamics indeed act in the same pathway. Moreover, standard siRNA rescue experiment such as SCG10 overexpression should be included.
7. On p.9, the authors mention a central role of SCG10 degradation in axonal growth defects caused by prenatal THC exposure. This cannot be concluded from the experiments since the experiments on SCG10 degradation and neurite outgrowth were performed in THC-stimulated cultured neurons. The authors could address this issue by performing neurons cultured from offspring of THC treated mice.
8. Figure 4D, it looks as if THC exposure leads to increased SCG10 expression of both phosphorylated and un-phosphorylated. Please explain.
9. Figure 4F. How do CB1R receptor agonists and antagonists affect neuritic outgrowth? Additional control experiments would at least narrow down the specificity of THC actions on neuritic morphology to the CB1R.

10. Figure 5, the amount of acetylated tubulin is measured. These levels should be normalized to total tubulin levels to exclude the possibility that the observed effect is due to an overall increase in microtubule density. Moreover, what happens to dynamic tyrosinated MT? Does this correspond to SCG10 knock-down?

11. Figure 5A. What is the evidence that SCG10-DD is functionally inactive, and outcompeting endogenous SCG10? Is endogenous SCG10 down-regulated? Essential controls are lacking. Does SCG10-DD have the same phenotype as SCG10 knock-down? How does over-expression of SCG10 and nonphospho-SCG10 affect neurite morphology?

12. Throughout the manuscript indications on the number of repeated and independent experiments are lacking.

#### Minor points

1. Figures G1,G2 do not correspond to inset in G.
2. In figure 3, SCG10 mRNA levels in hippocampus are measured, however the text refers several times to mRNA levels in cerebellum.
3. Figure 4D, pCB1R band on Western blot is unclear.
4. Figure 5C is not clearly visible. Also show the separate channels.
5. Figure 5C2 shows the quantification of subcellular morphology of microtubules. The distinction between the two

1st Revision - authors' response

26 November 2013

#### Point-by-point comments to the Expert Referees (EMBOJ-2013-86035)

##### Referee #1:

Thank you for recognizing the public importance of our work, and considering our manuscript "*rigorous, comprehensive and mechanism-oriented*". We appreciate your specific comments and queries. We have addressed these experimentally. Please note that in some cases we felt that our additional data, whilst certainly exciting on their own right, would distract from the lead hypothesis of our manuscript. Therefore, we embedded corresponding figures into this document.

Q1: "the authors suggest that THC is acting as a functional antagonist in this experimental set-up, and indeed they provide both pharmacological and knockout mouse data to substantiate this hypothesis. However, the authors also suggest that THC effect might be due to counteraction of endocannabinoid tone at CB1 whereas the possibility also exists that chronic THC administration causes instead desensitization and decreased expression/targeting of CB1 receptors. Did the authors perform any experiment other than that shown in fig. 1 to distinguish between the two possibilities? Furthermore, what was the overall effect of THC treatment on CB1 mRNA and protein expression?"

We agree with you that these are important questions since THC can potentially act through a number of molecularly distinct mechanisms in the fetal brain. In revised Fig. 2C-D, we show that THC exposure decreases CB1 receptor mRNA and protein expression in the fetal cerebrum. Similarly, DAGL $\alpha$  mRNA expression is decreased. In contrast, MGL mRNA is increased. By Western analysis we find likewise decreased CB1 receptor, DAGL $\alpha$ , as well as MGL protein contents ( $n = 3$ /group). However, residual CB1 receptors in THC-exposed brains escape desensitization as shown by Erk1/2 phosphorylation upon acutely challenging CB1 receptors prepared from THC-exposed cortices at E18 (vs. vehicle; Fig. 2D). These data concord with recent observations by Ken Mackie's group showing that CB1 receptor desensitization only occurs after prolonged (i.e. 8 days *in vitro*) of pharmacological challenge (Straiker *et al.*, Neurobiol Dis, 2013). Considering these data we have carefully revised our conclusions and suggest that THC might act by 1) deregulating endocannabinoid signaling, and 2) reducing CB1 receptor availability for axonal growth and guidance in the developing brain.

Please note that by addressing Referee 2's questions on the signaling cascade linking CB1 receptor activation to SCG10 degradation, we provide additional evidence that THC induces errant signaling – rather than desensitization – at CB1 receptors (*see below*).

Q2: “The experiment carried out with cannabis is a very important one as it provides physiopathological relevance to the other experiments. Since street cannabis may contain also high levels of cannabidiol, and this non-CB1-acting cannabinoid might modify CB1-mediated effects of THC via other receptors, it would be important to see whether a 1:1 ratio between CBD and THC (higher than that normally found in contemporary strains of cannabis used for recreational purposes) produces at least some of the same effects as THC alone, or if instead the co-presence of CBD counteracts or reduces the effect of THC. This would also provide important information regarding Sativex, which is now approved in the EU for the treatment of spasticity in multiple sclerosis (although this drug cannot be prescribed to pregnant women);”

The cannabidiol (CBD) content of street cannabis can vary greatly. Selective cultivation resulted in subspecies almost entirely devoid of CBD, which is preferred for psychoactive street cannabis (Potter DJ, Drug Test Anal, 2013). Cannabidiol's mechanism of action, besides low-affinity binding at CB1 and CB2 receptors, may involve GPR55, serotonin, opioid and other receptors. Therefore, and even if CBD antagonizes/promotes THC's effect on SCG10/stathmin-2 degradation, CBD's mechanism of action may be vastly different from that of THC.

We performed the experiment you suggested in an *in vitro* setting: primary cortical neurons isolated from E17.5 mouse cortices were cultured for 48h before exposed to THC (10  $\mu$ M), CBD (10  $\mu$ M) or their equimolar (1:1) mixture for an additional 10 min. Thereafter, cells were lysed, and protein fractions blotted to determine JNK phosphorylation and SCG10/stathmin-2 levels. As shown in Fig. 1 (Data not shown - for referees only), CBD alone affected neither the state of JNK phosphorylation nor SCG10/stathmin-2 content in cortical neurons. In addition, CBD failed to modify the effect of THC on either parameter.

Despite their clarity, these data are difficult to interpret since no published account exists on CBD's action during fetal development. A number of scenarios may exist, including 1) ineffectiveness, 2) a specific concentration range for bioactivity, 3) a mechanism of action independent from THC, 4) modulation of the function of non-cortical neurons. Therefore, we are of the view that rigorous testing is required to evaluate CBD's activity in any developmental setting. This is clearly beyond the scope of our present study.

Q3: “The authors seem to have tested only one dose of THC. It would have been useful to see whether similar effects of THC can also be seen at lower dosages, which are possibly more relevant to the blood/placental concentrations of THC during marijuana smoking.”

We have tested three THC doses: 500 nM, 2  $\mu$ M (24 h treatments, neuronal morphology) and 10  $\mu$ M (acute pharmacology) *in vitro*. In morphogenic assays, 2  $\mu$ M THC was found to significantly disrupt both neurite outgrowth (Fig. 2A) and collateralization (Fig. 2A1). Therefore, we conducted all outgrowth assays (24 h) using 2  $\mu$ M final concentration of THC (Data not shown – for referees only).

For the *in vivo* experiments, we used a THC dose that is considered “golden standard” in the literature and are shown to be non-aversive (*see also answer to Referee 3's point 1*). We have extensively referenced available literature (papers from the groups of O Manzoni, V Cuomo and SA Deadwyler) to highlight the appropriate dose-range we used (including WIN55,212-2 and AM251 for pharmacological comparisons). Our molecular biology/pharmacology analysis of endocannabinoid signaling and CB1 receptor responsiveness (Fig. 2C-D), as well as the significant loss of SCG10 mRNA and *protein* in both mouse and human tissues (Figs. 3,4) unequivocally support the relevance of our experimental models to elucidating critical events of *human* nervous system development. We have extended the “Discussion” to appropriately address these points.

We hope our extensive and successful experimentation; revised figures and text edits meet your expectations, allowing you to recommend publication of our work.

**Referee #2:**

We appreciated your view that our hypothesis and specific questions are of high medical importance, and that you found most of our data and interpretations convincing. Please find our specific responses to your enumerated queries below:

Q1: “Proof is missing that counteracting reduced SCG10 expression rescues long-term effects of fetal THC treatment on cortical synapses and hippocampal synaptic plasticity. If reduced SCG10 mediates the effect of fetal THC treatment, than increased expression of SCG10, i.e. by transgenic overexpression or by inhibition of JNK, either genetically, i.e. by reducing JNK expression in JNK +/- mice, should counteract the observed effects *in vivo* and lead to normalization of synaptic density in cortex and LTD in Schaffer collaterals. In this context, the study by Jeanneteau *et al.* Nature Neuroscience 13, 1373-9, 2010 should be cited and discussed. MKP1 knockout mice show reduced JNK activation and reduced phosphorylation of Stathmin-1 and SCG10, and these mice could be used to study whether reduced JNK activation counteracts the effects of fetal THC exposure. Admittedly, this is difficult, but such data would greatly enhance the quality of these data. Alternatively, the authors have to dampen the conclusion that altered JNK and SCG10 activity is responsible for the long-term alterations in cortex and hippocampus shown in Fig. 1.”

Indeed, directly link a fetal pathogenic impact with postnatal deficits (months after the initial insult) is always challenging. You are correct in recognizing that these are very complex experiments. In fact, the above very concern (linking developmental changes to postnatal organization) motivated the original organization of our manuscript: we “set the stage” by highlighting that THC altered synaptic connectivity in the postnatal hippocampus, warranting our molecular dissection of possible candidates for this change, yet downplaying causality. In the revised manuscript, we have combined *in vivo* and *in vitro* tools to address your concerns. Firstly, we have combined organotypic slice cultures with molecular pharmacology to show that JNK inhibition (SP600125) acutely rescues SCG10 levels in corticofugal axons exposed to THC. We opted for this experimental setting since there is no data available on the cross-placental transfer of JNK inhibitors, and their effective doses, vital to the design of an *in vivo* strategy. Secondly, we used JNK1<sup>-/-</sup> mice to show that JNK1 loss-of-function *per se* induced developmental deficits of the corticofugal system. Since JNK1 has a number of targets, including SCG10, the JNK1<sup>-/-</sup> axonal phenotype may be unrelated to SCG10 deregulation. Nevertheless, our data clearly show that SCG10 in corticofugal axons is downstream from JNK1 since JNK1<sup>-/-</sup> mice showed increased axonal SCG10 content, a phenocopy of THC-induced deficits (Fig. 6). Thirdly, we used SCG10 knock down (siRNA approach *in vitro*; see also Referee 3's queries) to show that reduced SCG10 levels induce growth arrest and render THC ineffective (Fig. 7B-B2). We are confident that these experiments provide sufficiently broad mechanistic support to our hypothesis.

Nevertheless, we agree with you that toning down our “Discussion”, and highlighting that this may only be one of the mechanisms contributing to postnatal network deficits was necessary. This has been made, and includes a succinct discussion of the Jeanneteau *et al.*, 2010 paper.

Q2: “Altered JNK activation does not only act on SCG10, but also on other signaling pathways that modulate synaptic plasticity. In a previous paper published in 2005, the authors have shown that altered CB1R function regulates the migration and morphogenesis of developing neurons by transactivating Trk-B. Thus the question arises whether altered Trk-B signaling contributes to the effects shown in Fig. 1 on cortical synaptogenesis and hippocampal LTD in Schaffer collaterals. Is there any reduction of Trk-B phosphorylation in THC treated mouse embryos? This could easily be tested. If there is a difference, then the possibility that altered TrkB function modulates development and function of synapses also needs to be taken into account.”

Thank you for this suggestion. However, please note that TrkB is primarily expressed by cortical interneurons, while pyramidal cells are recognized as the source of BDNF. Therefore, and even if TrkB levels would change, their relevance to the pathfinding of long-range (excitatory)

corticofugal axonal errors may be limited.

Nevertheless, we performed the experiments you suggested with data shown in Fig. 3 (Data not shown – for referees only). We have used both an anti-panphospho-Tyr (Y99) antibody and a Y816-specific antiphospho-TrkB antibody (kindly provided by Dr. Eero Castren (Helsinki)). Quite unexpectedly, the total amount of TrkB content (normalized to  $\beta$ -actin) but not its phosphorylation changed upon THC exposure. This finding is genuinely exciting since it might confirm and extend our present knowledge on the molecular basis of THC-induced impairments of interneuron development. Nevertheless, it also warrants careful follow-up and in-depth analysis (interneuron subtype, developmental stage, BDNF concentrations, cannabinoid receptor specificity), which is clearly beyond the scope of our present study.

Q3: “It is not clear whether the effects of altered CB1R signaling on corticofugal axon fascicles are mediated through altered function of Stathmin-2. If THC inhibits SCG10 function, then SCG10 suppression or knockout should phenocopy the effects of THC treatment. Such evidence would be necessary to support the strong statement “The identified SCG10 is a novel “master effector” of morphogenetic CB1R signaling...” in the first paragraph of the discussion.”

Firstly, please note that this experiment in fact was done using SCG10-DD, which we show to outcompete endogenous SCG10 (also: *Referee 3's queries*; revised Fig.7A-A2). However, we have complied with your query and now show (Fig. 7B-B2) that SCG10 knock down phenocopies the effects of THC exposure, and renders THC ineffective to induce (additional) growth modulation.

Q4: “The data shown in Fig. 2F-G are not convincing. Resolution is relatively low, and it is not clear whether at what extent SCG10 and CB1R are colocalized in individual axons and growth cones in the cortical plate at E18.5. Fig. 1B and F point to altered synaptic morphology but it is not clear whether this is due to altered microtubule stability in presynaptic terminals, or whether it reflects a combination of pre- and postsynaptic effects. This needs to be worked out by more detailed analysis of SCG10 and CB1R distribution in developing cortex and hippocampus, and analysis whether alterations of microtubules in terms of distribution of acetylated and tyrosinated tubulin in pre- and postsynaptic terminals correlate with altered JNK phosphorylation in the pre- and postsynaptic parts of these synapses.”

We undertook high-resolution laser-scanning microscopy analysis of SCG10 and CB1R in fetal mouse cortices. As shown in Fig. 3D2,D3 (and *in vitro*; e.g. Fig. S6A) while the two molecules co-exist in the same processes, the likelihood of their close association is approximately 50% (“physical proximity without signal separation”). This is not unexpected since a cell-surface receptor and an intracellular effector associated with the cytoskeleton should be at considerable distances from one another, as supported by *in vitro* morphometric data (Fig. S6B1,B2). This point is also dealt with in the revised “Discussion”.

You suggested that remodeling affects both the pre- and postsynaptic sites. Please note, however, that the distribution of the CB1R is exclusively axonal/presynaptic (on the cell surface), suggesting a restricted site of action. Moreover, and as we have shown earlier (Keimpema *et al.*, J Neurosci, 2010), once the growth-cone stops and undergoes remodeling for the presynapse to emerge, endocannabinoid signaling changes (DAGLs redistribute to the dendrites, MGL intrudes into the growth cone) to adopt their postnatal molecular organization. Accordingly, we only find CB1R/JNK1/SCG10/acetylated-tubulin signaling in motile growth cones *in vitro*. In putative dendrites exposed to THC for 24h *in vitro*, acetylated tubulin but not SCG10 levels increase significantly. This suggests an alternative mechanism of action (i.e. *via* alternative receptors). In established pre-/post-synapses (excitatory subset revealed by postsynaptic PSD95 accumulation *in vitro*), we find no indication of tubulin acetylation after 24h THC exposure (Fig. S6C-D3). Cumulatively, these data suggest that the mechanism we uncovered is growth associated and specific to CB1R-containing axons.

Q5. “The authors make a strong point that SCG10 is reduced by JNK/ERK mediated phosphorylation and subsequent proteasomal degradation. However, the data shown for human fetal brain in Fig. 3 do not fit into this picture. These data provide some evidence that



the mRNA for SCG10 is reduced in the developing brain. It would be much more convincing to show that JNK phosphorylation is enhanced and that SCG10 protein levels are reduced in order to fit these data into the concept of proteasomal degradation of SCG10.”

These are clearly interesting thoughts. However, you certainly can appreciate the difficulty of obtaining human fetal tissues relevant to our study, and the complexity of the ensuing analysis. Given the often long (and most variable) *post-mortem* delay, it is unreasonable to expect that JNK phosphorylation would be preserved (please note that many phosphorylated proteins are degraded rapidly after death, particularly when the brain's pH changes). In contrast, SCG10 protein content may indeed be supportive of our concept. During our revisions, we have successfully extracted protein samples from the partially fixed tissues available for our analysis and probed them for SCG10. These data, shown in Fig. 4C,C1, confirmed that maternal cannabis smoking leads to reduced hippocampal SCG10 protein levels in human fetuses. This loss was significant after careful covariate analysis (Fig. 4C1). Moreover, SCG10 protein and mRNA levels correlated well (and both decreased), recapitulating data from our mouse model, unequivocally supporting SCG10 as a THC-regulated target.

### Referee #3:

Thank you for appreciating mechanistic details of our study, and highlighting it as relevant to medical care. Indeed, our work is amongst the very few, which establish a mechanistic link between GPCR activation and cytoskeletal modifications. Moreover, we appreciated your many comments in relation to specific details. As such, your input suggested to us – as every so often with comprehensive analysis – that many of our findings were thought provoking and worthy of follow-up. Please find our point-by-point responses to your queries below.

Q1: “The molecular mechanisms of 'life-long circuit modifications' in offspring of THC-treated mice remain largely unclear. In fact, the statement in the title and abstract implying that THC induced 'miswiring' of the brain is regulated through SCG10 degradation is a strong overclaim. This is not at all shown in this study. At best it might be discussion at the end of the manuscript that SCG10 is a factor that might be involved in modifying neuronal wiring. The only data showing that miswiring occurs after fetal THC exposure is the altered density of CB1R boutons in somatosensory cortex and hippocampus. However these data are not convincing and rather confusing. It is not clear whether the altered density is achieved by changes in cell-birth, migration, elongation, branching and/or synapse formation. To map the morphology of specific CB1R expressing cell types in the brain after THC exposure would partly address this issue.

Moreover, it is not at all clear whether this effect is caused by altered SCG10 expression. In fact it is very likely that many other factors and cellular processes play a role. For example, THC is known to control Rho signaling and the authors show clear effects of THC on actin cytoskeleton (Figure 5C). In summary, the authors should provide evidence that 1) SCG10 plays a role in the rewiring of the *in vivo* brain and 2) THC induced altered morphology of CB1R expressing neurons is a direct effect of SCG10 degradation and microtubule alterations.”

We agree with your thoughts (similar to Referee 1) that a direct causal relationship between THC-induced SCG10 modifications and postnatal circuit disruption is lacking. However, we have not stated this at any point in the manuscript. In fact, we exactly used the electrophysiology data (Fig. 1) to “set the stage” and justify our analysis but to not conclude what you might have implied. To make this point clear, we have revised the concluding sentences of the electrophysiology data, and our discussion.

We are of the view that the title was *per se* not misleading since it did not link developmental deficits with postnatal circuit modifications. Yet to de-emphasize network wiring, we replaced “connectivity” with “development”.

We increased the clarity of our manuscript by removing some of our neuroanatomy data

(which were prone to misinterpretations), and increased the amount of electrophysiology analysis to show that not only synaptic plasticity but also the likelihood of neurotransmitter release is altered in THC-exposed brains.

We have previously shown (Mulder *et al.*, PNAS 2008) that genetic manipulation and pharmacological antagonism of CB1Rs induce an axonal phenotype similar to what we describe here. Moreover, we showed that CB1R manipulation affects neurogenesis and cell migration. We discussed these findings in the revision, addressing your concerns. We have previously established that CB1Rs couple to Rho signaling in developing neurons (Berghuis *et al.*, Science, 2007). Therefore, we could not agree more that there may be other co-existent, cell-stage and context-dependent pathways that are co-modulated by THC-induced CB1R activation. Nevertheless, it is clear that SCG10 degradation is one of those, and sufficient to change neuronal morphology. This is directly shown by the molecular manipulation of SCG10 (siRNA; point mutagenesis). Therefore, we revised the “Discussion” of our manuscript by removing the statement on “master regulator/-on” and expanding the description of alternative pathways.

Q2: “1. In figure 1, the authors conclude that THC administration during pregnancy effects normal connectivity and functionality of the cortical circuitry of the offspring. However, there is substantial evidence that THC administration to mice alters their behavior. Therefore the observed effects in the offspring could also be assigned to differences in maternal care. To rule out this possibility extra control experiments are needed, i.e. switch offspring from treated and non-treated mice.

We do not concur with this concern: 1) The dose of THC used in the current study did not change maternal behavior or physiological measures such as catatonia or alteration of maternal body weight as would be predicted from high-dose THC intoxication. 2) Since THC treatment ceased 3 days prior to delivery, it is unlikely that the drug itself or its metabolites acutely altered maternal behavior. 3) The sex ratio or body weight of the pups was not different between the THC and vehicle groups (as reported in the manuscript). 4) We used agonist (WIN55,212-2) and antagonist (AM 251) groups as controls, and show that THC induces an intermediate phenotype, as expected based on pharmacological properties. 5) We controlled our *in vivo* experiments genetically. 6) THC-exposed adult offspring were of the same body weight, without apparent behavioral dysfunction, as vehicle controls. Therefore, we are convinced that our experiments are well controlled and relevant to the human condition.

Q3: “2. Furthermore, the authors show both a decrease and increase in the amount of CB1R positive boutons in the cortex or striatum, respectively. How can this be explained? How do these opposite results relate to SCG10 degradation pathway?”

There clearly is a misunderstanding at this point, since we did not study the striatum. We studied strata (layers; stratum/layer) of the hippocampal formation. Therefore, our data implied mistargeting of CB1R-containing axons in both cortical structures. We have condensed our revised data and focus on the hippocampal formation to prevent any further misinterpretation on this subject.

Q4: “3. From figure 2A,B, the authors claim that THC can act as a functional antagonist for CB1R. This cannot be concluded from these experiments and additional data should be provided.”

As per Reviewer 1’s query, we addressed this point by showing mRNA and protein expression profiles for enzymes metabolizing 2-AG and CB1Rs, and by studying the functionality of CB1Rs. Clearly, there is a dysregulated endocannabinoid signaling network but not CB1R desensitization. Therefore, we have expanded our discussion of this point, and revised relevant wording.

Q5: “4. Human fetuses are used to investigate the effect of maternal cannabis use on SCG10 mRNA levels in hippocampus. The results in Figure 3 show a decrease in SCG10 mRNA levels of cannabis-exposed fetuses, however it remains questionable whether this is a

specific effect or due to loss of cellular density.”

In accord with Referee 2’s request, we determined SCG10 protein levels, which were normalized to  $\beta$ -actin. These clearly show reduced SCG10 protein content in cannabis-exposed human fetuses. Moreover, we have correlated SCG10 mRNA and protein levels, excluding histochemical bias due to e.g., different cell numbers, in cannabis-exposed brains.

Q6: “5. It is confusing that THC acts as a CB1R agonist on JNK recruitment and SCG10 degradation but as a functional antagonist during development. This might imply two different mechanisms, suggesting that the altered connectivity might not due to increased SCG10 degradation.”

Please note that “functional antagonism” does not imply antagonist ligand properties. It instead identifies that a low-affinity agonist displaces a high-affinity endogenous ligand, and disrupts its time course of action. We have (see also above) removed this phrase. Moreover, our additional data show that THC is an agonist at CB1Rs both *in vivo* and *in vitro*, pointing towards a single mechanism of action.

Q7: “6. The authors use cultured cortical neurons to investigate the effect of THC application on SCG10 levels and neuronal morphology. Additional knock-down experiments of CB1R and SCG10 could be performed to confirm that CB1R, JNK, SCG10 and microtubule dynamics indeed act in the same pathway. Moreover, standard siRNA rescue experiment such as SCG10 overexpression should be included.”

We appreciated your suggestions. Indeed, we performed additional experiments using organotypic slice cultures and pharmacology (combined THC and SP600125 administration with SCG10 degradation as read-out; Fig. 6A-B4), the analysis of JNK1<sup>-/-</sup> mice (phenotyping; Fig. 6C-D1), and SCG10 knock down (siRNA experiments; Fig. 7B-B2). These data, together with the use of WIN55,212-2 and AM 251 as pharmacological controls *in vivo*, clearly strengthen our concept and experimentally address your specific questions.

Q8: “7. On p.9, the authors mention a central role of SCG10 degradation in axonal growth defects caused by prenatal THC exposure. This cannot be concluded from the experiments since the experiments on SCG10 degradation and neurite outgrowth were performed in THC-stimulated cultured neurons. The authors could address this issue by performing neurons cultured from offspring of THC treated mice.”

If this is an active receptor mediated mechanism (and not an epigenetic phenomenon, which we find unlikely given our negative data in Fig. S1) then normal growth rates can be expected in neurons pre-treated with THC. Moreover, experiments shown in revised Fig. 7 significantly elaborate the role of SCG10 in THC-induced growth defects.

Q9: “8. Figure 4D, it looks as if THC exposure leads to increased SCG10 expression of both phosphorylated and un-phosphorylated. Please explain.”

Please note that only phosphoproteins were pulled down in the experiment you referred to. As per definition by the Shin *et al.*, PNAS 2013 paper, the lower bands likely correspond to mono-phosphorylated SCG10 with the higher molecular weight proteins being polyphosphorylated. We have expanded our “Discussion” on this matter.

Q10: “9. Figure 4F. How do CB1R receptor agonists and antagonists affect neuritic outgrowth? Addition experiments control would at least narrow down the specificity of THC actions on neuritic morphology to the CB1R.”

We (Berghuis *et al.*, PNAS, 2005; Mulder *et al.*, PNAS, 2008, Keimpema *et al.*, J Neurosci, 2010) and others (Vitalis *et al.*, Eur J Neurosci, 2008) have reported on the effects of CB1R agonists and antagonists on neurite outgrowth. For cortical pyramidal cells we showed that MGL inhibition induces 2-AG-mediated neurite outgrowth. Similarly, anandamide (CB1R agonist action) induced the outgrowth of the lead neurite in an AM251-sensitive manner. These data clearly show that CB1R modulation affects neurite morphology.

Q11: “10. Figure 5, the amount of acetylated tubulin is measured. These levels should be normalized to total tubulin levels to exclude the possibility that the observed effect is due to an overall increase in microtubule density. Moreover, what happens to dynamic tyrosinated MT? Does this correspond to SCG10 knock-down?”

Additional control experiments using  $\beta$ -III-tubulin as loading control are shown in Fig. S5B. We also performed SCG10 knock down with data shown in Fig. 7B-B2.

Q12: “11. Figure 5A. What is the evidence that SCG10-DD is functionally inactive, and outcompeting endogenous SCG10? Is endogenous SCG10 down-regulated? Essential controls are lacking. Does SCG10-DD have the same phenotype as SCG10 knock-down? How does over-expression of SCG10 and nonphospho-SCG10 affect neurite morphology?”

Indeed, we cannot entirely rule out that SCG10-DD is active. Yet it is just unresponsive to JNK-mediated phosphorylation. For your question on endogenous SCG10 down regulation, we transfected PC12 cells (for increased transfection efficacy vs. primary neurons) with SCG10-DD-GFP (PC12 cells express SCG10; see Sobczak *et al.*, BBA, 2011). Next, we blotted cell lysates and determined endogenous SCG10 (non-GFP-tagged) and SCG10-DDGFP levels by Western blotting. As Fig. 7A2 shows, SCG10-DD transfection for various time periods competed out the endogenous SCG10 levels, with GFP-tagged SCG10 species predominating in these preparations. In addition, we show that SCG10 siRNA knock down (Fig. 7B-B2) replicates the phenotype imposed by SCG10-DD on cortical neurons. Therefore, we are confident that SCG10 is central to the mechanism we dissected.

Q13: “12. Throughout the manuscript indications on the number of repeated and independent experiments are lacking.”

We have clearly identified these in the methods section. During the revision, we added in some of the key measures in the figure legends.

Q14: “Minor points 1. Figures G1,G2 do not correspond to inset in G.”

These panels were replaced (as per Referee 1’s queries).

Q15: “2. In figure 3, SCG10 mRNA levels in hippocampus are measured, however the text refers several times to mRNA levels in cerebellum.”

The text refers to the “cerebrum” (and not cerebellum), which is the sum of the neocortex and archicortex (including the hippocampus). These statements are therefore correct.

Q15: “3. Figure 4D, pCB1R band on Western blot is unclear.”

This is an extremely challenging experiment. Indeed, this band is weak, and this is the very point showing that not all proteins will get phosphorylated and along the same time course as SCG10. In other words, pCB1R is our negative “control” data. Therefore, the image has not been modified.

Q16: “4. Figure 5C is not clearly visible. Also show the separate channels.”

We provided single-channel images in revised Fig. 7 as requested.

Q17: “5. Figure 5C2 shows the quantification of subcellular morphology of microtubules. The distinction between the two sorts of morphology is unclear.”

We have referenced the different types of growth cones (Berghuis *et al.*, Science, 2007; Morii *et al.*, J Neurobiol, 2006).

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10 December 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your revised manuscript has now been re-reviewed by referees #1 and 2. As you can see below, referee #1 is happy with the revised paper as is, while referee #2 would like to see some more data to support that reduced SCG10 expression is responsible for the effects of fetal THC exposure. While I recognize that it would be nice to have such additional data, I also find the paper strong and insightful enough as is. I am therefore very pleased to accept the paper for publication in The EMBO Journal. Please consider if you want to respond to the point about Trk-B expression with text changes. You can send me a modified text by email.

Thank you for submitting your interesting work to The EMBO Journal.

## REFEREE REPORTS

Referee #1:

The authors have revised the article according to my specification. The article has been significantly improved. I have no further suggestions

Referee #2:

The authors have revised their manuscript and addressed some of my original points of criticism. It is unfortunate that the authors did not go further to provide proof that reduced SCG10 expression is responsible for the effects of fetal THC exposure. The data provided in the original and revised version of the manuscript only allow the conclusion that SCG10 is one of the targets and a potential candidate mediator for the altered synaptic wiring and activity in the brain of THS treated mice and humans. However, it could also be that other targets which are not yet known also contribute. As it stands, this paper, in the authors' words, "sets the stage", but one also could say it makes a suggestion without providing proof that the proposed mechanism is indeed responsible the postnatal neuronal alterations observed in this study. The demonstration that JNK inhibition rescues SCG10 levels in axons does not exclude the possibility that JNK inhibition also rescues other effectors which potentially are more important for the neuronal deficits.

The authors state that Trk-B is primarily expressed in cortical interneurons, but this is not correct. Trk-B is also expressed in pyramidal cells, and recent publications (Medina et al., EMBO J 2004, Bartkowska et al., Development 2007, Puehringer et al., Nat. Neuroscience 2013) have shown that Trk-B activation plays an essential role for migration of cortical pyramidal cells in late embryonic/early postnatal stages. The demonstration that THC exposure induces Trk-B expression would support that Trk-B signaling is involved in this process. The new data shown in Fig. 3 do not rule out that transactivation of Trk-B by CB1R activation in embryonic brain contributes to the phenotype. TrkB phosphorylation needs to be studied at a developmental stage when transactivation is expected to be at high levels, i.e. at late embryonic and early postnatal stages.

2nd - authors' response

10 December 2013

### Point-by-point response to Expert Referee #2 (EMBOJ-2013-86035R)

Thank you for appreciating the revisions we have made. Please find our point-to-point responses to your remaining queries below.

Q1: "The authors have revised their manuscript and addressed some of my original points of criticism. It is unfortunate that the authors did not go further to provide proof that reduced SCG10 expression is responsible for the effects of fetal THC exposure. The data provided in the original and revised version of the manuscript only allow the conclusion that SCG10 is one of the targets and a potential candidate mediator for the altered synaptic wiring and activity in the brain of THS treated mice and humans. However, it could also be that other targets which are not yet known also

contribute. As it stands, this paper, in the authors' words, "sets the stage", but one also could say it makes a suggestion without providing proof that the proposed mechanism is indeed responsible for the postnatal neuronal alterations observed in this study. The demonstration that JNK inhibition rescues SCG10 levels in axons does not exclude the possibility that JNK inhibition also rescues other effectors which potentially are more important for the neuronal deficits."

Thank you for raising this point, which we feel is indeed at the heart of any experimental research. We certainly cannot exclude the possibility that other mechanisms, maybe at developmental stages other than those studied here, are critical in rendering neurons vulnerable to THC's effect. However, we are confident that our focus was on a relevant and significant mechanism, particularly since SCG10/Stathmin-2 is one of the primary targets discovered by high-throughput quantitative proteomics. *All our data support the critical involvement of SCG10 degradation in THC-induced developmental impairments.* Indeed, the other >30 targets we have identified might also exert significant contributions to aspects of cell-autonomous or inter-cellular (trans-axonal) signaling events sensitive to THC exposure. Yet each of these warrants future investigations, at appropriate depth and power, to define the global – and likely hierarchical – molecular architecture of THC-induced neuronal modifications in the developing brain.

Q2: "The demonstration that THC exposure induces Trk-B expression would support that Trk-B signaling is involved in this process. The new data shown in Fig. 3 do not rule out that transactivation of Trk-B by CB<sub>1</sub>R activation in embryonic brain contributes to the phenotype. TrkB phosphorylation needs to be studied at a developmental stage when transactivation is expected to be at high levels, i.e. at late embryonic and early postnatal stages."

We appreciate this point. However, and as discussed previously, we have measured TrkB phosphorylation at late embryonic stages, and cannot find increased phosphorylation, which would be suggestive of a transactivation mechanism. Equally importantly, and from a mechanistic standpoint, what the Reviewer queries is whether CB<sub>1</sub>R activation would induce TrkB phosphorylation and in fact TrkB would facilitate JNK1 phosphorylation. Most certainly, TrkB involvement would add an extra element into the signal transduction cascade. However, this would neither call our hypothesis or data in question nor appears indispensable to transduce THC's developmental effects.