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Synaptic activity-induced glycolysis facilitates membrane lipid provision and neurite outgrowth

Marc Segarra-Mondejar, Sergi Casellas-Díaz, Marina Ramiro-Pareta, Claudia Müller-Sánchez, Alejandro Martorell-Riera, Ismaïl Hermelo, Manuel Reina, Julián Aragonés, Ofelia M. Martínez-Estrada and Francesc X. Soriano.

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(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.)

1st Editorial Decision 23rd June 2017

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 from the comments, the referees find the analysis interesting. However, they also find that more work is needed to support the key findings and that metabolic changes affect neurite growth. Should you be able to address the raised concerns then I would like to invite a revised manuscript. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the key points at this stage.

Also, I see that extensive revisions are needed and I can extend the revision time to 6 months if that is helpful. You will get an automatic chaser at 3 months and at that time point just let me know how the revisions are coming along.

REFEREE REPORTS

Referee #1:

Review of manuscript by Segarra-Mondejar et al.

In this article, the authors investigate the link between synaptic activity, glucose consumption and metabolism at the transcriptional level and correlate it to activity-dependent neurogenesis.

Using primary cortical neuronal cultures they first show that after a period of 48 hours stimulation, achieved by means of treating neurons with bicuculline and 4-AP, the cultures increased their incorporation of labeled glucose into lipids. Further, they also observed an increased production of acetyl-coA and found that interfering with the expression levels of Glut3 (glucose transporter)

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prevents activity-dependent neuritogenesis.
They proceed to investigate the metabolism of the neurons, which were previously activated and find an increase glucose uptake and lactate release, consistent with an increase glycolytic flux. Next, they analyze the expression of Glut3 and of 4 main enzymes involved in glycolysis at the mRNA and protein level. A recent paper was published on The Journal of Biological Chemistry (Bas-Orth et al, 2017), showing that prolonged activity of neurons brings to an increase of the glycolytic flux and of the expression of some of the genes analyzed also in this manuscript. The authors do cite the former paper in the discussion, but only in relation to expression of genes in astrocytes versus neurons. The content of this previously published article should be better acknowledged as it shows and supports what the authors of the present article are claiming (prolonged synaptic activity brings to an increase of glycolysis flux and expression of same key relevant genes).
The authors then proceed to show an increased stabilization of the transcription factor HIF1-a followed caused by synaptic activity and mediated by Siah2. To this, they use a series of elegant molecular tools and well-designed experiments. They also observe that HIF1-a is necessary for the activity-dependent induction of glycolytic genes, with the exception of Glut3. In the next figure, they demonstrate that the induction of the expression of Glut3 is controlled by the transcription factor CREB and that CREB is also indirectly responsible for the stabilization of HIF1-a by mediating the expression of Siah2.
Finally, they show that HIF1-a expression is higher during developmental stages where neuritogenesis takes place and nicely show that HIF1-a plays a role in developmental as well as activity-dependent neuritogenesis.

Overall, the article clearly shows that prolonged synaptic activity causes an increase glycolytic rate in neurons, via the regulation of expression of key proteins. They demonstrate how this phenomenon is regulated by transcription factors CREB and HIF1-a and that HIF1-a is important for developmental and activity-dependent neuritogenesis. However, performing additional experiments to better clarify some links and assumptions made by authors could strengthen the article.

Major:

1- Overall, the morphometric analysis should show be separated for dendrites and axons. As most of the in vitro experiments appear to be done at DIV10-11, when the majority of the dendrites are mostly stable, and neurites can be easily categorized between axon or dendrites, it is important to differentiate the results in order to better assess which morphological compartment is affected. This is particularly relevant in light of the fact that in vivo data on HIF1a are related to dendrites while the rest of the in vitro experiments are most likely representing axons.

2-Fig2, the authors state that, after a 24hrs stimulation period, neurons were washed for 30' to "allow restoration of ion gradients before analyzing glucose uptake". Prolonged stimulation of neurons can result in an activity rate of the cells even after removal of bicuculline. Thus, there is the potential risk that the analyzed neurons in these experiments are still experiencing robust bursting, which would explain the observed increased glucose uptake and lactate release. The authors should confirm these findings by showing, with electrophysiology or calcium imaging, the activity state of the analyzed cultures in these conditions (24hrs treatments, then 30' wash).

3-The authors show that HIF1-a stabilization by Siah2 is responsible for the increased expression of enzymes crucial for the augmented glycolysis triggered by synaptic activity. However, they do not show that this ultimately affects neuronal metabolism as they do not measure glucose uptake, lactate release, glucose incorporation into lipid or acetyl-coA production (as in Fig1 and 2) in conditions where Siah2 expression and/or HIF1a activity is impaired. These experiments are necessary to ultimately make the link between the observed changes in expression levels and cellular events.

4-The finding that Siah2 expression is regulated by CREB and synaptic activity is of interest for several implications. The link to morphological rearrangements remains, however, indirect via the regulation of HIF1-a. Do manipulations of expression of Siah2 (overexpression, siRNA) affect activity-dependent neuritogenesis and developmental neuritogenesis?

Minor:

1-What happens to activity-dependent neuritogenesis when the cultures are fed 2-DG? Is it

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blocked?

2- what is the reason for variating the time of stimulation treatments from 48hrs (i.e. fig1) to 24hrs (i.e. fig.2)?

3-Prolonged incubation with cycloheximide might result in cellular suffering and toxicity. What is the viability of the neuronal cultures after 24hrs cycloheximide treatment?

4-Albeit is it of course true that BDNF causes neurite growth, it was also shown that it can modulate the activation of neurons (Lau et al., 2015). Thus, it could be that the BNDF-related results of the present study are due to an increase activation of the neurons (in a similar way as to what bicuculline does) and not to the fact that neuritogenesis is the primum movens. Both events are correlated and almost impossible to distinguish but authors should consider this option and list the increased activity of neurons followed BDNF treatment as one possible reason for their observed effects.

5-HIF1-a pattern of expression in relation to bicuculline treatment is different from Bas-Orth et al. 2017, what do the authors think is the reason behind this discrepancy?

Referee #2:

In this manuscript, Segarra-Mondejar and colleagues dissect the mechanisms underlying the increased glucose uptake occurring upon neuronal activation and its importance for neuronal development. Using in vitro cultures of rat cortical neurons and stimulations with Bicuculline and 4-aminopyridine, they demonstrate that glucose uptake is required for activity-induced neurite outgrowth. They found that this increased glucose uptake is mediated by the upregulation of the glucose transporter GLUT3 via CREB. They further show that CREB also regulates Siah2, which stabilizes the HIF-1a protein leading to the induction of glycolysis genes. Finally, the authors demonstrate, in vivo, that HIF-1a activation is required for proper neuronal development.

The coupling between neuronal activity and metabolism is a key question in neuroscience. Synaptic signaling has long been associated with increases in energy demand and this observation is at the basis of functional brain imaging techniques but the precise molecular mechanisms linking synaptic activity, increased glucose uptake and neurite outgrowth are still unknown. Here, the authors very convincingly demonstrate the central role played by the stabilization of the HIF-1a protein in the induction of glycolysis genes and subsequent lipid synthesis to support neurite outgrowth. The experiments are very well designed and the conclusions are solid. Although this work was mostly performed in vitro, the authors could confirm the importance of HIF-1a in vivo. Taken together, this is a very solid study with important data, which will - together with other data that were recently published such as Ashrafi et al 2017 - be useful for future investigations of this mechanism in vivo.

Referee #3:

General
The study of Segarra-Mondejar et al. demonstrates a link of neurite growth and dendritic expansion via HIF-1• mediated increase in lipid synthesis. Mechanistically, the authors demonstrate that Ca2+ influx-induced transcription factor CREB, which elevates Siah2 expression, causing stabilization and subsequent activation of HIF-1•. The resulting increased rate of glycolysis upon neurite stimulation and HIF-1• activation is proposed to enhance lipid synthesis, necessary for membrane expansion during neurite outgrowth. The link between CREB and neurite growth as well as between HIF-1• and glycolysis/lipid synthesis have been already reported extensively (Ma et al. 2014, J Biol Chem; Puram & Bonni 2013, Development; Denko 2008, Nat Rev Cancer). Furthermore, the direct stabilization and activation of HIF-1• by Siah2 (in a hypoxia-independent manner by directly reducing HIF-prolyl-hydroxylase (PHD) abundance) has also been documented (Nakayama et al. 2004, Cell). This study represents a novel link of those known mechanisms. However, at least in its current state, the manuscript lacks sufficient evidence that the reported metabolic mechanisms

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indeed promote membrane production in stimulated neurites.
Questions related to lipid synthesis

1. Overall, there is insufficient convincing evidence and information that activated neurons use glucose (via glycolysis) for de novo lipid synthesis. What was the final concentration of 14C-glucose in the medium - relative to the final cold glucose concentration? The authors should also perform 13C-labeled glucose tracing coupled to LC-MS or GC-MS analysis in order to demonstrate incorporation of glucose into lipids (including cholesterol). The lipid synthesis assay needs to be normalized to protein levels as was done for the other assays. The rather small increase in lipid synthesis observed should be further validated by testing changes in protein levels of FASN and/or phosphorylation of acetyl-CoA carboxylase (p-ACC), which is the major regulated step in lipid synthesis. Does silencing FASN abrogate the activity-induced lipid biosynthesis? To further prove that this increased de novo lipid synthesis is used for the generation of membranes, the radioactive tracer assay should be done in combination with membrane fractionation. Further, the authors should consider cholesterol as key component of membranes generated from acetyl-CoA, as cholesterol is an essential metabolite for neuronal physiology (Zhang & Liu 2015, Protein Cell) and neuronal cholesterol homeostasis was demonstrated to be regulated by CREB (Lemberger et al. 2008, FASEB J.).

2. Acetyl-CoA levels need to be normalized to CoA levels. 2DG abrogates any use of glucose for further downstream metabolism, and forces cells to evoke adaptations, and is thus not a physiological blocker of glycolysis alone. More specific silencing of glycolytic genes should be tested to determine that lipid synthesis relies on glycolysis. Is the increase in cytosolic acetyl-CoA levels and lipid synthesis blocked by silencing ACLY?

3. Figure 4 lacks any functional evaluation of glycolysis and lipid synthesis. If the proposed mechanism is regulated by CREB, then inhibition via ICER or A-CREB should also inhibit glycolysis and lipid synthesis. Does CREB activation by Forskolin recapitulate the effect observed with Bic+4AP? Also, the degree of ICER overexpression in Figure 4D should be provided in the supplemental data.

4. The final concentration of glucose in the culture medium is unclear (27 or 30 mM?). In any case, this is supraphysiological (in fact pathological as in diabetes). Question is thus whether the need for glycolysis is not artifically induced by the experimental culture conditions. This is an important issue, which needs to be resolved by appropriate in vitro or in vivo conditions (13C-tracing studies in vivo, etc).

5. Is glucose the only substrate fuelling lipid synthesis - what is the relative role of glucose versus glutamine in fatty acid synthesis, given that the glutamine concentrations in the culture medium are much more physiological than those of glucose? Further, does ACLY silencing block the glycolysis-dependent lipid synthesis.

Questions related to glycolysis

6. Lactate can be produced from other sources than pyruvate - the sentence that is a sole surrogate marker of glycolysis needs to be downtuned.

7. Neurons are generally not considered to have high glycolytic flux rates, which have been even related to toxicity (see data from J. Bolanos). Does this increase in glycolysis renders the cells more prone to toxic damage?

8. The authors give the impression that glycolysis is required for ATP synthesis in neurons - is that correct? If they want to maintain this statement, then some evidence for the relative production of ATP by the different energy-generating metabolic pathways should be given.

9. Figure 1E: The knock down efficiency of Glut3 should be provided (RNA & protein levels).

10. Fig. 2A: The method for measuring glucose uptake is unclear. Was the 2-NBDG intensity normalized to cell size (neurite growth)? Since the described method is using fluorescent microscopy to detect 2-NBDG in cells, the authors could present representative images for further

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improvement or repeat the experiments using flow cytometry.

Questions related to HIF regulation & hypoxia signaling

11. Hypoxia also stabilizes HIF1a, but how do the authors reconcile that the same transcription factor promotes neurite growth and dendritic expansion in normoxia, and is activated in hypoxia, which is known to impair rather than stimulate neurite growth and dendritic expansion. How can these contradictory findings be explained?

12. Why do the authors exclude a possible role for hypoxic inactivation of the PHDs - thus in addition to the proposed Siah2-mediated inactivation of PHD1 and PH3? Have they excluded the possibility that synaptic activity could increase oxygen consumption, leading to intracellular hypoxia, and secondarily HIF-activiation and increased glycolysis? This seems very plausible, given that oxidative metabolism provides most energy for other high energy-consuming processes in neurons. The authors would also need to show that PHD2, which is not a target for Siah2, is sufficiently inactivated to allow HIF1a acivation. Or, is PHD2 not involved (should be demonstrated by silencing experiment? Can the findings be reproduced by single/combined silencing of PHD1/PHD3?

13. Figure 3: In line, it remains unclear how the authors arrived to the conclusion that Siah2 is the only responsible mechanism for HIF-1• stabilization. Other parameters such as lactate/pyruvate accumulation and ROS stabilize HIF-1•: this should be tested in order to clearly demonstrate that only Siah2 stabilizes HIF-1• in stimulated neurites. Is HIF2 involved in activity-induced glycolysis upregulation?

14. Siah proteins have been reported to mediate breakdown of PHDs in hypoxia, while limited evidence suggests that they may also mediate breakdown of FIH in normoxia? Is FIH involved in the shown phenotype?

15. Can some more convincing in vivo evidence be provided that HIF1a in neurons (not globally) mediates neurite outgrowth in a glycolysis dependent manner? Also, the link between HIF-1• and glycolysis/lipid synthesis in neurons in vivo is entirely absent. Evidence hererfore would greatly increase the impact.

16. Figure 3G: It is unclear why two shRNA sequences for Siah2 were used. Alternatively, the authors should provide qPCR of Siah2 knock down.

17. Is Siah1 also increased upon Bic or BDNF stimulation in neurons? In line, is HIF-2• involved in the Bic or BDNF stimulated neurons? To be included in 3A for clarification.

18. The phenotype of Siah2 silenced neurites is incompletely described: do those neurons have fewer dendrites? Are HIF-prolyl-hydroxylases (PHD1/3) indeed directly regulated by Siah2 and more abundant in Siah2 silenced neurons? What is the consequence of Siah2 silencing on glycolysis or lipid synthesis in neurons?

19. The graphical abstract in Figure 6 should be structured more clearly on the left panel as the arrows seem confusing.

20. Figure S1B: gene expression data upon BDNF stimulation should also be provided for 24h to test the consistency with the transient GLUT3 upregulation.

Others

21. Figure S1E: neuronal stainings (TuJ-1) should be provided for comparison with the glial content shown.

22. Throughout the manuscript, densitometry should be provided for the quantification of all western blot analysis.

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Minor comments:

1. The error bars on control panels are missing and should be included.

2. Typo of 'lenght' instead of 'length' in the axis labeling of figure figure 5B,D.

3. Images in figure 5A are not labeled.

4. To keep the order of figure panels consistent figure 5C and 5D should be switched.

5. Figure S2A and 3A seem to be redundant.

6. Control conditions should be consistently abbreviated as C or CT. Use this abbreviation instead of "-".

7. PFKFB3 was not correctly spelled in the text (page 7, line 10).

8. Material & Methods referring to "Glucose incorporation into lipids" should be more extensively described.

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Referee #1:

Review of manuscript by Segarra-Mondejar et al.

In this article, the authors investigate the link between synaptic activity, glucose consumption and metabolism at the transcriptional level and correlate it to activity-dependent neurogenesis.

Using primary cortical neuronal cultures they first show that after a period of 48 hours stimulation, achieved by means of treating neurons with bicuculline and 4-AP, the cultures increased their incorporation of labeled glucose into lipids. Further, they also observed an increased production of acetyl-coA and found that interfering with the expression levels of Glut3 (glucose transporter) prevents activity-dependent neuritogenesis.
They proceed to investigate the metabolism of the neurons, which were previously activated and find an increase glucose uptake and lactate release, consistent with an increase glycolytic flux. Next, they analyze the expression of Glut3 and of 4 main enzymes involved in glycolysis at the mRNA and protein level. A recent paper was published on The Journal of Biological Chemistry (Bas-Orth et al, 2017), showing that prolonged activity of neurons brings to an increase of the glycolytic flux and of the expression of some of the genes analyzed also in this manuscript. The authors do cite the former paper in the discussion, but only in relation to expression of genes in astrocytes versus neurons. The content of this previously published article should be better acknowledged as it shows and supports what the authors of the present article are claiming (prolonged synaptic activity brings to an increase of glycolysis flux and expression of same key relevant genes).
The authors then proceed to show an increased stabilization of the transcription factor HIF1-a followed caused by synaptic activity and mediated by Siah2. To this, they use a series of elegant molecular tools and well-designed experiments. They also observe that HIF1-a is necessary for the activity-dependent induction of glycolytic genes, with the exception of Glut3. In the next figure, they demonstrate that the induction of the expression of Glut3 is controlled by the transcription factor CREB and that CREB is also indirectly responsible for the stabilization of HIF1-a by mediating the expression of Siah2.
Finally, they show that HIF1-a expression is higher during developmental stages where neuritogenesis takes place and nicely show that HIF1-a plays a role in developmental as well as activity-dependent neuritogenesis.

Overall, the article clearly shows that prolonged synaptic activity causes an increase glycolytic rate in neurons, via the regulation of expression of key proteins. They demonstrate how this phenomenon is regulated by transcription factors CREB and HIF1-a and that HIF1-a is important for developmental and activity-dependent neuritogenesis. However, performing additional experiments to better clarify some links and assumptions made by authors could strengthen the article.

We thank the referee for their helpful comments. All points are addressed in full below.

Major:

1- Overall, the morphometric analysis should show be separated for dendrites and axons. As most of the in vitro experiments appear to be done at DIV10-11, when the majority of the dendrites are mostly stable, and neurites can be easily categorized between axon or dendrites, it is important to differentiate the results in order to better assess which morphological compartment is affected. This is particularly relevant in light of the fact that in vivo data on HIF1a are related to dendrites while the rest of the in vitro experiments are most likely representing axons.

Now, we have represented separately dendrites and axons length and found that at DIV 10-11 synaptic activity increases axonal growth with no effect or minimal effect on dendritic growth. The activity-dependent axon growth is impaired by knocking down Glut3, ACLY, Siah2 or expressing a dominant negative HIF, in agreement with the proposed model in this study.

The in vivo experiments were performed in the stage when is produced dendritic growth, thus, as the referee indicated, are related mainly to dendrites. We explain this apparent discrepancy because enhanced glycolysis does not trigger axonal or dendritic growth per se but it is necessary to supply the building blocks necessary to enlarge the membranes regardless of whether the neurons are in axonal or dendritic growth state.

2-Fig2, the authors state that, after a 24hrs stimulation period, neurons were washed for 30' to "allow restoration of ion gradients before analyzing glucose uptake". Prolonged stimulation of neurons can result in an activity rate of the cells even after removal of bicuculline. Thus, there is the potential risk that the analyzed neurons in these experiments are still experiencing robust bursting, which would explain the observed increased glucose uptake and lactate release. The authors should confirm these findings by showing, with electrophysiology or calcium imaging, the activity state of the analyzed cultures in these conditions (24hrs treatments, then 30' wash).

We have performed Fluo-4 live calcium imaging and confirmed that neurons stimulated for 24 hours followed by 30 minutes washout do not show different bursting than control unstimulated neurons. These results are included in the new Fig. E2VA and B.

3-The authors show that HIF1-a stabilization by Siah2 is responsible for the increased expression of enzymes crucial for the augmented glycolysis triggered by synaptic activity. However, they do not show that this ultimately affects neuronal metabolism as they do not measure glucose uptake, lactate release, glucose incorporation into lipid or acetyl-coA production (as in Fig1 and 2) in conditions where Siah2 expression and/or HIF1a activity is impaired. These experiments are necessary to ultimately make the link between the observed changes in expression levels and cellular events.

We have performed new experiments showing that both Siah2 knockdown or HIF-DN expression impairs synaptic activity-mediated increased lactate release and glucose

incorporation into lipids. These results are now shown in new figures 3E and F and 4I and J. Additionally, we have also analyzed the effect of A-CREB expression on lactate release and glucose incorporation into lipids with stronger effects on lactate release that those produced by HIF-DN or Siah2 KD, as shown in Fig 5L and M. This is probably because, unlike HIF1α/Siah2, A-CREB not only blocks the expression of key glycolytic genes but also glucose transporter Glut3 expression. The effect of blocking HIF1α/Siah2 on glucose incorporation into lipids is greater than the effect on lactate release, indicating that only when glucose metabolism has covered the essential physiologic functions it is used to provide lipids to enlarge the membranes.

4-The finding that Siah2 expression is regulated by CREB and synaptic activity is of interest for several implications. The link to morphological rearrangements remains, however, indirect via the regulation of HIF1-a. Do manipulations of expression of Siah2 (overexpression, siRNA) affect activity-dependent neuritogenesis and developmental neuritogenesis?

We have overexpressed Siah2 in immature neurons and found that this is sufficient to increase neurite growth. These results are shown in the new Fig 6B. To study the effect of Siah2 in activity-dependent neuritogenesis we have knocked it down and as shown in the new Fig 6F we found reduced activity-dependent growth in Siah2 KD neurons.

Minor:

1-What happens to activity-dependent neuritogenesis when the cultures are fed 2-DG? Is it blocked?

We have done these experiments which are shown in the new Fig EV1I and found that, similarly to Glut3 knockeddown neurons, activity-dependent neuritogenesis is blocked when glucose metabolism is inhibited.

2- what is the reason for variating the time of stimulation treatments from 48hrs (i.e. fig1) to 24hrs (i.e. fig.2)?

We reasoned that neurite growth is a long process that requires synthesis and assemblage of the components of new formed membranes, thus we decided to check it after 48 h stimulation. Giving that 48 hours stimulation produced significant differences in neurite growth and glucose incorporation into lipids the enzymes and metabolites needed to extend membranes should be regulated and synthesized previously, and for that reason we analyzed lactate release, acetyl-CoA levels and mRNA and protein expression after 24 hours stimulation. Following the same logic we observed that 4 hours stimulation produced changes in the transcriptional regulation of key glucose metabolism enzymes, thus the transcription factors involved in this regulation should be already active after 4 hours stimulation.

3-Prolonged incubation with cycloheximide might result in cellular suffering and toxicity. What is the viability of the neuronal cultures after 24hrs cycloheximide treatment?

We understand the referee's concern. When we performed the initial experiments, microscopic observation did not show any evident neuronal damage after 24 hours cycloheximide treatment. Now we have analyzed neuronal viability counting piknotic nuclei and we can confirm that cycloheximide treatment did not affect neuronal viability. These results are included in the new Figure EV2E.

4-Albeit is it of course true that BDNF causes neurite growth, it was also shown that it can modulate the activation of neurons (Lau et al., 2015). Thus, it could be that the BNDF-related results of the present study are due to an increase activation of the neurons (in a similar way as to what bicuculline does) and not to the fact that neuritogenesis is the primum movens. Both events are correlated and almost impossible to distinguish but authors should consider this option and list the increased activity of neurons followed BDNF treatment as one possible reason for their observed effects.

We thank the reviewer for pointing this possibility. Since acute application of exogenous BDNF increases neuronal activity and synaptic transmission in cultured neurons we cannot exclude the possibility that the BDNF effect observed is due to increased neuronal activity. In the new version we mention this possibility.

5-HIF1-a pattern of expression in relation to bicuculline treatment is different from Bas-Orth et al. 2017, what do the authors think is the reason behind this discrepancy?

Here we can only speculate the reason of the discrepancies found in Bas-Orth et al (which has been better acknowledged in the new version of the manuscript) and ours studies. Given that the media and stimulation protocol is the same we believe that the reason is the quality of the antibody used to detect HIF-1α. Bas-Orth et al show as positive control increased levels of HIF-1α after hypoxia, however this band do not seem to be as strong as expected. Compare hypoxia induced HIF-1α accumulation in Fig. 2A in Bas-Orth et al with CoCl2 induced HIF-1α accumulation (Fig. EV3A). That would suggest that the antibody used by Bas-Orth et al can only detect HIF-1α when it is strongly activated and cannot detect the intermediate activation induced by synaptic activity. We are fully confident of HIF-1α activation by synaptic activity, in addition to our reliable western blots (9 figures representing a total of 40 independent western blots), HIF-1α activation by synaptic activity is also shown using HRE-luciferase reporter assay (3 figures representing 13 independent luciferase assays) and blockage of glycolysis genes induction by dominant negative HIF-1a.

Referee #2:

In this manuscript, Segarra-Mondejar and colleagues dissect the mechanisms underlying the increased glucose uptake occurring upon neuronal activation and its importance for neuronal development. Using in vitro cultures of rat cortical neurons and stimulations with Bicuculline and 4-aminopyridine, they demonstrate that glucose uptake is required for activity-induced neurite outgrowth. They found that this increased glucose uptake is mediated by the

upregulation of the glucose transporter GLUT3 via CREB. They further show that CREB also regulates Siah2, which stabilizes the HIF-1a protein leading to the induction of glycolysis genes. Finally, the authors demonstrate, in vivo, that HIF-1a activation is required for proper neuronal development.

The coupling between neuronal activity and metabolism is a key question in neuroscience. Synaptic signaling has long been associated with increases in energy demand and this observation is at the basis of functional brain imaging techniques but the precise molecular mechanisms linking synaptic activity, increased glucose uptake and neurite outgrowth are still unknown. Here, the authors very convincingly demonstrate the central role played by the stabilization of the HIF-1a protein in the induction of glycolysis genes and subsequent lipid synthesis to support neurite outgrowth. The experiments are very well designed and the conclusions are solid. Although this work was mostly performed in vitro, the authors could confirm the importance of HIF-1a in vivo. Taken together, this is a very solid study with important data, which will - together with other data that were recently published such as Ashrafi et al 2017 - be useful for future investigations of this mechanism in vivo.

We are pleased the referee considers this is a very solid study with important data.

Referee #3:

General

The study of Segarra-Mondejar et al. demonstrates a link of neurite growth and dendritic expansion via HIF-1• mediated increase in lipid synthesis. Mechanistically, the authors demonstrate that Ca2+ influx-induced transcription factor CREB, which elevates Siah2 expression, causing stabilization and subsequent activation of HIF-1•. The resulting increased rate of glycolysis upon neurite stimulation and HIF-1• activation is proposed to enhance lipid synthesis, necessary for membrane expansion during neurite outgrowth. The link between CREB and neurite growth as well as between HIF-1• and glycolysis/lipid synthesis have been already reported extensively (Ma et al. 2014, J Biol Chem; Puram & Bonni 2013, Development; Denko 2008, Nat Rev Cancer). Furthermore, the direct stabilization and activation of HIF-1• by Siah2 (in a hypoxia-independent manner by directly reducing HIF-prolyl-hydroxylase (PHD) abundance) has also been documented (Nakayama et al. 2004, Cell). This study represents a novel link of those known mechanisms. However, at least in its current state, the manuscript lacks sufficient evidence that the reported metabolic mechanisms indeed promote membrane production in stimulated neurites.
Questions related to lipid synthesis

We appreciate the thoroughness of their review and comments. We have addressed each of their specific points.

1. Overall, there is insufficient convincing evidence and information that activated neurons use glucose (via glycolysis) for de novo lipid synthesis. What was the final concentration of 14C-glucose in the medium - relative to the final cold glucose concentration? The authors should also perform 13C-labeled glucose tracing coupled to LC-MS or GC-MS analysis in order to demonstrate incorporation of glucose into lipids (including cholesterol). **The lipid synthesis assay needs to be normalized to protein levels as was done for the other assays.** ***The rather small increase in lipid synthesis observed should be further validated by testing changes in protein levels of FASN and/or phosphorylation of acetyl-CoA carboxylase (p-ACC), which is the major regulated step in lipid synthesis. Does silencing FASN abrogate the activity-induced lipid biosynthesis? To further prove that this increased de novo lipid synthesis is used for the generation of membranes, the radioactive tracer assay should be done in combination with membrane fractionation. Further, the authors should consider cholesterol as key component of membranes generated from acetyl-CoA, as cholesterol is an essential metabolite for neuronal physiology (Zhang & Liu 2015, Protein Cell) and neuronal cholesterol homeostasis was demonstrated to be regulated by CREB (Lemberger et al. 2008, FASEB J.).***

Now we provide additional evidences that support our claim that as a consequence of activity-mediated enhanced glucose metabolism more lipid precursors were provided for neurite growth:

1. The protein levels of FASN and ACLY, two key enzymes involved in lipid synthesis, are increased in active neurons (new Fig EV1A-C).
2. Knockdown of ACLY, the enzyme that links glucose metabolism with lipid synthesis, results in impairment of glucose incorporation into lipids in active neurons (new Fig 1F).
3. Knockdown of ACLY results in blockage of activity-mediated neurite growth (new Fig. EV1J).
4. Knockdown of GPI, the second enzyme in the glycolysis, results in impairment of glucose incorporation into lipids in active neurons (new Fig 1B).
5. Inhibition of glucose metabolism with 2-DG blocks neurite growth (new Fig. EV1I).
6. Glutamine incorporation into lipids is not increased by synaptic activity (new Fig. EV1E).
7. CREB is a key regulator of the process by regulating Glut3 expression and HIF-1α stabilization. Dominant negative A-CREB expression blocks glucose incorporation into lipids in active neurons (new Fig 5M).
8. Over-expression of HIF-DN blocks glucose incorporation into lipids in active neurons (new Fig 3F).
9. Siah2 knockdown blocks glucose incorporation into lipids in active neurons (new Fig 4J).

10. Siah2 knockdown blocks activity-mediated neurite growth (new Fig. 6F).
11. Siah2 overexpression promotes neurite growth in immature neurons (new Fig. 6B).
12. Administration in vivo of the PFKFB3 inhibitor 3PO during the period of maximal activity-mediated neurite growth results in reduced dendritic complexity (new Fig. 6H and J).

The aim of this study was to study the metabolic adaptations required for neuronal membrane extension. We absolutely agree with the referee that the understanding of the neuronal lipidome, how it is modified by synaptic activity and the physiologic consequences derived are outstanding questions in the field that we believe need to be addressed in an independent study and, thereby, it is beyond the aim of this study.

In this new version of the manuscript the glucose incorporation into lipids assay was done adding 0.8 μCi/ml 14C-(U)-glucose to have a final concentration of 3.04 μM radioactive glucose in medium with the 2.4 mM cold glucose that is the physiologic concentration of glucose in rat brain [1]. The results were normalized to protein levels. All this information is now better explained in the supplementary material and methods section.

2. Acetyl-CoA levels need to be normalized to CoA levels. 2DG abrogates any use of glucose for further downstream metabolism, and forces cells to evoke adaptations, and is thus not a physiological blocker of glycolysis alone. More specific silencing of glycolytic genes should be tested to determine that lipid synthesis relies on glycolysis. Is the increase in cytosolic acetyl-CoA levels and lipid synthesis blocked by silencing ACLY?

We have analyzed CoA levels and found that stimulation did not produce changes in CoA levels. These results are now shown in the new Fig. E1F.

We have knocked down GPI and this results in reduced glucose incorporation into lipids (new fig. 1B).

ACLY is the enzyme that links glucose and lipid metabolism. ACLY knockdown results in reduced glucose incorporation into lipids and blocked activity-mediated neurite growth (new fig. 1F). Additionally, ACLY KD blocks activity-mediated neurite growth (new fig. E1J). These results strongly support the concept that activity-mediated increase in glucose transport and metabolism fuels lipid synthesis required to enlarge membranes during neuritogenesis.

3. Figure 4 lacks any functional evaluation of glycolysis and lipid synthesis. If the proposed mechanism is regulated by CREB, then inhibition via ICER or A-CREB should also inhibit

glycolysis and lipid synthesis. Does CREB activation by Forskolin recapitulate the effect observed with Bic+4AP? Also, the degree of ICER overexpression in Figure 4D should be provided in the supplemental data.

New experiments shown in figs. 5L and 5M show that A-CREB blocks activity-mediated lactate release and glucose incorporation into lipids.

Forskolin treatment produces increased lactate release but not glucose incorporation into lipids (Fig. EV5B and C). These results are coherent with the fact that rise in cAMP levels strongly potentiates neuritic growth in response to neurotrophic factors but cannot promote neuritic growth per se [2].Thereby, increased glucose uptake and metabolism is necessary for neuritic growth but it is not the first cause of neuritogenesis.

Transfection efficiency in primary neurons is around 5% and so not high enough to show overexpression by western blot or qPCR. We apologize for not making this clear, it is now mentioned in the supplemental methods. We suppose the referee asks to show ICER overexpression levels because s/he is concerned that ICER is so highly expressed that the repressive effect shown is unspecific. In the new Fig E5A is shown that ICER over-expression has no effect on the activation of MEF2, a well known activity-dependent transcription factors.

4. The final concentration of glucose in the culture medium is unclear (27 or 30 mM?). In any case, this is supraphysiological (in fact pathological as in diabetes). Question is thus whether the need for glycolysis is not artifically induced by the experimental culture conditions. This is an important issue, which needs to be resolved by appropriate in vitro or in vivo conditions (13C-tracing studies in vivo, etc).

This is an important point raised by the referee. Primary cortical cultures is a very useful model to dissect molecular mechanism of molecular processes since they allow the design of experimental approaches that difficulty, if possible, could be done in vivo. Unfortunately, primary neurons are a very sensitive type of cells that need to be cultured in very specific media that not always mimic the physiological conditions. The glucose concentration used in our experiments is 27.5 mM much higher than the 2.4 mM physiological concentration in rat brain [1] but not much different to the 25 mM of Neurobasal, the most commonly used neuronal culture medium. To rule out the possibility that the unphysiologic glucose concentration was mediating the metabolic effects observed we have done all the glucose incorporation into lipids experiments shown through the manuscript, and lactate release in media in Fig. EV2D with physiologic 2.4 mM glucose and the results observed are the same that in our high glucose medium.

5. Is glucose the only substrate fuelling lipid synthesis - what is the relative role of glucose versus glutamine in fatty acid synthesis, given that the glutamine concentrations in the culture medium are much more physiological than those of glucose? Further, does ACLY silencing block the glycolysis-dependent lipid synthesis.

We analyzed glutamine incorporation into lipids and this was not changed by synaptic activity (new Fig EV1E). Taking all our data together and the fact that glucose is the main provider of

lipogenic acetyl-CoA in resting neurons [3] lead us to conclude that activity-mediated glucose incorporation into lipids is paramount for membrane expansion required for neurite growth. This, obviously, does not exclude that other metabolites could contribute in this process.

Questions related to glycolysis

6. Lactate can be produced from other sources than pyruvate - the sentence that is a sole surrogate marker of glycolysis needs to be downtuned.

We believe the referee meant that lactate is produced by the conversion of pyruvate to lactate by LDH but glucose is not the only source of pyruvate. Now we have explained that although in different type of cells the glycolysis derived lactate accounts 82-90 % [4] lactate release is a commonly used surrogate of glycolysis [5].

7. Neurons are generally not considered to have high glycolytic flux rates, which have been even related to toxicity (see data from J. Bolanos). Does this increase in glycolysis renders the cells more prone to toxic damage?

It is well know the opposite indeed. Synaptic activity activates signaling pathways and transcriptional programs that confer neuroprotection to different insults, including oxidative stress, excitotoxicity and apoptosis [6]. In one of his papers [7] they found that overexpression of PFKFB3 decreased use of glucose in the PPP what caused oxidative stress. We and others have previously shown that synaptic activity regulates transcriptional programs that boost antioxidant defenses by regulating the thioredoxin-peroxiredoxin and glutathione systems [8, 9]. Another aspect of Bolaños' paper that is worth to mention here is that they first reported using tripsinized neurons and analyzing by FACS that glycolitic flux in neurons was very low. Follow up studies by his lab. [10] analyzed glucose metabolism in intact neurons and found that they had underestimated glucose metabolism around 5 fold probably because resuspended neurons had lost neurites, thus most of the neuronal volume.

8. The authors give the impression that glycolysis is required for ATP synthesis in neurons - is that correct? If they want to maintain this statement, then some evidence for the relative production of ATP by the different energy-generating metabolic pathways should be given.

There is no doubt that almost the totality of ATP produced by the neuron comes from the OXPHOS. However, several reports in vitro and in vivo show that under certain conditions when is needed fast production of ATP, such as synaptic activity, glycolysis also provide ATP [11-14]. In the first version of the manuscript we already provided experimental data that agree with what has been describe in the literature. In fig. 2B of the first version (new Fig 2C) we show that just 30 minutes of mitochondrial uncoupling causes a sharp reduction in the ATP levels that is even greater in absence of glucose. These experiments indicate: 1) neurons are

extremely dependent of OXPHOS for ATP production; 2) although limited, glycolysis also can contribute to ATP production.

9. Figure 1E: The knock down efficiency of Glut3 should be provided (RNA & protein levels).

As mentioned above, transfection efficiency in neuronal cultures is around a 5%, and so not high enough for analysis of siRNA efficiency by western blot or qPCR. To knockdown Glut3 we have used a pool of 4 different commercial siRNAs. The manufacturer guarantees that 3 in 4 siRNA effectively knockdown the targeted gene, thus the probability that none of the 4 siRNAS works is only 0.39 %. Given that Glut3 knocked down neurons show the same phenotype regarding activity-mediated neurite growth that when glucose metabolism is inhibited with 2-DG (new Fig E1I) we are fully confident that the commercial pool effectively worked.

10. Fig. 2A: The method for measuring glucose uptake is unclear. Was the 2-NBDG intensity normalized to cell size (neurite growth)? Since the described method is using fluorescent microscopy to detect 2-NBDG in cells, the authors could present representative images for further improvement or repeat the experiments using flow cytometry.

We apologize for not making this clear in the methods section, it is now more detailed described. We create same surface ROI in the soma of neurons and analyze fluorescence intensity in these ROIs. Representative images are now shown in new figure E2C.

Questions related to HIF regulation & hypoxia signaling

11. Hypoxia also stabilizes HIF1a, but how do the authors reconcile that the same transcription factor promotes neurite growth and dendritic expansion in normoxia, and is activated in hypoxia, which is known to impair rather than stimulate neurite growth and dendritic expansion. How can these contradictory findings be explained?

HIF-1α is best known as a mediator of response to hypoxia, however its activation is not limited to hypoxic conditions. The literature showing normoxic stabilization of HIF-1α is extensive. The fact that HIF-1α is activated in hypoxia and by synaptic activity does not mean that the signaling events are the same. Neurite growth supposes an important energetic cost; hypoxia prevents ATP generation by OXPHOS while synaptic activity still produces ATP mainly by OXPHOS (Fig. 2C). Although complete hypoxia impairs neurite growth, HIF-1α is a central player in regeneration [15], including axonal regeneration [16].

12. Why do the authors exclude a possible role for hypoxic inactivation of the PHDs - thus in addition to the proposed Siah2-mediated inactivation of PHD1 and PH3? Have they excluded the possibility that synaptic activity could increase oxygen consumption, leading to intracellular hypoxia, and secondarily HIF-activiation and increased glycolysis? This seems very plausible, given that oxidative metabolism provides most energy for other high energy-

consuming processes in neurons. The authors would also need to show that PHD2, which is not a target for Siah2, is sufficiently inactivated to allow HIF1a acivation. Or, is PHD2 not involved (should be demonstrated by silencing experiment? Can the findings be reproduced by single/combined silencing of PHD1/PHD3?

That is an interesting possibility. We have used the fluorescent hypoxic sensor Image-iT Red Hypoxia Reagent (Thermo Fisher) and we could not detect hypoxia generation by synaptic activity (new Fig. EV4J). These results indicate that the mechanism beyond activity-dependent HIF-1α stabilization does not seem to involve intracelular hypoxia.

Siah2 is induced in a CREB-dependent manner by synaptic activity and both CREB dominant negative and Siah2 KD are sufficient to block HIF-1α activation. This indicates that Siah2 is central to activity-mediated HIF-1α stabilization. Siah2 is best known to mediate HIF-1α stabilization by degrading PHD1 and PHD3 which hydroxilate and target HIF-1α for degradation). We have analyzed protein levels of HIF prolyl hydroxilases PHD1, PHD2, PHD3 and FIH-1 after inducing synaptic activity for 4 hour. The only change observed is an increase in PHD2 which is a HIF-1α target gene itself [17]. Despite the increased expression of PHD2, obviously, this is sufficiently inactivated, otherwise HIF-1α would not be stabilized and activated as we have shown in a 9 representative western blots (40 independent experiments) and 3 HRE-luciferase assays (13 independent experiments) . The increase in PHD2 levels is probably responsible of the later decay in HIF-1α. Many papers assume that Siah2-dependent HIF-1a stabilization relies on PHDs degradation but other mechanisms have been described [18], moreover exist different possibilities that need to be experimentally linked, for instance, Sprouty2 promotes HIF-1α ubiquitination [19], is regulated by Siah2 [20] and its downregulation promote axonal growth [21-23]. Acid pH and LDH promotes the conversion of 2-oxoglutarate to L-2-HG which functions as a potent inhibitor of PHDs [24-26]. Disruption of OGDHC results in accumulation of oxoglutarate with the subsequent increase in L-2-HG and HIF-1α stabilization [25]. Siah2 has been reported to disrupt OGDHC complex [27, 28], however we could not detect changes in the protein levels of two components of the OGDHC. Future studies need to better characterize the mechanisms involved in Siah-2-dependent stabilization of HIF-1α.

13. Figure 3: In line, it remains unclear how the authors arrived to the conclusion that Siah2 is the only responsible mechanism for HIF-1• stabilization. Other parameters such as lactate/pyruvate accumulation and ROS stabilize HIF-1•: this should be tested in order to clearly demonstrate that only Siah2 stabilizes HIF-1• in stimulated neurites. Is HIF2 involved in activity-induced glycolysis upregulation?

The results that lead us to conclude a central role of Siah2 in HIF-1α stabilization has been already explained in point 12. We have reworded some sentences to indicate the role of Siah2 as key, hence not excluding the possibility of other mechanisms also participating in HIF-1α stabilization.

The fact that the strong antioxidant Trolox does not prevent activity-mediated HIF-1α stabilization (new Fig. EV4 K and L) argue against the possibility that oxidative stress is involved in activity-dependent HIF-1α stabilization.

We found that LDH inhibition also blocks HIF-1a stabilization (new Fig. 4K and L). Since, both, Siah2 KD and LDH inhibition almost completely blocked activity-mediated HIF-1α stabilization they may be part of the same regulatory pathway. As mentioned above, one possibility is Siah2 dependent degradation of OGDHC causes accumulation of 2-oxoglutarate that is converted to the potent PHDs inhibitor L-2-hydroxyglutarate by noncanonical LDH activity, but we could not detect by western blot changes in two components of the OGDHC which have been described to by targeted by Siah2 for degradation (Fig. EV4H and I).

We have also analyzed HIF-2α protein levels and these do not change with synaptic activity (new Fig. EV3E and F), indicating an unlikely role of HIF-2α in activity-induced glycolysis upregulation.

14. Siah proteins have been reported to mediate breakdown of PHDs in hypoxia, while limited evidence suggests that they may also mediate breakdown of FIH in normoxia? Is FIH involved in the shown phenotype?

As the referee correctly indicates, the role of Siah2 degrading FIH is very limited. The search in PubMed Siah2 and FIH-1 only produce 7 results, including a retracted paper and a retraction notice. We have analyzed FIH-1 protein levels in resting and active neurons and there is not changes (Fig. EVH and I) despite increased Siah2 levels what argues against the idea that Siah-2 mediates FIH-1 degradation and that FIH-1 plays a role mediating HIF-1α stabilization by synaptic activity.

15. Can some more convincing in vivo evidence be provided that HIF1a in neurons (not globally) mediates neurite outgrowth in a glycolysis dependent manner? Also, the link between HIF-1• and glycolysis/lipid synthesis in neurons in vivo is entirely absent. Evidence hererfore would greatly increase the impact.

HIF-1α deletion in neural precursor cell progenitors leads to atrophy of the cerebral cortex [29], indicating an important role for HIF-1α in neuronal development in vivo. We show that HIF-1α deletion in early postnatal mice, a period of maximal afferent innervations and neurite growth, disturbs neurite architecture in the adult. Now, we have performed dual immunostaining revaling colocalization of HIF1α with the neuronal marker NeuN, and complete absence in astrocytes expressing GFAP (new Fig. 6K). This together with all the in vitro data showing a central role of HIF-1α in neurite growth strongly support the view that the defects observed in neurite architecture in early postnatal HIF-1α KO mice are due to the absence of neuronal HIF-1α.

The generation of animal models that unequivocally link HIF-1a/glycolysis/lipid synthesis in neurons in vivo would exceed the time frame of the revision process. However, we have added

new data supporting that glycolysis is necessary for postnatal neurite growth. In new fig. 6H it is shown that administration of the PFKFB3 inhibitor 3PO to postnatal rats reduces neuronal complexity.

16. Figure 3G: It is unclear why two shRNA sequences for Siah2 were used. Alternatively, the authors should provide qPCR of Siah2 knock down.

shRNA are 21 nt double strand RNAs with a loop sequence. The 21 nt sequence are identical to the targeted gene and although shRNA are designed to avoid offtarget knockdown this cannot be ruled out. Hence, it cannot be excluded the possibility that a single shRNA is making its effect rather than for knocking down the gene that was designed for for knockingdown unspecifically another gene. However the possibility that two different shRNA against a same gene also knockout unspecificilly another gene to produce a phenotype is even more remote. In summary, the reason to use two different shRNAs is to confidentially exclude the possibility of an unspecific effect.

In new Fig. EV4C and D we provide quantification of Siah2 mRNA in AAV-sh-sc and sh-Siah2 transduced neurons.

17. Is Siah1 also increased upon Bic or BDNF stimulation in neurons? In line, is HIF-2• involved in the Bic or BDNF stimulated neurons? To be included in 3A for clarification.

Synaptic activity does not upregulate Siah1 mRNA nor increase HIF-2α protein levels. These data are included in new fig. EV4A and EV3E and F.

18. The phenotype of Siah2 silenced neurites is incompletely described: do those neurons have fewer dendrites? Are HIF-prolyl-hydroxylases (PHD1/3) indeed directly regulated by Siah2 and more abundant in Siah2 silenced neurons? What is the consequence of Siah2 silencing on glycolysis or lipid synthesis in neurons?

It is shown in new Fig. 6B that over-expession of Siah2 is enough to induce neurite outgrowth in immature neurons. On the other hand, Siah2 KD blocks activity-dependent neurite growth (new Fig. 6F).

Siah2 silencing also reduces glycolysis and incorporation of glucose into lipids (new Fig. 4I and J). All these results are in accordance with the proposed model in which Siah2 regulates HIF-1α to increase glycolysis that is necessary for neurite growth.

19. The graphical abstract in Figure 6 should be structured more clearly on the left panel as the arrows seem confusing.

We thank the referee for telling us that graphical abstract is not clear. Now we have changed it and it is shown in new Fig. 7

20. Figure S1B: gene expression data upon BDNF stimulation should also be provided for 24h to test the consistency with the transient GLUT3 upregulation.

As requested these new data are shown in Fig EV2F.

21. Figure S1E: neuronal stainings (TuJ-1) should be provided for comparison with the glial content shown.

In the new Fig E2J it is shown NeuN staining as well.

22. Throughout the manuscript, densitometry should be provided for the quantification of all western blot analysis.

As requested all western blots are accompanied by the densitometric analysis.

Minor comments:

1. The error bars on control panels are missing and should be included.

All control bars show now error bars.

2. Typo of 'lenght' instead of 'length' in the axis labeling of figure figure 5B,D.

Thank you for spotting this typo. It has been corrected now.

3. Images in figure 5A are not labeled.

We apologize for this oversight. It has been corrected.

4. To keep the order of figure panels consistent figure 5C and 5D should be switched.

We have introduced a number of new panels in former Fig 5 and others we believe the order of the panels is consistent now.

5. Figure S2A and 3A seem to be redundant.

The reason to introduce former Fig S2A, now Fig EV3A, is to show the specificity if our HIF-1α antibody. Nonetheless, we would not object its removal if the reviewer and editor consider it necessary since that would not affect the general message of our study.

6. Control conditions should be consistently abbreviated as C or CT. Use this abbreviation instead of "-".

Control is now labeled as CT.

7. PFKFB3 was not correctly spelled in the text (page 7, line 10).

We apologize for this typo. It has been corrected.

8. Material & Methods referring to "Glucose incorporation into lipids" should be more extensively described.

We have described this method with more detail in the supplementary methods section.

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2nd Editorial Decision 16th Febuary 2018

Thank you for submitting your revised manuscript to The EMBO Journal. Your paper has now been seen by referees #1 and 3 and their comments are provided below.

As you can see both referees find that the analysis has been strengthened. Referee # 3 finds that some of the initially requested experiments have not been adequately addressed ((i) radiolabeled substrate incorporation into the cell membrane and (ii) 13C-labeled glucose tracing coupled to GC-MS and LC-MS analysis to measure glucose incorporation into lipid intermediates). I have looked carefully at the comments and your response. While I agree with the referee that it would have been good to have data along such lines, I also see your point and I am OK to move forward without these experiments. Make sure you have a balanced discussion of potential experimental shortcomings.

The other points raised by the referees should be addressed. I have provided you with a link below that you can use to upload the revised version. Let me know if we need to discuss anything further

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REFEREE REPORTS

Referee #1:

Review of manuscript-resubmission by Segarra-Mondejar et al.

In their revised article, the authors have satisfactorily answered to the experiment-related concerns I had raised.

It still remains to be better highlighted, however, the discrepancy between the dendrite vs axonal remodeling. Albeit it is a likely explanation what the authors state in their rebuttal letter (= that the investigated phenomenon provides building blocks for either dendrites or axons, whenever growth is needed).
I believe that this explanation/conclusion should be more strongly featured in the text both in results and discussion.

Minor but necessary:

-For the in vivo/Golgi morphological assessment it should be stated clearly somewhere which neurons were analyzed. In particular, please clearly state from which subcortical brain area and from which layer neurons were analyzed. Although it might appear as a detail, it is becoming increasingly important in the field to be as specific as possible as evidences that morphological rearrangements are cell type-specific are accumulating. Were there any regions were the morphological impairment was not taking place?

-Please correct carefully the manuscript. It is decorated with typos/wording mistakes. Examples: often when quoting the additional figures (i.e. EV2, E2V...). Or another example page 10 "by which Siah2 could HIF1a is mediating"; "Redmond et al" on page 11 is missing the year..and so on,
This was also true for the rebuttal letter where the authors were referring i.e. to panels E2Va and B (which btw should be labeled as EV2) for the calcium imaging experiments, which are instead featured in the appendix as EV2 is related to ICER experiments.

Referee #3:

General:

For the second submission of the manuscript entitled "Synaptic activity-mediated neurite growth requires HIF-1α stabilization" the authors have performed an extensive revision. The rebuttal letter displays a comprehensive point-by-point answer to many of the raised questions. To the reviewers' opinion, the current revised study has now improved, however some of the critical

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concerns/questions were not addressed.

Major comments:

- Various requested experiments were still not done: (i) radiolabeled substrate incorporation (glucose and glutamine) into the cell membrane (via membrane fractionation experiments) and (ii) 13C-labeled glucose tracing coupled to GC-MS and LC-MS analysis to measure glucose incorporation into lipid intermediates (including cholesterol). Also, various other comments were neglected or not adequately addressed.

- The authors should provide the evidence of the knockdown efficiency of GPIKD and ACLYKD in cell models used in this study as a control of the experiments (mRNA level at least). They claim that the transfection efficiency is only 5% - does this refer to only 5% of the cells that were transfected (and for how much?) or to a 5% reduction of target mRNA levels in the entire population?). In any case, this is highly problematic.

Minor comments:

- It appears odd that the error bars are not always centralized relative to the upper edge of the bar graph (e.g. Fig. 1F, 4E, S3L and other locations). Also, the Y-axes often do not display the upper end of the axis but appear to be cut.

- In the Figure 7 "Lypid synthesis" should be changed to: Lipid synthesis.

- Throughout the manuscript, the text would benefit from some linguistic improvements.

- The labeling of figure panels is often not congruent with the manuscript text (eg. EV1E should be labeled as S1E; figure EV4 does not exist). This should be improved.

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This has now been explained in the results section corresponding to Fig. 6 and the discussion.

Minor but necessary:

-For the in vivo/Golgi morphological assessment it should be stated clearly somewhere which neurons were analyzed. In particular, please clearly state from which subcortical brain area and from which layer neurons were analyzed. Although it might appear as a detail, it is becoming increasingly important in the field to be as specific as possible as evidences that morphological rearrangements are cell type-specific are accumulating. Were there any regions were the morphological impairment was not taking place?

For the Golgi staining, we only analyzed layer 5 pyramidal neurons of the somatosensory cortex that have shown postnatal growth by depolarization, and in a CREB-dependent manner (Aizawa et al, 2004; Chen & Ghosh, 2005; Redmond et al, 2002) and have high HIF-1α expression. This information is now included in the appendix supplementary methods section and the figure legend of Fig. 6.

-Please correct carefully the manuscript. It is decorated with typos/wording mistakes. Examples: often when quoting the additional figures (i.e. EV2, E2V...). Or another example page 10 "by which Siah2 could HIF1a is mediating"; "Redmond et al" on page 11 is missing the year..and so on,

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- Various requested experiments were still not done: (i) radiolabeled substrate incorporation (glucose and glutamine) into the cell membrane (via membrane fractionation experiments) and (ii) 13C-labeled glucose tracing coupled to GC-MS and LC-MS analysis to measure glucose incorporation into lipid intermediates (including cholesterol). Also, various other comments were neglected or not adequately addressed.

In the first version of the manuscript we claimed that activity-mediated enhanced glycolysis was necessary to provide lipids for neurite growth. That conclusion was supported by experiments showing 1) increased glucose incorporation into lipids in active neurons; 2) increased production of the lipid precursor acetyl-CoA in active neurons, which was dependent of glucose metabolism; 3) reduced activity-mediated neurite growth when Glut3, the main neuronal glucose transporter, was knocked down; 4) we also showed that activity-mediated induction of glycolysis genes were HIF-1α-dependent and that expression of a dominant negative HIF-1 blocked activity-dependent neurite growth in vitro. Furthermore, HIF-1α knock out in postnatal mice reduced dendritic architecture in vivo.

The referee considered all these were insufficient convincing evidences and suggested several experiments. In the revised version we provided 12 new additional evidences that are described in the previous rebuttal letter which we will not repeat here again, in summary we show that pharmacological inhibition of glycolysis affects neurite growth in vitro and in vivo; knockdown of the second enzyme in the glycolysis blocks both glucose incorporation into lipids and activity-mediated neurite growth; knockdown of ACLY, the enzyme linking glycolysis with fatty acid metabolisms blocks both glucose incorporation into lipids and activity-mediated neurite growth; genetic manipulation of CREB, HIF-1α and Siah2 (three proteins we have shown to be central in activity-mediated increase in glucose metabolism) block both glucose incorporation into lipids and activity-mediated neurite growth. If we look at all the evidences shown as a whole rather that individually we believe these strongly indicate that enhanced activity-mediated glycolysis is necessary to provide lipids for neurite growth. The possibility that the glucose-derived lipids, which levels are increased by synaptic activity, are stored intracellulary and the lipids required for neurite growth are derived from metabolites other

than glucose and glutamine (glutamine incorporation into lipids is not induced by synaptic activity) seems very unlikely. Now, in the discussion of the latest version of the manuscript we highlight that we have not analyzed glucose incorporation into lipids in the growth cone but the evidences strongly support the view that these are used to enlarge membranes.

Since the aim of this study was to study the metabolic adaptations required for neuronal membrane extension and the mechanism involved, we believe that the study of the lipidic composition of neuronal growing membranes is very important and should be addressed in detail in an independent study.

The authors should provide the evidence of the knockdown efficiency of GPIKD and ACLYKD in cell models used in this study as a control of the experiments (mRNA level at least). They claim that the transfection efficiency is only 5% - does this refer to only 5% of the cells that were transfected (and for how much?) or to a 5% reduction of target mRNA levels in the entire population?). In any case, this is highly problematic.

In appendix Fig. S1D, S1G and S1H we already provided evidences of knockdown efficiency of sh-GPI and sh-ACLY.

In cell biology, transfection efficiency refers to the amount of transfected cells in a given population. Thereby, 5% transfection efficiency means that of every 100 cells in a culture only 5 are transfected. Depending on the technique used this low transfection efficiency is not problematic at all. For instance, luciferase assay is a very sensitive method and since mammal cells do not express luciferase, luminiscence is only produced in luciferase-transfected cells and there is no interference from non transfected cells. In other cases low transfection is not problematic but it is actually desired. Neurites from different neurons intercross forming a dense mesh that makes it impossible to distinguish whether a neurite belongs to a neuron or any other from the same or different fields. Thus, to analyze neurite length it is necessary to have very low transfection efficiency to be sure that the traced neurites belong to one single neuron. However, there are other techniques in which the analysis is made in the entire neuronal culture, such as western blot or qPCR where with just 5% transfection efficiency it is impossible to detect changes. For those kinds of experiments we transduced the neurons with AAV which easily transduces 70-90% of the neuronal culture.

Minor comments:

- It appears odd that the error bars are not always centralized relative to the upper edge of the bar graph (e.g. Fig. 1F, 4E, S3L and other locations). Also, the Y-axes often do not display the upper end of the axis but appear to be cut.

We apologize for this. We have now corrected it.

The reason sometimes the Y-axes do not display the upper end of the axis is merely aesthetic, to not leave too much empty space above the bar or fill the Y-bar with too many numbers.

Since there are no modifications in the scale we do not see how this could affect the interpretation of the results but we certainly will change it if the editor considers it is misleading.

- In the Figure 7 "Lypid synthesis" should be changed to: Lipid synthesis.

We apologize for this typo. It has been corrected now.

- Throughout the manuscript, the text would benefit from some linguistic improvements.

The text has now been checked by an English native speaker.

- The labeling of figure panels is often not congruent with the manuscript text (eg. EV1E should be labeled as S1E; figure EV4 does not exist). This should be improved.

We apologize for these oversights. Now they have been corrected.

References

Aizawa H, Hu S-C, Bobb K, Balakrishnan K, Ince G, Gurevich I, Cowan M, Ghosh A (2004) Dendrite Development Regulated by CREST, a Calcium-Regulated Transcriptional Activator. *Science* **303:** 197-202

Chen Y, Ghosh A (2005) Regulation of dendritic development by neuronal activity. *Journal of Neurobiology* **64:** 4-10

Redmond L, Kashani AH, Ghosh A (2002) Calcium Regulation of Dendritic Growth via CaM Kinase IV and CREB-Mediated Transcription. *Neuron* **34:** 999-1010

Accepted 5th March 2018

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at it and I appreciate your response to the remaining concerns. I am therefore very pleased to accept the manuscript for publication here.

Just a few remaining things to sort out:

We encourage the publication of source data, particularly for electrophoretic gels and blots. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

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Corresponding Author Name: Francesc X. Soriano
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-97368R1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No sample size was chosen. If after 4 independent experiments there was a clear tendency to be significant but not reached yet because the inter-experimental variability another repeats were done
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We tried to use the fewer number of animals possible starting from 3-4.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The microscopic analysis was performed blindly
For animal studies, include a statement about randomization even if no randomization was used.	Tamoxifen or 3PO or vehicle administration was performed randomly
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The microscopic analysis was performed blindly
4.b. For animal studies, include a statement about blinding even if no blinding was done	Microscopic analysis of brain slices was performed blindly
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analysis was performed with GraphPad Prism 5 program
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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http://www.ncbi.nlm.nih.gov/gap

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	This information is provided in appendix supplementary methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Sprague-Dawley rats were used with no distinction of gender. The age is indicated in the figure lengends. C57BL/6J mice possessing loxP sites on either side of exon 2 of the HIF1A gene expressing or not the CRE-ERT under the ubiquitin promoter. There was no distinction of gender Rats and mice were kept under specific pathogen-free conditions at the animal facility at the University of Barcelona and Autonomus University of Madrid
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All the experimental procedures were approved by the Research Ethics Committee at the UAM (Autonomous University of Madrid) and University of Barcelona and they were carried out in accordance with Spanish and European guidelines (B.O.E, 18 March 1988, and 86/609/EEC European Council Directives).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	This study did not involve human research participants or samples.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	This study did not involve human research participants
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	This study did not involve human research participants
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	This study did not involve human research participants
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	This study did not involve human research participants
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	This study did not involve human research participants

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18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No data was deposited in a public database
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G- Dual use research of concern

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