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Cholesterol loss during glutamate-mediated excitotoxicity

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1st Editorial Decision 16 March 2011

Thank you for submitting your manuscript to the EMBO Journal. Three referees have now evaluated your manuscript and their comments are provided below. While referee #2 is fairly supportive of the work, I am afraid that the other two referees do not share this opinion. Referee #1 raises concerns regarding the conclusiveness of some of the data and referee #3 finds that further data would be need to support that the observed reduction in cholesterol is functionally significant. As also indicated in their comments both referees are not able to support publication in the EMBO Journal. Given these significant concerns and as the EMBO Journal only accepts manuscripts that receive an enthusiastic response from at least a majority of the referees upon initial review, I am afraid we can not offer to publish it here.

I thank you in any case for the opportunity to consider this manuscript. I am sorry that we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

with best regards

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Sodero et al describe a series of investigations concerning the effects of glutamate-mediated excitotoxicty on the cholesterol content of neuronal and synaptosomal membranes. They observe a small loss of cholesterol from these membranes in response to NDMA treatment, although there does not appear to be a clear dose-response relationship. They further explore the role of Ca2+ in this process and present limited data from a STIM gene-ablated mouse model in support of an essential role for Ca2+. They hypothesise that translocation of the cholesterol catabolising protein cholesterol 24-hydroxylase from the ER to the plasma membrane, where its catalytic domain may be exposed to plasma membrane cholesterol, is essential for the observed cholesterol loss. I do not believe that the data presented in the manuscript is sufficiently robust to substantiate the authors' claims of a 'mechanistic link' between disturbed cholesterol homeostasis and excessive glutamate receptor activation.

Major points:
1. The authors normalise membrane cholesterol content to membrane protein concentration as detected by using a commercial Bradford based approach (BioRad Protein Microassay). I am concerned about the reliability of these assays as the authors state that they buffer used to resuspend the membrane pellet contained 0.2% SDS, a substance known to interfere with this assay (this is double the permitted levels for the assay). I thus have doubts about the robustness of any sterol data normalised using this approach. As this will ahve profound effects on all of the sterol data and the conclusions drawn from it, the authors should, as a matter of urgency, clarify if this is indeed the methodology used or if they used a detergent compatible reagent.
2. A critical issue is a lack of any data on the exact mechanism by which the cholesterol is lost from the critical membranes in question. The authors hypothesise that cholesterol is converted to 24S-hydroxycholesterol in response to the translocation of the cholesterol 24-hydroxylase but provide no evidence that the enzyme is indeed functional in its new site. Given that such activity would require an appropriate reductase (e.g. POR) it is highly doubtful if the enzyme would indeed be active at the plasma membrane. The authors should provide chemical evidence that the cholesterol is either converted into 24S-hydroxycholesterol or that it is released from the membranes as cholesterol. The authors should also discuss the possibility that, as the enzyme system used will detect any 3beta-hydroxy sterol, they are potentially following a movement of existing 24S-hydroxycholesterol from the membranes. Finally, there is the issue of the acceptor particles for the sterols - 24S-hydroxycholesterol is more polar than cholesterol but it is not considered to be water soluble.
3. The immunoblotting data concerning the exposure of cholesterol 24-hydroxylase is unconvincing - there is an effect in the order of 10% with statistics based on an n equal to three. At the very least this should be repeated several more times and confirmed with additional antibodies.
4. The units for cholesterol are not consistent. Notably, Figure 3 D refers to a synaptosomal lysate and I was confused by the decision to express the cholesterol as a molar concentration. I was unable to find a clear explanation of the origin of this fraction in the methods and it appears that it is identical to the resuspended membrane pellet normalised to protein (technical issues aside) in other areas.

Minor points:
1. The entire manuscript would benefit from a careful revision with regards to language, grammar and layout - there are sections which repeat information unnecessarily and there is some methodological information provided for which there are no mention of results (e.g. STIM genotyping).
2. Cyp46 should be replaced with Cyp46a1 or CYP46A1 as appropriate throughout the manuscript.
3. The reference list does not seem to be entirely up-to-date. They authors are encouraged to make a more systematic review of the literature in this area.

Referee #2

This work by Sodero et al. presents novel mechanistic information on the regulation of cholesterol metabolism at synapses in response to neuronal activity. Specifically, the authors demonstrate that acute activation of excitatory synaptic transmission triggers a down-regulation of cholesterol levels at the synaptic plasma membrane, both in vitro and in vivo. This down-regulation is mediated by a translocation of the cholesterol hydroxylating enzyme Cyp46 from the endoplasmic reticulum, where it usually resides, to the plasma membrane. The regulated mobilization of Cyp46 requires an

initial calcium entry via glutamatergic receptors, which then appears to be amplified by calcium release from intracellular stores. The relevance of cholesterol metabolism for neuronal function is well established, as well as its alteration during pathological conditions such as Alzheimer's disease or acute brain injury. However, the mechanisms linking neuronal activity with the regulation of cholesterol metabolism were unknown. This manuscript provides mechanistic insight to fill this important gap, and therefore should be of general interest for the neuroscience and cell biology communities. Nevertheless, this work would be strengthened by providing some additional details on the coupling mechanisms between glutamate receptors and Cyp46 translocation. These are my specific comments:

1. The authors propose that cholesterol reduction is triggered by glutamate receptor activation, because this effect is induced by NMDA or AMPA treatment. However, widespread activation of NMDA or AMPA receptors is likely to produce global neuronal depolarization, action potential firing and secondary calcium entry from voltage-gated channels. To strengthen the conclusion that cholesterol loss is directly due to postsynaptic receptor activation, the authors should repeat the NMDA and/or AMPA treatment while blocking voltage-gated sodium channels, for example with tetrodotoxin.
2. It is intriguing that the extent of cholesterol loss upon glutamate receptor activation is similar in synaptosomes and in total membrane fractions. Does this imply that cholesterol down-regulation is widespread, that is, not specific for synaptic membranes? And related to this point, what fraction of total membrane cholesterol is typically associated to synaptosomes? These points are important to understand the physiology of cholesterol regulation in neurons.
3. An important conclusion from this work is that cholesterol down-regulation is mediated by Cyp46 translocation to the plasma membrane in a calcium-dependent manner. However, the calcium source mediating this effect is not conclusively demonstrated in this work. The calcium-free buffers used in Fig. 3A would preclude the entry of extracellular calcium as well as the release from intracellular stores (as these will be depleted in the absence of extracellular calcium). Therefore, these experiments demonstrate the requirement of calcium, but do not unambiguously identify the calcium source. Similarly, the release of calcium from mitochondrial stores (Ru360 treatment) demonstrate that a rise in intracellular calcium is sufficient for cholesterol loss, but do not identify the endogenous source triggered by glutamate receptor activation. The involvement of calcium intracellular stores is mostly based on the results with the STIM2 knock-out neurons, which are characterized by low calcium levels at intracellular stores because of their defective capacitative calcium entry. Nevertheless, additional pharmacological evidence targeting calcium intracellular stores would be important to strengthen this point. This could be done using caffeine- or thapsigargin-pretreated synaptosomes to deplete intracellular stores.
4. The presence of basal levels of Cyp46 at the plasma membrane is an important new piece of information revealed by this study. It would be important to provide a quantitative interpretation for this observation. In principle, these numbers could be obtained from the electron microscopy experiments or from the surface biotinylation assays.
5. Total levels of Cyp46 have been proposed to increase upon brain injury. Judging from the western blots in Fig. 5, total Cyp46 levels do not appear to change in response to KCl, but this is not explicitly stated in the text or figure. This would be an important observation, which should be expressed in a quantitative manner in Fig. 5. On a similar line, it would be interesting to test whether other membrane-associated proteins follow similar or different behaviors. For example, are AMPA or NMDA receptors delivered to the synaptic surface with this treatment? What about other ER-associated proteins, such as SERCA? This information would be important to evaluate the specificity of this regulatory mechanism for Cyp46.
Minor points:
6. The TIRF images shown in Fig. 6 do not directly monitor the translocation of Cyp46 to the cell surface, but rather its close proximity to the plasma membrane, as acknowledged by the authors in the Discussion section. Therefore, the wording describing Fig.6 in the Results section should be modified to state that the increase in TIRF signal is "consistent with" (but does not "indicate") Cyp46 translocation to the cell surface.
7. In page 9, it is stated that the active site of P450s faces the luminal domain, but the opposite is stated at the beginning of page 12.
8. I find rather confusing the explanation in page 12 of the topology of Cyp46 based on the surface biotinylation experiments and the use of a C-terminal antibody. Why should the antibody recognition sequence matter for this assay? Isn't surface labeling based on the biotinylation step (and not the antibody labeling)? Does this imply that there are no surface exposed lysine residues at the

N-terminus for the biotin reagent to react with? This point should be clarified.

Referee #3

The authors find that cholesterol content of neuronal cultures and synaptosomes is reduced upon treatment with depolarizing KCl or NMDA in vitro and by kainate-induced neuronal activity in vivo. This effects seems to depend on the ER resident Stim2 and mitochondrial Ca storage, and somewhat correlated with translocation of the P450 cytochrome Cyp46 to the plasma membrane.

It seems to me that the presented data are too limited and not of broad enough interest for publication in EMBO J. In support of this notion, most of the cited earlier studies on regulation of neuronal cholesterol content and its effects on neuronal functions have appeared in highly specialized neuroscience journals, which might be a better option for the presented work. Further, several effecst are rather small, often barely larger than 10%. This point is the more striking as the different stimulus paradigms are very strong non-physiological stimuli (KCl for 30 min causes very long unphysiological depolarizations; kainic acid, which causes very sever seizures in rodents; NMDA can cause epileptogenic activity in neurons-all of that should be carefully monitored for a comprehensive study). It is unclear from the presented studies whether this modest reduction in cholesterol is of any functional relevance, an aspect that would have to be addressed for publication in EMBO J. In addition the following technical concerns exist.

1. The experiments on neuronal culture are only meaningful if the neurons were of high-quality - for instance, do they showed clearly discernible dendritic spines? Respective images should be provided for cultures in which changes in cholesterol has been demonstrated upon certain treatments for the various conditions-does their appearance, perhaps spine density, change? What happens to glutamate receptors and other synaptic, dendritic, and axonal markers?

2. Synaptosomes typically consist of pinched off and re-sealed nerve terminals with postsynaptic structures that are open and not re-sealed attached to it (the postsynaptic density)-the postsynaptic site is thus not a closed structure and stimulation with KCl or NMDA should not lead to any influx of Ca-the PSD would be exposed to extracellular Ca all the time independent of NMDA or KCl. It is thus puzzling how NMDA can have an effect on cholesterol content unless it acts via the rather rare presynaptic NMDAR or through off target effects. At the minimum the synaptosomal preparations have to be well documented by EM and the above considerations carefully addressed (e.g., are there functional presynaptic NMDARs?).

3. Blocking the mitochondrial Ca uniporter with Ru360 will have numerous side effects as mitochondrial functions will be affected and any involvement of Ca storage by mitochondria has to be carefully documents with additional experiments, which are typically considered tricky and therefore time consuming. The currently extremely limited dataset on mitochondrial Ca release is thus by far not sufficient to support the proposed model that Ca release from mitochondria is involved in cholesterol metabolism. In addition, mitochondria need to be loaded with Ca by preceding depolarizations before they can be stimulated to release substantial amounts of Ca-it is my understand that under resting conditions there is only a small amount of Ca in mitochondria.

4. Similarly, support for a role of Stim2, which is an ER resident protein involved in capacitative Ca entry upon depletion of Ca in the ER stores, is very limited and based only on the finding that kainate-induced cholesterol loss is less in Stim2 KO mice. A role of Stim2 and how that ER resident connects to mitochondrial Ca has to be supported through several additional lines of evidence.

5. The specificity of the antibody used for immunogold EM for Cyp46 needs to be established, ideally by use of KO mice or knockdown in cultures. The localization to the plasma membrane has to be somehow quantified perhaps before and after kainate treatment to functionall connect it to a change in cholesterol.

6. KCl-induced redistribution of Cyp46 towards the plasma membrane is marginal (~10%) and only obvious following 5 min of KCl treatment. It is not obvious, possibly reversed, after 10 and 15 min treatments and does not match the 30 min KCl treatment used to show the modest decrease in cholesterol. The link between this redistribution and cholesterol depletion is thus at best tenuous.

Specificity for surface labeling is not monitored (absence of biotinylation of an abundant cytosolic protein, e.g., actin) and the surface signals appear so weak that trust in their quantification is limited.

7. The redistribution of Cyp46 in HEK cells should be documented in neurons. Also rather than using ionomycin the authors should use more physiological stimuli or at least match the stimuli they use for cholesterol depletion (KCl, NMDA).

Resubmission 27 June 2011

Reviewer #1

General comment:
Sodero et al describe a series of investigations concerning the effects of glutamate-mediated excitotoxicity on the cholesterol content of neuronal and synaptosomal membranes. They observe a small loss of cholesterol from these membranes in response to NDMA treatment, although there does not appear to be a clear dose-response relationship.

Answer:
Our data indicate that excitatory neurotransmission induces an on-off response. In addition, we show that this response is due to activation of excitatory, but not inhibitory, neurotransmission, showing the functional specificity of the phenomenon. The term "dose-response" has been eliminated from the text.

Clarification: If this referee meant to use the term "small" to indicate that 10-20% reduction of cholesterol may not be biologically relevant, we would like to argue that moderate significant oscillations of pleiotropic molecules (like cholesterol in this case) will certainly have biological consequences, not necessarily dose-dependent. We had shown in the past that a similar loss of cholesterol plays a role in the activation of the Trk survival pathway. Now we show that cholesterol loss plays an important role in the control of intracellular calcium peaks after the constant (30 minutes) exposure to NMDA (new Figure 3).

They further explore the role of Ca2+ in this process and present limited data from a STIM gene-ablated mouse model in support of an essential role for Ca2+.

The STIM2 KO mice were used as a tool to test the possible involvement of SER-membrane proximity in cholesterol loss. Following the observation that cholesterol loss is prevented in STIM2 KO mice we further investigated ER-membrane association, by three other approaches: surface biotinylation, electron microscopy and TIRF microscopy. All these aspects are now further strengthened with new data.

Major point 1*. The authors normalize membrane cholesterol content to membrane protein concentration as detected by using a commercial Bradford based approach (BioRad Protein Microassay). I am concerned about the reliability of these assays as the authors state that the buffer used to resuspend the membrane pellet contained 0.2% SDS, a substance known to interfere with this assay (this is double the permitted levels for the assay). I thus have doubts about the robustness of any sterol data normalised using this approach. As this will have profound effects on all of the sterol data and the conclusions drawn from it, the authors should, as a matter of urgency, clarify if this is indeed the methodology used or if they used a detergent compatible reagent.*
Answer:
I am afraid this comment is due to a misunderstanding: the SDS concentration used in the samples to measure protein is 0.04% (0.2% is the concentration used to solubilize the initial pellet; this is then diluted 5 times). In all the experiments, protein levels were assessed by triplicate, in order to normalize cholesterol levels. We have never observed significant changes in the protein levels after the different treatments (typically performed for 30 minutes). This made us confident about the possibility of normalizing cholesterol levels by the amount of protein.

Major point 2. *A critical issue is a lack of any data on the exact mechanism by which the cholesterol is lost from the critical membranes in question. The authors hypothesize that cholesterol is converted to 24S-hydroxycholesterol in response to the translocation of the cholesterol 24-*

hydroxylase but provide no evidence that the enzyme is indeed functional in its new site. Given that such activity would require an appropriate reductase (e.g. POR) it is highly doubtful if the enzyme would indeed be active at the plasma membrane. The authors should provide chemical evidence that the cholesterol is either converted into 24S-hydroxycholesterol or that it is released from the membranes as cholesterol.

Answer:
We are thankful for this comment. We certainly attempted to measure 24S-hydroxycholesterol in the medium of our neurons, always without success, possibly due to either binding of this oxysterol to the tissue culture substratum required for the attachment of our cells (poly-L-lysine) or to its oxidation in a fast non-enzymatic manner, which could have made it undetectable with our methodology (performed in the laboratory of Professor Paul Van Veldhoeven, K.U.Leuven). This, added to the fact that we could not detect an increase in cholesterol in the medium, moved us to study the dynamics of the cholesterol 24-hydroxylase, as it is thought to be the main enzyme for cholesterol removal in the brain (see Russell et al., Ann. Rev. Biochem. 78: 1017-40, 2009). In defense of this rationale and possibility, we now provide new data showing 1) approximation of this enzyme to the plasma membrane in stimulated neurons (new Figure 8), and 2) increase in CYP46A1 after stimulation, both by surface biotinylation (new Figure 6) and by electron microscopy (see Results).

We understand the concern of this referee about the unlikely possibility that CYP46A1 works at the plasma membrane. We envision that the concern arises from the demonstration that the NADPH reductase is restricted to the ER (Brignac-Huber et al., Mol. Pharmacol. 79: 549-57, 2011). However, these results were obtained in non-neuronal cells, in which the cytochrome enzyme is not present at the plasma membrane. Since we found that in neurons a pool of endogenous CYP46A1 exists at the plasma membrane (demonstrated by electron microscopy and surface biotinylation experiments, see new Figures 5 and 6) and that its levels increase after stimulation (demonstrated by three means: TIRF microscopy, surface biotinyation and electron microscopy), we find it reasonable to propose local activity of this enzyme as a possibility. On the other hand, we now give more weight to this reviewer's concern and state that cholesterol loss could be also due to translocation of this sterol from the plasma membrane to the ER tubules that move to the proximity of the plasma membrane, from where it would be eliminated as 24S-hydroxycholesterol (see Results and Discussion). To address this issue in a more exact way we will have to wait until the development of new tools to allow the purification of neuronal plasma membrane, without contamination from internal membranes or membranes from the other cell types of the brain (i.e. astrocytes). Only in such systems one would be able to study membrane-specific activity.

The authors should also discuss the possibility that, as the enzyme system used will detect any 3beta-hydroxy sterol, they are potentially following a movement of existing 24S-hydroxycholesterol from the membranes.

Although the enzymatic kit used in this work can potentially detect 24S-hydroxycholesterol, we have performed our measurements in total membrane extracts from the hippocampus. The reviewer has to take into account that brain 24S-hydroxycholesterol is 1000 fold less abundant than cholesterol (Heverin et al., J. Lipid Res. 45: 186-93, 2004).

Major point 3. *The immunoblotting data concerning the exposure of cholesterol 24-hydroxylase is unconvincing - there is an effect in the order of 10% with statistics based on an n equal to three. At the very least this should be repeated several more times and confirmed with additional antibodies.*

Answer: We have increased the number of biotinylation experiments and the result stays at a similar level of significance (*before:* average increase: 11.9±4.1%; p = 0.045; *now:* average increase: 16.0±5.1%, p = 0.019). A suitable explanation for this is that a considerable amount of CYP46A1 is already present at the plasma membrane in steady state conditions (20.1±2.3% of the total cellular pool). Therefore, just a small increase in surface CYP46A1 could account for the 20% cholesterol reduction if the activity is high.

We would like to clarify that surface biotinylation does not depend on the antibody used for the detection of the streptavidin pull-down protein; it depends on the capacity of the biotin to react with lysine residues exposed to the extracellular space. As expected, experiments in which the detection

was performed with a commercial CYP46A1 antibody (ABCAM) showed a similar result to those obtained in our initial experiments, with the antibody produced in the laboratory of Dr. D. W. Russell. These data have been included in the current version (see new Figure 6).

Major point 4. *The units for cholesterol are not consistent. Notably, Figure 3 D refers to a synaptosomal lysate and I was confused by the decision to express the cholesterol as a molar concentration. I was unable to find a clear explanation of the origin of this fraction in the methods and it appears that it is identical to the resuspended membrane pellet normalised to protein (technical issues aside) in other areas.*

We have followed this referee's suggestion and now show cholesterol variations normalized by protein, like in the other figures. Indeed, we were referring to the re-suspended membrane pellets from purified synaptosomes. We apologize for the lack of clarity.

Minor comments:
1. The entire manuscript would benefit from a careful revision with regards to language, grammar and layout - there are sections which repeat information unnecessarily and there is some methodological information provided for which there are no mention of results (e.g. STIM genotyping).

Corrected. Redundancies and mistakes have been corrected to the best of our capacities.

2. Cyp46 should be replaced with Cyp46a1 or CYP46A1 as appropriate throughout the manuscript.

CYP46A1 is now used throughout the manuscript.

3. The reference list does not seem to be entirely up-to-date. The authors are encouraged to make a more systematic review of the literature in this area.

We do not understand if the reference list is not up-to-date regarding lipid/cholesterol metabolism, glutamate activity, excitotoxicity or calcium modulation, all aspects experimentally tackled in our work. We would be happy to include any reference this referee considers appropriate.

Referee #2

General comment

The relevance of cholesterol metabolism for neuronal function is well established, as well as its alteration during pathological conditions such as Alzheimer's disease or acute brain injury. However, the mechanisms linking neuronal activity with the regulation of cholesterol metabolism were unknown. This manuscript provides mechanistic insight to fill this important gap, and therefore should be of general interest for the neuroscience and cell biology communities. Nevertheless, this work would be strengthened by providing some additional details on the coupling mechanisms between glutamate receptors and Cyp46 translocation. These are my specific comments:

Specific comment 1. *The authors propose that cholesterol reduction is triggered by glutamate receptor activation, because this effect is induced by NMDA or AMPA treatment. However, widespread activation of NMDA or AMPA receptors is likely to produce global neuronal depolarization, action potential firing and secondary calcium entry from voltage-gated channels. To strengthen the conclusion that cholesterol loss is directly due to postsynaptic receptor activation, the authors should repeat the NMDA and/or AMPA treatment while blocking voltage-gated sodium channels, for example with tetrodotoxin.*

Answer. We have followed the suggestion of this referee and now show that cholesterol loss induced by KCl stimulation still occurs in the presence of tetrodotoxin (see new Figure S1). These results reinforce the idea that the reported cholesterol loss is largely post-synaptic.

Specific comment 2*. It is intriguing that the extent of cholesterol loss upon glutamate receptor activation is similar in synaptosomes and in total membrane fractions. Does this imply that*

cholesterol down-regulation is widespread, that is, not specific for synaptic membranes? And related to this point, what fraction of total membrane cholesterol is typically associated to synaptosomes? These points are important to understand the physiology of cholesterol regulation in neurons.

The changes in total membrane cholesterol the referee is referring to arise from studies in cultured neurons. Hence, since the same loss is obtained in purified synaptic structures we feel tempted to conclude that the loss in whole cells reflects the loss at synapses. Unfortunately, a "big hole" in the lipid field is the lack of tools to make valid quantitative assumptions about cholesterol ratios in different membrane sub-domains. Moreover, it is not possible to make these quantitative measurements in membrane fractions from brain because of the presence of contaminating material from non-neuronal cells.

Specific point 3. An important conclusion from this work is that cholesterol down-regulation is mediated by Cyp46 translocation to the plasma membrane in a calcium-dependent manner. However, the calcium source mediating this effect is not conclusively demonstrated in this work. The calcium-free buffers used in Fig. 3A would preclude the entry of extracellular calcium as well as the release from intracellular stores (as these will be depleted in the absence of extracellular calcium). Therefore, these experiments demonstrate the requirement of calcium, but do not unambiguously identify the calcium source. Similarly, the release of calcium from mitochondrial stores (Ru360 treatment) demonstrate that a rise in intracellular calcium is sufficient for cholesterol loss, but do not identify the endogenous source triggered by glutamate receptor activation. The involvement of calcium intracellular stores is mostly based on the results with the STIM2 knock-out neurons, which are characterized by low calcium levels at intracellular stores because of their defective capacitative calcium entry. Nevertheless, additional pharmacological evidence targeting calcium intracellular stores would be important to strengthen this point. This could be done using caffeine- or thapsigargin-pretreated synaptosomes to deplete intracellular stores.

We now present new data showing that treatment of synaptosomes with 10 μM thapsigargin in calcium-free buffer leads to a reduction of 8% in membrane cholesterol (new Figure 4). Since thapsigargin raises intracellular calcium by blocking the pumping of calcium at the level of the ER, this new result, together with the presented data on STIM2, suggest the requirement of the ER calcium depletion for the observed NMDA-mediated cholesterol effect. In fact, cholesterol loss in NMDA-stimulated synaptosomes is 10%.

Specific comment 4. *The presence of basal levels of Cyp46 at the plasma membrane is an important new piece of information revealed by this study. It would be important to provide a quantitative interpretation for this observation. In principle, these numbers could be obtained from the electron microscopy experiments or from the surface biotinylation assays.*

We have followed this referee recommendation and now show new quantifications in the new Figure 6. Surface biotinylation assays indicate that 20.1±2.3% of the cellular CYP46A1 is present at the plasma membrane in cultured neurons at steady-state situation. Upon stimulation, this quantity increases 16.0±5.1%, without changes in the total cellular pool of the enzyme. Moreover, quantification of the EM images revealed that 17.8% of the labeled CYP46A1 is at the plasma membrane *in vivo*, in steady-state conditions (saline-injected mice).

Specific comment 5. *Total levels of Cyp46 have been proposed to increase upon brain injury. Judging from the western blots in Fig. 5, total Cyp46 levels do not appear to change in response to KCl, but this is not explicitly stated in the text or figure. This would be an important observation, which should be expressed in a quantitative manner in Fig. 5. On a similar line, it would be interesting to test whether other membrane-associated proteins follow similar or different behaviors. For example, are AMPA or NMDA receptors delivered to the synaptic surface with this treatment? What about other ER-associated proteins, such as SERCA? This information would be important to evaluate the specificity of this regulatory mechanism for Cyp46.*

We now include the variation in total CYP46A1 (see new Figure 6). The lack of change in total CYP46A1 after high KCl stimulation is now stated in the corrected text, as suggested.

Minor points: 6. *The TIRF images shown in Fig. 6 do not directly monitor the translocation of*

Cyp46 to the cell surface, but rather its close proximity to the plasma membrane, as acknowledged by the authors in the Discussion section. Therefore, the wording describing Fig.6 in the Results section should be modified to state that the increase in TIRF signal is "consistent with" (but does not "indicate") Cyp46 translocation to the cell surface.

We have followed this reviewer indication and corrected the text accordingly.

Minor point: 7. *In page 9, it is stated that the active site of P450s faces the luminal domain, but the opposite is stated at the beginning of page 12.*

This mistake has been corrected.

Minor point 8*. I find rather confusing the explanation in page 12 of the topology of Cyp46 based on the surface biotinylation experiments and the use of a C-terminal antibody. Why should the antibody recognition sequence matter for this assay? Isn't surface labeling based on the biotinylation step (and not the antibody labeling)? Does this imply that there are no surface exposed lysine residues at the N-terminus for the biotin reagent to react with? This point should be clarified.*

In fact, the only aspect that matters in surface biotinylation assays is the presence of exposed lysine residues, which are absent in the N-terminus of CYP46A1. We did not explain this fact properly. Since we detect the protein by surface biotinylation we reason that the active site (C-terminus) may become exposed to the outer of the cells, which would be consistent with the occurrence of a true fusion event. We have clarified this issue.

Referee #3

General comment:

It seems to me that the presented data are too limited and not of broad enough interest for publication in EMBO J. In support of this notion, most of the cited earlier studies on regulation of neuronal cholesterol content and its effects on neuronal functions have appeared in highly specialized neuroscience journals, which might be a better option for the presented work.

It is correct that the consequences of cholesterol loss were published in specialized journals. On the other hand, the actual fact of lipid changes in neurons, both cholesterol and sphingomyelin, and the functional relevance were published in more general interest journals: Molecular Biology of the Cell (Martin et al., 2008) and Journal of Cell Science (Trovo et al., 2011). We felt The EMBO Journal was appropriate for the current results because of the elucidation of the cellular mechanism by which cholesterol may be removed from neurons, which could also be relevant to other cell types, as it is an intracellular calcium raise-dependent event. In this regard, our demonstration that excessive excitatory neurotransmission triggers a change in synaptic cholesterol content and that this leads to the translocation/approximation of ER/CYP46A1 membrane carriers was, to us, a cell biology advance, suitable therefore for The EMBO Journal.

Further, several effects are rather small, often barely larger than 10%. This point is the more striking as the different stimulus paradigms are very strong non-physiological stimuli (KCl for 30 min causes very long un-physiological depolarizations; kainic acid, which causes very severe seizures in rodents; NMDA can cause epileptogenic activity in neurons-all of that should be carefully monitored for a comprehensive study). It is unclear from the presented studies whether this modest reduction in cholesterol is of any functional relevance, an aspect that would have to be addressed for publication in EMBO J.

We understand this concern. However, a loss of 10% of a pleiotropic molecule like cholesterol (involved in receptor signaling strength, micro-domain formation and ion permeability, just to mention some functions) is likely to have important consequences. In agreement with this possibility, we now show that neurotransmitter-mediated cholesterol loss plays a role in the control of intracellular calcium levels under excitatory stimulation (see new Figure 3). This new piece of information adds functional weight to our work.

We agree with this referee in that we used non-physiological conditions of stimulation. However, this was exactly the aim of our work: to use conditions to dissect the mechanisms operating in situations like stroke or epileptic seizures. In fact, the title of our manuscript is *"Cholesterol loss during glutamate-mediated excitotoxicity"*. Moreover, it is well established in science that some intrinsic biological activities can only be revealed through experimental exaggerations (i.e. protein over-expression). We think that through these "non-physiological conditions" we have unveiled part of the dynamics of cholesterol in neurons. Whether the reported observations also apply to other cells under high intracellular calcium conditions is an interesting venue for research, derived from our work.

Specific comment 1. *The experiments on neuronal culture are only meaningful if the neurons were of high-quality - for instance, do they showed clearly discernible dendritic spines? Respective images should be provided for cultures in which changes in cholesterol has been demonstrated upon certain treatments for the various conditions-does their appearance, perhaps spine density, change? What happens to glutamate receptors and other synaptic, dendritic, and axonal markers?*

As mentioned above, we are utilizing a paradigm of neuronal stress, therefore neurons cannot be of "high-quality" after treatment, and numerous biochemical and structural changes occur. On the other hand, they are viable and functional and therefore suited for the type of studies we performed. To prove this, we now provide the following new data.
a) Field images of mature hippocampal neurons (14 days *in vitro*) treated with 10 μM NMDA for 30 minutes. The images reveal that treated neurons do not show dramatic morphological changes or abnormal cell loss (Supplementary Figure S2-Panel A).
b) Western blot of several synaptic proteins show that levels of expression are not affected by the treatment (Supplementary Figure S2-Panel B).
c) Fura-2 calcium experiments show that KCl-treated neurons present a proper calcium response to a second pulse of high KCl, 30 minutes after the initial pulse (new Figure 3A).
Furthermore, in Schubert et al. (J. Cell Biol. 172: 453-67, 2006) we have demonstrated that this type of experimental paradigm does not result in synaptic loss but in spine size changes. This could certainly be, among others, another consequence of the cholesterol loss here reported.

Specific point 2. *Synaptosomes typically consist of pinched off and re-sealed nerve terminals with postsynaptic structures that are open and not re-sealed attached to it (the postsynaptic density)-the postsynaptic site is thus not a closed structure and stimulation with KCl or NMDA should not lead to any influx of Ca-the PSD would be exposed to extracellular Ca all the time independent of NMDA or KCl. It is thus puzzling how NMDA can have an effect on cholesterol content unless it acts via the rather rare presynaptic NMDAR or through off target effects. At the minimum the synaptosomal preparations have to be well documented by EM and the above considerations carefully addressed (e.g., are there functional presynaptic NMDARs?).*

We believe this comment may arise from previous experiences of this reviewer with "bad" synaptosomal preparations. We have followed a well-established protocol for the purification of synaptosomes that renders sealed pre and post-synaptic compartments, as determined by electron microscopy (see Nagy & Delgado-Escueta, J. Neurochem. 43: 1114-23, 1984; Bagni et al., J. Neurosci. 20: 1-6, 2000). We now provide EM data, for this referee only, showing that using this protocol, most synaptic structures are sealed.

Specific comment 3. *Blocking the mitochondrial Ca uniporter with Ru360 will have numerous side effects as mitochondrial functions will be affected and any involvement of Ca storage by mitochondria has to be carefully documents with additional experiments, which are typically considered tricky and therefore time consuming. The currently extremely limited dataset on mitochondrial Ca release is thus by far not sufficient to support the proposed model that Ca release from mitochondria is involved in cholesterol metabolism. In addition, mitochondria need to be loaded with Ca by preceding depolarizations before they can be stimulated to release substantial amounts of Ca-it is my understand that under resting conditions there is only a small amount of Ca in mitochondria.*

We do not make any claim that cholesterol loss is due to calcium release from mitochondria. We only claim, and this was our unique aim in this regard, that cholesterol loss requires the raise in

intracellular calcium. This was the reason for testing the effect of Ru360. In fact, we demonstrate that a rise in intracellular calcium by this mean is "sufficient" to trigger the cholesterol loss. To make this point even stronger, we now present new data showing that a raise in intracellular calcium as consequence of the ER depletion (through the use of thapsigargin) is also "sufficient" to induce a loss of cholesterol of a similar extent to that produced by NMDA (See new Figure 4).

Specific comment 4. *Similarly, support for a role of Stim2, which is an ER resident protein involved in capacitative Ca entry upon depletion of Ca in the ER stores, is very limited and based only on the finding that kainate-induced cholesterol loss is less in Stim2 KO mice. A role of Stim2 and how that ER resident connects to mitochondrial Ca has to be supported through several additional lines of evidence.*

The STIM2 KO experiments were performed to determine the possible involvement of the ER membrane fusion events in relation to the cholesterol loss, not to determine the role of ER calcium in this process. However, we realize that by doing this experiments we should have also addressed to which extent calcium from the ER plays a role. We now show that thapsigargin is able, per se, to induce cholesterol loss from synaptosomes.

Specific comment 5. The specificity of the antibody used for immunogold EM for Cyp46 needs to be established, ideally by use of KO mice or knockdown in cultures. The localization to the plasma membrane has to be somehow quantified perhaps before and after kainate treatment to functionally connect it to a change in cholesterol.

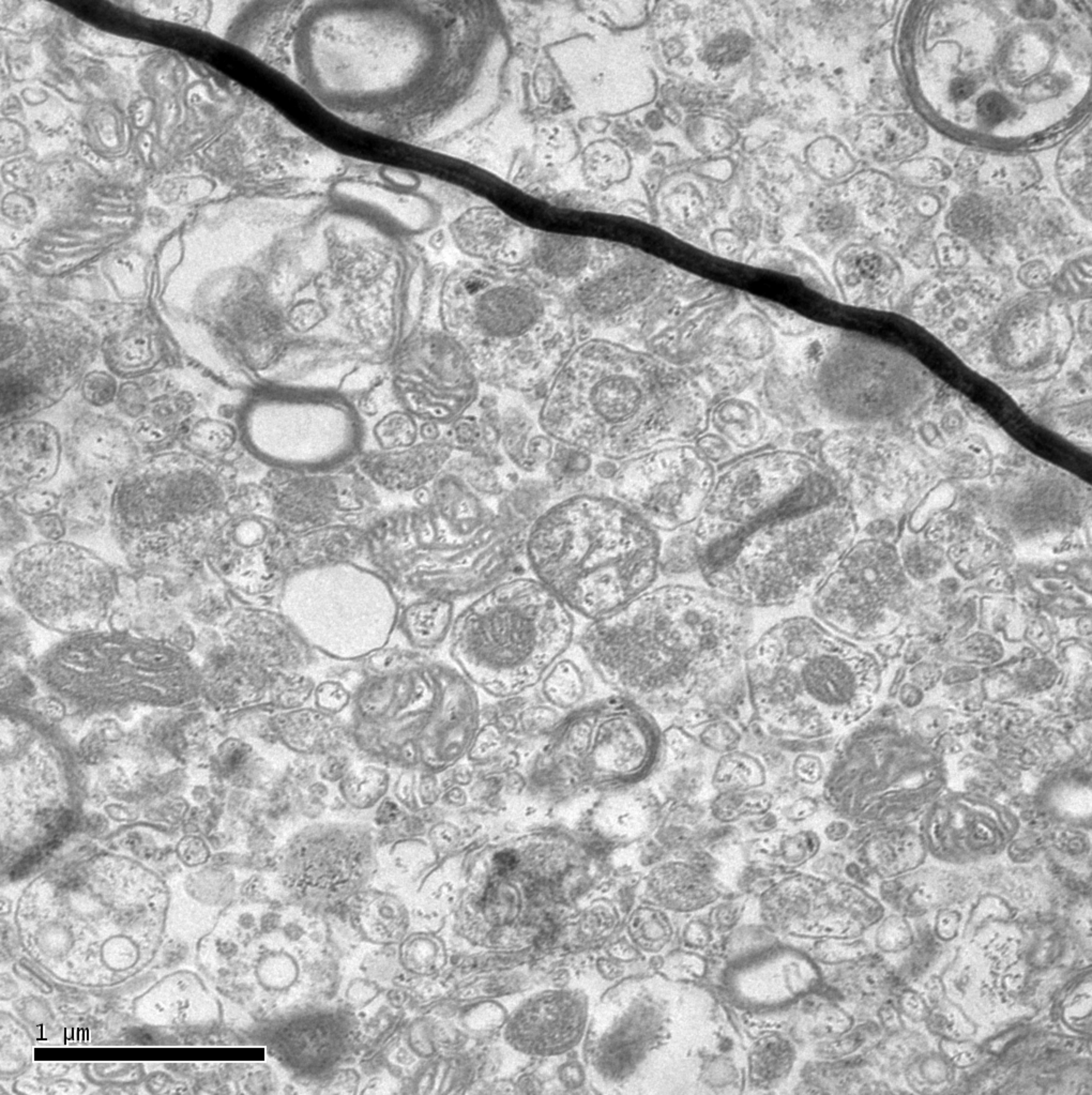
The specificity of the antibody used for the immunogold EM has been previously demonstrated, in the laboratory of Dr. D. W. Russell (see Lund et al., PNAS 96: 7238-43, 1999). In any event, we now provide new surface biotinylation experiments with a different antibody (new Figure 6), which confirmed the results obtained with the original antibody. Moreover, new EM data confirm the enrichment of the studied protein in the ER tubules and the plasma membrane (new Figure 5), and that the amount of protein in the plasma membrane increases upon stimulation with kainic acid (see Results).

Specific point 6. *KCl-induced redistribution of Cyp46 towards the plasma membrane is marginal (~10%) and only obvious following 5 min of KCl treatment. It is not obvious, possibly reversed, after 10 and 15 min treatments and does not match the 30 min KCl treatment used to show the modest decrease in cholesterol. The link between this redistribution and cholesterol depletion is thus at best tenuous. Specificity for surface labeling is not monitored (absence of biotinylation of an abundant cytosolic protein, e.g., actin) and the surface signals appear so weak that trust in their quantification is limited*.

We have increased the number of surface biotinylation experiments. The new results more clearly show the presence of enzyme at the plasma membrane in steady-state conditions and its increase after stimulation. Quantitatively, 20.1±2.3% of the neuronal CYP46A1 is present at the plasma membrane in steady-state conditions. Notably, a similar percentage was obtained after quantification of the electron microscopy experiments (17.8%). Upon high KCl stimulation, the surface amount increases 16.0±5.1%, without changes in the total cellular pool of the enzyme. We also show that the abundant cytoplasmic protein β-actin is not biotinylated. Finally, we now show CYP46A1 detection with two different antibodies (Russell's laboratory and ABCAM). We do not have a solid explanation for the fast increase in the levels of the enzyme in cultured hippocampal neurons. We now suggest that this could be related to the kinetics of cholesterol loss after hydroxylation, which may involve endocytosis and binding to transporters. These aspects are now considered in Discussion.

Specific point 7. *The redistribution of Cyp46 in HEK cells should be documented in neurons. Also rather than using ionomycin the authors should use more physiological stimuli or at least match the stimuli they use for cholesterol depletion (KCl, NMDA).*

We provide new TIRF data in hippocampal neurons that shows CYP46A1 mobilization towards the plasma membrane upon KCl stimulation (new Figure 8 and Supplementary Movies 2 and 3).


1 µm

2nd Editorial Decision 22 August 2011

Thank you for submitting your manuscript to the EMBO Journal. This is a resubmission of MS # 77261 that was rejected post review earlier this year. Your resubmission has now been seen by the original three referees that are listed in the same order below.

As you can see, while referee #3 is still not persuaded that the advance and insight provided is sufficient to consider publication in the EMBO Journal, referee #1 and 2 are much more supportive. The main concern raised by referee #1 is that we need further support for that plasma membrane associated Cyp46a1 is functional and that 24S-hydroxysterol is produced. Given the mixed assessment from the referees, I decided to get further advice on the study from a good expert in the field. I have now heard back from the advisor and the advice is listed below. The advisor finds the analysis interesting and suitable for publication here, but is also in agreement with referee #1 that we need good support for that 24S-hydroxysterol is actually produced. This is one of the key messages of the paper. Given all the available input, I would like to invite you to submit a revised manuscript, should you be able to provide more definitive support for that plasma membrane associated Cyp46a1 is functional and that 24S-hydroxysterol is produced. This is the main issue that has to be addressed in a revised manuscript. As you can see below, the advisor has 2 other comments, but they can be addressed with appropriate text changes. Please also respond appropriately to the specific concerns raised by referee #3 either in the point-by-point response or were needed in the text and figures.

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.

Editor
The EMBO Journal

REFEREE REPORTS

Advice:

1) The authors suggest that cholesterol release is synaptic but there is actually no data in the paper that it occurs preferentially at synapses. The % loss of cholesterol is similar in membranes and synaptosomes - if there was a preferential loss in synapses the loss of cholesterol should be bigger in these preparations. This is not a central/major concern but should be addressed/discussed in the text.

2) The stimulation methods used in this study are rather non- physiological - KA injections in vivo and KCl treatment in vitro are very strong hyperphysiological stimuli - it can not be excluded that the phenomenon described here is only present under these extreme conditions and has limited physiological relevance.

3) The lack of evidence that 24S-hydroxysterol is actually produced is a major problem. As this is a major part of the hypothesis and message of the paper I feel that this needs to be addressed - either by testing whether CYP46A1 function is required for cholesterol loss or by improving methods to detect this metabolite in the medium (or in vivo). I agree with reviewer 1 that this is a key issue.

Referee #1

Sodero et al present data in support of a link between excitatory neurotransmission, cholesterol

homeostasis and localisation of cholesterol 24-hydroxylase. This is the second time I have seen this manuscript and I would like to commend the authors for their efforts in addressing the reviewers' comments and improving the manuscript. While many of my previous issues have been dealt with there are a few points which necessitate clarification by the authors, centred on the mechanistic explanation for the decrease in cholesterol levels.

Theoretically there are three metabolic 'fates' for hippocampal free cholesterol - removal from the cell via cytochrome P450 catalysed mechanism(s), removal via lipoprotein mediated pathways and storage as steryl esters. The focus of the authors is on the P450 route and they have concentrated on Cyp46a1, a major neuronal cytochrome P450.

The authors admit that they have no evidence that plasma membrane associated Cyp46a1 is functional - they have attempted to identify the enzymatic product (24S-hydroxycholesterol) in the medium of their cell experiments, but have been unable to detect it. The magnitude of the decrease in the cells - up to 25% or so in some experiments - implies that, if 24S-hydroxylation is the reason for the decreased cholesterol, that there will be a significant increase in oxysterol content in the medium which should be detectable as an equivalent increase in the cells or medium. The authors speculate that they are unable to detect it due to either rapid non-enzymatic oxidation or binding to the culture plasticwear. Given that 24S-hydroxycholesterol is produced at about 100 pg/hr/µg cell DNA and that medium was conditioned by (presumably) 150,000 cells, each containg about 10pg of DNA, only 80-100 pg or so of 24S-hydroxycholesterol could have been produced in the treatment period by each dish, levels which are below the detection limit of most methods. If all of the decrease is accounted for by transformation into 24S-hydroxycholesterol, then it should be possible to detect this using existing methodology. Moreover, 24S-hydroxycholesterol is abundant in the hippocampus and should be readily detectable in the KA treated animals.

The alternative explanations have not been excluded by the authors, namely that unmodified cholesterol is leaving the cells and accumulating in the medium or that cholesterol is being redistributed within the cell. In the absence of demonstration that there is an increase in 24S-hydroxycholesterol is important that these two possibilities are excluded as much as possible. With regards to the first possibility the authors should provide data on the levels of cholesterol in the medium which is well within the capacity of the sterol determination method(s) at their disposal. It has been reported (see Hutter-Paier et al, Neuron, 2004) that as much as 25% of the cholesterol content of hippocampal neurons is esterified. This is a substantial pool which the authors' method does not detect and which is essential to exclude intracellular redistribution as a possible explanation, particular given the discussion of plasma and ER membrane proximity and enhanced sterol exchange.

In the data shown in figure three the authors state that they use methyl-β-cyclodextrin at 0.1 mM. At this concentration MβCD will not influence the steady-state levels of cholesterol in the medium of the cells; rather it will influence the rate of transfer of cholesterol between extracellular acceptors (e.g. lipoproteins) and the plasma membrane of the cells. It is also unclear what the format of the cholesterol delivery system is - I am unclear as to how much MβCD is present in the 30 uM solution which is applied with a 5.2 mM cholesterol solution (does this contain lipoproteins and/or phospholipids?). Delivery of cholesterol via MβCD typically requires concentrations in excess of 5mM. It is highly unlikely that incubation of cells under these conditions would have specific effects on cholesterol levels, particularly given the short time frame and the authors should clarify the methodology and data interpretation.

Referee #2

This revised manuscript by Sodero et al. proposes a molecular mechanism for the loss of cholesterol in neurons in response to glutamatergic neurotransmission. This mechanism involves the translocation of Cyp46A1, a cholesterol degrading enzyme, from intracellular compartments to the plasma membrane. This translocation is triggered by glutamate activation and membrane depolarization, followed by release of calcium from intracellular stores. Specifically, this work has been significantly strengthened by the inclusion of new experiments addressing:
- Presynaptic versus postsynaptic role of depolarization in cholesterol loss (glutamate receptor activation in the presence of tetrodotoxin, Fig. S1).

- Source of calcium rise upon neuronal activation (thapsigargin experiments in Fig. 4).
- Translocation of Cyp46A1 to the plasma membrane in activated neurons (new Fig. 8).
In addition, the authors now show that the activity-induced loss of cholesterol modulates calcium signaling within the neuron (new Fig. 3).

These are novel and significant results, which should be of general interest for the readership of EMBO Journal.

Referee #3

Although I appreciate the extra effort the authors dedicated to revise this manuscript, the newly added experiments are not that much more conclusive than the original results and the specific responses are unconvincing. A number of issues could have been more rigorously approached than presented. For instance, Reviewer 1 asks whether 0.2% SDS in the samples could affect the protein determination. Rather than showing protein assay curves at different SDS concentrations the authors just state that the actual SDS concentration was 0.04% in the assay-they should have shown that 0.04% SDS does not affect the assay - a rather simple experiment!

I maintain that the presented data are too limited and not of broad enough interest for publication in EMBO J. Specific concerns that remain from the previous submission or arise from newly added data and statements are as follows:

1. As stated previously, neuronal cultures have to be of high quality to be meaningful for testing either physiological or pathological effects. I had specifically asked whether they showed clearly discernible dendritic spines and that respective images are be provided. The authors refer to supplemental figure 2, in which the magnification is far too low to evaluate spine integrity. If untreated (control) neurons don't have spines it will be questionable whether data from these cultures or generally applicable.

2. As stated previously, synaptosomes typically consist of pinched off and re-sealed nerve terminals that may (or may not) have attached to it postsynaptic structures that are typically open and not re-sealed. This has been worked out by Whittaker and co-workers more than 50 years ago. In fact, the citation Nagy and Delgado-Escueta 1984, which I know well, is in full agreement with this: all (!) structures shown in Fig. 4 (depicting their synaptosomal fraction) are clearly presynaptic nerve terminals as they are filled with synaptic vesicles and often containing mitochondria; these elements are very rarely discernable in postsyanptic sites be EM and leave no doubt that Nagy and Delgado-Escueta had only re-sealed pre- but not post-synaptic structures. There is not a single vesicular structure in the Nagy figure that resembles the postsynaptic structure, which can be identified by postsynaptic densities. The Bagni citation is given incorrectly and I could not find it. The synaptosomal figure provided to reviewers is a) not a very clean synaptosomal preparation but contains many more non-defined structures than the Nagy figure; b) does not contain any (!) obvious structure that looks like expected for a re-sealed postsynaptic structure. The authors certainly did not label which structure they think would reflect re-sealed postsynaptic sites. Accordingly, there are no closed postsynaptic structures and stimulation with KCl or NMDA should not lead to any influx of Ca - the PSD would be exposed to extracellular Ca all the time independent of NMDA or KCl. I should add that in the EM by the authors there is little evidence for any PSDs so postsynaptic sites are a minor component. This is what I would consider a very serious flaw of this manuscript.

3. There is no causative link between CYP46A redistribution and cholesterol loss. As pointed out by Reviewer 1, CYP46A would require a second enzyme (POR) for catalytic activity. The authors fail to provide evidence for CYP46A being actually active at the plasma membrane and I don't see any evidence for a causative link of CYP46A relocalization and cholesterol loss, a minimal requirement for publication in EMBO J.

4. Evidence for a role of Stim2, which is an ER resident protein involved in capacitative Ca entry upon depletion of Ca in the ER stores, is very limited and based only on the finding that kainate-induced cholesterol loss is less in Stim2 KO mice. As stated before, a role of Stim2 and how that ER resident connects to mitochondrial Ca has to be supported through several additional lines of evidence. The thapsigargin experiments do not really address this issue in a convincing manner.

5. As stated before, KCl-induced redistribution of Cyp46 towards the plasma membrane is marginal (~10%) and only obvious following 5 min but not 10 and 15 min of KCl treatment. It does not match the 30 min KCl treatment used to show the modest decrease in cholesterol. The link between this redistribution and cholesterol depletion is thus at best tenuous, an aspect of my previous critique that has not really been discussed in the authors' reply.

6. The redistribution of Cyp46 in HEK cells and neurons monitored by TIRF is in general a reasonable addition but to be honest the video images are not as convincing as desirable, in part because to build confidence in the interpretation of the observed signals one would want to perform a number of additional experiments. Also, this analysis requires overexpression of CYP46 which could result in artificial redistribution although the surface biotinylation experiments alleviate this concern to some degree.

7. the use of a second CYP46 antibody is reassuring but a more convincing approach would have included an evaluation of the specificity of both antibodies (e.g., IF and immunoblotting for CYP46 upon manipulation of its abundance-overexpression or knock down, which should result in altered immunoreactivities).

1st Revision - authors' response 30 November 2011

Advisor

The lack of evidence that 24S-hydroxysterol is actually produced is a major problem. As this is a major part of the hypothesis and message of the paper I feel that this needs to be addressed - either by testing whether CYP46A1 function is required for cholesterol loss or by improving methods to detect this metabolite in the medium (or in vivo). I agree with reviewer 1 that this is a key issue.

In the current version we provide new data demonstrating the increase in 24S-hydroxycholesterol upon stimulation with NMDA (Supplementary Figure 3 and underlined text page 10). We also show that stimulation-mediated cholesterol loss is prevented by the knockdown of CYP46A1 (Supplementary Figure 3 and underlined text, page 10).

Referee #1

Sodero et al present data in support of a link between excitatory neurotransmission, cholesterol homeostasis and localization of cholesterol 24-hydroxylase. This is the second time I have seen this manuscript and I would like to commend the authors for their efforts in addressing the reviewers' comments and improving the manuscript. While many of my previous issues have been dealt with there are a few points, which necessitate clarification by the authors, centered on the mechanistic explanation for the decrease in cholesterol levels.
Theoretically there are three metabolic 'fates' for hippocampal free cholesterol - removal from the cell via cytochrome P450 catalyzed mechanism(s), removal via lipoprotein mediated pathways and storage as steryl-esters. The focus of the authors is on the P450 route and they have concentrated on CYP46A1, a major neuronal cytochrome P450.
The authors admit that they have no evidence that plasma membrane associated CYP46A1 is functional - they have attempted to identify the enzymatic product (24S-hydroxycholesterol) in the medium of their cell experiments, but have been unable to detect it. The magnitude of the decrease in the cells - up to 25% or so in some experiments - implies that, if 24S-hydroxylation is the reason for the decreased cholesterol, that there will be a significant increase in oxysterol content in the medium, which should be detectable as an equivalent increase in the cells or medium. The authors speculate that they are unable to detect it due to either rapid non-enzymatic oxidation or binding to the culture plastic-wear. Given that 24S-hydroxycholesterol is produced at about 100 pg/hr/ÎŒg cell DNA and that medium was conditioned by (presumably) 150,000 cells, each containing about 10 pg of DNA, only 80-100 pg or so of 24S-hydroxycholesterol could have been produced in the treatment period by each dish, levels which are below the detection limit of most methods. If all of the decrease is accounted for by transformation into 24S-hydroxycholesterol, then it should be

possible to detect this using existing methodology. Moreover, 24S-hydroxycholesterol is abundant in the hippocampus and should be readily detectable in the KA treated animals.
The alternative explanations have not been excluded by the authors, namely that unmodified cholesterol is leaving the cells and accumulating in the medium or that cholesterol is being redistributed within the cell.
In the absence of demonstration that there is an increase in 24S-hydroxycholesterol is important that these two possibilities are excluded as much as possible.
With regards to the first possibility the authors should provide data on the levels of cholesterol in the medium, which is well within the capacity of the sterol determination method(s) at their disposal.

We thank this referee for this helpful comment. By means of LQ/MS we are now able to prove that excitatory neurotransmission induces the release into the medium of 24S-hydroxycholesterol (see page 10 and Supplementary Figure 3).
The levels of 24S-hydroxycholesterol were normalized to the levels of phospholipids, which do not change upon NMDA stimulation.

It has been reported (see Hutter-Paier et al, Neuron, 2004) that as much as 25% of the cholesterol content of hippocampal neurons is esterified. This is a substantial pool which the authors' method does not detect and which is essential to exclude intracellular redistribution as a possible explanation, particular given the discussion of plasma and ER membrane proximity and enhanced sterol exchange.

We appreciate this comment, to help us define the outcome of glutamate-mediated cholesterol loss. Although the use of an esterase in our enzymatic assay favors the conclusion that most of our cholesterol reduction corresponds to "free cholesterol", we cannot at this point rule out that excitatory stimulation led to small changes in the rate of cholesterol esterification. Irrespective of this, and in line with our hypothesis and conclusions, the increased levels of 24S-hydroxycholesterol in the medium of stimulated cells and the prevention of the cholesterol loss in a CYP46A1 loss-of-function experiment strongly indicate that the loss due to excitatory neurotransmission occurs via the mobilization/activation of the CYP46A1 enzyme.

In the data shown in figure three the authors state that they use methyl-β-cyclodextrin at 0.1 mM. At this concentration MBCD will not influence the steady-state levels of cholesterol in the medium of the cells; rather it will influence the rate of transfer of cholesterol between extracellular acceptors (e.g. lipoproteins) and the plasma membrane of the cells.

We agree with this referee. Indeed, our intention was to affect the amount of plasma membrane cholesterol to a minimum, so to mimic the effect of the NMDA stimulation, which leads to a 20% membrane cholesterol loss. Higher concentrations of MBCD can induce a greater loss, which in turn leads to cell membrane compromise and death.

Is also unclear what the format of the cholesterol delivery system is - I am unclear as to how much MBCD is present in the 30 μM solution which is applied with a 5.2 mM cholesterol solution (does this contain lipoproteins and/or phospholipids?).
Delivery of cholesterol via MBCD typically requires concentrations in excess of 5 mM. It is highly unlikely that incubation of cells under these conditions would have specific effects on cholesterol levels, particularly given the short time frame and the authors should clarify the methodology and data interpretation.

We acknowledge the criticism of this referee, which led us to identify a typographic error.
The concentration of cholesterol (used in combination with 30 μM of Sigma® water-soluble cholesterol) was "5.2 μM", and not "5.2 mM". This has been corrected in the text and the corresponding legend for Figure 3.
So, the cholesterol delivery solution contains a combination of Sigma® water-soluble cholesterol (that is cholesterol stabilized with MBCD) and cholesterol, at final concentrations of 30 μM and 5.2 μM, respectively.
Water-soluble cholesterol has been shown to be effective for the delivery of cholesterol to the "*plasma membrane"* in cell culture conditions. The addition of "free cholesterol" to the delivery

solution has the aim to stabilize the water-soluble cholesterol, shifting the equilibrium towards the formation of the complex with MBCD.
Our delivery solution does not contain lipoproteins or phospholipids.

Referee #2

This revised manuscript by Sodero et al. proposes a molecular mechanism for the loss of cholesterol in neurons in response to glutamatergic neurotransmission. This mechanism involves the translocation of CYP46A1, a cholesterol-degrading enzyme, from intracellular compartments to the plasma membrane. This translocation is triggered by glutamate activation and membrane depolarization, followed by release of calcium from intracellular stores. Specifically, this work has been significantly strengthened by the inclusion of new experiments addressing:
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- Source of calcium rise upon neuronal activation (thapsigargin experiments in Fig. 4).
- Translocation of CYP46A1 to the plasma membrane in activated neurons (new Fig. 8).
In addition, the authors now show that the activity-induced loss of cholesterol modulates calcium signaling within the neuron (new Fig. 3).
These are novel and significant results, which should be of general interest for the readership of EMBO Journal.

We thank the positive comments of this referee.

Referee #3

Although I appreciate the extra effort the authors dedicated to revise this manuscript, the newly added experiments are not that much more conclusive than the original results and the specific responses are unconvincing. A number of issues could have been more rigorously approached than presented. For instance, Reviewer 1 asks whether 0.2% SDS in the samples could affect the protein determination. Rather than showing protein assay curves at different SDS concentrations, the authors just state that the actual SDS concentration was 0.04% in the assay-they should have shown that 0.04% SDS does not affect the assay - a rather simple experiment!

We did not show SDS curves and we only said that 0.04% SDS does not affect the protein assay because this has been established many years ago and is well accepted in the biochemistry routine. Furthermore, the higher SDS concentration that is compatible with the protein method we used is 0.1%. As a matter of fact, Reviewer #1 was satisfied with our answer. His/her question simply arose because our lack of clarity: we only mention the SDS concentration used for the membrane solubilization, but not the SDS concentration of the final reaction volume for protein measurement.

I maintain that the presented data are too limited and not of broad enough interest for publication in EMBO J. Specific concerns that remain from the previous submission or arise from newly added data and statements are as follows:
1. As stated previously, neuronal cultures have to be of high quality to be meaningful for testing either physiological or pathological effects. I had specifically asked whether they showed clearly discernible dendritic spines and that respective images are be provided. The authors refer to supplemental figure 2, in which the magnification is far too low to evaluate spine integrity. If untreated (control) neurons don't have spines it will be questionable whether data from these cultures or generally applicable.

Experimental paradigms based on “excess”, whether it is gain or loss-of-function, through protein over-expression or down-regulation or via drugs that activate or inhibit proteins or lipids, are a common and useful tool to try to elucidate “normal” cellular-molecular responses. Indeed, unless one is interested in establishing temporal/spatial correlations, the function of a protein or a lipid in any given cellular context can be only investigated through the use of “extreme” experimental paradigms. This is exactly how we got to know the organization of different pathways. Concerning our topic, the use of an excess of neurotransmitter (excitoxicity paradigm) has allowed us to discover the dynamics and organization of the actin cytoskeleton at dendritic spines, and the

existence of a local synthesis machinery, just to mention a few essential biological pathways known thanks to the use of a similar strategy. Equally important to answer to this reviewer, much of this kind of work has been published in Cell Biology journals.

It seems that this reviewer did not understand that our work is based on an **excitotoxity** paradigm (Title of our manuscript: Cholesterol loss during glutamate-mediated excitotoxicity). Consequently, it precludes physiological maintenance (as a matter of fact, we published in The Journal of Cell Biology showing that dendritic spines collapse under this condition, through a glutamate receptor-RhoA mechanism). Naturally, any paradigm based on "excess" (there are just few "ideal" paradigms) breaks homeostasis, therefore physiology. On the other hand, without our "excess" approach we would not knew the "possibility" that cholesterol could be removed from the plasma membrane following the mobilization of CYP46A1. Whether or not this event and underlying mechanism occur in the steady-state condition remains to be established, upon the development of the right tools.

2. As stated previously, synaptosomes typically consist of pinched off and re-sealed nerve terminals that may (or may not) have attached to it postsynaptic structures that are typically open and not re-sealed. This has been worked out by Whittaker and co-workers more than 50 years ago. In fact, the citation Nagy and Delgado-Escueta 1984, which I know well, is in full agreement with this: all (!) structures shown in Fig. 4 (depicting their synaptosomal fraction) are clearly presynaptic nerve terminals as they are filled with synaptic vesicles and often containing mitochondria; these elements are very rarely discernable in postsyanptic sites be EM and leave no doubt that Nagy and Delgado-Escueta had only re-sealed pre- but not post-synaptic structures. There is not a single vesicular structure in the Nagy figure that resembles the postsynaptic structure, which can be identified by postsynaptic densities. The Bagni citation is given incorrectly and I could not find it. The synaptosomal figure provided to reviewers is a) not a very clean synaptosomal preparation but contains many more non-defined structures than the Nagy figure; b) does not contain any (!) obvious structure that looks like expected for a re-sealed postsynaptic structure. The authors certainly did not label which structure they think would reflect re-sealed postsynaptic sites. Accordingly, there are no closed postsynaptic structures and stimulation with KCl or NMDA should not lead to any influx of Ca - the PSD would be exposed to extracellular Ca all the time independent of NMDA or KCl. I should add that in the EM by the authors there is little evidence for any PSDs so postsynaptic sites are a minor component. This is what I would consider a very serious flaw of this manuscript.

We find of extremely little relevance to enter into discussion about the usefulness of an experimental tool widely utilized in Neuroscience. Irrespective of the liking of this reviewer, synaptosomes are used for the study of synaptic vesicle filling, composition of the pre- and post-synaptic specializations (active zone and post-synaptic density) and control of local mRNA translation. If for all these works the use of synaptosomes is acceptable, then it comes that its use is also acceptable to investigate lipid content under different experimental conditions. Moreover, the claim of this reviewer is also technically unfounded, as we have followed a well-established protocol for the purification of synaptosomes, used for other scientists at The EMBO Journal and Cell (Pilo Boyl et al, EMBO J 26: 2991-3002, 2007; Napoli et al, Cell 134(6): 1042-54, 2008).
In the actual version of the manuscript we have eliminated the citation Nagy & Delgado-Escueta, to avoid confusion. In fact, we did not follow strictly their gradient protocol with Percoll. Initially, we referenced this paper because these authors were the first ones describing how to purify synaptosomes using Percoll gradients.
It is true that the citation Bagni et al (J Neurosci 20: 1-6, 2000) is not given in the manuscript: it was just included in the previous point-by-point response in order to clarify that pre and post sealed-synaptic structures can associate. Indeed, this is demonstrated by electron microscopy in the Figure 1-A of this paper, in which very interesting functional studies in synaptosomes are reported.
Finally, in the worst scenario, and accepting the criticism that this type of preparation does not clearly define pre- and post- synaptic compartments, which for us is an unfounded opinion, our experiments with the specific NMDA receptor antagonist AP-5 in cultured hippocampal neurons demonstrate that much of the stimulation-mediated cholesterol loss is post-synaptic.

3. There is no causative link between CYP46A1 redistribution and cholesterol loss. As pointed out by Reviewer 1, CYP46A1 would require a second enzyme (POR) for catalytic activity. The authors fail to provide evidence for CYP46A1 being actually active at the plasma membrane and I don't see

any evidence for a causative link of CYP46A re-localization and cholesterol loss, a minimal requirement for publication in EMBO J.

We provide new data showing that CYP46A1 is active at the plasma membrane: NMDA-stimulated neurons show and increased efflux of 24S-hydroxycholesterol. In addition, knockdown of CYP46A1 prevents KCl-induced cholesterol loss in cultured neurons (Supplementary Figure S3).

4. Evidence for a role of Stim2, which is an ER resident protein involved in capacitive Ca entry upon depletion of Ca in the ER stores, is very limited and based only on the finding that kainate-induced cholesterol loss is less in Stim2 KO mice. As stated before, a role of Stim2 and how that ER resident connects to mitochondrial Ca has to be supported through several additional lines of evidence. The thapsigargin experiments do not really address this issue in a convincing manner.

STIM2 experiments were performed to determine the possible involvement of this molecule in the ER membrane fusion events in relation to the cholesterol loss, not to elucidate the particular mechanisms by which the internal stores contribute to this process. We show: a) that the ER calcium sensor STIM2 is required for cholesterol loss; b) that the ER calcium depletion process participates in this process.

5. As stated before, KCl-induced redistribution of Cyp46 towards the plasma membrane is marginal (~10%) and only obvious following 5 min but not 10 and 15 min of KCl treatment. It does not match the 30 min KCl treatment used to show the modest decrease in cholesterol. The link between this redistribution and cholesterol depletion is thus at best tenuous, an aspect of my previous critique that has not really been discussed in the authors' reply.

We do not understand this comment. First of all, “marginal” was significant, therefore of a likely biological relevance. A “marginal” and significant loss of cholesterol will have a “non-marginal” effect on cation influx, just to mention how something “marginal” can have dramatic consequences. One should be very careful to not underscore the relevance of a “marginal” (and significant) change. Secondly, the “marginal” redistribution of CYP46A1 that gave rise to this comment came from a study performed in synaptosomes. In the revised manuscript we presented data from cultured hippocampal neurons, showing higher surface levels of CYP46A1, detected with two different antibodies. In other words, this question was answered in the revised version.

6. The redistribution of Cyp46 in HEK cells and neurons monitored by TIRF is in general a reasonable addition but to be honest the video images are not as convincing as desirable, in part because to build confidence in the interpretation of the observed signals one would want to perform a number of additional experiments. Also, this analysis requires overexpression of CYP46 which could result in artificial redistribution although the surface biotinylation experiments alleviate this concern to some degree.

With the first version, this reviewer specifically requested to add TIRF experiments in neurons. We had not done so because the point was to simply test if CYP46A1 movement was sensitive to an intracellular calcium rise. In any event, we did the TIRF experiments in neurons stimulated with high potassium. Quite contradictorily, this reviewer now argues that over-expression of CYP46A1 may lead to artificial redistribution. One then wonders what moved this reviewer to ask for this experiment. Irrespective of motivation, it is not possible to perform TIRF microscopy on endogenous CYP46A1. Finally, we do not reach conclusions based only on a single experiment (i.e. TIRF microscopy).

7. The use of a second CYP46 antibody is reassuring but a more convincing approach would have included an evaluation of the specificity of both antibodies (e.g., IF and immunoblotting for CYP46 upon manipulation of its abundance-overexpression or knock down, which should result in altered immunoreactivities).

The specificity of the antibody, broadly accepted in any case, is now illustrated in the Supplementary Figure S3, showing reduced amounts of CYP46A1 in shCYP46A1 knockdown neurons.

Additional Correspondence 10 January 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I asked referee #1 to review the revised version and have now received the comments back. As you can see below, the referee appreciates the introduced changes and support publication here. I am therefore pleased to proceed with the acceptance of the paper for publication here. You will receive the formal acceptance letter shortly.

Best Karin

Editor
The EMBO Journal

REFEREE REPORT

Referee #1:

Sodero et al present a revised manuscript detailing their studies of cholesterol loss during excitotoxicity which includes new data. Critically it now includes data on the levels of 24S-hydroxycholesterol in the medium of cultured cells. This is essential to draw the conclusion that the loss of cholesterol from the PM is due to the action of CYP46A1. While I would prefer to see the changes displayed as amounts, I think that the 24S-OHC/ PL ratio is acceptable. The author also present data on changes in hippocampal cholesterol in an experimental animal model which is consistent with their cell work. Several methodological aspects have also been clarified which increases the clarity of the paper. While I have some concerns about the biological relevance I do think that it is important that this data be published and scrutinised and discussed by the oxysterol community at large.

