February 3, 2021

Nam Nguyen Ph.D.,
Manager of Review,
JoVE

Dear Dr. Nguyen,

Thank you for reviewing our manuscript entitled, "Molecular modulation by lentivirus-delivered specific shRNAs changes neurons' vulnerability to neurite atrophy induced by endoplasmic reticulum stress", by Carolina Morales, Mariano Bisbal and Mariana Bollo. We have revised the manuscript in accordance with the reviewers and editor's suggestions and we are resubmitting it following their recommendations.
We have addressed all the issues raised by the review. The specific changes to the manuscript are presented below. Editor and reviewer's comments are in "quotes" and italicized.

Response to the editor's comments:

"Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues."
Response: In the revised manuscript we have addressed spelling and grammar issues, and made some other corrections (either highlighted in yellow or crossed out), as follows:
1- The omission of number 1.0 was corrected (under the title "**Primary rat cortical neuron cultures**").
2- "ml" was changed to "mL" (in 1.1)
3- 290 was changed to 293 (in 3.4.1).
4- A parenthesis was deleted (in 1.4.).
5- Periods were added after some abbreviations (highlighted in yellow)
6- In the Table of materials, some missing information was included (typed in red in the revised version of the manuscript)

"2. Please revise the title for conciseness. The title should not be a complete sentence".
Response: The title has been changed as follows (highlighted in yellow in the revised manuscript):

Molecular modulation by lentivirus-delivered specific shRNAs in endoplasmic reticulum stressed neurons.

"3. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?"
Response: In the revised manuscript, we have addressed this including more details particularly in points 2.3, 2.4, 2.5, 2.6, 3.1 and 3.3 (highlighted in yellow and copied below). Also, minor changes have been made in the text in points 1.3, 1.4, 3.1 and 3.4.1 (highlighted in yellow in the revised manuscript).

2.3 Clone the shRNA insert into lentiviral vector pLKO.3G, with GFP as a green fluorescent marker. For this, digest 1 µg of vector with Eco RI and PacI restriction enzymes by mixing in a sterile tube:

Sterile ionized water	16 µL
10X Restriction enzyme buffer (100 mM Bis-Tris Propane-HCl, pH 6.5, 100 mM $MgCl_2$, 1 mg/mL Bovine serum albumin)	2 µL
pLKO3.G (1µg/µL)	1 µL
ECoR I (10 U/ µL)	0.5 µL
Pac I (10 U/ µL)	0.5 µL

Mix gently by pipetting. Incubate the reaction for 3-4 h at 37 °C. Note: overnight digestion is generally unnecessary and may result in DNA degradation.

2.4 For ligation, mix the digested vector and insert in a molar ratio of 3:1 and incubate it with the T4 ligase (**Table of materials**) and ligase buffer (provided by manufacturer) overnight at 16 °C.

At this point, ligation reaction can be stored at 4 °C until further use.

2.5 Transform DH5α competent cells (see **Table of materials**) or another substitutable *E.coli* strain with the ligation reaction as follows:

2.5.1: Mix competent *E. coli* with the total volume of ligation reaction and chill it on ice for 15 min, place it in a 42 °C bath for 2 min and then chill it on ice again for 15 min.

2.5.2: Transfer the total volume subjected to heat shock to a Falcon 15 mL tube and complete the volume to 1000 µL with Luria-Bertani (LB) liquid medium that has been previously maintained at 37 °C. Shake the bacteria for 90 min at 37 °C in an orbital shaker at approximately 330 rpm.

2.5.3: Centrifuge them at 5000 g for 5 min at 4 °C and discard 900 µL of supernatant. Use the remaining supernatant to resuspend the pellet. Spread bacteria suspension evenly over a solid ampicillin (100 µg/mL) LB plate. Incubate at 37 °C ON, then check bacterial growth. At this point, the bacteria may be stored at 4 °C for 4-5 days.

2.5.4. Pick a single colony to transfer into a Falcon 50 mL tube with 10 mL of LB liquid medium and ampicillin (100 µg/mL). Shake the bacteria ON at 37 °C at approximately 330 rpm. Centrifuge the culture to pellet the bacteria at 5000 g for 5 min at 4 °C, discard the supernatant and conserve the bacterial pellet. This can be stored at -20 °C.

°C.

2.6 Purify the plasmid with a DNA purification kit from bacteria pellet following manufacturer's instructions (see **Table of materials**). Calculate the DNA concentration by measuring the absorbance at 260 nm and multiplying by the dilution factor, using the following relationship: A_{260} of 1.0 = 50 µg/mL pure double strand (ds) DNA.

3.2 Mix 14 µg pKLO.3G vector with specific insert, 10.5 µg of packing plasmid psPAX and 3.5 µg of envelope plasmid pMD2.G (see **Table of materials**). Dilute 45 uL of plasmid transfection reagent (see **Table of materials**) in 700µL of reduced serum

media (see **Table of materials)** and incubate for 5 min at room temperature. Then combine the DNA mixture with the diluted plasmid transfection reagent, mix gently, and incubate for 20 min at room temperature.

3.3 Meanwhile, change for fresh DMEM the medium of the culture dish of HEK 293 cell cultures (70 % confluent at the time of transfection). Then add the entire volume of the DNA solution drop by drop. Rock the dish gently. It is not necessary to add/change medium after transfection. Incubate the cells at 37 °C, 5 % CO_2 for 48 h- 72 h to allow shRNAs to reach their optimum transduction, checked by GFP fluorescence using a fluorescence microscope. Afterwards, collect the medium with lentivirus and store at 4 °C.

"4. Was any anesthesia used before euthanasia?"
Response: To address this concern. The sentence changed (in 1.0) is copied below (either highlighted in yellow or crossed out in the revised manuscript)

Anesthetize E18 Wistar pregnant ~~E18 Wistar~~ rats in a CO_2 chamber with admixture of 80% CO_2/20% O_2 for 60 s of exposure and then sacrifice then by dislocate the cervical vertebrae.

"5. Please specify all surgical instruments used"
Response: In the revised manuscript, we have addressed this including more details particularly in points 1.0 and 1.1, as follow:

1.0 The following steps are performed in a laminar flow hood. Extract encephalon from the embryos with a forceps style # 3 and straight sharp small spring scissors (see **Table of material**) to Hanks solution in cell culture dishes.

1.1 Dissect the tissue under 20X magnifying glass, using two fine-tippeds forceps style #5, separating the frontal cortex from the meninges.

"6. 1.1: How much trypsin-EDTA is used? Please specify all volumes used throughout."
Response: We apologize for this omission. In the new version of the manuscript this information was included in point 1.1 (highlighted in yellow). Some wrong information was also deleted in this point (crossed out).

7. "2.3-2.5: How are these steps done? Please provide reaction recipes and conditions."
Response: We apologize for this lack of information. In the revised version, the reaction recipes and conditions were included (please see point "*3*").

"8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique Analisis de datos
c) Any limitations of the technique

d) The significance with respect to existing methods
e) Any future applications of the technique"

Response: To address this concern, we included the sentences copied below (highlighted in yellow in the revised manuscript) and the corresponding references.

It is important to note that, if primary cortical neuron cultures at 7-8 days *in vitro* already show high neurite outgrowth, these may be used to induce stress and perform the subsequent analysis, instead of 15 days *in vitro* cultures.

With respect to the neurite atrophy analysis procedure, this paper presents an alternative methodology that requires less manual work than specific Image J plug-ins that help to trace neurites. Commonly, these require that processes be individually traced for each neuron, while the steps followed in this paper trace the processes of multiple neurons using 5 sets of common accessible Image J commands at once. Both plug ins and this protocol procedure enable the value of all the processes in the sample image to be quantified.

We previously demonstrated a novel cytoprotective role for the ubiquitous CN in the early phase of UPR, triggered either by pharmacological tools or ischemic processes [1] [2] [3]. Also others and we described the impact of the inhibition of CHOP expression on cell survival in different systems [3] [4] [5]. Here we propose a methodology in which manipulating the expression of any of these genes enables to opposite modulates the beginning of the neuritic atrophy process in a cellular model of ER stress to be modulated in the opposite mode.

We envision that this methodology can be used to evaluate the susceptibility of neurons to undergo neuritic atrophy in different cell system models of ER stress and thus, contribute to understanding the molecular bases of the transition between acute and chronic phases of UPR in diverse neurodegenerative disease models.

"9. Please spell out journal titles in the references."
Response: To address this we modified the references to the citation style named "Superscripted Number" (EndNote), indicated for JoVE journal.

"We are awaiting the completion of your video as well. Please upload a high-resolution video here:"
https://www.dropbox.com/request/nSpeztiWnCEx3B63tj0u?oref=e

Response: We apologize for this delay. During the past year, because of the pandemic, we have been restricted from even accessing the Institute. This year we are slowly getting permits. We plan in March, after the summer recess, to complete the video.

Response to reviewer 1

"Manuscript Summary:
In this article, Morales et al. demonstrate that individual knockdown of calcineurin and CHOP controls cytoprotective and pro-apoptotic effects in cultured rat cortical neuronal cells. The authors used a lentiviral mediated shRNA approach to knockdown calcineurin and CHOP. The authors claim that this lentiviral-mediated approach could be useful for other cell culture models of ER stress for studying neurite atrophy. The manuscript in its current form needs rigorous improvement, particularly with images. There are also several typos and labeling errors that need to be addressed."

"Major Concerns:
The discussion part is short. It needs to be elaborated."
Response: As we mentioned before, to address this concern we included the sentences copied above in "8" (highlighted in yellow in the revised manuscript) and the corresponding references.

"The experiments are sketchy to prove the conclusion. Additional, methodological approaches need to be considered."
Response: We have addressed these issues, as indicated above. We have made specific changes, including more information in points 2.3, 2.4, 2.5, 2.6, 3.1 and 3.3 (highlighted in yellow).

"Minor Concerns:
1. In figure 1, replace the western picture with a good quality image, notably include all the groups in the same membrane. Also, there is a typo in the figure label. GAPDH labeled as GADPH."
Response: These figures are republished from our group's previous publication. For this, reprint permission was obtained from the publisher (indicated in the figures). The original article was cited in the revised version of the manuscript following the indications of the JoVE Author's guide. We apologize for the typo, which was corrected in the new version.

"2. The actin band in Figure 2 is not legible in the control group, so I suggest the authors replace the figure with another image."
Response: We agree that the actin band intensity in the control group is lower than the other actin bands and difficult to perceive, but it is possible to measure its intensity by densitometry and hence use it as loading control.

"3. Also, replace figure-3 and 4 with a different set of images."
Response: Figures 3 and 4 are republished from a previous publication of the group. The original article was cited in the revised version of the manuscript (please see Figures 3 and 4) following the indications of the JoVE Author's guide.

"4. Mention the viral titration used in this paper."
Response: The viral titration was made by flux cytometry. This is mentioned in point 3.4.1 in the original version of the manuscript.

"5. GM2 to be replaced with "ganglioside" when it first appears in the text."
Response: "Ganglioside" was included and is highlighted in yellow in the revised version of the manuscript (points 4 and 4.1.1).

"6. Line-63: mention the name of three UPR sensors."
Response: To address this, we have modified two paragraphs in the Introduction section (pasted below), including the new information requested (highlighted in yellow) and deleted some words, as follows:

Endoplasmic reticulum (ER) stress is defined as any perturbation that compromises protein-folding capacity in the organelle. The accumulation of unfolded proteins within the ER lumen activates a transduction cascade signal called the unfolded protein response (UPR). This complex signaling pathway is orchestrated by three stress sensors: PERK (protein kinase RNA [PKR]-like ER kinase), IRE1 (inositol-requiring enzyme 1) and ATF6 (activated transcription factor 6). All together these attempt to restore homeostasis in the organelle. But if stress persists, the UPR eventually induces cell death by apoptosis [6] .

~~One of the three pathways is controlled by (PKR)-like-ER kinase (~~PERK~~)~~, an ER transmembrane protein, upon ER stress, ~~PERK activation~~ leads the phosphorylation of eukaryotic initiation factor-2 alpha (eIF2α), reducing global protein synthesis and thus protein load in the ER [7].

"7. The study lacks rigor and statistical analysis."
Response: Statistical significance was determined by one-way ANOVA, as was indicated in the corresponding figure legends.

"8. Line-109 include the final concentration of trypsin used for digestion."

Response: We apologize for this omission. In the revised version of the manuscript, we include this information (highlighting in yellow and deleting some words; the sentence is pasted below).

1.1. Dissect the tissue under 20X magnifying glass, separating the frontal cortex from the meninges. Transfer the frontal cortex to a 15 ml conical tube and incubate the tissue with 3-4 mL of Trypsin-EDTA (0.25 %) ~~diluted in Hanks solution~~ for 15 min, at 37 °C for chemical digestion.

"9. Section 1.4, line-120, please check the culturing or incubation time after trypsin digestion. Two hours of incubation may not be enough to settle cells in the culture dish."

Response: Actually, 2 hours of incubation is enough to settle the cells in the culture dish. We followed the protocol described by Kaech S and Banker G (Nat. Protoc. 1 (2006) 2406–2415).

"10. It would be easier for the readers if the authors organized a table of materials in one page."

Response: We thank the reviewer for this suggestion, but to make this table we used the template provided by the journal.

"11. Section-3.1, whether authors used HEK293 cells or HEK293T, please clarify."
Response: We used HEK293 cells.

"12. Include the steps involved in the concentration of viral particles from supernatants."
Response: This information is detailed in points 3.4.2 and 3.4.3.

"13. Include the information about the origin or procurement details of HEK293 cells."
Response: We apologize for the omission of this information in the previous version of the manuscript. In the revised version it is included in the Table of materials.

14. In addition to CN-Aα and CHOP, it would be interesting to know other PERK signaling protein levels, which mediate the ER stress.
Response: We agree with the reviewer that it would be interesting to know whether another PERK signaling component, in addition to *CN-Aα* and CHOP, might modulate neurite atrophy. But we consider that this information is beyond the scope of this paper.

Response to reviewer 2

Major Concerns:
Since GM2 accumulation and UPR response also occurs in other intracellular organelles, it is important to address their significance and contribution to neuronal atrophy in this model.
Response: We agree with this reviewer that, in GM2-loaded cells, in addition to the ER membrane, ganglioside accumulates in other membranes (intracellular as well as external). Indeed, we tested the intracellular GM2 by densitometry after performing the thin layer chromatography (TLC) overlay, described by Lopez P H and Schnaar RL (*Methods Enzymol, 2006, 417:205*). The anti-GM2 antibody was used to immuno-stain the TLC. Thus, we quantified the GM2 localized in the membrane fraction enriched in plasma membrane, mitochondria and lysosome (Fig. 1 and Table 1).

Fig 1: TLC immunostained with anti-GM2 antibody

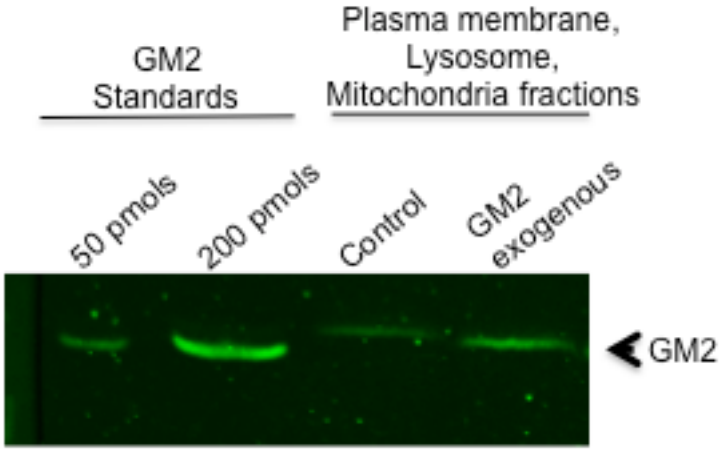

Table 1 : GM2 localized in Plasma membrane, lysosome and mitochondria fractions

Conditions	picomoles/mg wet weight
Control	162
GM2 exogenous	262

We also examined the presence of this glycosphingolipid in the microsomal fractions by TLC-immunoverlay and immunofluorescence (see Fig. 1 from Virgolini MJ et al. 2019). It is important to note that GM2 is not a normal constituent of the ER membrane (as observed in controls, 0 picomoles/100 mg wet weight of microsomes), but GM2-loaded cells showed high levels of ganglioside in the microsomal fraction (48 picomoles/100 mg wet weight of microsomes). These data indicate the increase of this ganglioside in GM2-loaded cells, which is even greater in endoplasmic reticulum membranes. Consequently, we have focused on studying the impact of ER membrane lipid perturbation by GM2 overload on PERK activation, an ER transmembrane protein and one of the UPR sensors.

"Minor Concerns:
1. Write a suitable subtitle for section 1.4.1 such as culture dish preparation/coating, etc"
Response: This was done in the revised version of the manuscript with the subtitle "Organic matter removal and poly-L-Lysine coating" (highlighted in yellow in the revised manuscript).

"2. Delete the word anti-mouse antibody in section 4.3.2"
Response: "Anti-mouse antibody" was deleted in the revised manuscript.

“3. 15 day in vitro culture is not too dense with axons and dendrites to score neurite outgrowth as mentioned in section 4? Isn't it a good idea to study at 7 days?”

Response: We thank the reviewer for this suggestion. In the revised manuscript we include in point 4.1 the following information (highlighted in yellow).

Note: Primary cortical neuron cultures of 7-8 days *in* could also be used if the cells show high neurite growth.