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TITLE:

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

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KEYWORDS:

Neurodegenerative disorders, Unfolded protein response (UPR), Protein kinase RNA [PKR]-like ER kinase (PERK), Ganglioside GM2, Calcineurin (CN), CHOP (CCAAT/enhancer binding protein homologous protein), Microtubule-associated protein 2 (MAP2).

SUMMARY:

In the present study, the expression is knocked down of two downstream signaling components of the PERK pathway, the cytoprotective calcineurin and the pro-apoptotic CHOP, by using specific shRNAs. In opposite ways, these modulate the susceptibility of primary cortical neurons to neurite atrophy after induction of endoplasmic reticulum stress.

ABSTRACT:

The accumulation of unfolded proteins within the endoplasmic reticulum (ER), caused by any stress condition, triggers the unfolded protein response (UPR) through the activation of specialized sensors. UPR attempts first to restore homeostasis; but if damage persists the signaling induces apoptosis.

There is increasing evidence that sustained and unresolved ER stress contributes to many pathological conditions including neurodegenerative diseases. Because the UPR controls cell fate by switching between cytoprotective and apoptotic processes, it is essential to understand the events defining this transition, as well as the elements involved in its modulation.

Recently, we demonstrated that abnormal GM2 ganglioside accumulation causes depletion of ER Ca^{2+} content, which in turn activates PERK (PKR-like-ER kinase), one of the UPR sensors. Furthermore, PERK signaling participates in the neurite atrophy and apoptosis induced by GM2 accumulation. In this respect, we have established an experimental system that allows us to

molecularly modulate the expression of downstream PERK components and thus change vulnerability of neurons to undergo neuritic atrophy.

We performed knockdown of calcineurin (cytoprotective) and CHOP (pro-apoptotic) expression in rat cortical neuronal cultures. Cells were infected with lentivirus-delivered specific shRNA and then treated with GM2 at different times, fixed and immunostained with anti-MAP2 (microtube-associated protein 2) antibody. Later, cell images were recorded using a fluorescence microscope and total neurite outgrowth was evaluated by using the public domain image processing software ImageJ. The inhibition of expression of those PERK signaling components clearly made it possible to either accelerate or delay the neuritic atrophy induced by ER stress.

This approach might be used in cell system models of ER stress to evaluate the vulnerability of neurons to neurite atrophy.

INTRODUCTION:

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 attempt to restore homeostasis. But if stress persists, UPR eventually induces cell death by apoptosis[1].

PERK, an ER transmembrane protein, upon ER stress, leads the phosphorylation of eukaryotic initiation factor-2 alpha (eIF2α), reducing global protein synthesis and thus protein load in the ER[2]. We demonstrated that calcineurin A/B (CNA/B), a heterodimer Ca^{2+} phosphatase, directly binds the cytosolic domain of PERK, increasing its auto-phosphorylation and significantly enhancing inhibition of protein translation and cell viability[3,4]. Interestingly, CNA/B is abundant in the mammalian brain, distinguishing two isoforms of the subunit A of CN: α and β.

Under sustained ER stress, the PERK signaling pathway is the only UPR branch that remains activated, thus mediating both the pro-survival and the apoptotic response. In the chronic phase, one major downstream event is the induction of the transcription factor, CHOP (CCAAT/enhancer binding protein homologous protein)[5]. Chronic ER stress is also increasingly recognized as a common contributor to an extensive range of pathological disorders, including neurodegenerative diseases[6]. It is important to understand how UPR can facilitate cytoprotective signaling instead of cell death [7]. However, at present little is known about the exact mechanism controlling the transition between these two UPR phases.

Recently, we found that, in cultured neurons, ganglioside GM2 accumulates in ER membranes and induces luminal calcium depletion. This in turn activates PERK signaling, which mediates neurite atrophy and apoptosis [8]. In this study, the GM2 build-up in cultured neurons is used as a cell system model of ER stress-induced neurite atrophy. Specifically, two PERK factor

expressions are manipulated, CN-Aα and CHOP, which switches the transition between early/protective events and a chronic/apoptotic phase. To accomplish this, the respective genes are silenced; thus, primary cortical neuron cultures are infected with lentivirus-delivered specific shRNA. Western blot analysis reveals a significant reduction of CN-Aα and CHOP expression levels in comparison with the control cells, which are infected with lentivirus that carry a scrambled shRNA. After this treatment, neurons are subjected to different incubation times of exogenous GM2, fixed, and immunostained with anti-microtubule associated protein 2 (MAP2) antibody[9]. Images are obtained with an epifluorescence microscope. The total neurite outgrowth is evaluated relative to total cell number.

PROTOCOL:

The animal procedures are performed following approved protocols of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Approval to conduct the study is granted by the Animal Care and Ethics Committee (CICUAL) of INIMEC-CONICET-UNC (Resolution numbers 014/2017 B and 006/2017 A).

1. Primary rat cortical neuron cultures

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

1.2. Perform the following steps in a laminar flow hood. Extract encephalon from the embryos with a forceps style # 3 and straight sharp small spring scissors (**Table of Materials**) to Hanks solution in cell culture dishes.

1.3. Dissect the tissue under 20x magnifying glass, using two fine-tipped forceps style #5, 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%) for 15 min, at 37 °C for chemical digestion.

1.4. After digestion, wash it 3 times with Ca^{2+}/Mg^{2+} free Hanks Balanced Salt solution and 0.1% glucose. The duration of each wash is 5 min.

1.5. Mechanically dissociate the tissue 10 times by pipetting up and down using a 1000 µL tip, in Dulbecco's modified eagle medium (DMEM) plus 10% fetal calf serum (FCS) (**Table of Materials**). Then centrifuge the homogenate at 100 x g for 1 min, collect the supernatant and complete the remaining volume to 1000 µL.

1.6. Plate the cell suspension on cell culture dishes at a density of 520 cell/mm^2 with 2 mL of DMEM containing 10% FCS, 1% penicillin/streptomycin and 1% of an essential aminoacid additive (see **Table of Material**). Then incubate at 37 °C in a humidified environment with 5% CO_2 for 2 h.

1.6.1. Organic matter removal and poly-L-lysine coating of cell culture dishes

1.6.1.1. Remove the organic matter by placing the glass material in a reaction chamber and add 96% ethyl alcohol to just about cover the material. Then add 68% (w/v) nitric acid drop by drop until brownish-greenish bubbles start to appear. Once this happens, partially cover the container and allow the reaction to continue until explosion ceases.

NOTE: Remove the organic matter under a gas extraction cabin to avoid exposure to noxious gases.

1.6.1.2. Before poly-L-lysine coating, rinse the glasses with sterile Mili-Q water about ten times. Then incubate the culture glasses and dishes with 0.1 μg/μL poly-L-lysine during 4 h before using them. After incubation, rinse the glasses with sterile Mili-Q water about ten times.

1.7. To allow neural cell differentiation, change the medium for a serum-free neurobasal medium, with the serum-free supplement (**Table of Materials**). Keep cultures at 37 °C with 5% CO_2 until treatment.

2. Lentivirus production

NOTE: The oligonucleotides containing the sequences targeting the 3'UTRs of either the CN-Aα isoform or CHOP and with the non-targeting sequences (scrambles) are listed in **Table 1**.

2.1. For annealing, mix two single-stranded oligonucleotides with complementary sequences in equal molar amounts, using Annealing Buffer (10 mM Tris-HCl, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA).

2.2. Heat at 95 °C for 2 min and then cool slowly by transferring samples from the heat block or water bath to a bench-top at room temperature. Dilute the resulting product [short hairpin RNA, (shRNA) with cohesive ends] to a final concentration of 0.5 μM, aliquot them and store at -20 °C.

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 EcoRI and PacI restriction enzymes by mixing the following in a sterile tube: 2 μL of 10x restriction enzyme buffer (100 mM bis-Tris propane-HCl, pH 6.5, 100 mM $MgCl_2$, 1 mg/mL bovine serum albumin), 1 μL of 1 μg/μL pLKO3.G, 0.5 μL of 10 U/μL ECoRI, 0.5 μL of PacI (10 U/μL), and 16 μL of sterile ionized water.

2.3.1. Mix gently by pipetting. Incubate the reaction for 3-4 h at 37 °C.

NOTE: Overnight digestions are 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 and ligase buffer (provided by manufacturer) overnight at 16 °C.

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

2.5. Transform DH5α competent cells (**Table of Materials**) or another suitable *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 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 5,000 x 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.

2.5.4. Incubate at 37 °C overnight, then check bacterial growth. At this point, the bacteria may be stored at 4 °C for 4-5 days.

2.5.5. Pick a single colony to transfer into a 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.

2.5.6. Centrifuge the culture to pellet the bacteria at 5,000 x g for 5 min at 4 °C, discard the supernatant and conserve the bacterial pellet. This can be stored at -20 °C.

2.6. Purify the plasmid with a DNA purification kit from bacteria pellet following manufacturer's instructions (**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. Lentivirus infection

NOTE: Perform the lentivirus generation procedure in a Class II laminar flow hood.

3.1. 24 h before transfection, seed 1-5 × 10^6 HEK 293 cells into 100 mm of diameter culture dishes in 8 mL of growth medium and incubate them at 37 °C, 5% CO_2 overnight.

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

3.4. 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.

3.5. Filtrate viral supernatant with a 0.45 µm nylon membrane. Aliquot the flow-through in 1 mL. Next, centrifuge at 17,000 x g for 4 h. Discard the supernatant and conserve the pellet. Allow invisible pellet to dry, and, once dried, store at – 80 °C.

3.5.1. Perform viral titration by flux cytometry. Seed 4 x 10^5 HEK 293 cells in 24-well plates and incubate at 37 °C for 24 h. Then resuspend the viral particles in 200 µL of DMEM culture medium (**Table of Materials**) and add 0.05, 0.02, and 0.005 µL to each well.

3.5.2. Incubate viral infection for 72 h, remove medium and trypsinize (0.05% trypsin, 1 mL) for 3 min. Add 1 mL of DMEM in 10% FCS solution and centrifuge at 500 x g for 5 min. Discard the supernatant and wash the pellet twice with PBS.

3.5.3. Resuspend the pellet with 100 µL of PBS and fix the cells with 100 µL of 2% paraformaldehyde (PFA) in PBS. Analyze the cells with a flux cytometer and determine the percentage of GFP fluorescent cells. Collect this data to calculate viral titration using the following formula:

$$Viral\ Titration$$

$$\frac{GFP\ Cells\ number\ \times initial\ cells\ number}{total\ virus\ volume} = X\ \times dilution\ factor$$

4. Primary neuron cultures stressed with ganglioside GM2 accumulation and immunocytochemistry using anti-MAP2 antibody

4.1. Infect the primary cortical neuron cultures (15 days in vitro) with specific shRNA oligonucleotides targeting the 3' UTRs of either CN-Aα or CHOP and incubate them at 37 °C with 5% CO_2 for 1 day.

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

4.1.1. Prepare a 500 µM stock solution of ganglioside GM2 in ethanol and sonicate it for 1 h.

4.1.2. Add GM2 stock solution to the culture dishes medium at a final concentration of 2 µM. Allow to incubate at 37 °C with 5% CO_2 for 16, 24, and 48 h.

4.2. Use some cultures to prepare cellular homogenate for western blot analysis (for antibody specification, see the **Table of Materials**).

4.3. Follow steps 1.4. and 1.5. and then fix the cells with 4% PFA and 120 mM sucrose in PBS for 20 min at 37 °C, and then wash it with PBS. Perform this procedure on an anti-slipping membrane in a wet chamber. Afterwards, add permeabilization solution (0.2% Triton X-100 in PBS) for 5 min and wash with PBS.

4.3.1. Use 5% BSA diluted in PBS for 45 min to allow blocking, then incubate with anti-MAP2 antibody, diluted 1:800 in blocking solution, at 4 °C overnight.

4.3.2. At the end of the incubation time, wash twice with PBS and incubate with secondary antibody (**Table of Materials**) for 1 h. Then wash the cells with PBS and mount the glasses onto slides using an aqueous mounting medium (**Table of Materials**).

5. Neurite atrophy analysis

5.1. Obtain images with a 20x air lens (NA 0.8) using an epifluorescence-inverted microscope equipped with a CCD-camera. Analyze images with ImageJ plug-ins. For this, load the picture to ImageJ and subtract the background by clicking on **Process** | **Subtract Background**.

5.2. Click on **Image | Adjust | Threshold** or type the following keys: **Ctrl (or Cmd)** + **Shift** + **T**. A window pops open to adjust minimum values, allowing path and soma tracings to be distinguishable. Click on the binary image (neurites and somas are white colored). On this image, use freehand selection to delete somas; the selected soma area turns black again.

5.3. Select traces and click **Edit** | **Selection** | **Create Selection**. Get the ROI by clicking **Ctrl** (or **Cmd)** and **T** keys of the selection and conserve it. Open the original image, then click on the ROI manager panel and select the belonging ROI, previously obtained. Then click on the original image.

5.4. Once the ROI traces on the image, type **Ctrl (or Cmd)** and **M** keys (**Analyze** | **Measure**). A window pops open; copy the mean value (arbitrary units a.u.) to process statistical analysis.

NOTE: To obtain values as µm, click on **Analyze | Set Scale**. A window pops up open to set values. Be sure to add the correct scale (**Analyze** | **Set Scale**) depending on the characteristics of the microscope used.

REPRESENTATIVE RESULTS:

Here, we address the question of whether silencing two PERK downstream components affects the transition phase of UPR in an ER stress cell model. To achieve this, we silence the CN-Aα gene as well as the CHOP gene by two specific shRNA sequences for each (**Table 1**) in primary neuron cell culture for 1 day[10]. The expression is analyzed by Western blotting (**Figure 1** and **Figure 2**). A clear inhibition is observed of ER stress-mediated CN-Aα and CHOP increase in knockdown cells, but not in control cells (shRNA scrambles). It should be noted that the basal CN expression level is not affected with this treatment condition.

We also examine the possible link of GM2 accumulation-induced ER stress with neuronal degeneration by performing MAP2 immunostaining of primary neuronal cultures (**Figure 3** and **Figure 4**).

Neurite atrophy is analyzed as total neurite outgrowth relative to the total cell number. This increases significantly after inducing ER stress by incubation of GM2 at 16–48 h. Interestingly, silencing of CN-Aα expression significantly enhances neurite atrophy, particularly at 16 h of GM2 accumulation, relative to GM2-untreated groups (**Figure 3**). Thus, CN-Aα knockdown accelerated the degeneration processes in neurons, corroborating the pro-survival effect of CN during the early phase of UPR. Conversely, CHOP knockdown resulted in significantly diminished neurite atrophy relative to controls, specifically at 16-24 h (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1. **Data analysis for CN-Aα silencing efficiency.** Knockdown is evaluated by Western blot analysis using antibody against CN-Aα and GADPH (loading control). The primary antibody is visualized by near-infrared fluorescence imaging system. Numbers below represent relative intensity ratio between CN-Aα and GADPH bands. This figure has been republished from Virgolini, M.J. et al.[4].

Figure 2. Data analysis for CHOP silencing efficiency. Knockdown is evaluated by Western blot analysis using an antibody against CHOP and β actin (loading control). The primary antibody is visualized and then the data are analyzed as indicated in **Figure 1**. This figure has been republished from Virgolini, M.J. et al.[4].

Figure 3. (Top) Data analysis for neuritic atrophy imaging in CN-Aα knockdown cells. Cultured primary neurons are loaded with GM2. Representative images of MAP2 immunostained cells are shown under control and experimental conditions. Images are recorded with an epifluorescence-inverted microscope equipped with a CCD-camera. Scale bars: 150 μm (regular images), 75 μm (magnified images). **(Bottom)** Histogram (mean ± SEM) represents neurite outgrowth with respect to total cells, analyzed by ImagJ plug-ins. ******* and **###**: $p \leq 0.0001$ for comparison with the control, from one-way ANOVA. This figure has been republished from Virgolini, M.J. et al.[4].

Figure 4. (Top) Data analysis for neuritic atrophy imaging in CHOP silencing cells. Primary neuron cultures are treated as indicated in **Figure 3(top)**. **(Bottom)** Histograms and statistical

notations as in **Figure 3(Bottom)** except **##:** $p \leq 0.001$. This figure has been republished from Virgolini, M.J. et al.[4].

Table 1. Forward construct sequences inserted to pKLG0.3 viral vector. Scrb: scramble.

DISCUSSION:

We describe an experimental system that enables molecular modulation of the transition from survival to apoptotic UPR phases in a neuronal cell model.

For a proper analysis of neurite atrophy, it is essential to obtain primary neuron cultures with numerous, long, highly branched processes[9,11]. This facilitates the examination of neuron process extension, allowing the clear differences between treatments to be detected. 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.

Moreover, the inhibition percentage of ER stress-mediated CHOP overexpression should be about 65-70% or higher. Importantly, silencing of the CN-Aα expression level should affect only the ER stress-induced CN-Aα expression increase without modifying its basal level. This is to minimize the impact on CN functions not associated with PERK signaling[12,13].

With respect to the neurite atrophy analysis procedure, this paper presents an alternative methodology that requires less manual work than specific ImageJ 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 ImageJ commands at once. Both plug ins and this protocol 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[3,4,8]. Others and we described the impact of the inhibition of CHOP expression on cell survival in different systems[8,11,14]. 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.

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DISCLOSURES:

The authors have no competing financial interests.

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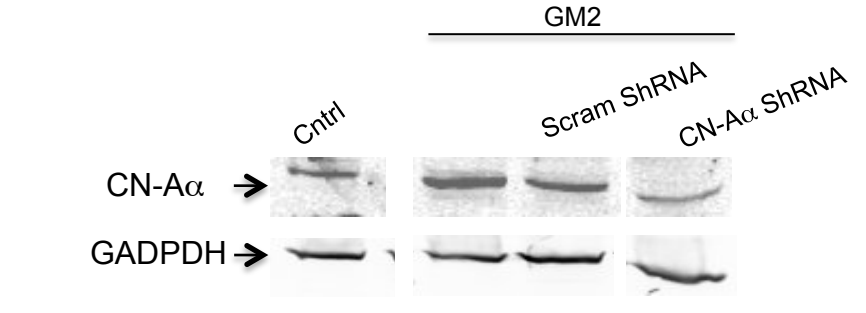
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Cntrl
Scram ShRNA
CN-Aα ShRNA
CN-Aα
GADPDH
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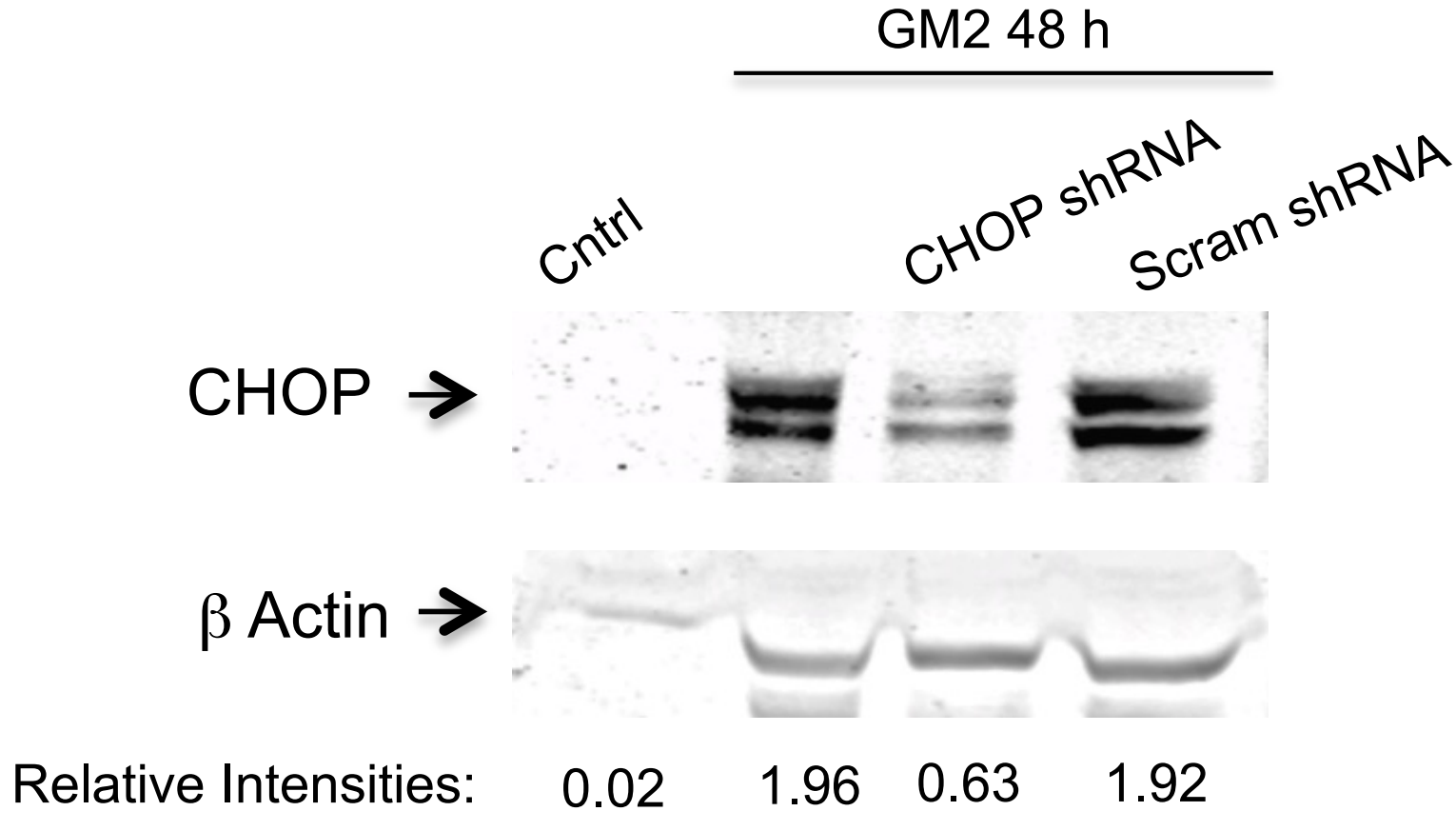
6.99
4.72
3.44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Cntrl
CHOP shRNA
Scram shRNA
CHOP
β Actin
Relative Intensities:
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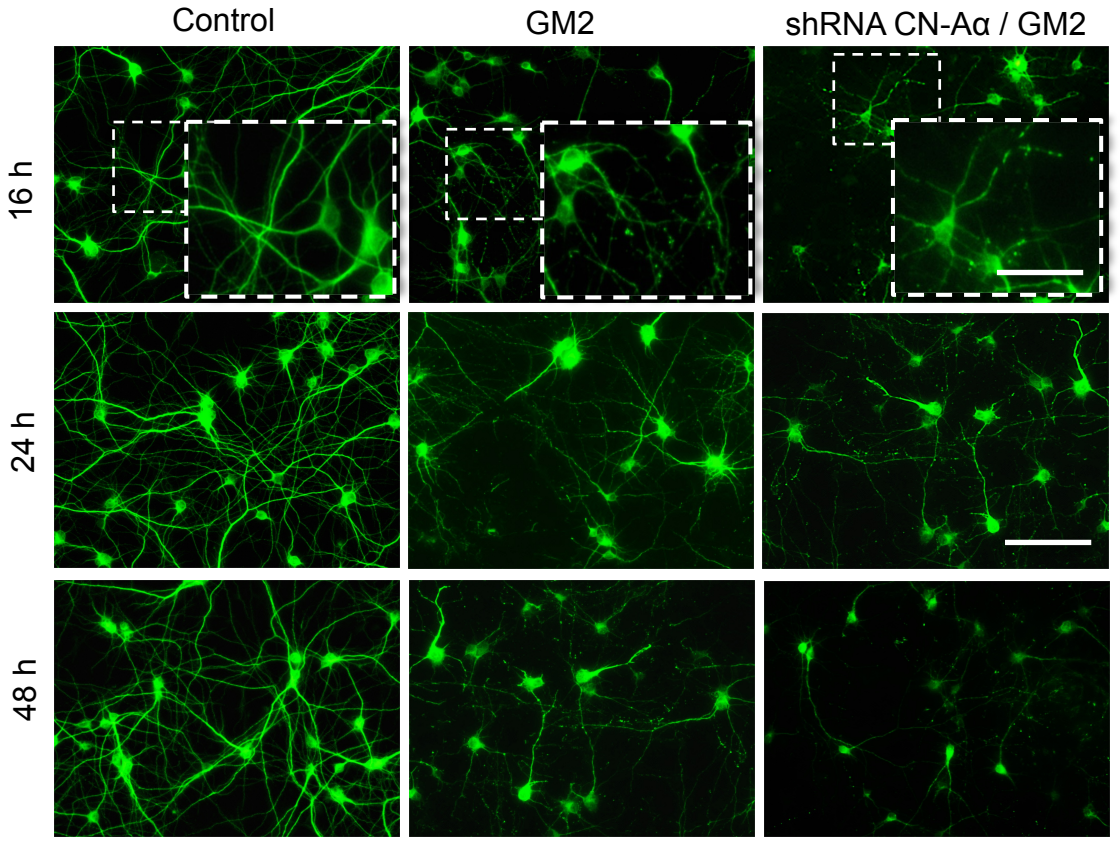
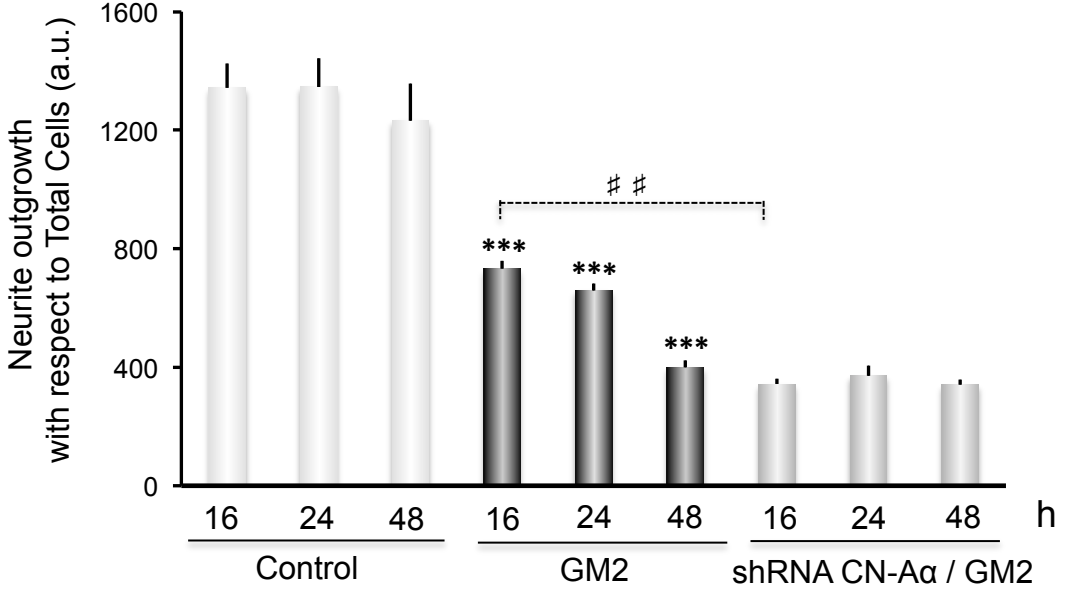
1.92


Control
GM2
shRNA CN-Aα / GM2
16 h
24 h
48 h


Neurite outgrowth
with respect to Total Cells (a.u.)
1600
1200
800
400
0
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16
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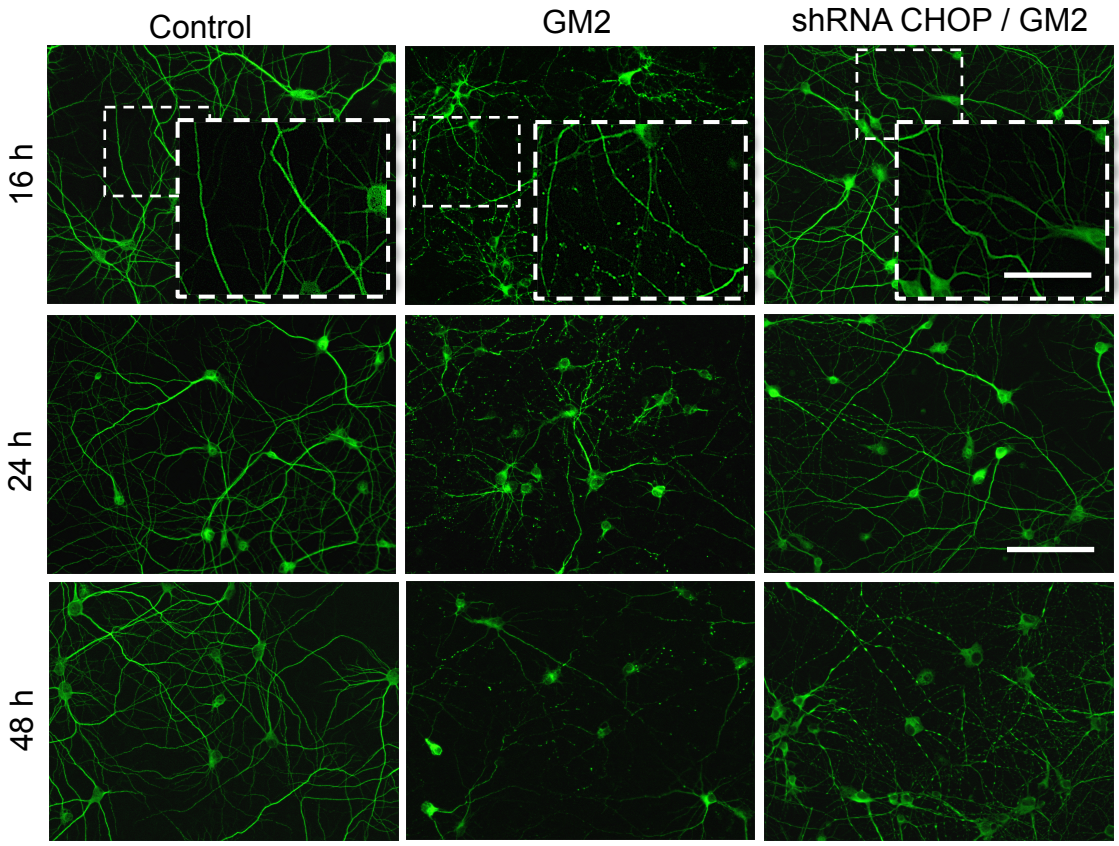
GM2
shRNA CN-Aα / GM2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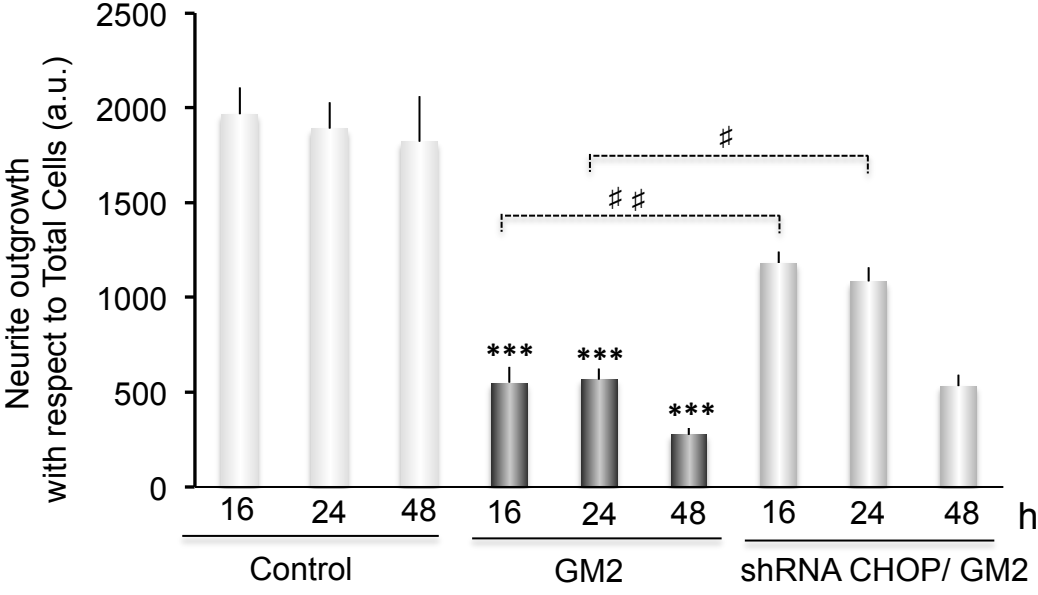
Neurite outgrowth
with respect to Total Cells (a.u.)
2500
2000
1500
1000
500
0
#
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***
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16
24
48
16
24
48
16
24
48
h
Control
GM2
shRNA CHOP/ GM2

Table 1

Constructs	Sequence 5’ to 3’ shRNA
CN-Aα 1	AATTGCCAGGAATTGGATTCAGTTTCTCGAGAAACTGAATCCAATTCCTGGCTTTTTTTAT
CN-Aα 2	AATTCGCCAACCTTAACTCCATCAACTCGAGTTGATGGAGTTAAGGTTGGCGTTTTTTTAT
CN-Aα Scrb	AATTGAGTGAATTGTCGCTCTAAGTCTCGAGACTTAGAGCGACAATTCACTCTTTTTTTAT
CHOP 1	AATTGGTCCTGTCCTCAGATGAAATCTCGAGATTTCATCTGAGGACAGGACCTTTTTTTAT
CHOP 2	AATTTGAAGAGAACGAGCGGCTCAACTCGAGTTGAGCCGCTCGTTCTCTTCATTTTTTTAT
CHOP Scrb	AATTGAAGAGAGAAAGCGAACAATACTCGAGTATTGTTCGCTTTCTCTCTTCTTTTTTTAT

Table of Materials

Click here to access/download;Table of Materials;MoralesC-TableofMaterials-edited.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alexa Fluor 488 anti-Mouse	Thermo Fisher Scientific	#R37120	
anti-CHOP	Thermo Fisher	# MA1 - 250	
Anti-CN-Aα	Millipore	# 07-067	
Anti-GM2	Matreya	#1961	
anti-MAP2	Sigma Aldrich	# M2320	
anti-β-actin	Thermo Fisher	# PA1 - 183	
aprotinin	Santa Cruz Biotechnology	#3595	
Axiovert 200 epifluorescence microscope	Zeiss		
B27 supplement	Life Technologies	#17504944	
Dulbecco's Modified Eagle's Medium (DMEM)	Life Technologies	#11966025	
EcoRI	Promega	#R6011	
Fetal Calf Serum (FCS)	Life Technologies	#16000044	
Fine-tippeds forceps style #5	Dumont		
Forcep style #3	Dumont		
HEK 293	ATCC	#CRL-1573	
IRDye 680 CW secondary antibody	LI-COR Biosciences	#92632221	
IRDye 680 secondary antibody	LI-COR Biosciences	#92632220	
IRDye 800 CW secondary antibody	LI-COR Biosciences	#92632210	
IRDye 800 CW secondary antibody	LI-COR Biosciences	#92632211	
lentiviral envelope plasmid pMD2.G	Addgene	#12259	
lentiviral packing plasmid psPAX2	Addgene	#12260	
lentiviral vector pLKO.3G	Addgene	#14748	
Leupeptin hemisulfate	Santa Cruz Biotechnology	#295358	
Lipofectamine LTX & Plus Reagent (plasmid transfection reagent)	Life Technologies	#A12621	
MISSION shRNA	Sigma Aldrich		
Monosialoganglioside GM2	Matreya	#1502	
NanoDrop 2000	Thermo Scientific		
Neurobasal Medium	Life Technologies	#21103049	
Nitrocellulose membrane 0.45 µm	BIO-RAD	#1620115	
Odyssey infrared imaging system	LI-COR Bioscience		
OneShot Top 10	Life Technology	#C404010	

Opti-MEM (Reduced serum media)	Life Technologies	#105802
PacI	BioLabs	#R0547S
penicillin-streptomycin	Life Technologies	#15140122
Pepstatin A	Santa Cruz Biotechnology	#45036
phenylmethylsulfonyl fluoride	Santa Cruz Biotechnology	#329-98-6
Poly-L-lysine	sigma aldrich	P#2636
Straight sharp small spring scissors	Fine Science Tools	
T4 DNA Ligase	Promega	#M1801
Trypsin-EDTA 0.25 %	Life Technologies	#25200056
Vibra-Cell Ultrasonic Liquid Processor (VCX 130)	Sonics	
Wizard plus SV Minipreps DNA purification system	Promega	#A1330

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

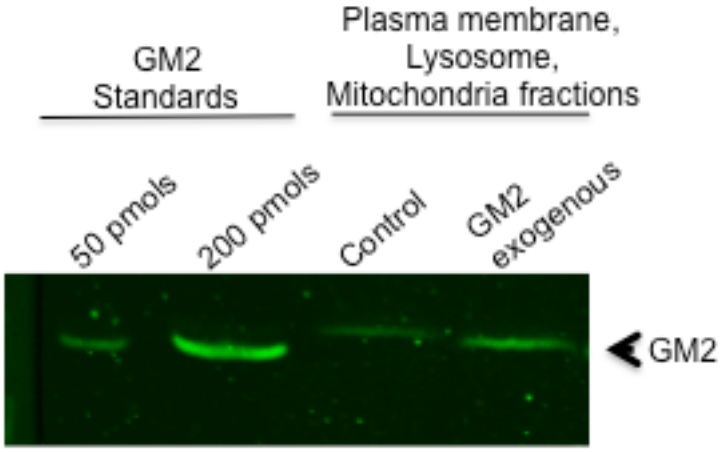

Table 1 : GM2 localized in Plasma membrane, lysosome and mitochondria fractions

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.