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Protocol status: Working
We use this protocol and it's working

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Testing ER stress induction in Cultured Induced Neurons via measuring ATF4 protein level or XBP-1 mRNA splicing

In 1 collection

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ABSTRACT

The endoplasmic reticulum (ER) has a vast proteomic landscape to preform many diverse functions including protein and
lipid synthesis, calcium ion flux, and inter-organelle communication. The ER proteome is remodeled in part through membrane-embedded receptors linking ER to degradative autophagy machinery (selective ER-phagy). A refined tubular ER network is formed in neurons within highly polarized dendrites and axons. Autophagy-deficient neurons *in vivo* display axonal ER accumulation within synaptic ER boutons, and the ER-phagy receptor FAM134B has been genetically linked with human sensory and autonomic neuropathy. However, mechanisms and receptor selectivity underlying ER remodeling by autophagy in neurons is limited. Here, we combine a genetically tractable induced neuron (iNeuron) system for monitoring extensive ER remodeling during differentiation. To test if any of our knockout iNeurons lead to induction of ER stress programs, we either left iNeurons with different genotypes untreated or treated them with tunicamycin as a positive control for ER stress induction. We then compared ATF4 protein induction and *XBP-1* splicing levels in untreated versus tunicamycin treated iNeurons.

MATERIALS

	A	B	C	D
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**	

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	A	B	C	D
	Cell line			
	NGN2 inducible embryonic stem cells	See protocol dx.doi.org/10.17504/protocols.io.br9em93e	CVCL_9773 (modified from this source line)	
	Chemicals			
	Dulbecco's MEM (DMEM), F12	Thermo Fisher Scientific	11330057	
	Phosphate Buffered Saline 1X	Corning	21-031-CV	
	E8 components	See protocol dx.doi.org/10.17504/protocols.io.bsacnaaw		
	ND1 and ND2 components	See protocol dx.doi.org/10.17504/protocols.io.br9em93e		
	Tunicamycin	Cell Signaling	12819S	
	RNAeasy Qiagen kit	Qiagen	74104	
	Qiashredder columns	Qiagen	79654	
	DNAseI	Thermo Fisher Scientific	EN0521	
	Superscript III reverse transcriptase master mix	Invitrogen	18080-051	
	oligo dT20 primers	Invitrogen	79654	
	dNTP solution mix	NEB	N0447L	
	Urea	Sigma-Aldrich	U5378	
	Sodium Chloride (NaCl)	Sigma-Aldrich	S9888	
	Protease Inhibitor Cocktail	Roche	11873580001	
	PhosSTOP tablets	Roche	4906845001	
	Pierce‰ BCA Protein Assay Kit	Pierce	23227	

	A	B	C	D
	4x Laemmli Sample Buffer	Biorad	1610747	
	2-Mercaptoethanol	Sigma-Aldrich	M6250	
	CREB-2/ATF-4 Mouse Monoclonal Antibody (B-3)	Santa Cruz	SC-390063	
	Goat anti-Mouse IgG HRP conjugate	BioRad	1706516	
	hFAB‰ Rhodamine Anti-Tubulin Antibody	BioRad	12004166	
	Primers			
	GAPDH cDNA forward	5'GGATGATGTTCTGGAGAGCC3',		
	GAPDH cDNA reverse	5'CATCACCATCTTCCAGGAGC3'		
	XBP1 cDNA forward	5' CCTTGTAGTTGAGAACCAGG 3',		
	XBP1 cDNA reverse	5'GGGGCTTGGTATATATGTGG 3'		
	Hardware			
	NanoDrop2000	Thermo Fisher Scientific		
	Sonicator			
	Thermal Cyler	BioRad	C1000	
	BioRad ChemiDoc imager	BioRad		
	Software			
	Image Lab BioRad Software 6.1	BioRad	RRID:SCR_014210	

Genetically modify Ngn2-inducible embryonic stem (ES) cell H...

1 Genetic editing of Ngn2-inducible ES cells is done using the following protocol "Electroporation of Cas9 protein into human pluripotent stem cells" (dx.doi.org/10.17504/protocols.io.br87m9zn)

Differentiate Stable Cell ES H9 line to induced neurons (iN)

2 Differentiation to induced neurons (iN) is done by following the protocol "Neural differentiation of AAVS1-TREG3-NGN2 pluripotent stem cells (dx.doi.org/10.17504/protocols.io.br9em93e)

iN Treatment

3 iN Treatment with or without tunicamycin

3.1 At day 12, iN with each genotype are left untreated or treated with tunicamycin (variable concentrations and times can be used but we found that optimal ER stress response was 1.0 microgram/ml) tunicamycin, 6h treatment).

3.2 After treatment, all iN are scrapped off the dishes, pelleted and washed three times with 1X Phosphate Buffered Saline (PBS)

3.3 Determine Number of cells. Pellet cells into two tubes-one for subsequent western blot analysis and the other for mRNA analysis (each pellet should have >1x10^6 cells)

3.4 Snap freeze pelleted cells in liquid nitrogen. Pellets can be stored at -80C for a few days before use.

ATF4 protein level detection

4 ATF4 protein level detection

4.1 Thaw
cell pellets and resuspend them in Urea lysis buffer (8M Urea, 75mM NaCl, 150mM Tris pH 7.4, 1X protease inhibitors, 1X phosphatase inhibitors). Approximately 75 µL per sample. Sonicate pellets 2x for 5 sec each.

4.2 Clarify the lysate: Spin down 4C for 20,000xg 10min, keep supernatant

4.3 Measure protein concentration using a BCA assay (follow kit directions).

4.4 Create samples with equal concentrations of protein, add 4x Laemmli Sample Buffer and 2-Mercaptoethanol to 1X in each sample. Heat samples at 65C for 10min

4.5 With equal amounts of each sample perform a western blot and probe for ATF-4 (B-3) 1:1000 with Goat anti-Mouse IgG HRP conjugate 1:3000 and hFAB™ Rhodamine Anti-Tubulin 1:10,000. Image blot on BioRad ChemiDoc imager and quantify levels with Image Lab BioRad Software 6.1

XBP-1 splice isoform detection

5 *XBP-1* splice isoform detection

5.1 Thaw cell pellets and resuspend them in freshly prepared RLT buffer (350 µL per sample for $1x10^6$ cells cells) from the RNAeasy Qiagen kit

5.2 Add Dnase1 digestion buffer to cells and subsequently lyse via passage through a Qiashredder column

5.3 Add One volume of 70% ethanol to the lysate and transfer the lysate-EtOH solution to a RNAeasy spin column

5.4 Perform the following spins:
1) on column DNAseI (Thermo EN0521) digestion,
2) buffer washes, and
3) RNA elution via the RNAeasy Qiagen kit directions

5.5 Measure final extracted RNA concentration for each condition using a NanoDrop

5.6 Perform reverse transcription reactions for each condition (using the same amount of starting µg of RNA, 0.5 µg, in each reaction) with Superscript III reverse transcriptase master mix using oligo dT_{20} primers and dNTPs to create complementary DNA (cDNA). Incubate at 50 or 55˚C for 50 minutes, then quench the reaction by heating to 85˚C for 5 minutes. Freeze these samples as cDNA at -20 until ready to use.

5.7 With the cDNA, perform PCR reactions to amplify cDNA from *GAPDH* mRNA (forward 5'GGATGATGTTCTGGAGAGCC3', reverse 5'CATCACCATCTTCCAGGAGC3') ; or to amplify cDNA from unspliced or spliced *XBP1* mRNA (forward 5'CCTTGTAGTTGAGAACCAGG 3', reverse 5'GGGGCTTGGTATATATGTGG 3') (as performed in van Schadewijk et al 2012, Yoshida et al 2001).

5.8 Perform gel electrophoresis on PCR products using a 2.5 percent agarose gel, run for 30min at 100V. Image gel on BioRad ChemiDoc imager and quantify levels with Image Lab BioRad Software 6.1 GAPDH levels should be even in all samples. The size difference between the spliced and the unspliced XBP1 is 26 nucleotides.