Supplementary Information

1. Materials and methods

1.1 Mice and locomotor testing

BALB/c mice carrying a heterozygous mutation in the NPC1 gene were kindly donated by Dr. Peter Pentchev. Genotypes were identified by using PCR-based screening as described previously [1]. Animal protocols had been reviewed and approved by the animal studies review boards at our institutions. Locomotor coordination was evaluated weekly by two tests; the hanging and the beam tests, as previously described [1].

1.2 Histological analysis

Cerebellar sections from 4% paraformaldehyde fixed brains were cut into 40 μm slices and analyzed by the following: a. ***Immunohistochemical procedures***: anti-calbindin D-28K antibodies (AB1778; 1:1000) (Chemicon International, Temecula, CA) and revealed with the avidin biotin horseradish peroxidase complex (ABC) method (Vector Laboratories, Burlingame, CA), anti KDEL (10C3, 1:200) and PDI (1D3, 1:200) (StressGene, San Diego, CA). Secondary antibodies conjugated with antimouse- Alexa Fluor-488 or anti-rabbit-Alexa Fluor-594 were used at 1:1000 dilution. (Molecular Probes, OR, USA). b. ***Filipin staining:*** cerebellar sections or N2a cells fixed with 4% paraformaldehyde were incubated with $NaBH_4$ 10mg/ml for 10 min and then incubated with 50μg/ml of filipin overnight at 4°C. Later, sections were washed in PBS, mounted on gelatine pre-coated slides and covered with Fluoromount G (Southern Biotech, Birmingham, AL). c. ***In situ apoptosis detection:*** The TUNEL assay was performed using the apoptosis detection kit purchased from Roche Molecular Biochemicals (Mannheim, Germany), following the manufacturer's instructions.

1.3 Western blot analysis

Tissue proteins were prepared as previously described [1]. 40 μg of cerebellum protein extracts were resolved on SDS-PAGE. The following antibodies and dilutions were used: anti-Grp78/Bip and anti-PDI, 1:2,000 (StressGene, San Diego, CA), anti-CHOP (1:2000) (Santa Cruz) and anti tubulin 1:5,000 (Sigma).

1.4 Real-time PCR and XBP-1 splicing assay.

Real-time PCR was performed using methods and primers previously described in [2, 3] employing SYBR green fluorescent reagent in an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). The relative amounts of mRNAs were calculated from the values of comparative threshold cycle by using α-actin as control. Primer sequences were designed by Primer Express software (Applied Biosystems, Foster City, CA) or obtained from the Primer Bank (http://pga.mgh.harvard.edu/primerbank/index.html). Real time PCR primers are described in [3]. *xbp-1* mRNA splicing assay was performed as previously described [4].

1.5 Knockdown of Npc1 expression

We generated stable motoneuron cell lines with reduced levels of NPC1 using methods previously described [5] by targeting the respective mRNA with shRNA using the lentiviral expression vector pLKO.1 and puromycin selection. As controls, empty vectors or a shRNA against the *luciferase* gene were employed. Constructs were generated by The Broad Institute (Boston, USA) based on different criteria for shRNA design (see http://www.broad.mit.edu/genome_bio/trc/rnai.html). We screened a total of five different constructs for NPC1 and selected the most efficient one for further studies. Targeting sequences identified for mouse *npc1* mRNA is CCCGTCTTACTCAGTTACATA.

2. Supplementary Discussion

The hallmark feature of NPC is the abnormal accumulation of cholesterol, associated with neuronal dysfunction and apoptosis, leading to motor impairment and premature death [6]. Recent evidence indicates that chronic ER stress may be involved in lysosomal storage diseases such as GM1-gangliosidosis and Infantile Neuronal Ceroid Lipofuscinoses. However, a recent report indicated no signs of ER stress on a cellular and mouse model of Gaucher disease [7]. Based on the observation that cholesterol overload leads to ER stress-induced apoptosis [8, 9], that the UPR [10, 11], and particularly XBP-1, has a critical role in cholesterol metabolism in the liver [4], and that primary neuronal cultures exposed to a cholesterol transport-inhibiting agent that mimics the NPC phenotype show activation of ER stress markers [12] in the present study we have investigated the possible engagement of ER stress responses in NPC models. Despite initial predictions in favor of a role played by the UPR in NPC, we did not find evidence of ER stress in a well established NPC mouse model analyzed at an age in which neurodegeneration and locomotor impartment were evident (fig. 1 and S1) and recapitulating major pathological alterations observed in NPC patients. Our results were also corroborated in a cellular NPC model that we have developed here, where Npc1 was knocked down with shRNA, leading to intracellular accumulation of cholesterol.

Increased expression of ER stress markers is observed in post-mortem brain tissues from patients affected with diseases such as Parkinson's disease, ALS**,** Alzheimer's disease, and Creutzfeldt-Jacob's disease in addition to mouse models [13]. However, most of the studies linking the occurrence of ER stress and neurodegeneration are correlative. Depending on the levels of ER stress, activation of the UPR has completely different consequences: adaptation to protein misfolding stress or cell death by apoptosis. To functionally address the role of the UPR in the nervous system we recently generated a brain-specific XBP-1 knockout mouse and tested its susceptibility to Prion disease [2]. Despite evident activation of ER stress responses [14, 15], disease pathogenesis was not affected by XBP-1 deficiency [2]. Interestingly, ablation of XBP-1

protected against familial Amyotrophic Lateral Sclerosis, increasing life span and motoneuron survival associated with lysosome-mediated degradation of mutant SOD1 [3]. Thus predicting the function of the UPR in a certain disease setting is complex due to the homeostatic nature of the pathway. In the case of lysosomal storage diseases where ER stress has been reported, no direct functional data has yet been provided to directly asses the role of the pathway toward the disease.

The UPR controls cell survival and cell death through complex regulation of its signaling. Sustained PERK activation under chronic ER stress leads to apoptosis associated with the upregulation of the UPR transcription factor CHOP, which also modulates the expression of the pro-apoptotic BCL-2 family member BIM [16]. In addition, ER stress-induced apoptosis has been linked to the transcriptional upregulation of other BCL-2 family members such as PUMA [17]. Here, we observed a slight increase in the mRNA levels of BIM, and CHOP in NPC mice. However, it is important to mention that a greater mRNA upregulation is normally described in conditions of ER stress mediated apoptosis (more than 10 fold, see *chop* mRNA levels in supplemental fig.S2) [2]. Besides, BIM and PUMA are general pro-apoptotic genes that are upregulated by a diversity of intrinsic cell death stimuli that are not related to ER stress. There is emerging evidence that supports the idea that oxidative stress is present in NPC cells [18], raising the possibility that the fluctuations of CHOP induction in the NPC cerebellum are due to this oxidative stress and not ER stress. Supporting this idea we have found that the c-Abl/p73 pro-apoptotic pathway is activated in the NPC-mouse cerebellum [1] and c-Abl activation is induced by several types of pro-apoptotic stimuli, including oxidative stress [19].

Over 200 different mutations in the Npc1 gene have been reported in NPC patients [20], and the most prevalent is $Npc1^{I1061T}$ which misfolds upon expression [21]. Although we have not found clear evidence of ER stress in NPC1 knockout mice, we cannot discard the potential role of ER stress in the $Npc1^{I1061T}$ condition. Consistent with our findings no signs of UPR and ER stress were observed in hepatocytes and macrophages from $NPC1^{-/-}$ mice [22, 23]. In conclusion, our findings describe a lack of general UPR activation in NPC *in vitro* and *in vivo* models. These

findings are in agreement with a recent report in neuropathic models of Gaucher disease. Thus, the role of the UPR in the pathogenesis of NPC and possibly other lysosomal storage diseases may have to be evaluated.

Fig. S1. NPC mice disease progression. **a** Cerebellum from wild-type (WT) and NPC mice at 8 weeks of age were analyzed by the TUNEL assay. White arrows indicate examples of apoptotic cells. **b** Beam test using control and NPC mice with a total of 6 mice per group. The data show the mean ± SEM. *, $p<0.05$, p value was calculated using Student's t-test. **c** The survival curve of NPC was assed over time. As control, wild-type mice were monitored.

Fig. S2. Real time PCR primers control. To control the real time PCR primers employed in this study, we monitored the mRNA levels of indicated ER stress-related genes by real-time PCR in MEFs treated or not with 1 μg/ml of tunicamycin for 8h. Mean and standard deviations are presented for two determinations.

3. Supplementary References

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