Published in final edited form as:

J Neuroimmunol. 2006 December ; 181(1-2): 34–45.

The Role of the Proteasome-Ubiquitin Pathway in Regulation of the IFN- γ Mediated Anti-VSV Response in Neurons¹

Jingjun Yang^{*}, Derin Tugal^{*}, and Carol Shoshkes Reiss^{*,†,‡,§,2}

^{*} *Department of Biology and*

[†] *Center for Neural Science, New York University, New York, NY 10003;*

[‡] *NYU Cancer Institute and Department of Microbiology, New York University School of Medicine, New York, NY 10016;*

[§] *Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029.*

Abstract

Pharmacologic inhibition of the proteasome resulted in increased NOS-1 protein levels and increased NO production by neuronal cells. This correlated with an increased antiviral effect of IFN- γ against the replication of vesicular stomatitis virus (VSV) replication *in vitro*. We also observed that a regulatory protein, Protein Inhibitor of NOS-1 (PIN) was down-regulated by IFN- γ treatment, and more ubiquitinated PIN accumulated in IFN- γ treated neurons. In cells of the reticuloendothelial system, IFN- γ treatment induces the expression of a set of low molecular weight MHC-encoded proteins (LMPs), which replace the β -subunit of the proteasome complex during the proteasome neosynthesis, resulting in a complex termed the immunoproteasome. LMP-2, -7, and -10 were induced and the immunoproteasome was generated by IFN- γ treatment in neuronal cells. Importantly, we observed that IFN- γ induced inhibition of VSV protein synthesis was not dependent on ubiquitination.

Keywords

NOS-1; PIN; Ubiquitination; VSV

Introduction

IFN- γ is a critical antiviral mediator in eliminating viruses from the CNS (Komatsu *et al*, 1996). IFN- γ upregulates NOS-1 expression posttranscriptionally and increases NO production, which is essential in the clearance of VSV infection by neurons (Bi and Reiss, 1995; Komatsu, *et al*, 1996; Chesler *et al*, 2004). VSV is not unique; other neurotropic viruses whose replication is inhibited by NO include CMV (Cosugi, *et al*, 2002), HIV (Hori *et al*, 1999), HSV-1 (Kodukula, *et al*, 1999), MHV (Lane, *et al*, 1997), Poliovirus (Komatsu, *et al*, 1996), and Reovirus (Goody, *et al*, 2005). But how this antiviral process is regulated largely remains unknown.

We propose that IFN- γ enhanced antiviral activity in neurons through alteration of the proteasome degradation of host proteins to induce NOS-1 expression and activity which can inhibit VSV protein synthesis (Bi and Reiss, 1995). Protein Inhibitor of NOS-1 (PIN) is a

¹This work was supported by a NYU Research Challenge Fund award N5385, and NIH grants DC003536 and NS039746 to C.S.R.

²Address correspondence and reprint requests to: Dr. Carol Shoshkes Reiss, Biology Department, Silver Center Room 1009, M/s 5181, New York University, 100 Washington Square East, New York, NY 10003-6688. E-mail address: Carol.Reiss@nyu.edu.

protein which complexes with NOS-1, preventing functional homodimer formation (Jaffrey and Snyder, 1997; Fan, *et al*, 1998). Previous studies indicated that treatment of neurons with prostaglandin E₂ (PGE₂), an inhibitor of NOS-1 activity, induces PIN expression in neurons. Conversely, pharmacologic inhibition of cyclooxygenase-2 *in vitro* and in mice enhances NOS-1 activity, down-regulates PIN expression, and enhances clearance of virus and survival (Chen and Reiss, 2002).

The proteasome is an intracellular complex that degrades polyubiquitin-conjugated proteins (Ciechanover, 1994). Previous studies showed that ubiquitination of NOS-1 occurs (Bender, *et al*, 2000) and that irreversible inactivators of NOS enhance the proteolytic turnover of the enzyme by a mechanism involving the proteasome (Noguchi, *et al*, 2000). IFN treatment may regulate the expression or activity of E3 Ub-ligases or de-Ubiquitinases, thus altering the functional half-life of the target proteins. The proteasome-ubiquitin pathway might be involved in IFN- γ mediated regulation of NOS-1 in neurons.

MHC-encoded IFN- γ -inducible low molecular weight proteins (LMP2, LMP7 and LMP10) replace the β -subunits of the proteasome complex in antigen presenting cells (Groettrup, *et al*, 1996), altering its substrate specificity (Van den Eynde, *et al*, 2001). Immunoproteasomes containing the IFN- γ -inducible β -subunits produce class I-binding peptides more efficiently than those containing only constitutive subunits. However, neurons do not participate in antigen processing and presentation, and are generally incapable of expressing MHC molecules (Gogate *et al*, 1996; Joly and Oldstone, 1992).

The immunoproteasome has been observed in neurons in three human neurodegenerative diseases: Huntington's Disease (Diaz-Hernandez *et al*, 2003), Amyotrophic Lateral Sclerosis (Cheroni *et al*, 2005), and Alzheimer's Disease (Mishto *et al*, 2006). This induction of the LMPs in neurons may be secondary to local secretion of inflammatory cytokines including IFN- γ by T cells and NK cells during inflammatory diseases.

In this study, we examined ubiquitination of PIN, NOS-1 and VSV proteins and the generation of the immunoproteasome in neurons following IFN- γ treatment. The results demonstrate that IFN- γ treatment of neurons increases PIN ubiquitination and degradation. NOS-1 levels and production of NO increases. Ubiquitin-modification and protein degradation does not contribute to the suppression of VSV protein accumulation in IFN- γ -treated neuronal cells. The immunoproteasome is generated by treatment of neuronal cells with IFN- γ .

Results

Pharmacologic inhibition of the proteasome increases the antiviral effect of IFN- γ *in vitro*

In order to determine if the proteasome degradative pathway contributed to the inhibition of accumulation of VSV proteins in IFN- γ -treated neuronal cells (Komatsu *et al*, 1996; Chesler *et al*, 2004), we examined the effect of two proteasome inhibitors Lactacystin (LC) and Geldanamycin (GA) on VSV infection in neurons. LC inhibits proteasome directly by modifying the proteasome's catalytic β -subunits (Craiu *et al*, 1997). GA reduces proteasome-mediated antigen presentation through inhibition of Hsp90 (Imai *et al*, 2003). In order to determine the optimal concentration of inhibitors to be used in our studies, neuronal cells were treated with a range of doses of inhibitors. The MTS assay was used to measure mitochondrial activity as a surrogate for cell viability following LC or GA treatment (Fig 1A). The suppression of proteasome activity by the two inhibitors was confirmed by an assay of proteasome cleavage of Z-GGL-pNA (Fig 1B). Treatment of cells with LC or GA alone results in inhibition of VSV replication in NB41A3 cells (Figure 2A). When we combined the two drugs, viral replication was almost totally eliminated. In IFN- γ -treated NB41A3 cells, treated with LC or GA, the antiviral effect of IFN- γ was enhanced.

Inhibition of proteasome results in increased NO production and NOS-1 accumulation

NO is an important cellular inhibitor of viral replication. The NOS-1 product, NO, can spontaneously combine with superoxide to form peroxynitrite (ONOO⁻), which readily degrades to NO₂⁻ (Love, 1999). To investigate the regulation of NOS-1 activity by the proteasome, we measured NO₂⁻ production by IFN- γ or LC treated neuronal cells. We treated NB41A3 neuroblastoma cells for 48 hours with IFN- γ to allow accumulation of NO₂⁻ in the media. Figure 2B shows NO₂⁻ production is increased by IFN- γ or LC treatment, consistent with the NOS-1 expression. This correlates well with the inhibition of VSV replication (Figure 2A).

Ubiquitination and proteasome degradation of NOS-1 has been observed in transfected HEK cells (Bender, et al, 2000). To determine if this were also true in mouse neuronal cells which constitutively express NOS-1, NB41A3 cells were treated for IFN- γ and/or LC for 24 hours; the expression of NOS-1 mRNA was detected by RT-PCR and the protein by Western blot. In all related experiments, double treatment of IFN- γ and LC provide us the information of how IFN- γ effects the cell when proteasome activity was blocked. As expected, the mRNA level of NOS-1 is unaltered by IFN- γ or LC treatment (Figure 3A). Similar results were observed at earlier time points (3h, 12h; data not shown). While NOS-1 protein levels increased four- to six-fold with IFN- γ or LC treatment (Figure 3B; Komatsu, *et al*, 1996; Chesler, *et al*, 2004). The induction of NOS-1 protein in the presence of LC treatment was also observed in primary neuron cultures (Figure 3C). This indicates both treatments of neuronal cells led to an accumulation of NOS-1 at the post-transcriptional level.

IFN- γ treatment inhibits NOS-1 degradation but ubiquitination is unchanged

The level of ubiquitination and degradation of NOS-1 following IFN- γ treatment of neurons was examined by immunoprecipitation (IP) and by pulse-chase experiments. Cells treated with LC served as a control for accumulation of NOS-1. Tubulin III was used as housekeeping and loading control for the IP experiment. As shown in Figure 3D, unchanged levels of ubiquitinated NOS-1, molecular mass ~25KD higher than the NOS-1 monomer, were observed in both IFN- γ -treated neurons and the untreated cells. When the proteasome was inhibited, more Ub-NOS was observed in LC treated samples, but this was not affected by IFN- γ treatment.

Pulse-chase was performed to measure the degradation of NOS-1 in the presence or absence of IFN- γ or LC. As previously observed (Chesler DA, *et al*, 2004), the half-life of NOS-1 increased 33%, from ~18 hours to ~24 hours in IFN- γ -treated neuronal cells (Figure 3E). NOS-1 accumulation in IFN- γ -treated neuronal cells is secondary to slower degradation. We do not know at present whether activity of an E3 ligase is suppressed or whether a de-ubiquitinase specific for NOS-1 is upregulated in IFN- γ -treated neurons. Work is in progress to examine gene expression in these cells under differing conditions to elucidate this question.

IFN- γ post-transcriptionally down-regulates PIN

To determine if loss of PIN were involved in the IFN- γ -mediated activation of NOS-1, we examined the expression of PIN following IFN- γ treatment in neuronal cells. RT-PCR indicated that the ratio of PIN mRNA to β -tubulin mRNA was unchanged by IFN- γ treatment (Figure 4A), while the protein level was significantly reduced in the IFN- γ -treated group (Figure 4B). Earlier time points (3h to 12h, data not shown) were also examined with consistent results. Thus IFN- γ treatment of neuronal cells down-regulates PIN accumulation at the posttranscriptional level.

IFN- γ treatment enhances ubiquitination and degradation of PIN

To further investigate the mechanism of post-transcriptional down-regulation of PIN by IFN- γ , we performed an immunoprecipitation experiment to detect ubiquitination of PIN in both control and IFN- γ -treated neuronal cells. We precipitated ubiquitin- conjugated proteins using anti-ubiquitin pAb and blotted with anti-PIN mAb. As shown in Figure 4C, PIN migrated at a molecular mass band of ~34kD; we interpret this to mean three ubiquitin units. Four-fold more ubiquitinated PIN was found in IFN- γ treated neuronal cells.

To measure the degradation of PIN, we performed a pulse-chase experiment. Based on preliminary results, the kinetics of PIN in cells was distinct from that of NOS-1. In comparison with control, PIN was degraded more rapidly in IFN- γ treated neurons (Figure 4D). The half-life of PIN was reduced 40%, from ~5 hours to ~3 hours. As a control, LC treatment prolonged its half-life 50%, to ~7.5 hours. We interpret these data to be consistent with IFN- γ induction of a Ub-ligase which modifies PIN and results in its degradation by the proteasome. The loss of PIN increases the active fraction of NOS-1.

Ubiquitin is not involved in IFN- γ -mediated inhibition of accumulation VSV proteins

IFN- γ treatment of neurons inhibits the production of VSV proteins ~75% and the release of infectious VSV by 30- to 1000-fold (Komatsu, *et al*, 1996; Chesler, *et al*, 2003). The inhibition of VSV protein synthesis is not associated with the dsRNA-activated protein kinase PKR which can suppress protein translation by phosphorylation of eIF2 α (Chesler *et al*, 2003). Viral proteins can be degraded more rapidly when they are Ub-modified (Aviel, *et al*, 2000).

NB41A3 cells were treated with IFN- γ and infected with VSV. Whole cell lysates were obtained at 5 hours p.i. and a direct immunoblotting was performed to estimate total amount of VSV proteins in IFN- γ -treated as well as control cells (Figure 5A). As previously observed (Komatsu *et al*, 1996; Chesler *et al*, 2003), IFN- γ treatment of neuronal cells resulted in inhibition of accumulation of VSV proteins (G, N, P, M), indicating that *either* viral protein synthesis is inhibited by IFN- γ treatment *or* viral proteins are rapidly degraded in IFN- γ treated neuronal cells. To determine if VSV proteins were targeted to the proteasomal pathway, we performed immunoprecipitations of ubiquitinated proteins and of viral proteins. Ubiquitinated VSV proteins were detected following immunoprecipitation with anti-ubiquitin by Western blot using anti-VSV pAb. As indicated in Figure 5B, only M protein was found to be Ubiquitin modified in neuronal cells, which is consistent with the observation in BHK-21 cells of Harty and coworkers (2001). In IFN- γ -treated group, less Ub-M was detected, which correlated with decreased level of VSV proteins observed in NB41A3 cells following IFN- γ treatment. Ub-M was not over-represented.

Similar results were observed in a reciprocal IP in which anti-VSV pAb was used for precipitation and anti-ubiquitin mAb used for Western blotting (Fig 5B). Thus IFN- γ treatment did not alter the ubiquitination of VSV M protein and proteasomal degradation cannot account for the IFN- γ -mediated inhibition in VSV protein accumulation.

A pulse-chase study was performed on VSV proteins in media and IFN- γ -treated cells to determine if the half-life of the viral proteins were shorter than in untreated neurons. If it were, this might indicate a proteolytic mechanism distinct from the ubiquitination-to-proteasome pathway, possibly autophagy, were active in the neurons. The result indicates that the degradation of VSV proteins is unchanged by IFN- γ treatment. As shown in Figure 5C, the half-lives of VSV M, G, and NP proteins vary from 2 to 5 hours, and there are no significant differences in between the control and IFN- γ -treated groups.

Immunoproteasome is induced by IFN- γ in murine neurons *in vitro*.

The IFN- γ -induced immunoproteasome plays a major role in generating MHC I presenting peptides in antigen presenting cells. We investigated whether IFN- γ treatment resulted in the induction of the immunosubunits of the proteasome in murine neurons *in vitro*. Figure 5A shows the induction of LMP2 mRNA expression and LMP7 protein by IFN- γ in NB41A3 cells. Similar results were observed in all three immunosubunits LMP2, LMP7 and LMP10. Untreated cells show no or extremely low levels of LMP expression while IFN- γ induced their expression at both mRNA and protein levels significantly. The mRNA induction was rapid and can be observed within 3 hours of IFN- γ treatment (data not shown). All three components display maximal induction of expression at about 24 hours treatment. To confirm the immunosubunits are incorporated into the immunoproteasome, we isolated and immunoprecipitated the proteasome/immunoproteasome 20S complex from IFN- γ -treated and untreated NB41A3 cells and used Western blot to detect the incorporation of the three immunosubunits. Figure 5B indicates that immunosubunits are found as components of the proteasomes isolated from IFN- γ -treated samples. The constitutive subunit α_5 was consistent in all samples. Similar observations were made using primary cultured neurons (not shown). Our results demonstrate that the immunoproteasome is generated in murine neurons by *in vitro* IFN- γ treatment.

Discussion

In this study, the contribution of proteasome-ubiquitin pathway in the IFN- γ -mediated anti-VSV response in neuronal cells was examined. We found that PIN is polyubiquitinated and rapidly degraded by proteasome in neurons (Figure 4). IFN- γ treatment of neuronal cells leads to increased synthesis and slower degradation of NOS-1 (Figure 3), resulting in production of NO and inhibition of viral replication (Figure 2). Ub-modification and degradation of VSV proteins was unchanged in IFN- γ -treated neurons (Figure 5). Immunoproteasome components LMP-2, -7, and -10 are induced rapidly and incorporated into the proteasome in IFN- γ -treated neurons (Figure 6).

Among VSV proteins, only M was polyubiquitinated, but its ubiquitination profile is unchanged by IFN- γ treatment (Figure 5). Among the three isoforms of NOS, NOS-1 is the only type expressed in neurons. The mechanisms by which the viral replication is inhibited NO remain to be clarified. Peroxynitrite, ONOO(-), the product of superoxide and NO, may attack tyrosines, serines and cysteines of proteins and results in NO₂Y, NO₂S, and NO₂C (Gu, *et al*, 2002;Radi, 2004). Such modifications may alter viral polymerase activity, protein interactions, or morphogenesis (Simon, *et al*, 1996;Saura, *et al*, 1999). NO₂⁻ modified proteins may not be optimal substrates for Ub-modification, as well.

In eukaryotic cells, one of the primary pathways for the degradation of proteins in the cytosol and nucleus is via the proteasome-ubiquitin pathway (Zwickl, *et al*, 1999). IFN- γ treatment altered the ubiquitination of PIN, likely through up-regulation of Ub-ligases and/or dampening of specific deUBases. It was reported 4 or more Ub units may be required for degradation of proteins by the proteasome(Hicke *et al*, 2001; Lederkremer *et al*, 2005). We found that inhibition of proteasome prolonged half-life of PIN. However, co-IP experiment only identified triply-Ub-modified PIN, this could due to the rapid degradation of poly-ubiquitin chain and/or the affinity of the commercial antibodies used. Identifying the specific enzymes critical for this pathway will help elucidate some of the mechanisms of IFN-mediated anti-viral responses.

Ubiquitination of viral proteins have been found in several virus infections (Hu, *et al*, 1999; Kim, *et al*, 2003; Eom, *et al*, 2004). The ubiquitination of VSV M protein is involved in regulation of viral budding (Harty, *et al*, 2001) and has been shown to be critical for morphogenesis of other viruses (Khor, 2003). Our examination of ubiquitinated VSV proteins

in neurons showed only M protein is significantly ubiquitinated and the amount of Ub-M was unchanged by IFN- γ treatment. Thus ubiquitin does not appear to contribute to the more rapid degradation of VSV proteins in IFN- γ -treated infected neurons. However, degradation of VSV by proteasome/immunoproteasome is still possible. Ubiquitin conjugation may not be required for the degradation of oxidized proteins by proteasome (Shringapure, *et al*, 2003). VSV infection induces oxidative stress in neurons (Komatsu, *et al*, 1999).

A major function of the immunoproteasome is to produce antigenic peptides presented to CD8⁺ T cells (Kloetzel and Ossendorp, 2004). However, little has been studied about the substrate specificity of the immunoproteasome. The immunoproteasome has been observed in three human neurodegenerative diseases: ALS, AD and HD (Diaz-Hernandez *et al*, 2003; Cheroni *et al*, 2005; Mishto *et al*, 2006). It is unclear what the effect of substitution of constitutive subunits with immunoproteasome LMPs has on the neuron's physiology in these diseases or in our studies. This alteration is likely to be secondary to infiltration of IFN- γ - secreting inflammatory cells from circulation in response to parenchymally-produced chemoattractants. Clearly, however, this is not necessary for processing and presentation of immunogenic short peptides to be presented on class I MHC molecules, as the vast majority of neurons are not able to express MHC and even when artificially induced to express MHC class I, incapable of presenting to T cells (Joly and Oldstone, 1992).

Although the impact of the immunoproteasome on inhibition of VSV replication remains unknown, it may be involved in degradation of VSV proteins through ubiquitin-independent pathways. A number of related ubiquitin-like proteins, including SUMO, Nedd8 and ISG15 have been identified that can modify to proteins and modulate proteins at the posttranslational level (Hochstrasser, 2000). ISG15 is the best defined Ub-like protein that is induced by IFN and microbial challenge, including virus infection. Upon induction, ISG15 conjugates with a variety of proteins, and may prevent the ligation of ubiquitin (Liu, *et al*, 2003). The gain of function modulation of Jak-1, STAT-1 proteins by ISG15 has been observed (Malakhova, *et al*, 2003) implying a role of ISG15 in cytokine induced cellular alterations. Work is in progress on the contribution of these homologues in IFN- γ -treated neurons on the inhibition of VSV replication.

Another degradative pathway is autophagy (Suhy *et al*, 2000; Schmid and Munz 2005; Levine and Yuan, 2005), in which cytoplasmic domains are engulfed and fuse with the lysosome for destruction and recycling of proteins. Although we initially chose to focus on the proteasome, work is in progress to determine the contribution of autophagy in cytokine-treated neurons.

Our study indicated that, in addition to its key role in cellular protein degradation, the proteasome-ubiquitin pathway is involved in many aspects of IFN- γ regulated antiviral responses in murine neurons *in vitro*. We also report generation of immunoproteasome in the murine neurons in culture. Ubiquitin and ubiquitin-like-proteins, with their roles in modulation of IFN- γ signaling components may be critical mediators in the IFN- γ -regulated anti-viral responses. IFN- γ treatment results in increased translation and longer half-life of NOS-1. At the same time, there is depletion of a NOS-1 regulatory protein, PIN by the ubiquitin-proteasomal pathway. Viral proteins accumulate more slowly in IFN- γ -treated neurons, but this is apparently not due to Ub-modification and degradation.

Experimental procedures and materials

Cell cultures and Reagents

NB41A3 cells, RAW 264.7 cells and L929 cells were purchased from ATCC. NB41A3 cells were maintained in F-12K medium containing 15% horse serum (Summit Biotech), 2.5% fetal bovine serum (FBS, Atlanta Biologicals), and 1% penicillin/streptomycin at 37°C/5% CO₂.

RAW 264.7 cells were grown in DMEM low glucose media with 10%FBS and 1% penicillin/streptomycin at 37°C/5% CO₂. L929 cells were maintained in DMEM low glucose media containing 10%FBS, 1 mM L-glutamine, HEPES buffer and 1% penicillin/streptomycin at 37°C/5% CO₂. Unless otherwise stated, all tissue culture reagents were obtained from Mediatech. VSV, Indiana serotype, San Juan strain, originally obtained from Alice S. Huang (The Childrens' Hospital, Boston) was used in all viral studies. Recombinant murine IFN- γ and IFN- β were purchased from R&D Systems and used at a concentration of 20ng/ml and 100units/ml in all experiments. Lactacystin (LC, EMD Biosciences) and Geldanamycin (GA, Sigma) were used at concentration of 5 μ M unless otherwise stated.

Primary neuron cultures

Newborn TgNSE-D^b mice (the gift of Michael B.A. Oldstone, The Scripps Research Institute; reference Joly and Oldstone, 1992) that constitutively express H-2D^b driven by the neural specific enolase promoter in neurons were used as a source of neurons for primary culture. They were positively selected by panning using Mouse anti-H-2D^b mAb (hybridoma cell lines HB36 and HB19 purchased from ATCC). Poly-D-lysine-coated 24 well plates (BD) were precoated with 100 μ l goat-anti-mouse IgG (1:50, Vector) at 37°C for 3 hours. The plates were then coated with 200 μ l anti-H-2D^b monoclonal antibody (3mg/ml) at 37°C overnight and rinsed twice with HBSS prior to use. Otherwise primary cultures were prepared as previously described (Chesler *et al* 2003). Olfactory bulbs were dissected from P0 (<24 h old) TgNSE-D^b mice, dissociated by enzymatic digestion (porcine trypsin 2.5%; Life Technologies, NY), and manual trituration using flame polished Pasteur pipettes. Cultures were plated in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS), 50 μ g/ml gentamicin (Life Technologies, NY), 10 mM KCl, 10 μ M L-glutamine, and 10 μ M AraC (Sigma) for 1h at 37°C/5% CO₂. Remove the media with floating cells after 1 hour and add same media with AraC. After 24h, the medium was replaced with Neurobasal Medium (Life Technologies, NY) with 1% N2 supplement (Life Technologies), 0.5 mM L-glutamine, 25 μ M L-glutamic acid, 10 mM KCl, 50 μ g/ml gentamicin, and 10 μ M AraC. All experiments were performed on day 6 of culture.

Cell viability (MTS) assay

Cell viability was determined by the MTS (Promega, Madison, WI) colorimetric assay following LC or GA treatment. The assay is based on soluble formazan production by dehydrogenase enzymes found in metabolically active cells. Cells were cultured with 100 μ l media in 96 well plates and samples were seeded in three wells per time point at 2.5×10^3 cells per well. 100 μ l of freshly mixed MTS reagent was added into each well and incubated for 1 hour. Absorbance was determined at 490 nm using a micro-plate reader (Model 550, Bio-Rad) and the results expressed as the mean absorbance of triplicate experiments $\pm$ SE.

Assay of Proteasome activity

Cell lysates containing active 20S proteasome were prepared from NB41A3 cells by 20S lysis buffer containing 25 mM Tris $\times$ Cl, pH 7.4, 1 mM EDTA, 1 mM NaN₃. The assay is based on the proteasome-catalyzed cleavage of *p*-nitroaniline from *N*-benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide (Z-GGL-pNA; Sigma) (Current protocols in protein science, 21.6). 200 μ g of cell lysate was diluted in 240 μ l 0.05 M Tris $\times$ Cl (pH 7.5), mixed with 10 μ l of 10 mM Z-GGL-pNA and incubated in 37°C for 30 min. The reaction was stopped by adding 0.25 ml of 10% TCA. Samples were centrifuged at and 0.25 ml clear supernatant was mixed with 0.25 ml of 0.1% NaNO₂, incubating at room temperature for 3 min. 0.25 ml of 0.5% ammonium sulfamate was added and mixed for 2 min. Then 0.5 ml of 0.05% NNEDA was added to the sample following by vigorous vortex. Liberated *p*-nitroaniline was converted to a red-violet colored product and the absorbance of the chromogen was read in a microplate reader (model 550; Bio-

Rad) at 540 nm against the blank. A standard curve (10 to 100 nmol *p*-nitroaniline) was established.

Plaque Assay

Viral titers were determined in triplicates using serially diluted culture culternatants on monolayers of L929 cells as previously described (Komatsu, *et al*, 1996). Data are presented as Geometric Mean Titer $\pm$ SEM, log (pfu).

RT-PCR

mRNA levels were determined by multiplex RT-PCR using the Qiagen One-Step RT-PCR kit. Total RNA (100ng) was used in each reaction. The following program was used to carry out the reaction: 50°C for 30 min, 95°C for 15 min, 32 cycles of 94°C for 1 min, 58°C for 30 sec, 72°C for 1 min, then 72°C for 10 min, and 4°C hold. The primers were used in these reactions are listed in Table 1. Products were resolved by 1% agarose gel electrophoresis, stained using the SybrGold dye (Molecular Probes), and visualized using a UV transilluminator. Results were digitally photographed using a Kodak DC290 camera controlled by the Kodak 1D analytic software (Eastman Kodak, Rochester, NY). DNA band densities were determined using the UN-SCAN-IT software.

Griess assay

The concentration of NO₂⁻ in culture supernatants was determined by assaying the stable end product of NO (Hewett, 1999). Briefly, equal volumes (100 μ l) of experimental sample and Griess reagent (1 part 0.1% sulfanilamide in 60% acetic acid, 1 part 0.1% naphthylenediamine dihydrochloride [Sigma] in distilled H₂O) were mixed and incubated at room temperature for 10 min. The reaction produces a pink color, which was quantified at 540 nm by microplate reader. Each sample was read in triplicate. A standard curve was made from sodium nitrite dissolved in Griess reagent.

Western Blot

NB41A3 cells or primary neurons were cultured and treated with IFN- γ or LC for indicated period of time. Cells were lysed and protein concentrations were determined using the Bio-Rad Dc protein assay, and 2mg/ml solutions were prepared and boiled for 5 min in dissociation buffer. Samples were resolved by 7.5%, 10% or 12% SDS-PAGE electrophoresis, transferred to Transblot membranes (Bio-Rad), and probed for 1 h at ambient temperature or overnight at 4°C simultaneously with primary antibodies, and then probed with horseradish peroxidase (HRP)- conjugated secondary antibodies for 1h at ambient temperature. Membranes were visualized with Pierce PicoWest EnhancedTM Chemiluminescent Reagents. Film exposure was on Kodak BioMax film for 30 sec to 2 min. Protein band densities were quantitated by UN-SCAN-IT software.

Antibodies

Mouse anti-mouse NOS-1 monoclonal antibody (mAb) (1:2000) and mouse anti-mouse PIN (1:100 for Western blot, 1:30 for IP) mAb were purchased from BD Pharmingen. Rat anti-mouse PIN mAb (1:1000 for Western blot, 1:100 for IP) were purchased from Alexis. Mouse anti-mouse class III β -tubulin (1:10,000) mAb was obtained from RDI Diagnostics; rabbit anti- β -tubulin III pAb (1:600) was purchased from Covance. Rabbit anti-mouse LMP2 (1:300), anti-LMP7 (1:1000), anti-LMP10 (1:1000), anti-proteasome 20S (1:100) pAb and mouse anti-mouse α ₅ mAb were purchased from Biomol. Anti- β -actin pAb (1:2000) was purchased from Abcam. Mouse anti-mouse β -actin (1:100,000) was obtained from Sigma. Mouse anti-mouse ubiquitin mAb (1:1000) and rabbit anti-mouse ubiquitin (1:300) pAb were purchased from AB Reagent and Dako, respectively. Sheep anti-VSV pAb was a generous gift from Dr. Alice S.

Huang (when at The Children's Hospital, Boston). HRP conjugated anti-mouse secondary antibody (1:2000) was purchased from Vector, Donkey anti-sheep (1:4000) and Donkey anti-rabbit (1:40,000) secondary antibodies, both HRP conjugated, were purchased from Jackson. β -actin and / or class III β -tubulin were used as loading controls since both of them have been used in related approaches (Habelhah *et al*, 2004; Golde *et al*, 2003) and neither was reported to interact with the proteasome-ubiquitin pathway .

Co-immunoprecipitation

NB41A3 cells were plated and treated with IFN- γ and/or Lactacystin for 24 hours. Whole cell lysates were extracted and 400 μ g protein from each sample was used. Immunoprecipitations of NOS-1 and β -tubulin from whole cell lysates with rabbit anti-NOS-1 pAb(1:40) and rabbit anti- β -tubulin III pAb were performed at 4°C overnight. Then, 20 μ l protein A/G beads (Pierce) were added and rotated at 4°C for minimum 3 hours. Beads were collected and washed with lysis buffer for 3 times, prior to boiling with dissociation buffer for 5 mins. The precipitate was detected by Western blot using mouse anti-Ubiquitin mAb and mouse β -tubulin mAb. Quantitation of the Ubi-NOS1: β -tubulin ratio was performed using UN-SCAN-IT software. At the same time, IP of Ubiquitin and β -tubulin from whole cell lysate using anti-ubiquitin pAb and anti- β -tubulin pAb. The precipitate was detected in Western blots with anti-NOS-1 mAb, anti-Ubiquitin mAb and anti- β -tubulin mAb. The Ubi-NOS1: β -tubulin ratio was determined. Three independent experiments, with each sample in duplicate, were performed to achieve statistical significance.

Co-IP of PIN and ubiquitin was performed in the same manner, excepted for using anti-PIN antibody instead of anti-NOS-1 antibody. For the VSV-ubiquitin co-immunoprecipitation, NB41A3 cells treated with IFN- γ and/or Lactacystin was infected with VSV at MOI=0.1 and lysed at 5 hours pi. 200 μ g of whole cell lysate was used in each reaction. Anti-Ubi pAb and anti-Tubulin β III pAb were used for IP. Anti-VSV pAb and anti-Tubulin III mAb were used in the immunoblotting. Ubi-VSV: β -tubulin ratio was quantitated.

Metabolic Labeling and Immunoprecipitation

NB41A3 cells were cultured in 6-well plates (2.5×10^5 cells/well) and incubated with 1.0 ml of Met/Cys free DMEM medium containing 10% dialyzed FBS for 30 min, and then pulsed with Met/Cys-free DMEM medium containing 100 μ Ci of [35 S]Met[35 S]Cys mix. Following the 1 or 2 hours labeling period, the medium was changed to chasing medium (starvation medium with 2mM Met/Cys added back). At appropriate time intervals, cell pellets were collected, freezed and stored at -80°C.

Cells were lysed and centrifuged at 14,000 rpm. Equal amounts of radioactive lysates were precleared with 50 μ l protein A/G beads (Pierce) at 4°C for 1 hour. Antibodies against NOS-1, PIN or VSV were added and incubated under agitation 4°C overnight. Subsequently, 25 μ l of protein A/G beads were added to the radioactive material and incubated for 1 hour at 4°C. Then beads were washed 3 times in lysis buffer, denatured in SDS-containing sample buffer and loaded on a 10% or 12% SDS-polyacrylamide gel. The gel was dried at 60°C for 1.5 hour and exposed at -80°C for 1-7 days. The radioactivity was quantified by UN-SCAN-IT software.

Statistical methods

Student's *t* test was used in all statistical analyses. Unless otherwise stated, all data were presented in mean $\pm$ SEM.

Acknowledgements

We are grateful to Dr. Alice S. Huang for the gift VSV and sheep anti-VSV antibody, Michael BA Oldstone for the mice constitutively expressing MHC I in neurons, to Dr. Michele Pagano (NYU School of Medicine) and Dr. Jane A. McCutcheon (NYU College of Dentistry) for helpful discussions.

References

- Aviel S, Winberg G, Massucci M, Ciechanover A. Degradation of the epstein-barr virus latent membrane protein 1 (LMP1) by the ubiquitin-proteasome pathway. Targeting via ubiquitination of the N-terminal residue. *J Biol Chem* 2000;275:23491–9. [PubMed: 10807912]
- Bender AT, Demady DR, Osawa Y. Ubiquitination of neuronal nitric-oxide synthase in vitro and in vivo. *J Biol Chem* 2000;275:17407–11. [PubMed: 10751385]
- Bi Z, Reiss CS. Inhibition of vesicular stomatitis virus infection by nitric oxide. *J Virol* 1995;69:2208–13. [PubMed: 7533852]
- Chen N, Reiss CS. Distinct roles of eicosanoids in the immune response to viral encephalitis: or why you should take NSAIDS. *Viral Immunol* 2002;15:133–46. [PubMed: 11952135]
- Cheroni C, Peviani M, Cascio P, Debiasi S, Monti C, Bendotti C. Accumulation of human SOD1 and ubiquitinated deposits in the spinal cord of SOD1G93A mice during motor neuron disease progression correlates with a decrease of proteasome. *Neurobiol Dis* 2005;18:509–22. [PubMed: 15755678]
- Chesler DA, McCutcheon JA, Reiss CS. Posttranscriptional regulation of neuronal nitric oxide synthase expression by IFN-gamma. *J Interferon Cytokine Res* 2004;24:141–9. [PubMed: 14980078]
- Chesler DA, Munoz-Jordan JL, Donelan N, Garcia-Sastre A, Reiss CS. PKR is not required for interferon-gamma inhibition of VSV replication in neurons. *Viral Immunol* 2003;16:87–96. [PubMed: 12725691]
- Chesler DA, Reiss CS. The role of IFN-gamma in immune responses to viral infections of the central nervous system. *Cytokine Growth Factor Rev* 2002;13:441–54. [PubMed: 12401479]
- Ciechanover A. The ubiquitin-proteasome proteolytic pathway. *Cell* 1994;79:13–21. [PubMed: 7923371]
- Craiu A, Gaczynska M, Akopian T, Gramm CF, Fenteany G, Goldberg AL, Rock KL. Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. *J Biol Chem* 1997;272:13437–45. [PubMed: 9148969]
- Eom CY, Heo WD, Craske ML, Meyer T, Lehman IR. The neural F-box protein NFB42 mediates the nuclear export of the herpes simplex virus type 1 replication initiator protein (UL9 protein) after viral infection. *Proc Natl Acad Sci U S A* 2004;101:4036–40. [PubMed: 15010529]
- Fan JS, Zhang Q, Li M, Tochio H, Yamazaki T, Shimizu M, Zhang M. Protein inhibitor of neuronal nitric-oxide synthase, PIN, binds to a 17-amino acid residue fragment of the enzyme. *J Biol Chem* 1998;273:33472–81. [PubMed: 9837926]
- Gogate N, Swoveland P, Yamabe T, Verma L, Woyciechowska J, Tarnowska-Dzidusko E, Dymecki J, Dhib-Jalbut S. Major histocompatibility complex class I expression on neurons in subacute sclerosing panencephalitis and experimental subacute measles encephalitis. *J Neuropathol Exp Neurol* 1996;55:435–43. [PubMed: 8786403]
- Golde S, Coles A, Lindquist JA, Compston A. Decreased iNOS synthesis mediates dexamethasone-induced protection of neurons from inflammatory injury in vitro. *Eur J Neurosci* 2003;18:2527–37. [PubMed: 14622153]
- Gomez RM, Yep A, Schattner M, Berria MI. Junin virus-induced astrocytosis is impaired by iNOS inhibition. *J Med Virol* 2003;69:145–9. [PubMed: 12436490]
- Goody RJ, Hoyt CC, Tyler KL. Reovirus infection of the CNS enhances iNOS expression in areas of virus-induced injury. *Exp Neurol* 2005;195:379–90. [PubMed: 16004984]
- Groettrup M, Kraft R, Kostka S, Standera S, Stohwasser R, Kloetzel PM. A third interferon-gamma-induced subunit exchange in the 20S proteasome. *Eur J Immunol* 1996;26:863–9. [PubMed: 8625980]
- Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 2002;297:1186–90. [PubMed: 12183632]

- Habelhah H, Takahashi S, Cho SG, Kadoya T, Watanabe T, Ronai Z. Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. *EMBO J* 2004;23:322–32. [PubMed: 14713952]
- Harty RN, Brown ME, McGettigan JP, Wang G, Jayakar HR, Huibregtse JM, Whitt MA, Schnell MJ. Rhabdoviruses and the cellular ubiquitin-proteasome system: a budding interaction. *J Virol* 2001;75:10623–9. [PubMed: 11602704]
- Hewett SJ. Interferon-gamma reduces cyclooxygenase-2-mediated prostaglandin E2 production from primary mouse astrocytes independent of nitric oxide formation. *J Neuroimmunol* 1999;94:134–43. [PubMed: 10376946]
- Hicke L. Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol* 2001;2:195–201. [PubMed: 11265249]
- Hochstrasser M. Evolution and function of ubiquitin-like protein-conjugation systems. *Nat Cell Biol* 2000;2:E153–7. [PubMed: 10934491]
- Hooper DC, Kean RB, Scott GS, Spitsin SV, Mikheeva T, Morimoto K, Bette M, Rohrenbeck AM, Dietzschold B, Weihe E. The central nervous system inflammatory response to neurotropic virus infection is peroxynitrite dependent. *J Immunol* 2001;167:3470–7. [PubMed: 11544340]
- Hori K, Burd PR, Furuke K, Kutza J, Weih KA, Clouse KA. Human immunodeficiency virus-1-infected macrophages induce inducible nitric oxide synthase and nitric oxide (NO) production in astrocytes: astrocytic NO as a possible mediator of neural damage in acquired immunodeficiency syndrome. *Blood* 1999;93:1843–50. [PubMed: 10068656]
- Hu Z, Zhang Z, Doo E, Coux O, Goldberg AL, Liang TJ. Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome complex. *J Virol* 1999;73:7231–40. [PubMed: 10438810]
- Imai J, Maruya M, Yashiroda H, Yahara I, Tanaka K. The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. *EMBO J* 2003;22:3557–67. [PubMed: 12853471]
- Jaffrey SR, Snyder SH. PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science* 1996;274:774–7. [PubMed: 8864115]
- Joly E, Oldstone MB. Neuronal cells are deficient in loading peptides onto MHC class I molecules. *Neuron* 1992;8:1185–90. [PubMed: 1610569]
- Khor R, McElroy LJ, Whittaker GR. The ubiquitin-vacuolar protein sorting system is selectively required during entry of influenza virus into host cells. *Traffic* 2003;4:857–68. [PubMed: 14617349]
- Kim JH, Kang S, Kim J, Ahn BY. Hepatitis B virus core protein stimulates the proteasome-mediated degradation of viral X protein. *J Virol* 2003;77:7166–73. [PubMed: 12805415]
- Kloetzel PM, Ossendorp F. Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Curr Opin Immunol* 2004;16:76–81. [PubMed: 14734113]
- Kodukula P, Liu T, Rooijen NV, Jager MJ, Hendricks RL. Macrophage control of herpes simplex virus type 1 replication in the peripheral nervous system. *J Immunol* 1999;162:2895–905. [PubMed: 10072539]
- Komatsu T, Bi Z, Reiss CS. Interferon-gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons. *J Neuroimmunol* 1996;68:101–8. [PubMed: 8784266]
- Komatsu T, Srivastava N, Revzin M, Ireland DD, Chesler D, Shoshkes Reiss C. Mechanisms of cytokine-mediated inhibition of viral replication. *Virology* 1999;259:334–41. [PubMed: 10388658]
- Kosugi I, Kawasaki H, Arai Y, Tsutsui Y. Innate immune responses to cytomegalovirus infection in the developing mouse brain and their evasion by virus-infected neurons. *Am J Pathol* 2002;161:919–28. [PubMed: 12213720]
- Lane TE, Paoletti AD, Buchmeier MJ. Disassociation between the in vitro and in vivo effects of nitric oxide on a neurotropic murine coronavirus. *J Virol* 1997;71:2202–10. [PubMed: 9032354]
- Lederkremer GZ, Glickman MH. A window of opportunity: timing protein degradation by trimming of sugars and ubiquitins. *Trends Biochem Sci* 2005;30:297–303. [PubMed: 15950873]
- Levine B, Yuan J. Autophagy in cell death: an innocent convict? *J Clin Invest* 2005;115:2679–88. [PubMed: 16200202]
- Liu M, Li XL, Hassel BA. Proteasomes modulate conjugation to the ubiquitin-like protein, ISG15. *J Biol Chem* 2003;278:1594–602. [PubMed: 12426315]
- Love S. Oxidative stress in brain ischemia. *Brain Pathol* 1999;9:119–31. [PubMed: 9989455]

- Malakhova OA, Yan M, Malakhov MP, Yuan Y, Ritchie KJ, Kim KI, Peterson LF, Shuai K, Zhang DE. Protein ISGylation modulates the JAK-STAT signaling pathway. *Genes Dev* 2003;17:455–60. [PubMed: 12600939]
- Mishto M, Bellavista E, Santoro A, Stolzing A, Ligorio C, Nacmias B, Spazzafumo L, Chiappelli M, Licastro F, Sorbi S, Pession A, Ohm T, Grune T, Franceschi C. Immunoproteasome and LMP2 polymorphism in aged and Alzheimer's disease brains. *Neurobiol Aging* 2006;27:54–66. [PubMed: 16298241]
- Noguchi S, Jianmongkol S, Bender AT, Kamada Y, Demady DR, Osawa Y. Guanabenz-mediated inactivation and enhanced proteolytic degradation of neuronal nitric-oxide synthase. *J Biol Chem* 2000;275:2376–80. [PubMed: 10644688]
- Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc Natl Acad Sci U S A* 2004;101:4003–8. [PubMed: 15020765]
- Reiss CS, Komatsu T. Does nitric oxide play a critical role in viral infections? *J Virol* 1998;72:4547–51. [PubMed: 9573217]
- Saura M, Zaragoza C, McMillan A, Quick RA, Hohenadl C, Lowenstein JM, Lowenstein CJ. An antiviral mechanism of nitric oxide: inhibition of a viral protease. *Immunity* 1999;10:21–8. [PubMed: 10023767]
- Schmid D, Munz C. Immune surveillance of intracellular pathogens via autophagy. *Cell Death Differ* 2005;12(Suppl 2):1519–27. [PubMed: 16247499]
- Shringarpure R, Grune T, Mehlhase J, Davies KJ. Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J Biol Chem* 2003;278:311–8. [PubMed: 12401807]
- Simon DI, Mullins ME, Jia L, Gaston B, Singel DJ, Stamler JS. Polynitrosylated proteins: characterization, bioactivity, and functional consequences. *Proc Natl Acad Sci U S A* 1996;93:4736–41. [PubMed: 8643472]
- Suhy DA, Giddings TH Jr, Kirkegaard K. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. *J Virol* 2000;74:8953–65. [PubMed: 10982339]
- Van den Eynde BJ, Morel S. Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. *Curr Opin Immunol* 2001;13:147–53. [PubMed: 11228406]
- Zwickl P, Voges D, Baumeister W. The proteasome: a macromolecular assembly designed for controlled proteolysis. *Philos Trans R Soc Lond B Biol Sci* 1999;354:1501–11. [PubMed: 10582236]

Fig 1. Sensitivity of neurons to Proteasome Inhibition

1A Cell viability (MTS) assay for NB41A3 cells treated with LC or GA. 2×10^6 NB41A3 cells were plated overnight before treatment of LC(24h) or GA(3h). Variable concentrations of LC or GA were used as indicated. Cell viability was measured using Promega MTS reagent. **1B** LC and/ or GA suppressed proteasome activity. NB41A3 cells were treated with LC and/or GA at indicated concentration and time. Cells were lysed and same amount of lysates from each sample were used to measure proteasome activity. Results are mean of triplicate samples.

Fig 2A. IFN- γ and proteasome inhibitors antagonize VSV replication in NB41A3 cells NB41A3 cells were incubated with or without IFN- γ for 24 hours; Lactacystin (10 μ M) or Geldanamycin (5 μ M) or both were added 3 hours prior to infection with VSV at M.O.I=0.1. At 7 hours post infection, supernatants were harvested, and viral titers were determined by plaque assay. Viral titers presented are geometric mean titers from triplicate samples of each treatment. Data are representative of two experiments with similar results. **B Lactacystin, IFN- γ , or both treatments induce NO production in NB41A3 cells.** NB41A3 cells were treated with LC (3 μ M, 24hours), IFN- γ (20ng/ml, 48hours) or both. Supernatants were collected and a Griess assay was performed to measure the production of NO. Each sample was measured

in triplicate. Results are representative of 3 independent experiments with similar trend.
(* $p < 0.01$)

Fig 3. Lactacystin or IFN-γ treatments induce NOS-1 expression posttranscriptionally with ubiquitination of NOS-1 unchanged

A, Lactacystin or IFN-γ treatment does not alter NOS-1 mRNA levels in NB41A3 cells. NB41A3 cells were treated in triplicate for 24 h with medium, LC, IFN-γ or both. Total RNA was isolated and NOS-1 mRNA levels were determined by RT-PCR. The relative densities of bands were measured, and differences between reactions were normalized by β-actin levels. Data are represented as means ± SEM; **B**, Lactacystin, IFN-γ, or both treatments induce NOS-1 expression in NB41A3 cells. NB41A3 cells were treated with LC, IFN-γ or both for 24 hours. Total protein was collected and expression of NOS-1 and β-actin was measured by Western blot using anti-NOS-1 mAb and anti-β-actin mAb. **C**, Lactacystin or IFN-γ treatment

induces NOS-1 expression in primary neurons. Primary cultured neurons were treated with LC or IFN- γ for 24 hours and expression of NOS-1 was detected by Western Blot. Results are representative of 3 independent experiments. (* $p < 0.01$) **D** NB41A3 cells were treated with media (control), IFN- γ or LC, or both for 24 hours. Ubiquitin conjugated proteins were precipitated using anti-ubiquitin pAb and blotted with anti-NOS-1 mAb. IP negative control (a sample with no antibody for IP step) was included. Each sample was loaded in duplicate. Results are representative of 3 independent experiments. **E** IFN- γ treatment results in prolonged half life of NOS-1. NB41A3 cells treated with IFN- γ (24h) or LC (3h) were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine mix for 2 hours and chased for the indicated times. Same treatment (IFN- γ or LC) was present throughout the experiment. The cells were lysed and equal amount of lysates was used for each chase time. NOS-1 was analyzed by immunoprecipitation with anti-NOS-1 antibody and SDS-PAGE. The gel was exposed and the radioactivity was quantified. The values are the mean $\pm$ S.E. of three independent experiments.

Fig 4. PIN is posttranscriptionally down-regulated by IFN- γ and more ubiquitinated PIN is found in IFN- γ treated neuronal cells

A, Treatment of neurons with IFN- γ does not alter PIN mRNA level. Total RNA was isolated from NB41A3 cells treated with IFN- γ for 0, 24, 48 or 72 hours. RT-PCR was performed. 100 ng of total RNA was used for each reaction. Product sizes are as follows: PIN-390bp; β -actin-540bp. Each sample was amplified in duplicate. Results are representative of 3 independent experiments. (* $p > 0.05$) **B**, Treatment of neurons with IFN- γ downregulates PIN protein level. Whole cell lysate was isolated from NB41A3 cells treated with IFN- γ for 0h, 24h, 48h or 72hours. The PIN protein level was determined by Western blot and normalized by β -actin. Results are representative of 3 independent experiments. (* $p < 0.05$). **C**, More

ubiquitinated PIN was found in IFN- γ treated neurons. Whole cell lysates were isolated from NB41A3 cells treated with LC or IFN- γ or both. Ubiquitin conjugated proteins were precipitated using anti-ubiquitin pAb and blotted with anti-PIN mAb. **D**, IFN- γ treatment reduces half-life of PIN. NB41A3 cells treated with IFN- γ or LC were metabolically labeled with [35 S]methionine/[35 S]cysteine mix for 2 hours and chased for the indicated times. Same treatment (IFN- γ or LC) was present throughout the experiment. The cells were lysed and PIN was analyzed by immunoprecipitation with anti-PIN antibody and SDS-PAGE. The gel was exposed and the radioactivity was quantified. The values are the mean $\pm$ S.E. of three independent experiments.

Fig 5. Ubiquitin is not involved in IFN-γ mediated inhibition of viral synthesis

NB41A3 cells were pretreated with media (control), IFN-γ or LC, or both for 24 hours prior to infection with VSV (MOI=0.1) and the 2 LC treated groups will be continuously treated with LC post infection. At 5 hours pi, whole cell lysates were extracted. **A**, The total amount of VSV proteins were determined by Western blot on duplicate samples with anti-VSV pAb, GAPDH was used as loading control. Results are representative of 3 independent experiments. **B**, Ubiquitin pAb and β-tubulin III pAb were used for immunoprecipitation prior to SDS-PAGE; samples were blotted with anti-VSV pAb and anti-β-tubulin III mAb, and visualized with Pierce ECL kit. For the same samples, we also did IP with anti-VSV pAb, blotted with anti-Ubiquitin mAb and similar results were observed. The relative densities of the gel bands

were quantitated and the amount of ubiquitinated M was normalized by total amount of β -tubulin. C, Half-lives of VSV proteins are not changed by IFN- γ treatment. NB41A3 cells were pretreated with media (control) or IFN- γ for 24 hours prior to VSV infection (MOI=1). After infection, cells were starved in Met/Cys free media for 30 mins, metabolically labeled with [35 S]Met/[35 S]Cys mix for 1 hour, and chased for indicated time. Equal amount of lysates were used and VSV proteins were immunoprecipitated with anti-VSV pAb and analyzed by SDS-PAGE. The gel was exposed and the radioactivity was quantified. The values are the mean $\pm$ S.E. of three independent experiments.

Fig 6A. LMPs are induced in NB41A3 cells by IFN- γ

Treatment of neurons with IFN- γ induces LMP2 mRNA level. Total RNA was isolated from NB41A3 cells treated with IFN- γ for 0, 12, 24 or 36 hours. RT-PCR was performed. Product sizes are as follows: LMP2-237bp; β -actin-540bp. Each sample was amplified in duplicate. Results are representative of 3 independent experiments. (* $p < 0.01$); IFN- γ induces LMP7 in NB41A3 cells. NB41A3 Cells were treated with IFN- γ 20ng/ml for 24 hours, 48hours and 72 hours. LMP 7 levels were tested by Western blot. Raw 264.7 cells treated with IFN- γ for 24 hours serve as positive control for anti-LMP7 antibody (not shown). Data are representative of three experiments with consistent trends. **B Immunoproteasome is generated in neurons with IFN- γ treatment.** NB41A3 cells were treated with media (control), IFN- γ (12hours and 24hours) and IFN- β (24hours). Whole lysates were extracted from each treatment. Proteasome 20S complex were purified from each lysate (200 μ g) by IP using anti-proteasome 20S pAb. The purified proteasome complex was boiled with dissociation sample buffer and the presence of the constitutive subunit α_5 and the immunoproteasome subunits LMP2, 7, 10 were detected by Western blot. Anti-GAPDH pAb was used in both IP and immunoblotting as assay control. To eliminate the heavy and light IgG chains in the immunoblotting, Rabbit Trueblot™ beads and secondary antibody set was used whenever a rabbit anti-mouse primary antibody was used

for blot. IFN- β treatment is used as positive control. Same results were observed in 3 independent experiments.

Table 1

Primers used for RT-PCR and size of products.

NOS-1	agcatgcctggcggaacgcctc	cagcaatgttaatctccacc	692bp
β -actin	gtgggccgctctaggcaccac	ctctttgatgtcacgcacgatttc	540bp
LMP2	aacgtgggtgaagaacatctc	gatggcatttgggtgaaac	237bp
LMP7	cacactcgcttcaagtcc	cttcaccaaccgtcttcc	551bp
LMP10	cttggttccgagatggagt	agagctgttgggtgaagcca	460bp
PIN	tagcgaccggctgtcttctg	ggatcactgggtgtttggca	392bp