Supplemental Data For:

Article Title : Overexpression of Nrf2 protects cerebral cortical neurons from ethanol - induced apoptotic death

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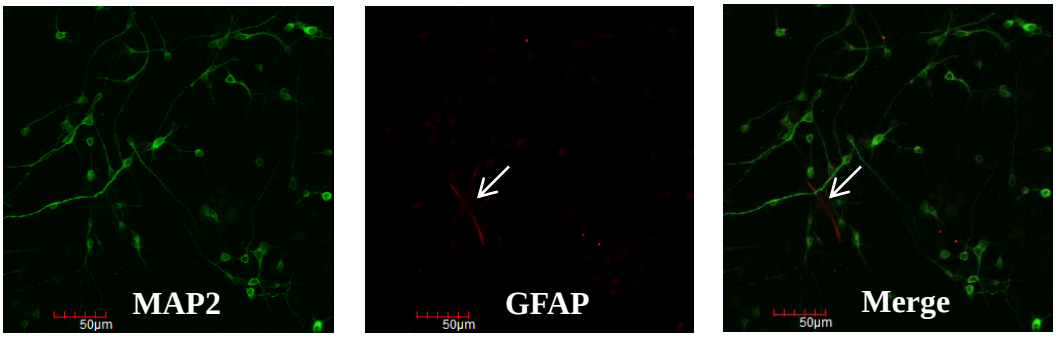
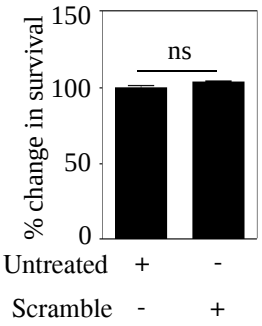
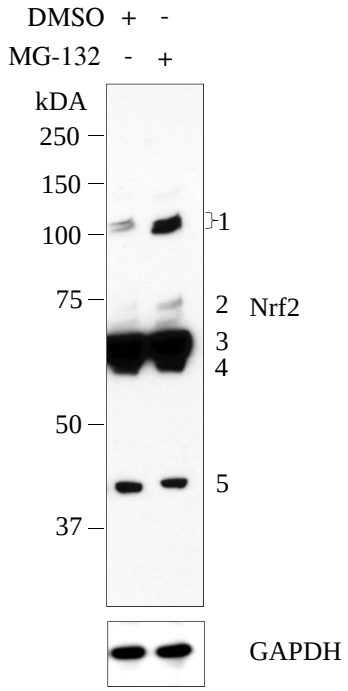


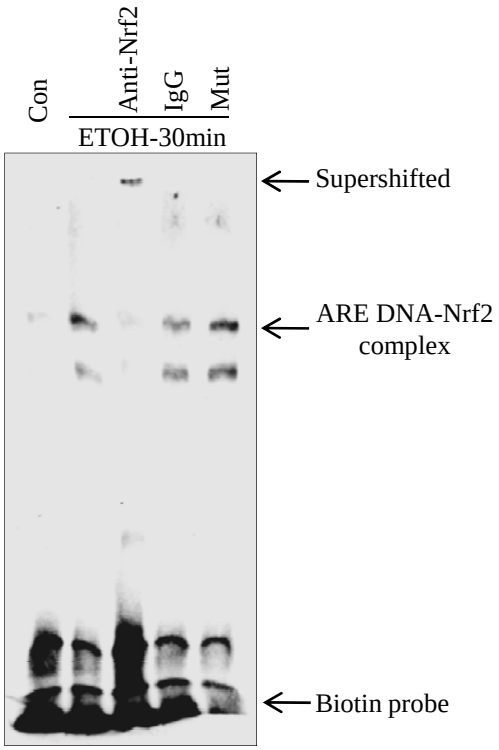
Supplemental Figure 1: Purity of isolated primary cortical neurons. Dual-immunofluorescence assay was performed using an anti-microtubule associated protein-2 (MAP-2) marker for neurons (Green) and possible contamination by astrocytes was assessed using anti-glial fibrillary acidic protein (GFAP) (Red). The cells were visusalised under confocal microscope and images captured using 40x magnification. Occasional one or two GFAP positive cells pointing out astrocyte contamination was observed similar to that shown in figure (white arrow in GFAP and merge panel). Five different fields were randomly counted for GFAP and MAP2 positive cells. Approximately 95% of the cells were neurons**.**



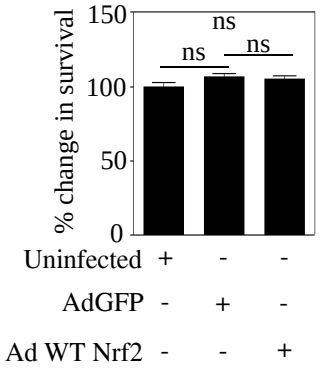
Supplemental Figure 2: Transfection agent, siPORT Amine did not have any toxicity in PCNs. As indicated in the materials and methods section, PCNs were transfected with scramble siRNA using siPORT amine and assessed for cell death, if any, using MTT assay. Briefly, at the end of 48 h after transfection, the cells were washed with RPMI-1640 phenol red free media and 1.5ml of 1/10 diluted 5mg/ml MTT solution was added to each well and incubated at 37^0 C for 2 h. MTT solution was then removed and the purple colored formazon complex formed was dissolved using 600 µL isopropanol/HCl/ Triton X-100 and measured at a wavelength of 570nm. The toxicity due to transfection agent is represented in terms of percentage change in survival between the non-transfected and transfected cells (n=4).



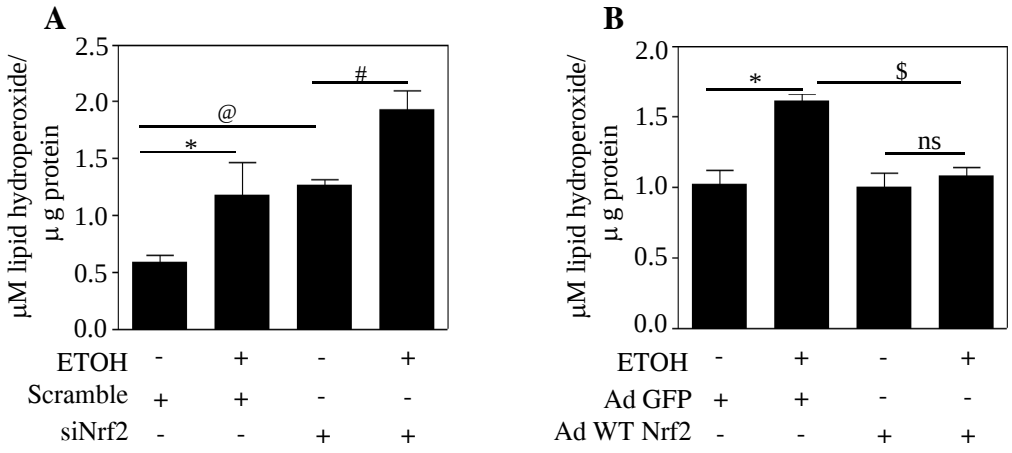
Supplemental Figure 3: MG-132 treatment stabilizes Nrf2 protein in PCNs. PCNs were treated with 10μM of proteasomal inhibitor, MG-132 for 4 h and western analysis were performed for Nrf2 using C-20 (sc-722) and GAPDH. A representative blot is given.



Supplemental Figure 4: Supershift analysis of ETOH treated PCNs using biotin based EMSA. NFE2 consensus oligonucleotide was end-labeled with biotin as per the instructions provided in the biotin 3' end DNA labeling kit (Thermo Scientific, Rockford, IL). 10 μg nuclear extracts from untreated and 30 min ETOH treated PCNs were used in the binding reaction containing 3 μL of biotinylated Nrf2 oligo and other constituents for 20 min as mentioned in LightShift chemiluminescnt EMSA kit (Thermo Scientific, Rockford, IL). Independently, for supershift analysis, nuclear extract from ETOH treated sample was preincubated for 30 min in ice either with 2.5 μL of rabbit monoclonal antibody against Nrf2 (TA303616, Origene, Rockville, MD) or IgG. This antibody incubated sample was then subsequently subjected to binding reaction along with biotinylated Nrf2 oligo for additional 20 min at RT. These samples were electrophoresed, transferred onto a positively charged Biodyne B nylon membrane (60209, Pall life sciences, Ann Arbor, MI). The membrane was then croslinked at 120mJ/cm^2 and the biotin-labeled DNA was detected using streptavidin-HRP/ chemiluminescence based autoradiography as mentioned in LightShift chemiluminescnt EMSA kit (Thermo Scientific, Rockford, IL).



Supplemental Figure 5: Adenovirus infection did not have any toxicity in PCNs. As indicated in the materials and methods section, PCNs were infected with AdGFP and Ad WT Nrf2 for 48 h and assessed for cell death, if any, using MTT assay. Briefly, at the end of 48 h after infection, the cells were washed with RPMI-1640 phenol red free media and 1.5ml of 1/10 diluted 5mg/ml MTT solution was added to each well and incubated at 37^0 C for 2 h. MTT solution was then removed and the purple colored formazon complex was dissolved using 600 µL isopropanol/HCl/Triton X-100 and measured at a wavelength of 570nm. The toxicity due to infection is represented in terms of percentage change in survival between the uninfected and adenovirus-infected cells (n=6).



Supplemental Figure 6: Lipid hydroperoxide measurement in Nrf2 knockdown (A) and adenovirus mediated overexpression of Nrf2 (B) in PCNs . As indicated in materials and methods section, PCNs were either downregulated with siRNA specific for Nrf2 or overexpressed using adenovirus encoding Nrf2 for 24 h, treated with ETOH for additional 24 h and assayed for lipid hydroperoxides, a measurement of lipid peroxidation. Briefly, the lysates were collected in HPLC grade water, sonicated and the collected supernatant is subjected to chloroform extraction as recommended in lipid hydroperoxide assay kit (705002, Cayman chemical company, Ann Arbor, MI). An aliquot of the samples were diluted with chloroform-methanol (2:1) to a volume of 950 μL and 50 μL of chromagen was added, incubated for 5 min at RT and read at 500 nm in a spectrophotometer. Non-hydroperoxide generated color in this assay is measured by reducing the hydroperoxides from samples with triphenylphosphine. These values were used to correct for any background absorbance in the samples. Before chloroform extraction, an aliquot of the samples were set aside and protein was estimated. The tripheylphosphine corrected LPO values were finally corrected to protein and represented (n=3). In A, B *P<0.05 compared with untreated control. In A, @ and # P<0.05 – vs control and siNrf2 respectively. In B, $ P<0.05 vs Ad GFP and ns - not significant vs Ad WT Nrf2.