

Resveratrol restores Nrf2 level and prevents ethanol-induced toxic effects in the cerebellum of a rodent model of Fetal Alcohol Spectrum Disorders

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**Abbreviations-**

ARE, antioxidant response element; bw, body weight; CGNs, cerebellar granule neurons; DAPI, 4',6-diamidino-2-phenylindole; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FASD, fetal alcohol spectrum disorders; GSH, glutathione; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; H<sub>2</sub>DCFDA, 2', 7'-dichlorodihydro-fluorescein diacetate; HO-1, heme oxygenase-1; HNE, 4-hydroxynonenal; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP-40, nonidet P-40; NQO1, NAD(P)H dehydrogenase (quinone)1; Nrf2, nuclear factor-erythroid derived 2-like 2; PBS, phosphate buffered saline; PD7, post-natal day 7; PMSF, phenylmethanesulfonylfluoride; Res, resveratrol; ROS, reactive oxygen species; SOD, superoxide dismutase; TFIID, transcription factor IID; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

## Abstract

In humans, ethanol exposure during pregnancy produces a wide range of abnormalities in infants collectively known as fetal alcohol spectrum disorders (FASD). Neuronal malformations in FASD manifest as postnatal behavioral and functional disturbances. The cerebellum is particularly sensitive to ethanol during development. In a rodent model of FASD, high dose of ethanol (blood ethanol concentration 80 mM) induces neuronal cell death in the cerebellum. However, the information on potential agent(s) that may protect the cerebellum against the toxic effects of ethanol is lacking. Growing evidence suggests that a polyphenolic compound, resveratrol, has anti-oxidant and neuroprotective properties. Here we studied if resveratrol (3,5,4'-trihydroxy-trans-stilbene, a phytoalexin found in red grapes and blueberries) protects the cerebellar granule neurons against ethanol-induced cell death. In the present study we show that administration of resveratrol (100 mg/kg bw) to postnatal day 7 rat pups prevents ethanol-induced apoptosis by scavenging reactive oxygen species in the external granule layer of the cerebellum and increases the survival of cerebellar granule cells. It restores ethanol-induced changes in the level of transcription factor nuclear factor-erythroid derived 2-like 2 (nfe2l2, also known as Nrf2) in the nucleus. This in turn retains the expression and activity of its downstream gene targets such as NADPH quinone oxidoreductase 1 (NQO1) and superoxide dismutase in cerebellum of ethanol-exposed pups. These studies indicated that resveratrol exhibits neuroprotective effects in cerebellum by acting at redox regulating proteins in a rodent model of FASD.

## Introduction

Exposure of the developing fetus to ethanol causes various birth defects in humans collectively known as Fetal Alcohol Spectrum Disorders (FASD) (Guerra et al., 2009). In the United States approximately 40,000 children are born with FASD each year costing an estimated 6 billion dollars in health, education, and social services (May and Gossage, 2001; Watterdorf and Muenke, 2005). While prenatal ethanol exposure affects the development of almost all organs, the brain and craniofacial features show the most severe defects and the cerebellum is particularly sensitive to the effects of ethanol during development (Bailey and Sokol, 2008; Guerra et al., 2009). The cerebellar granule neurons (CGNs) undergo apoptosis in response to ethanol exposure (Joshi et al., 2006). Ethanol-induced apoptosis in neurons is caused by reactive oxygen species generated due to the oxidative stress (Chu et al., 2007; Ikonomidou and Kaindl, 2011).

Neurons are most sensitive to the harmful effects of ethanol during the period of synaptogenesis. During this period (also called brain growth spurt stage) there is increased neuronal differentiation and synapse formation in the developing brain. Synaptogenesis phase in human fetus begins during third trimester of pregnancy and continues till the initial few years of postnatal life (Dobbing and Sands, 1979; Olney et al., 2000). This period in rodents corresponds to postnatal day 4 to 9 (P4 - P9). Exposure of rodents to high dose ethanol during this time frame depletes the neurons in the cerebellum (Bonthuis and West, 1990; Napper and West, 1995). For these reasons rodents are an excellent experimental animal model to study the mechanism of FASD. In our previous studies we have shown ethanol exposure to rats at postnatal day 7 inhibits the differentiation and induces apoptosis in the CGNs (Joshi et al., 2006).

Natural compounds with antioxidative properties are used in the treatment of various diseases. One of the such compound, resveratrol (3,5,4'- trihydroxy-trans-stilbene), exhibits a wide range of pharmacological effects including prevention of heart disorders, blocking of lipoprotein oxidation, free radical scavenging, prevention of apoptosis, and inhibition of platelet aggregation (Shakibaei et al., 2009). Because of the strong therapeutic potential, clinical trials for resveratrol in human for various diseases are currently being conducted (<http://clinicaltrials.gov/ct2/results?term=resveratrol>). Prior studies have shown that resveratrol prevented ethanol-induced apoptosis in embryonic stem cells and ethanol-disrupted mouse blastocyst development (Huang et al., 2007), and also protected ethanol-induce apoptosis in fetal rhombencephalic neurons (Antonio and Druse, 2008). In a recent study, resveratrol was found to improve ethanol-induced cognitive deficits in a rodent model of FASD (Tiwari and Chopra, 2011). Whether, resveratrol protects cerebellum and CGNs against the neurotoxic effects of ethanol, and the underlying mechanism is not known.

To study if resveratrol prevents ethanol-induced apoptosis we used P7 rodent model of FASD and demonstrated that ethanol increases oxidative stress and inhibits the activation of Nrf2 transcription factor in the cerebellum and in CGNs. Administration of resveratrol (100 mg/kg body weight (bw) before ethanol exposure restores the level of transcription factor Nrf2, prevents ethanol-induced oxidative stress in the cerebellum, and promotes survival of cerebellar granule cells. These studies show that resveratrol is a potent antioxidant that targets Nrf2 for protecting neurons against neurotoxic effects of ethanol.

## Materials and Methods

All animals used in these studies were handled in accordance with national guidelines for animal welfare and protocols were approved by the University of South Carolina Animal Care Committee. The present study was performed using randomly selected postnatal day 7 Long Evans rat pups (Charles River, Wilmington, MA). All the chemicals were used from Sigma (Sigma, St. Louis, MO) unless otherwise stated.

**Exposure of postnatal rat pups to ethanol and resveratrol administration.** For ethanol exposure, pups along with nursing dams were placed in an inhalation chamber for 5 h as reported previously (Joshi et al., 2006; Kumar et al., 2010). At the end of the inhalation period, we estimated blood alcohol concentration (BAC) with an alcohol reagent kit (Pointe Scientific, Canton, MI) according to the manufacturer's instructions. Mean BAC values were calculated from BACs obtained from three sets of pups (5 pups/set). Ethanol exposure according to this protocol produced 80 mM concentration in the blood (Joshi et al., 2006). A total of 226 pups were used for these studies. Pups were divided into ten groups: ethanol unexposed, ethanol unexposed plus resveratrol (2, 20, 40 or 100 mg/kg body weight), ethanol exposed, and ethanol exposed plus resveratrol (2, 20, 40 or 100 mg/kg body weight). Each group included at least four to six pups in individual studies. Resveratrol (Sigma, St. Louis, MO) was suspended in water and administered twice by oral gavage, 24 h and 1 h before ethanol exposure. After ethanol exposure pups were immediately sacrificed by decapitation, and cerebella were collected for isolation of whole cell extracts and preparation of CGN cultures.

**Isolation and primary culture of CGNs.** Primary culture of CGNs were prepared from at least six to eight pups per group in individual studies (Bhave and Hoffman, 1997; Joshi et al., 2006; Kumar et al., 2010). Briefly, cerebella isolated from different treatment groups

immediately after ethanol exposure were enzymatically digested with trypsin (Atlanta Biologicals, Lawrenceville, GA) at 37°C for 20 min, treated with soybean trypsin inhibitor (Worthington, Lakewood, NJ) and DNase I (Worthington, Lakewood, NJ) for 5 min at 37°C. Cells were dissociated by trituration, washed once with Basal Medium Eagles (BME) containing 25 mM KCl, and plated in BME containing 25 mM KCl, 10% fetal bovine serum (FBS) and 1% antibiotic solution. To prevent growth of non-neuronal cells, 10  $\mu$ M cytosine-D-arabinofuranoside (Sigma, St. Louis, MO) was added to the cultures after 12–14 h. With this protocol, nearly 95% of the cells were CGNs.

**Identification of total cerebellar proteins by Western blot analysis.** For Western blots at least four pups per group (ethanol unexposed, ethanol unexposed plus resveratrol, ethanol exposed, and ethanol exposed plus resveratrol) were used for the isolation of total cerebellum proteins. Tissue samples were homogenized and incubated for 30 min in 1x RIPA buffer (Cell Signaling, Danvers, MA) containing PMSF and protease inhibitors (aprotinin and leupeptin). Protein concentration was determined by BCA protein assay kit (Thermo Scientific, Waltham, MA). Extracted proteins were diluted with 5x Laemmli sample buffer and boiled for five minutes. The supernatants were subjected to electrophoresis and analyzed by Western blot. Briefly, after electrophoresis, proteins were transferred on PVDF membrane at 100 V for 3 h in cold room. Membrane was blocked with 5% non-fat dry milk/TBST (20 mM Tris-Cl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) followed by incubation in primary antibodies diluted in 2.5% non-fat dry milk/TBST for overnight at 4°C. After washing with TBST, membrane was incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG) diluted in 2.5% non-fat dry milk/TBST for 4 h at room temperature. Signals were detected by chemiluminescence detection kit (Pierce, Thermo Fisher Scientific, Rockford,

IL). Primary antibodies used were cleaved caspase-3 (Cell Signaling #9661, Danvers, MA), NQO1 (#34173, Abcam Inc, Cambridge, MA), HO1 (#SPA896, Stressgen, Plymouth Meeting, PA), caspase-7 (#56066), caspase-8 (#5263), SOD-2 (#133134), Nrf2 (#13032) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

**Isolation of nuclear proteins and electrophoretic mobility shift assay.** Nuclear proteins from cerebellar tissue were extracted as previously described (Kumar et al., 2010). Total four pups per group (ethanol unexposed, ethanol unexposed plus resveratrol, ethanol exposed, and ethanol exposed plus resveratrol) were used for the isolation of nuclear proteins from the cerebellum independently. Briefly, cerebella were homogenized in hypotonic buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM NaF, and 0.5 mM Na<sub>3</sub>VO<sub>4</sub>] followed by incubation for 10 min on ice. NP-40 was added to a final concentration of 0.5% and the cell lysate was centrifuged at 3,000xg for 10 min. The supernatant (cytoplasmic protein fraction) was collected, and nuclei pellet was washed once with hypotonic buffer. The pelleted nuclei were incubated with high salt buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 25% glycerol, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM NaF, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>) for 30 min on ice. Nuclei were then centrifuged at 12,000xg for 15 min. The extracted nuclear protein fraction was dialyzed to remove excess salt and stored at -80°C. Protein concentration was determined by the bicinchoninic acid method (Thermo Scientific, Waltham, MA).

For electrophoretic mobility shift assay (EMSA), rat NQO1 ARE (5'-TCT AGA GTC ACA GTG ACT TGG CAA AAT CTG A-3') was used as the Nrf2 binding site (Favreau and Pickett, 1991). Double stranded oligonucleotide was labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide



kinase (New England Biolabs, Ipswich, MA) and incubated with 10 µg of nuclear protein extract in binding buffer (10 mM HEPES (pH 7.9), 10 mM MgCl<sub>2</sub>, 0.02% NP-40, 0.5 mM DTT, 50 mM NaCl, 2 µg of poly(dI-dC), and 10% glycerol) at 25°C for 30 min. Binding reactions were resolved on 6% native polyacrylamide gels containing 0.5xTBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0) for 2.5 hours at 150 V. The gel was then dried and exposed to X-ray film. For competition experiments, excess unlabeled competitor (rat NQO1 ARE double stranded oligonucleotide) was pre-incubated with the nuclear extract for 15 min before labeled probe was added. For supershift assays, 1 µg of the anti-Nrf-2 antibody was added in binding reaction 30 min before addition of labeled probe.

**MTT assay.** To determine the viability of CGNs isolated from cerebellum treated with or without ethanol and/or resveratrol (three pups per group), MTT assay was carried out according to the manufacturer's instructions (Roche diagnostics corporation, Indianapolis, IN). CGNs were incubated with MTT reagent for 4 h and then overnight in solubilization buffer at 37°C. Absorbance of the formazan product was read at 575 nm. A reference wavelength of 690 nm was used to detect background.

**TUNEL staining.** TUNEL staining for paraffin-embedded brain sections were performed using the DeadEnd fluorometric TUNEL kit (Promega, Madison, WI). Brain sections (6 µm thick) were deparaffinized in xylene, rehydrated with ethanol, and washed with 0.85% NaCl. Tissue sections were fixed with 4% methanol-free formaldehyde soln. After PBS washing, sections were permeabilized with proteinase K solution (20 µg/ml) for 10 min at room temp. The nicked DNA was labeled with fluorescent labeled-dUTP nucleotide and rTdT enzyme mix for 60 min at 37°C. After washing with 2x SSC and PBS, slides were mounted with Vecta-shield

mounting media (Vector Laboratory, Burlingame, CA) and examined under fluorescence microscopy (Nikon E600).

**Detection of reactive oxygen species.** The production of reactive oxygen species (ROS) in CGNs from ethanol unexposed, ethanol exposed, and ethanol unexposed/exposed plus resveratrol treated pups (three pups per group) was detected using the fluorescence probe H<sub>2</sub>DCFDA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Briefly, CGNs from different group pups were cultured in Lab-Tek chamber slides (Fisher Scientific, Pittsburg, PA). After 45 min, cells were washed with HBSS (Hank's buffered salt solution) without phenol red and incubated with fluorescence probe carboxy-H<sub>2</sub>DCFDA for 30 min at 37°C in dark. DAPI was added to counter stain the nuclei. Cells were washed three times with HBSS, mounted and immediately examined (FITC filter) under Nikon-E600 fluorescence microscopy. Oxidation of H<sub>2</sub>DCFDA occurs almost exclusively in the cytosol, and generates a fluorescence that is proportional to ROS generation in that cell. CGNs without dye were processed in parallel as a negative control to detect autofluorescence. As a second negative control, we also monitored fluorescence in a cell free system in presence of ethanol, horseradish peroxidase (HRP) and carboxy-H<sub>2</sub>DCFDA dye at excitation and emission wavelength of 490 nm and 520 nm, respectively for photo- and/or atmospheric-oxidation (Myhre et al., 2003).

**Immunohistology.** To determine the alteration in protein expression profile in cerebellum, immunohistochemistry was performed (Kumar et al., 2010). At least four pups per group (ethanol unexposed, ethanol unexposed plus resveratrol, ethanol exposed, and ethanol exposed plus resveratrol) were used for immunohistochemical analysis. To detect apoptosis, cleaved caspase-3 antibody (1:500 dilutions, Cell Signaling #9661, Danvers, MA) was used as primary antibody. For 4-HNE staining, monoclonal anti-HNE antibody (1:250 dilutions, Abcam Inc,

Cambridge, MA) was used to detect adduct in tissue samples. Normal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used in place of primary antibody to assess nonspecific staining.

**Oxidative stress and anti-oxidant enzyme activity assay.** For enzyme-activity determination, four pups per group (ethanol unexposed, ethanol unexposed plus resveratrol, ethanol exposed, and ethanol exposed plus resveratrol) were used. After resveratrol and ethanol treatments, cerebella from pups were rapidly excised, thoroughly washed with ice-cold PBS to remove residual red blood cells, and homogenized in buffer containing 0.1 M Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.05% Triton X-100, and 0.5 mM PMSF. After centrifugation at 13,000 rpm at 4°C, supernatant was collected; protein was estimated by BCA kit (Thermo Scientific, Waltham, MA) and immediately used for biochemical assays. All the spectrophotometric readings were taken in spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA).

**a)- GSH level-** The glutathione level in tissue lysate was estimated by luminescence based GSH-Glo glutathione kit (Promega, Madison, WI) according to manufacturer's instructions. The luminescence was measured in a luminometer (Promega Biosystems, Sunnyvale, CA). The signal generated is proportional to the amount of glutathione present in the sample.

**b)- Total Thiol-** Total thiol in tissue lysate was estimated using 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) reagent according to Gularia et al. (Gularia et al., 2006). Briefly, tissue homogenate was mixed with 0.6 ml of the 0.25 M Tris - 20 mM EDTA buffer, pH 8.2 followed by addition of 40 µl of 10 mM DTNB and 3.16 ml of absolute methanol. After 20 min incubation, reaction mixture was centrifuged at 3,000xg for 10 minutes. The absorbance of the supernatant was measured at 412 nm. Total thiol in the sample was expressed in millimolar per

milligram protein in the lysate with a molar extinction coefficient of 13,600 mol/l per centimeter for calculation.

**c)- Lipid peroxidation-** The anti-lipid peroxidation effect of resveratrol in ethanol-treated cerebellum was determined by the formation of thiobarbituric acid reactive substances and expressed as the extent of malondialdehyde (MDA) produced due to lipid peroxidation (Guleria et al., 2006). The tissue lysate was mixed with 20% acetic acid, 8.1% SDS and 0.8% thiobarbituric acid. The reaction mixture was placed in a boiling water bath for 1 h. After cooling under tap water, reaction mixture was centrifuged at 3,000xg for 5 min and the absorbance of the supernatant was read at 532 nm. The molecular extinction coefficient of  $1.56 \times 10^5$  mol/l per centimeter is used to calculate nanomoles malondialdehyde (MDA) formed per milligram protein in the lysates.

**d)- Superoxide dismutase activity-** Superoxide dismutase (SOD) activity in tissue samples was determined using SOD determination kit (Sigma, St. Louis, MO) according to manufacturer's instructions. This assay utilizes a water-soluble tetrazolium, the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, to detect superoxide radical generated by xanthine oxidase, in the presence of superoxide dismutase.

**e)- 8-iso-PGF2 $\alpha$  assay-** 8-iso-PGF2 $\alpha$  levels reflecting the lipid peroxidation were estimated using a kit in accordance to manufacturer's instructions (Direct 8-iso-PGF2 $\alpha$  EIA kit, Enzo lifesciences, Plymouth Meeting, PA). In brief, after decapitation cerebellar tissue from resveratrol-fed ethanol treated or untreated pups were quickly stored in liquid N<sub>2</sub> until use. Tissue samples were powdered in liquid nitrogen, hydrolyzed with 1 ml of 2N NaOH at 45°C for 2 h followed by neutralization with an equal volume (1 ml) of 2N HCl. After centrifugation at 3000x rpm, clear supernatant was used to estimate 8-iso-PGF2 $\alpha$  using ELISA based Direct 8-iso-

PGF2 $\alpha$  EIA kit. The absorbance of developed yellow color was read at 405 nm in a spectrophotometer. The standards in range of 160 pg/ml to 100,000 pg/ml were also run in parallel.

**Data analysis.** Data are presented as the mean and standard deviation. Comparisons were made among the groups using the one-way ANOVA test followed by Tukey-Kramer ad hoc test (GraphPad software, La Jolla, CA). A  $p$ -value  $< 0.05$  was considered significant.

## Results

**Resveratrol prevents ethanol-induced apoptosis in the cerebellum.** Our previous studies have shown that ethanol at 80 mM concentration induces apoptosis in the external granule layer of cerebellum in PD7 pups (Joshi et al., 2006). To further test if resveratrol prevents apoptosis by preventing the activation of effector caspases, we isolated total proteins from the cerebellum of ethanol exposed or unexposed and/or resveratrol-fed pups, and performed Western blots. After activation, caspase-3 is cleaved to yield a smaller active fragment (19 kDa). The presence of a 19-kDa band in our Western blots of extracted proteins isolated from cerebellum of ethanol exposed pups indicated the activation of caspase-3 (Fig. 1A). The intensity of the active band (19 kDa) remained almost unchanged when 2, 20 or 40 mg/kg bw of resveratrol was administered before ethanol exposure. However, it was significantly reduced (~3.5 fold, compared to ethanol exposed pups) when higher dose of resveratrol (100 mg/kg bw) was used under similar conditions (Fig. 1A). Neither doses of resveratrol affected the intensity of caspase-7 and caspase-8 bands in protein samples isolated from the cerebellum of unexposed and ethanol exposed pups (Fig. 1A).

To detect the generation of cleaved active caspase-3 in the cerebellum, we also performed immuno-histochemistry with a specific antibody against cleaved caspase-3. In ethanol plus resveratrol (100 mg/kg bw) treated pups, the intensity of cleaved caspase-3 staining in the external granule layer (EGL) of cerebellum was significantly lower than that of ethanol exposed pups (Fig. 1B s, k). However, the fluorescence intensity for cleaved caspase-3 in the cerebellum of ethanol-exposed pups administered with lower doses of resveratrol fed (2, 20 or 40 mg/kg bw) was unaffected (Fig. 1B).

To determine if resveratrol protects against ethanol-induced apoptosis in cerebellum, we performed TUNEL assays to detect DNA fragmentation. Ethanol exposure for 5 h induced apoptosis in the external granule layer of the cerebellum as indicated by the TUNEL positive cells (Fig. 1C k). The staining with DAPI as well as fragmented DNA (TUNEL positive staining) in ethanol-exposed cerebellum overlapped in a single cell (shown in enlarged image) suggests that ethanol promotes DNA fragmentation and apoptosis in cerebellum (Fig. 1C u, v, w). After treatment with 100 mg/kg bw resveratrol, the number of TUNEL positive cells in the cerebellum were markedly reduced with ethanol exposure. Resveratrol alone had no effect on DNA fragmentation as shown in Fig. 1C, c-j; and low dose of resveratrol (2, 20 or 40 mg/kg bw) did not significantly reduce the number TUNEL-positive cells in response to 80 mM of ethanol.

**Resveratrol prevents ethanol-induced production of ROS in the cerebellum and in cerebellar granule cells.** Ethanol increases oxidation of lipids in the cerebellum that results in the formation of reactive aldehyde adduct 4-hydroxynonenal (4-HNE). By staining tissue samples with a specific antibody against HNE, we demonstrated increased lipid peroxidation in the external granule layer of cerebellum of ethanol treated samples (Fig. 2A k). Administration of 40 or 100 mg/kg bw (but not 2 or 20 mg/kg bw) resveratrol reduced the formation of HNE adduct in the cerebellum of ethanol exposed pups (Fig. 2A q, s). These data indicate that 40 or 100 mg/kg bw resveratrol prevents the ethanol-induced increase in lipid peroxidation.

To test whether resveratrol could suppress reactive oxygen species (ROS) formation, we performed H<sub>2</sub>DCFDA (2', 7'-dichlorodihydrofluorescein diacetate) staining in freshly isolated CGNs and counted the cells containing the dye (Karlsson et al., 2010). The assay, based on H<sub>2</sub>DCFDA oxidation by ROS and removal of acetate group by cellular esterases to fluorescent molecule CM-H<sub>2</sub>DCFDA, has been used frequently to measure oxidative stress and

representative staining is shown in Fig. 2B. In the present study, ~80% of CGNs isolated from ethanol exposed cerebellum stained positive for H<sub>2</sub>DCFDA, as compared with control ( $p < 0.01$  compared with ethanol unexposed). The percentage of stained CGNs from the ethanol plus 40 or 100 mg/kg bw resveratrol group was significantly reduced by ~30% or ~60%, respectively ( $p < 0.05$  compared with ethanol exposed, Fig. 2C), indicating an anti-oxidant effect of resveratrol. There was no staining when cells were processed without H<sub>2</sub>DCFDA dye (data not shown). As an additional control, we utilized cell free system with buffer containing dye, horseradish peroxidase, and ethanol (Myhre et al., 2003). As shown in Fig. 2D, ethanol alone was not able to oxidize H<sub>2</sub>DCFDA to fluorescent compound carboxy-DCF. After addition of horseradish peroxidase (5 unit/ml) in the mixture of buffer, dye (25  $\mu$ M), and ethanol (80 mM), the O.D. value increased by 4% after 15 sec when compared with dye plus HRP alone. It increased by ~13% after 6 min indicating the increased oxidation of H<sub>2</sub>DCFDA to fluorescent compound carboxy-DCF. The O.D. of only HRP plus buffer reaction was increased in first 15 sec and then remained constant. These data indicated that non-fluorescent H<sub>2</sub>DCFDA dye was oxidized in to fluorescent compound by ethanol in combination with cellular peroxidases.

**Resveratrol maintains high cell viability in ethanol-exposed cerebellar granule cells.** To evaluate a potential effect of resveratrol on cell viability, we also examined the morphology of CGNs under light microscope (phase contrast as dead cells and phase bright as live cells) and survival of CGNs by MTT assay. For studying cell morphology, CGNs were isolated from resveratrol fed ethanol untreated and ethanol treated pups and cultured for 2 days to assess the differentiation. In ethanol untreated CGNs, cell viability did not differ notably between different groups, as evaluated by number of phase contrast vs phase bright cells (Fig. 3A). The proportion of live cells isolated from untreated and 2, 20, 40 or 100 mg/kg bw resveratrol-fed pups were



94.8%, 95%, 96%, 95.7% and 95.3%, respectively. Ethanol exposure reduced the percentage of live CGNs (17.2%); however 18%, 20%, 59% and 86.9% of CGNs remains viable in 2, 20, 40 and 100 mg/kg bw of resveratrol fed ethanol-exposed pups, respectively (Fig. 3B). To further confirm the effect of resveratrol on viability of CGNs, we performed MTT assay (Fig. 3C). Similar OD values for cell viability were found among control groups (ethanol-unexposed, 2.9; and resveratrol fed 2 mg/kg bw, 2.8; 20 mg/kg bw, 2.8; 40 mg/kg bw, 2.6; and 100 mg/kg bw, 2.3) ( $p > 0.05$ ). The OD values were significantly decreased in ethanol exposed CGNs (OD 1.1,  $p < 0.05$  compared with ethanol unexposed pups) indicative of lower cells viability. Resveratrol (100 mg/kg bw) administration prevented the reduction in cell viability resulting from ethanol exposure in CGNs (OD 2.5,  $p < 0.05$  compared with ethanol exposed pups). Unlike the previous finding on morphological evaluation by light microscopy (Fig. 3B), resveratrol at 40 mg/kg bw did not prevent ethanol effect on survival of CGNs by MTT assay (Fig. 3C).

**Resveratrol prevents ethanol-induced oxidative stress in the cerebellum.** The reduced form of glutathione (GSH) and GSH-related enzymes are involved in the detoxification of  $H_2O_2$  and lipid peroxidation, and have been suggested to play important roles in protecting against oxidative stress. We therefore tested the effect of resveratrol on the total GSH levels in cerebellum. Ethanol exposure reduced total GSH levels in the cerebellum ( $p < 0.01$  compared with ethanol exposed pups) and this ethanol-induced GSH reduction was prevented when resveratrol was administered at 100 mg/kg bw ( $p < 0.01$  compared with ethanol exposed pups, Fig. 4A). The elevation in GSH level was not statically significant when resveratrol 2, 20 or 40 mg/kg bw was administered to ethanol exposed pups (Fig. 4A). Ethanol exposure significantly elevated total thiol levels in the cerebellum ( $p < 0.001$  compared with ethanol unexposed pups). Administration of resveratrol (40 or 100 mg/kg bw) significantly blocked the ethanol-induced

increase (For 40 mg/kg bw,  $p < 0.05$  and for 100 mg/kg bw,  $p < 0.001$  compared with ethanol exposed group) in total thiol levels in the cerebellum (Fig. 4B).

No significant differences in the malondialdehyde (MDA) levels, an indicator of lipid peroxidation, in cerebellum were observed between resveratrol and the control group. Ethanol exposure significantly increased ( $p < 0.05$  compared with ethanol unexposed) lipid peroxidation in the cerebellum as indicated by increased levels of MDA (Fig. 4C). Resveratrol (40 or 100 mg/kg bw) markedly suppressed (for 40 mg/kg bw,  $p = 0.05$ ; for 100 mg/kg bw,  $p < 0.05$  compared with ethanol exposed group) ethanol-induced MDA generation (Fig. 4C). We also measured the level of 8-epimer of prostaglandin F<sub>2</sub> $\alpha$  (8-iso-PGF<sub>2</sub> $\alpha$ ) in the cerebellum in response to ethanol exposure. The 8-iso-PGF<sub>2</sub> $\alpha$ , a stable F<sub>2</sub> isoprostane, is produced *in vivo* by the non-enzymatic peroxidation of arachidonic acid and has been considered a reliable oxidative stress marker to detect lipid peroxidation by free radicals in tissues (Gopaul et al., 2000; Morrow et al., 1990). In our experimental conditions, ethanol (80 mM) increased the level of 8-iso-PGF<sub>2</sub> $\alpha$  generation in cerebellum by approx. 2.5 fold as compared to ethanol-untreated pups. While resveratrol (40 mg/kg bw) reduced the levels of 8-iso-PGF<sub>2</sub> $\alpha$  in the cerebellum of ethanol exposed pups, higher dose of resveratrol (100 mg/kg bw) was more effective ( $p < 0.001$ , Fig. 4D). The altered levels of lipid peroxidation, GSH and total thiol in response to ethanol exposure were not significantly altered by administration of 2 mg/kg or 20 mg/kg bw resveratrol ( $p > 0.05$  vs ethanol-exposed pups).

**Resveratrol maintains the DNA-binding activity of Nrf2 upon ethanol exposure.** The nuclear factor-erythroid derived 2-like 2 (nfe2l2, also known as Nrf2) acts as transcription factor and binds to the antioxidant response element (ARE) of genes which protect against oxidative damage. To determine if resveratrol affects the nuclear distribution of Nrf2 in cerebellum, we

isolated nuclear proteins from the cerebellum of different treatment group of pups for Western blotting using an anti-Nrf2 antibody. The total as well as cytoplasmic level of Nrf2 did not change in response to ethanol and resveratrol ( $p > 0.05$  compared with ethanol unexposed pups, Fig. 5A and Ba). However, as shown in Fig. 5 Bb, the level of nuclear Nrf2 was decreased significantly in ethanol-exposed cerebellum ( $p < 0.001$  compared with ethanol unexposed pups), while administration of resveratrol (100 mg/kg bw) in ethanol-exposed pups prevented the loss of nuclear Nrf2 protein level ( $p < 0.001$  compared with ethanol exposed pups) and maintained Nrf2 levels almost similar to that of ethanol-unexposed cerebellum. Resveratrol less than 100 mg/kg bw (i.e. 2, 20 or 40 mg/kg bw) did not increase the level of Nrf2 in nuclear fraction of ethanol exposed cerebellar tissue. Similar results were obtained with nuclear proteins isolated from the CGNs prepared from different treatment groups of pups (Fig. 5 Bb).

To test if ethanol-induced changes in nuclear abundance of Nrf2 also affect expression of Nrf2-regulated proteins (such as NQO1, HO1 and SOD), we performed Western blots to determine their expression, and also the activity assay for SOD. In our western blots, the protein level of NQO1 and SOD2 was decreased in ethanol-treated cerebellum ( $p < 0.01$  compared with ethanol unexposed pups) while it was restored by 100 mg/kg bw of resveratrol in ethanol exposed pups (Fig. 5C, D). Administration of 40 mg/kg bw of resveratrol in ethanol-fed pups significantly increases SOD2 protein level ( $p < 0.01$ ) but not of NQO1 protein level in cerebellum ( $p > 0.05$ ). Neither 2 nor 20 mg/kg bw of resveratrol restored the protein level of NQO1 or SOD2 in cerebellum of ethanol exposed pups (Fig. 5C and D). The expression level for HO1 protein remains unchanged in different treatment groups ( $p > 0.05$  compared with ethanol unexposed pups, Fig. 5C, D). The activity of superoxide dismutase (SOD) was significantly decreased in ethanol exposed pups compared with untreated groups ( $p < 0.001$  compared with

ethanol unexposed pups), and administration of 40 or 100 mg/kg bw resveratrol resulted in significant elevation of SOD activity (for 40 mg/kg bw,  $p < 0.05$  and for 100 mg/kg bw,  $p < 0.001$  compared with ethanol exposed group) in ethanol exposed pups (Fig. 5G). We also measured the DNA-binding activity of Nrf2 by electrophoretic mobility shift assay (EMSA). Nuclear extracts prepared from cerebellum of ethanol-exposed pups exhibit decreased DNA-binding activity of Nrf2 to an oligonucleotide harboring the rat NADPH quinone oxidoreductase 1 (NQO1) ARE as compared to nuclear extract prepared from ethanol-untreated cerebellum (Fig. 5E, lane 4 and 16). The intensity of protein-DNA complexes was maintained near control levels in nuclear proteins isolated from ethanol-exposed cerebellum with resveratrol (100 mg/kg bw) administration (Fig. 5E, lane 5 and 20). The binding was specific, as addition of excess cold (unlabeled) oligonucleotide competed off the binding (lane 6). Inhibition of DNA-protein complexes by addition of Nrf2 antibody (1  $\mu$ g) in EMSA reaction further confirms the specificity of Nrf2 binding with the rat NQO1 ARE (Fig. 5E and F, lane 7-10). The band intensity of protein-DNA complexes in samples prepared from resveratrol (2, 20 or 40 mg/kg bw)-fed ethanol exposed pups was almost comparable to that of ethanol-exposed pups (Fig. 5E, lane 17-19). Similar results were obtained with nuclear proteins isolated from CGNs prepared from different treatment groups (Fig. 5F).

## Discussion

Ethanol is one of the most potent neurotoxic substances, and a high dose of ethanol during embryonic development causes dysfunction of the central nervous system (Olney et al., 2000). Previous reports have demonstrated that apoptosis is the major factor responsible for ethanol-induced neuronal cell death in the cerebellum, which may occur via the activation of caspase-3 as shown in our studies (Fig. 1A). The protein expression level of caspase-7 and -8 did not differ significantly in any group, suggesting that the caspase-7/8 is not involved in ethanol-induced apoptosis in the cerebellum. Our findings are consistent with previous reports showing that activation of caspase-3 is responsible for ethanol-induced neuronal cell death in the developing brain (Olney et al., 2002). Activated caspase-3 has been shown to induce multiple cellular events which trigger a range of downstream apoptotic events such as cell shrinkage, chromatin condensation, membrane blebbing, and DNA fragmentation (Nagata et al., 2003).

Currently resveratrol has been shown a promising natural compound containing anti-apoptotic, free radical scavenging and anti-lipoprotein peroxidation properties (Shakibaei et al., 2009). To test whether resveratrol has anti-apoptotic effect against ethanol in cerebellum, ethanol-exposed pups were fed with different doses of resveratrol (2, 20, 40 or 100 mg/kg bw). Fig. 1A clearly indicates that resveratrol administration at 100 mg/kg bw significantly prevented ethanol-induced activation of caspase-3 in the cerebellum, while the lower concentrations of resveratrol (2, 20 or 40 mg/kg bw) had no effect on caspase-3 activation. The reduced level of cleaved caspase-3 in ethanol exposed cerebellum by resveratrol suggests that the neuroprotective effect of resveratrol may result from reduced neuronal apoptosis in cerebellum. The increased level of activated caspase-3 trigger DNA fragmentation in ethanol-exposed cerebellum (as more TUNEL positive cells in ethanol-exposed cerebellum than control groups); however, resveratrol

(100 mg/kg bw; not lower concentrations) administration dramatically reduced DNA fragmentation in ethanol exposed cerebellum (Fig. 1C).

Using H<sub>2</sub>DCFDA staining, we further demonstrated that ethanol induced apoptosis is resulted from the generation of reactive oxygen species (ROS) in CGNs (Fig. 2B). However, Heaton et al detected no increase in ROS level at 2 h after ethanol exposure while ROS level was significantly decreased at 12 h or 24 h after ethanol exposure in whole cerebellum of P7 pups (Heaton et al., 2002). These differences in the results might be due to the analysis of ROS level in CGNs and different duration of ethanol exposure in our experiments. ROS, such as free radicals and peroxides, are the major cause of cellular oxidative stress which oxidizes the biomolecules such as oxidation of thiol groups, lipid peroxidation, and oxidation of bases in nucleic acids. All these events lead to harmful effects such as generation of highly reactive aldehyde by-products, protein aggregation, and degradation (Kannan and Jain, 2000). ROS generated by several metabolic pathways are detoxified by the SOD to prevent apoptosis in neurons (Greenlund et al., 1995). The results in the present study demonstrate that 40 and 100 mg/kg bw resveratrol restored protein expression (Fig. 5C), as well as enzymatic activity of SOD (Fig. 5G), decreased the level of total thiol, and reduced the oxidation of lipids in ethanol exposed cerebellum (Fig. 4). Additionally, treatment with resveratrol prevented ethanol-induced depletion of intracellular GSH level in the cerebellum (Fig. 4A). All these events indicated that anti-oxidative property of resveratrol is responsible for prevention of ethanol-induced oxidative stress in CGNs (Fig. 2B). The MTT assay, as well as morphological studies (by light microscopy), further revealed the protective role of resveratrol in the survival of ethanol exposed cerebellar neurons. In our experimental conditions, lower doses of resveratrol (2 or 20 mg/kg bw) were not able to restore the levels of GSH, total thiol, oxidation of lipids. Slightly higher

dose of resveratrol (40 mg/kg bw) was partially effective in preventing oxidative stress in the cerebellum. However, these effects were insufficient in preventing apoptosis and improving the viability of CGNs (Fig. 3A, C). High dose of resveratrol prevented ethanol effects on survival of CGNs, indicating that it was able to overcome the effects of ethanol on oxidative stress.

It is well documented that ethanol metabolism results in the formation of electrophilic aldehydes (4-hydroxynonenal and 4-oxo-2-nonenal) as by-products of peroxidation of lipid membrane (Niemela, 1999). 4-hydroxynonenal (HNE) exerts various biological effects in various cell types, such as alterations in cell proliferation and apoptosis (Ong et al., 2000). There is also evidence that HNE induces apoptosis in cultured cerebellar granule cells (Ito et al., 1999), in primary rat hippocampal neurons and PC12 cells (Kruman et al., 1997), and in fetal brain (Ramachandran et al., 2001). The toxic HNE has been shown to be detoxified by the conjugation with GSH and protecting PC12 and hippocampal neurons from apoptosis induced by oxidative stress as well as by HNE (Kruman et al., 1997). Our studies show that ethanol metabolism increases oxidative stress which enhances lipid peroxidation as well as the level of HNE in cerebellum (Fig. 2A). The elevated level of GSH (compared to the ethanol treated group) by administration of resveratrol (100 mg/kg bw) might detoxify the increased level of HNE and protects the neurons from ethanol-induced cell death.

To delineate the protective mechanism exerted by resveratrol against ethanol-induced toxicity, we focused on Nrf2-mediated signaling. Growing evidence supports a role of Nrf2 signaling in protecting cells from oxidative insults. Under normal conditions, Nrf2 remains associated with a Kelch-like ECH-associated protein1 (Keap-1) in cytoplasm (Kobayashi and Yamamoto, 2005). Nrf2 inducers such as antioxidants or electrophilic compounds dissociate the Nrf2/Keap-1 complex by modifying the thiol group of Nrf2 or Keap1 and/or phosphorylation by

PKC, as a result activated Nrf2 is transported to the nucleus where it is oligomerized with small proteins and interacts with antioxidant response elements (AREs) present upstream of several detoxifying enzymes such as catalase, superoxide dismutase, UDP-glucuronosyltransferase, NADPH quinone oxidoreductase 1, heme oxygenase 1, glutathione peroxidase,  $\gamma$ -glutamylcysteine synthetase, and modulate their expression (Jaiswal, 2000). These enzymes are involved in glutathione and NADPH production, as well as maintaining intracellular redox homeostasis.

To address the differential regulation of detoxifying and antioxidant enzymes in response to ethanol and resveratrol, we assessed the nuclear level as well as the DNA-binding activity of Nrf2. Our studies indicated that ethanol reduces nuclear abundance of Nrf2 as well as DNA binding activity of Nrf2 to ARE sequence of NQO1 when compared with ethanol untreated pups, while resveratrol prevents the loss of nuclear Nrf2 as well as its DNA binding activity in ethanol-treated cerebellum (Fig. 5B and E) as well as in CGNs (Fig. 5B and F). Contrary to previous observation on the transient Nrf2 accumulation in nucleus after oxidative insult (Jain and Jaiswal, 2007), we found ethanol-induced reduction in the levels of Nrf2 in the nucleus. It might be due to high dose ethanol and use of CGNs in our studies. In a related study, it was shown that direct activation of GSK3 $\beta$  results in nuclear exclusion of Nrf2 in CGNs. This unusual response to oxidative stress might be responsible for loss of tolerance to persistent oxidant exposure, as found in various neuropathologies (Rojo et al., 2008). Since, ethanol activates GSK3 $\beta$  in developing cerebellum and in CGNs (Luo, 2009), it might lead to reduction in Nrf2 levels in the nucleus.

Western blots of Nrf2-regulated downstream proteins (herein NQO1 and SOD) indicated that the ethanol exposure decreases the level of these proteins in cerebellum while it was restored in



the cerebellum of ethanol exposed resveratrol-fed (100 mg/kg bw) pups (Fig. 5C). In contrast to NQO1 and SOD expression, the protein level of HO1 remains unchanged in response to ethanol in cerebellum in our experimental conditions. HO1 enzyme activity, highly expressed in spleen and liver, is involved in heme biosynthesis and catalyzes intracellular heme into bilirubin. However, in guinea pig cerebellum ethanol exposure (in vitro, acute and chronic in vivo) has no effect on HO1 activity (Cook et al., 1997). Recently Yeligar et al has been reported that ethanol regulates HO1 and NQO1 transcription by different signaling pathways in kupffer cells (Yeligar et al., 2010). Thus besides Nrf2, other protein factor(s) interacting with regulatory elements might be involved in regulation of HO1 in response to ethanol-induced oxidative stress in the cerebellum.

Taken together these results, we hypothesize that reduced level of Nrf2 in the nucleus resulting from ethanol exposure alone decreases its binding to AREs which would in turn be expected to lower the expression of antioxidative enzymes as we found with NQO1 and SOD proteins (Fig. 5C). The lower level of antioxidant enzymes would fail to remove the reactive oxygen species and lead to neuronal cell death by ethanol. Resveratrol administration to ethanol-exposed pups restores the Nrf2 protein level in the nucleus to almost similar levels present in cerebellar nuclear fraction of ethanol-untreated pups. The ability of resveratrol to preserve Nrf2-DNA binding activity in ethanol-treated cerebellum as well as in CGNs (as shown in EMSA results, Fig. 5E and F) may maintain sufficient transcriptional activation of various antioxidant genes to balance the redox homeostasis in neurons, thereby promoting cell survival under ethanol-exposed conditions.

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### **Authorship Contributions**

*Participated in research design:* A. Kumar, U.S. Singh

*Conducted experiments:* A. Kumar

*Contributed new reagents or analytic tools:* C. K. Singh and H. LaVoie

*Performed data analysis:* A. Kumar, H. LaVoie, D. DiPette, U.S. Singh

*Wrote or contributed to the writing of the manuscript:* A. Kumar, H. LaVoie, D. DiPette, U.S.  
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### Legends for Figures

**Fig. 1.** Western blot analysis to determine the effect of different concentrations of resveratrol (2, 20, 40 or 100 mg/kg bw) on ethanol-induced activation of caspases in the cerebellum (**A**).

Whole cell extracts from the cerebellum of ethanol exposed or unexposed and/or resveratrol (Res)-fed pups were prepared using RIPA lysis buffer. Equal amount of proteins were used for Western blotting using anti-cleaved caspase-3, -7, and -8 antibodies.  $\beta$ -actin was used to check the loading difference. Bands were quantified by densitometric scanning and normalized to the level of  $\beta$ -actin. Data represent average fold change relative of control (ethanol unexposed pups) from at least three independent experiments (mean  $\pm$  S.D.). ns, non-significant compared to ethanol unexposed pups. Immuno-fluorescence staining for cleaved caspase-3 in the cerebellum (**B**). Resveratrol alone had no effect on the caspase-3 activation in ethanol unexposed control group (**c-j**). Ethanol induces activation of caspase-3 (**k**) while resveratrol at 100 mg/kg body weight (bw) reduced the activation of caspase-3 in the external granule layer (EGL, indicated by arrow) of ethanol exposed cerebellum (**s**). DAPI was used to stain nuclei; images were overlapped to monitor the boundary of cerebellum. The enlarged images **u** and **v** showing cleaved caspase-3 staining of EGL of ethanol-exposed cerebellum. Scale bar, 50  $\mu$ m. Pictures are representative of at least 3 independent experiments. TUNEL assays to study the effect of resveratrol on ethanol-induced apoptosis in the cerebellum (**C**). TUNEL staining was performed to study the DNA fragmentation in cerebellum of ethanol unexposed (**a-j**) and ethanol exposed pups (**k-t**) in the absence or presence of resveratrol. In ethanol unexposed control pups, resveratrol had no effect (**c-j**). Ethanol induced DNA fragmentation in EGL of cerebellum (**k**). The overlapped and individual images (u, v, w) showing a single cell with a typical apoptotic nucleus as stained with DAPI (blue) and TUNEL (green) in cerebellum from ethanol-exposed

pups (arrow-head indicates apoptotic/fragmented nucleus in a single cell and open arrow shows a normal nucleus). Resveratrol at 100 mg/kg bw prevented DNA fragmentation in ethanol exposed cerebellum (s). DAPI was used to counter stain the nucleus; images were overlapped to monitor the boundary of cerebellum. Pictures were taken randomly and are representative of at least 3 independent experiments. Scale bar, 50  $\mu$ m.

**Fig. 2.** Determination of oxidative stress. Immunofluorescence staining to determine the effect of resveratrol on ethanol-induced formation of 4-hydroxynonenal (4-HNE) protein adduct in the cerebellum (**A**). Nuclei were counter stained with DAPI; images were overlapped to monitor the boundary of cerebellum, and slides were examined under olympus stereo microscope (Olympus, Center Valley, PA). No significant staining was observed in control groups (**a, c, e, g** and **i**). Ethanol increased lipid peroxidation in EGL as shown by HNE staining (**k**) and resveratrol (40 and 100 mg/kg bw) prevented increased HNE staining (**q** and **s**). Scale bar, 1 mm. Pictures are representative of at least three independent experiments. H<sub>2</sub>DCFDA staining was performed to measure the reactive oxygen species (ROS) in CGNs (**B**). CGNs harvested from untreated and resveratrol-treated groups were cultured in chamber slides and stained with H<sub>2</sub>DCFDA dye as described in the Methods (**a-f**). Nuclei were counter stained with DAPI (**g-l**). Oxidation of H<sub>2</sub>DCFDA occurs almost exclusively in the cytosol, generating fluorescence that is proportional to ROS generation. Ethanol exposure increased the generation of ROS, indicated by the increased intensity of H<sub>2</sub>DCFDA dye in CGNs, as shown by arrows (**B b**). An enlarged image of a single CGN stained with DAPI and H<sub>2</sub>DCFDA dye is shown in **B h**. Reduced intensity of H<sub>2</sub>DCFDA in CGNs in **B f** and **l** indicates that resveratrol prevented generation of ROS. (**C**) Bar diagram shows the percentage of CGNs with H<sub>2</sub>DCFDA staining counted from treated and untreated groups. Pictures are representative of at least 3 independent experiments.

Scale bar, 50  $\mu$ m. Bars are mean and SD of three independent experiments. **(D)** Graph representing generation of fluorescent color of H<sub>2</sub>DCFDA dye was measured at 520 nm at different time points in cell free system in presence of ethanol, dye and HRP as described in method section for control.

**Fig. 3.** Effect of resveratrol on the survival of ethanol exposed CGNs. Phase contrast microscopy was used to examine the changes in morphology of CGNs isolated from unexposed, ethanol exposed, and ethanol exposed plus resveratrol (2, 20, 40 or 100 mg/kg bw)-fed pups **(A)**. CGNs isolated from ethanol-exposed pups had reduced survival as shown by phase contrast cell body **(b)**, while resveratrol (100 mg/kg bw) prevented ethanol-induced cell death in CGNs as shown by phase bright cell bodies **(f)**. Bar diagram shows the percentage of live and dead cells counted randomly from each treatment group **(B)**. Viability of cells from each group was measured by MTT assay as described in the Methods **(C)**. Absorbance of the formazan product was read at 575 nm. A reference wavelength of 690 nm was used to detect background signal. Pictures are representative of at least 3 independent observations. Bars are mean and SD of three independent experiments.

**Fig. 4.** The antioxidative effect of resveratrol on cerebellar glutathione (GSH) **(A)**, total thiol **(B)**, lipid peroxidation **(C)** and 8-iso-PGF<sub>2</sub> $\alpha$  level **(D)** in the cerebellum. Total cerebellar protein from untreated and resveratrol-treated plus ethanol exposed pups was isolated and biochemical assays were performed as described in the Methods. All the spectrophotometric readings were taken in Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA). For total thiol, the absorbance was measured at 412 nm and expressed in mM/mg protein with a molar extinction coefficient of 13,600 mol/l per cm. The effect of ethanol on lipid peroxidation was studied by the formation of thiobarbituric acid reactive malondialdehyde (MDA). The

absorbance of the supernatant was read at 532 nm. The molecular extinction coefficient of  $1.56 \times 10^5$  mol/l per cm was used to calculate nmoles MDA formed per mg protein. Bars represent mean and SD of three independent experiments.

**Fig. 5.** Resveratrol prevents ethanol-induced loss of Nrf2 level and its DNA-binding activity in the nucleus. Total cerebellar proteins were isolated, separated by SDS-PAGE and Western blot was performed with anti-Nrf2 antibody. Blot was probed to  $\beta$ -actin to check the loading differences (**A**, upper panel). Protein bands were quantified by densitometric scanning and normalized to the level of  $\beta$ -actin. Data represent average fold increase relative to control (ethanol unexposed pups) from at least three independent experiments (mean  $\pm$  S.D.). ns, non-significant compared to ethanol unexposed pups (**A**, lower panel). Determination of Nrf2 level in cerebellar proteins isolated from cytoplasmic and nuclear fractions (**B**). Equal amounts of cytoplasmic (**B a**) and nuclear (**B b**) proteins of cerebellar tissue (upper panel) and CGN (middle panel) isolated from ethanol and/or resveratrol-fed pups were separated by SDS-PAGE and subjected to Western blotting using antibodies for Nrf2 (upper panel) and  $\beta$ -actin or TFIID to assess loading (lower panel). Protein bands were quantified by densitometric scanning and normalized to the level of  $\beta$ -actin or TFIID. Data represent average fold change relative to control (ethanol unexposed pups) from at least three independent experiments (mean  $\pm$  S.D.). ns, non-significant compared to ethanol unexposed pups. The level of Nrf2-regulated proteins were assessed by Western blotting using total cerebellum proteins and probed with anti-NQO1, HO1 and SOD2 antibodies. Membranes were probed with  $\beta$ -actin as a control for equal loading (**C**). Bands were quantified by densitometric scanning and normalized to the level of  $\beta$ -actin. Data represent average fold change relative of control (ethanol unexposed pups) from at least three independent experiments (mean  $\pm$  S.D.). ns, non-significant compared to ethanol unexposed pups

**(D)**. EMSA was carried out to determine the DNA-binding activity of Nrf2. Nuclear protein (10  $\mu$ g) isolated from cerebellum **(E)** and from CGNs **(F)** of ethanol-unexposed (lane 2 and 11), ethanol-unexposed/ resveratrol (lane 3 and lane 12-15), ethanol-exposed (lane 4 and 16) and ethanol-exposed/resveratrol (lane 5 and lane 17-20) were used in EMSA reactions. To check the specificity of binding reaction EMSA was performed in presence of excess cold competitor (lane 6) and anti-Nrf2 antibody (lane 7-10). Figure is representative of at least three independent observations. SOD activity was determined in cerebellum in response to ethanol and/or resveratrol as described in methods **(G)**. Bars represent mean and SD of three independent experiments. ns, non-significant compared with ethanol unexposed pups. Putative model representing resveratrol neuroprotective effects **(H)**.

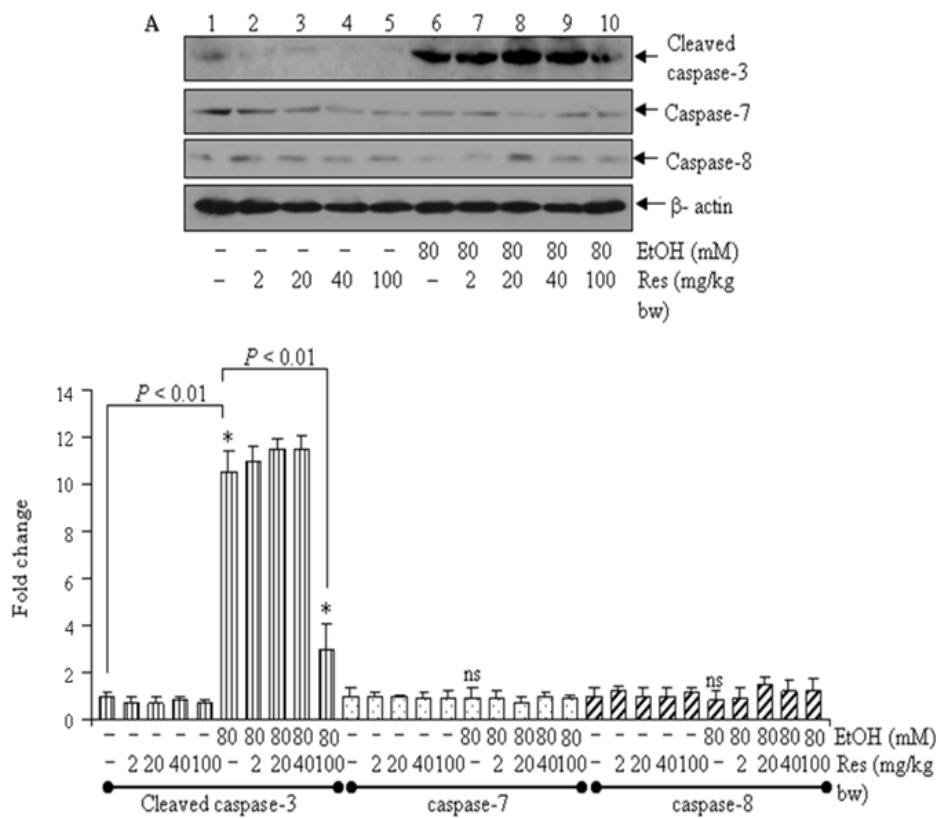


Fig. 1

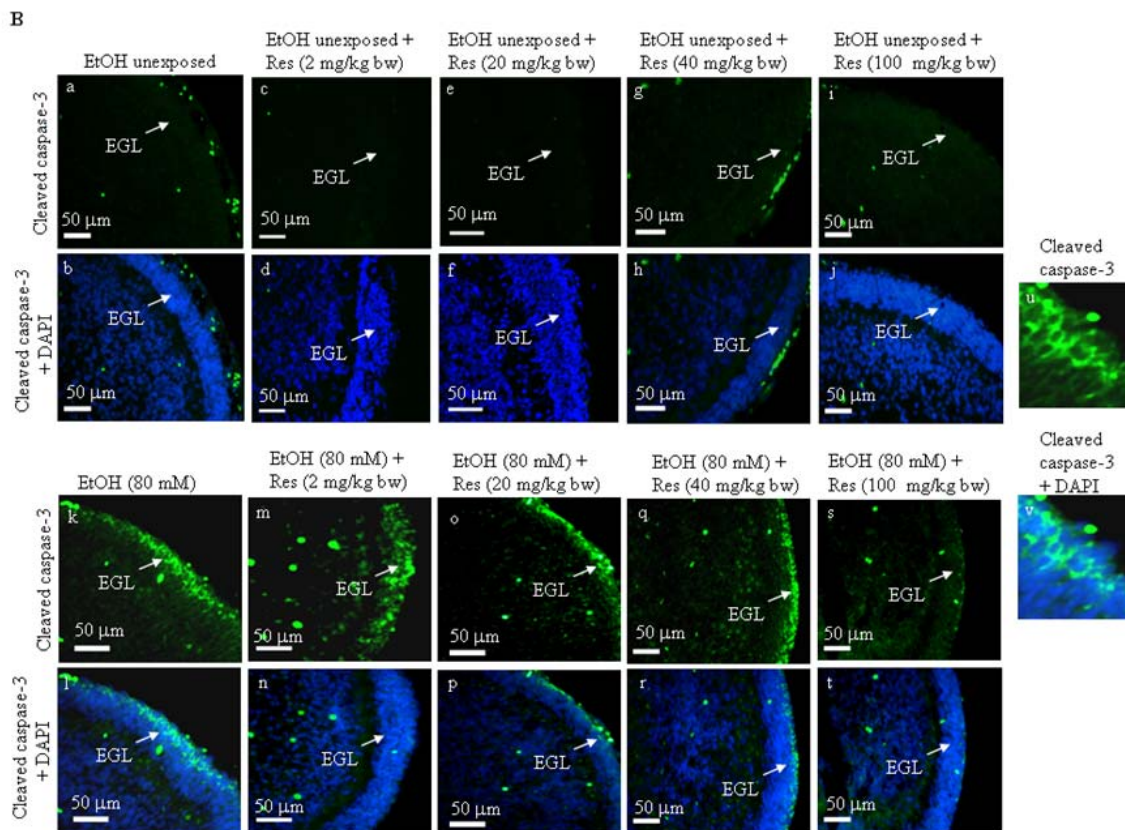


Fig. 1



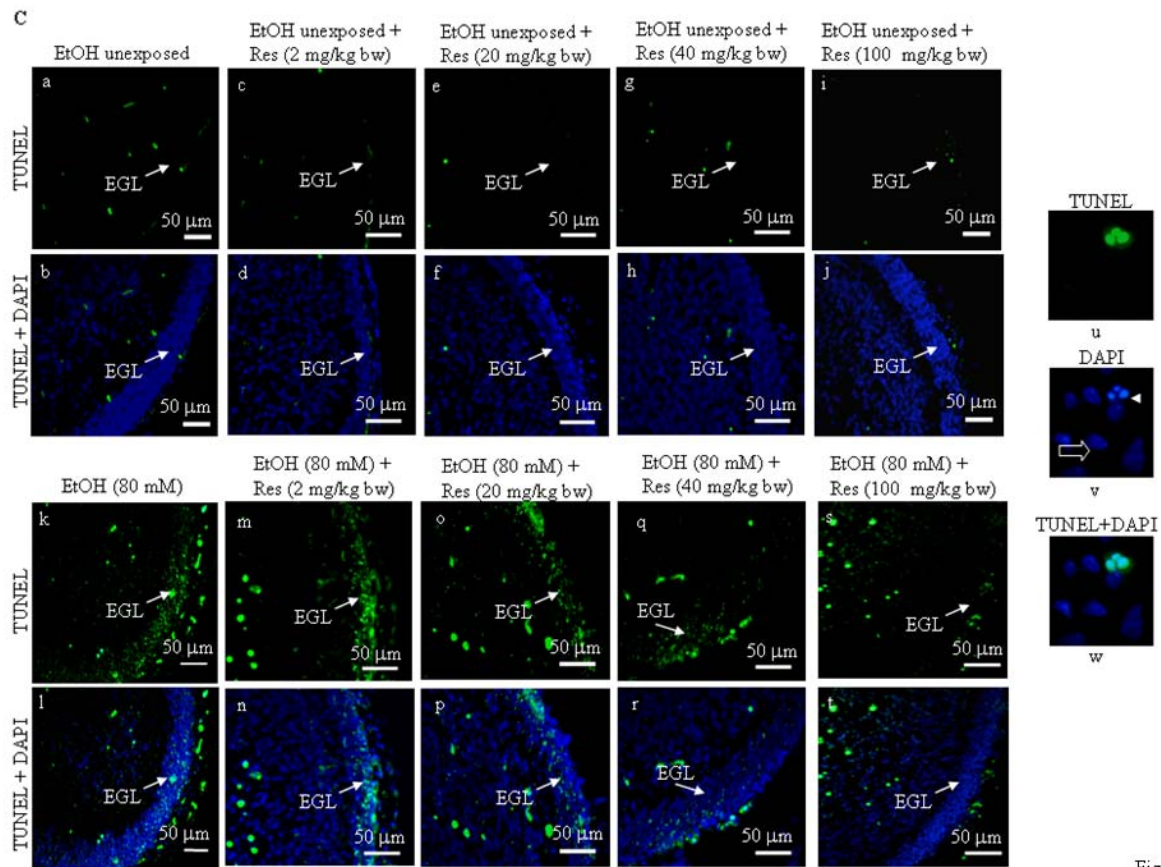


Fig. 1

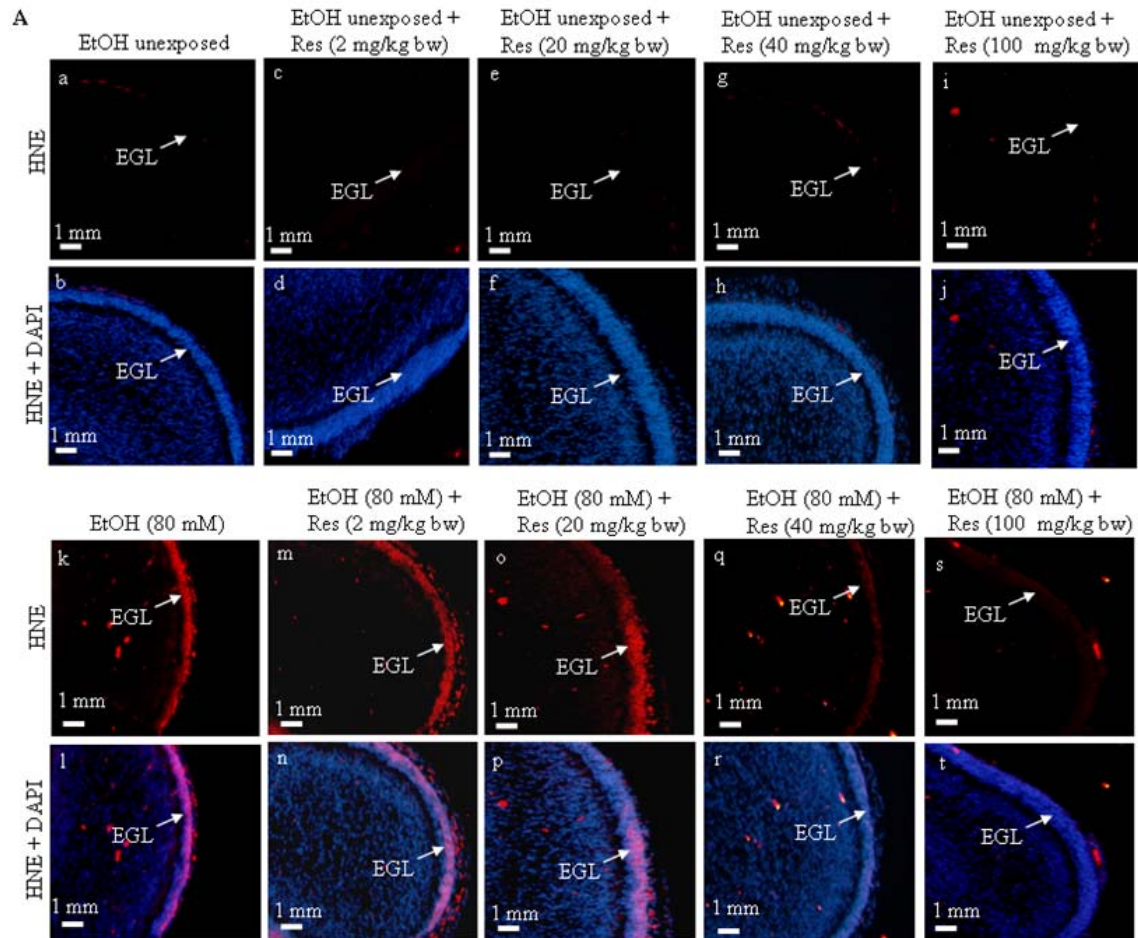


Fig. 2

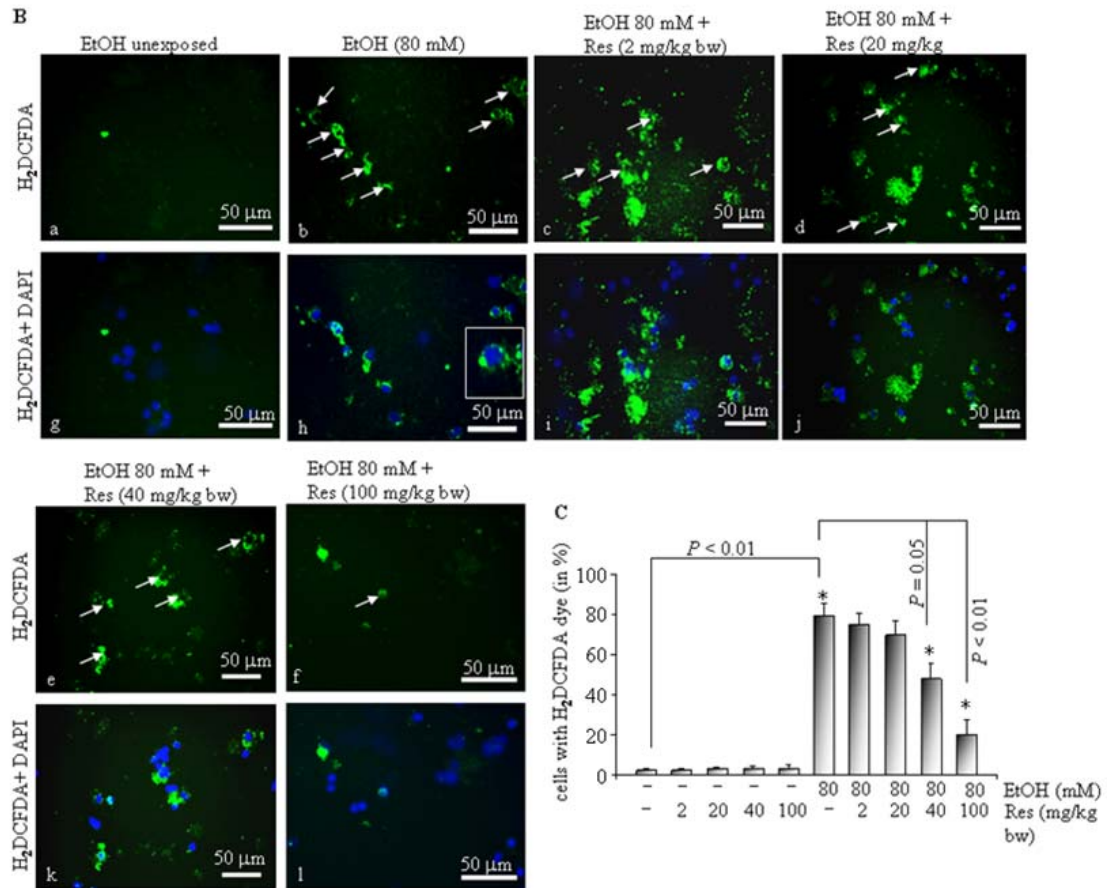


Fig. 2

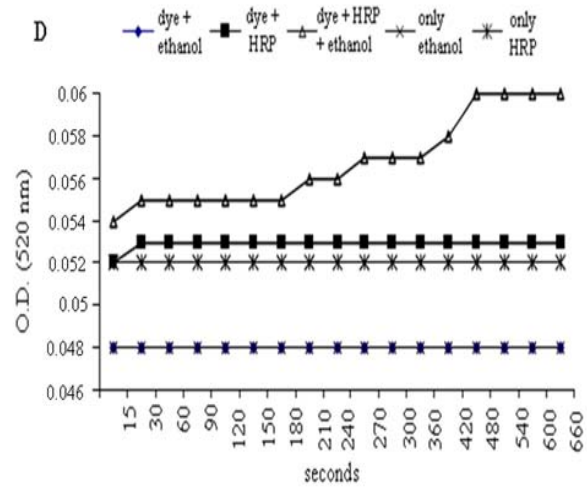


Fig. 2

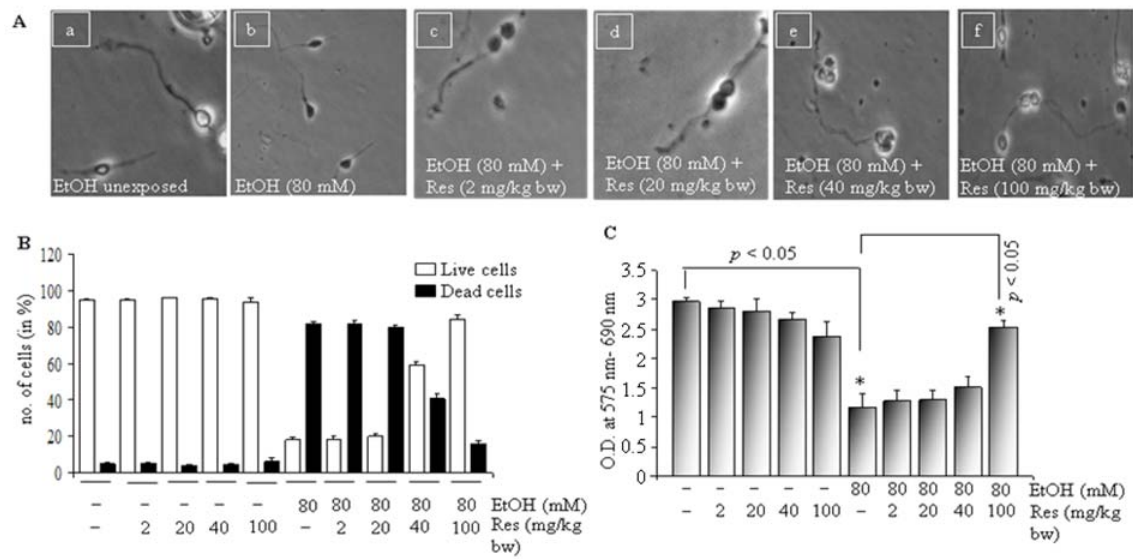


Fig. 3

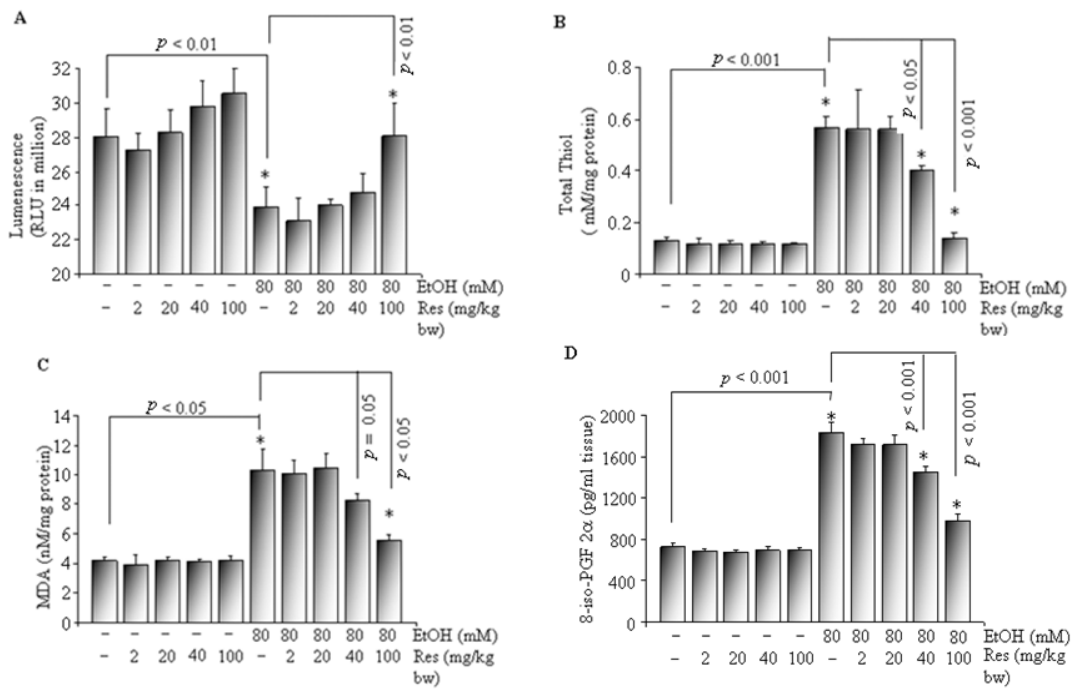


Fig. 4

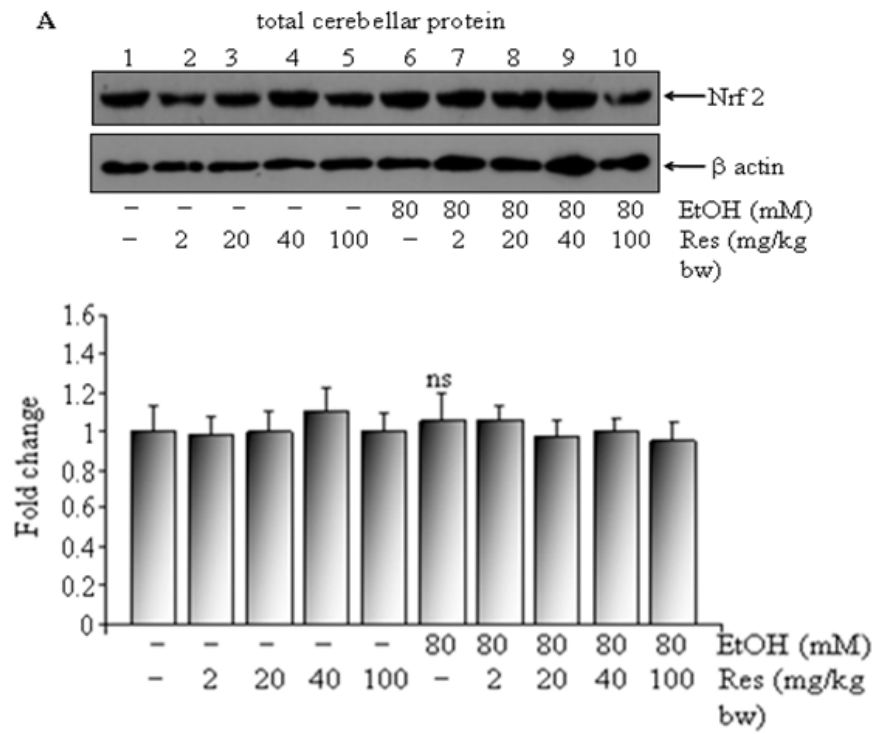


Fig. 5

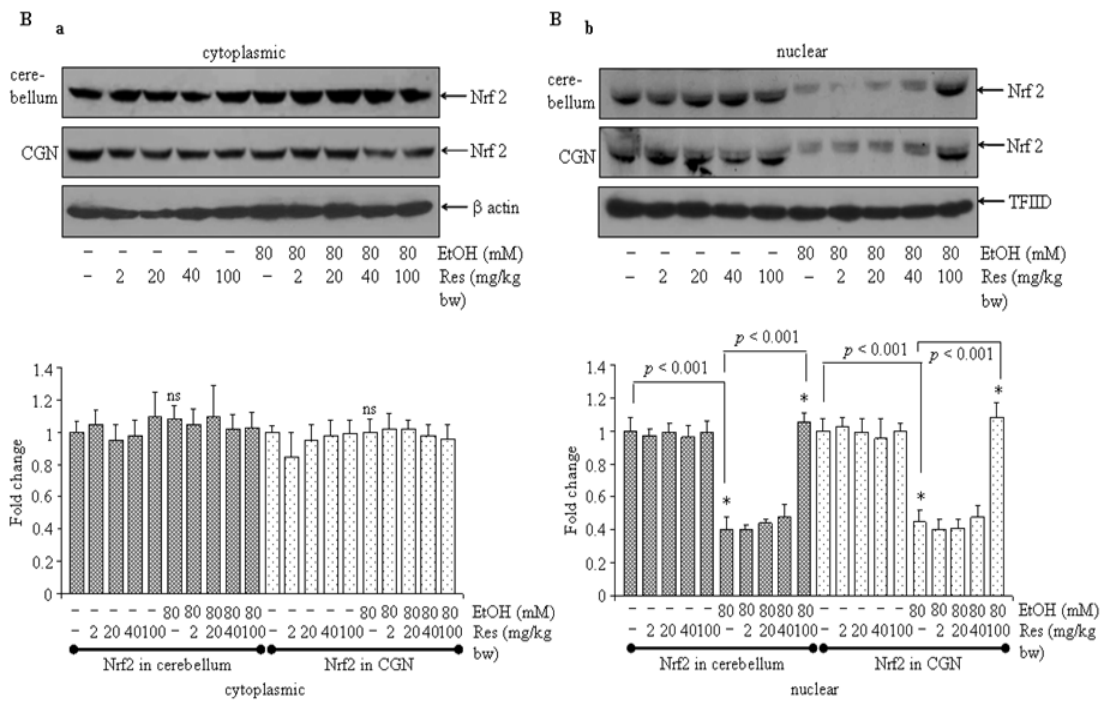


Fig. 5





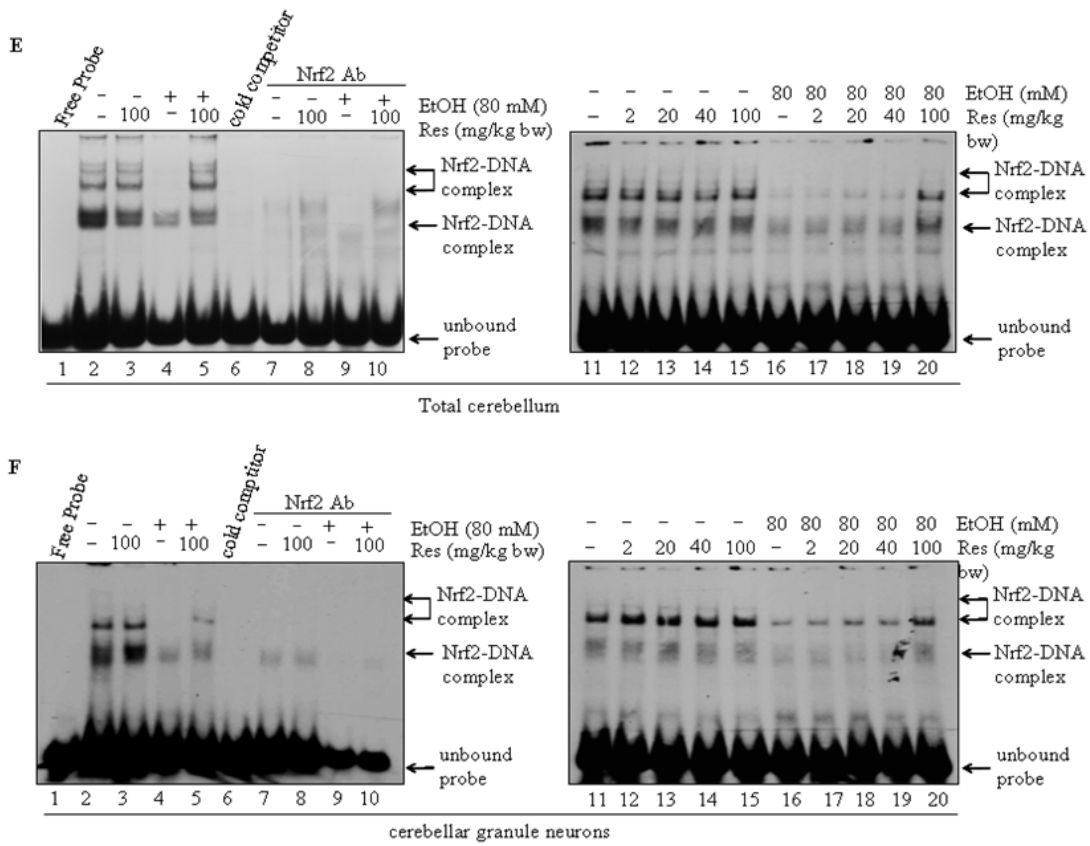


Fig. 5

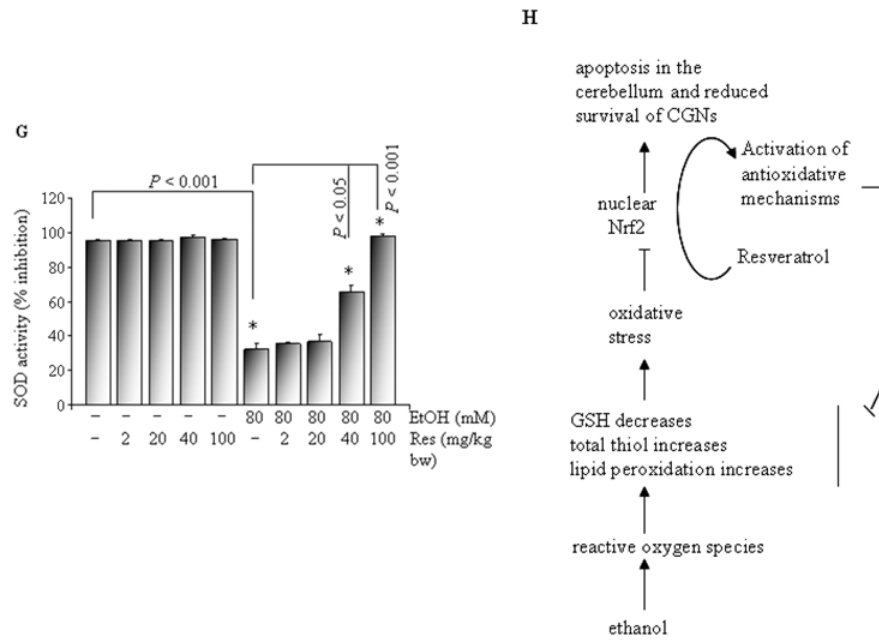


Fig. 5