Neurochemical Research (2018) 43:1841–1854
https://doi.org/10.1007/s11064-018-2600-1

ORIGINAL PAPER

Exposure of Rat Neural Stem Cells to Ethanol Affects Cell Numbers and Alters Expression of 28 Proteins

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Received: 23 February 2018 / Revised: 17 July 2018 / Accepted: 20 July 2018 / Published online: 24 July 2018

Abstract

Developing brain cells express many proteins but little is known of how their protein composition responds to chronic exposure to alcohol and/or how such changes might relate to alcohol toxicity. We used cultures derived from embryonic rat brain (previously shown to contain mostly neural stem cells; rat NSC, rNSC), exposed them to ethanol (25–100 mM) for up to 96 h and studied how they reacted. Ethanol (50 and 100 mM) reduced cell numbers indicating either compromised cell proliferation, cytotoxicity or both. Increased lipid peroxidation was consistent with the presence of oxidative stress accompanying alcohol-induced cytotoxicity. Proteomics revealed 28 proteins as altered by ethanol (50 mM for 96 h). Some were constituents of cytoskeleton, others were involved in transcription/translation, signal transduction and oxidative stress. Nucleophosmin (NPM1) and dead-end protein homolog 1 (DND1) were further studied by immunological techniques in cultured neurons and astrocytes (derived from brain tissue at embryonic ages E15 and E20, respectively). In the case of DND1 (but not NPM1) ethanol induced similar pattern of changes in both types of cells. Given the critical role of the protein NPM1 in cell proliferation and differentiation, its reduced expression in the ethanol-exposed rNSC could, in part, explain the lower cells numbers. We conclude that chronic ethanol profoundly alters protein composition of rNSC to the extent that their functioning—including proliferation and survival—would be seriously compromised. Translated to humans, such changes could point the way towards mechanisms underlying the fetal alcohol spectrum disorder and/or alcoholism later in life.

Keywords Alcohol · Proteomics · Stem cells · NPM1 · DND1 · FASD

Introduction

Development and maturation of brain tissue includes both pre- and postnatal neurogenesis [1–3]. The rate of neurogenesis varies as a function of brain maturity [4] but may also be influenced by anxiety, depression as well as by drug and alcohol intake [5].

Neural stem cells (NSC) can generate neurons, astrocytes and oligodendrocytes in the central nervous system (CNS; [6]). NSC first migrate to form specific brain regions while, later in the life, during the process of adult neurogenesis, may respond to functional demands or serve as a replacement for damaged cells. The normal cell generation may become compromised if the damage to the tissue is too great or the initial insult continues such as in an ongoing stress. The latter would include chronic drug use and alcoholism and, indeed, animal models have demonstrated that alcohol is toxic to the neuron-generating regions in brain [7]. Even the blood ethanol levels not exceeding 330 mg/dl (app. 72 mM) have been shown to decrease neurogenesis in adolescent rats [8, 9]. In this context, it is of interest to note that blood alcohol level in Indian-American (indigenous) mothers who gave birth to offspring with fetal alcohol syndrome (fetal alcohol spectrum disorder; FASD) was reported at 80 mM [10]; it is known that 100 mM alcohol can significantly influence human neurogenesis [11]. In another study, moderate doses of ethanol were shown to reduce the number of new neurons by two-thirds and significantly increased the rate of cell death in the dentate gyrus [9]. Administration of ethanol to 35–40 days old rats indicated that the neurogenesis in the adolescent brain is particularly sensitive

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to ethanol [12]. Moreover, prenatal exposure to ethanol may further compromise postnatal neurogenesis and this may have an additional impact on mental health later in life [13, 14].

Production of neurons proceeds in four stages; proliferation of NSC, differentiation, migration and selective neuronal death/survival. Certain proportion of cells will succumb to programmed cell death (apoptosis); this is an integral part of the differentiation process and may accompany even the adult neurogenesis. Ethanol interferes mainly at the stages one and four [15, 16]; it inhibits the NSC proliferation and increases the cell death. Longer exposures (a 4-day binge model) result in reduced cell proliferation and impaired survival [8, 17]. Moreover, ethanol at 50 mM concentration can affect both the proliferation of NSC and gliogenesis (glial phenotype); ethanol can alter intrinsic cellular mechanisms of NSC, eventually impacting on the structural and functional characteristics of both neurons and glial cells [18]. Such changes occur in prefrontal cortex, nucleus accumbens, and striatum [19, 20] thus potentially contributing to alcohol-induced functional deficits in those regions [21–23].

Protein composition is of key importance to the understanding of normal cellular functions and their changes in disease. The technique of proteomics can thus serve as a convenient indicator of the state of health of a biological system. Specifically, proteomic analyses of human brain tissue have identified changes in 238 proteins as associated with alcoholic disorders [24–31].

Proteomic analysis of human NSC detected thousands of proteins at the differentiation stage (review: [32]) but little is known of how this proteome is influenced by ethanol. The aim of the present study is to investigate in vitro changes in the proteome of rat embryonic neural stem cells (rNSC) following a chronic exposure to the concentrations of alcohol which could be encountered in vivo during heavy (or very heavy) drinking. Firstly, we looked at whether ethanol applied for 96 h at concentrations 25, 50 and 100 mM can influence the numbers of cultured rNSC and whether such changes are accompanied by an increased lipid peroxidation indicating the presence of oxidative stress. Secondly, we used a proteomic approach to study the protein composition of rNSC exposed to 50 mM ethanol for 96 h. Four of the proteins found to be significantly changed (two increased and two decreased) by the exposure to ethanol were estimated by Western blotting to verify the validity of the proteomic analysis. Additionally, two of the proteins strongly affected by the ethanol exposure (nucleophosmin; NPM1 and dead end homolog1; DND1; both classified as "nuclear proteins" and therefore deemed as potentially involved in the regulation of protein synthesis as well as cell proliferation and cell survival) were selected for further studies by both Western blotting and immunocytochemistry, using primary cultures of neurons and astrocytes derived from rat brains.

The data we present may contribute to a better understanding of how heavy alcohol drinking (particularly during pregnancy) alters the brain structure and function.

Materials and Methods

Neural Stem Cell Culture

Neural stem cells (rNSC) were prepared from 14-day-old (E14) rat embryos as described earlier [15, 33]. Animal experimental procedures were approved by the The University of Sydney Animal Ethics Committee (AEC Protocol Number: 2013/5742). The telencephalon was separated and blood vessels and meningae were removed while the tissue was submerged in ice-cold Hank's balanced salt solution (HBSS; Invitrogen, CA USA). After sectioning, the tissue was incubated with 0.05% trypsin solution for 30 min, triturated with a glass pipette and filtered through 70 µm nylon cell strainer (Falcon, USA). The resulting cell suspension was centrifuged (300 g for 5 min at 4 °C). The viability of the cells in the pellet was assessed by trypan blue (Invitrogen). The cells were seeded in complete neurobasal medium [(NBM, Invitrogen) + 2% B27 supplement (Invitrogen), + 0.5 mM L-glutamine + 20 ng/mL recombinant human fibroblast growth factor 2 (FGF-2) + 20 ng/mL recombinant human epidermal growth factor (EGF, Life Tech)] at a density of 1×10^6 cells per mL in culture dishes coated with poly-L-ornithine (Sigma). The dishes were maintained for a week at 37 °C in a 5% CO_2 atmosphere. For differentiation, the cells were washed and cultured in differentiating media (all components of complete neurobasal media except FGF-2 and EGF).

Neurons and Astrocytes

The brain tissue was obtained at the embryonic age (E) of either E15 (neurons) or E20 (astrocytes) and treated as above. Following recommendations by available culture protocols (e.g. https://www.thermofisher.com/au/en/home/references/protocols/cell-culture) the cells were cultured either in neurobasal medium (NBM, Invitrogen) + 2% B27 supplement (Invitrogen), + 0.5 mM L-glutamine with antibiotics (neurons) or in serum/antibiotics containing DMEM/F-12 (astrocytes) for 2 weeks at 37 °C in humidified 5% CO_2/95% air conditions. While most of the protocols usually recommend brain tissue harvested at embryonic ages E17 or E18 for the neuronal cultures and brains collected at later embryonic stages or from neonatal rats for the preparation of astrocytic cultures [34] we used E15 for the neuronal cultures (cf. also [35]) and E20 for the astrocytic culture with satisfactory

results, as indicated both by the appearance of the cells and expression of the characteristic markers (Figs. 3, 4).

Ethanol Treatment and Sample Collection

The cultured cells were exposed to ethanol (0, 25, 50 and 100 mM) added directly to the differentiation neurobasal media. In order to reduce the loss of ethanol by evaporation [36], we used a closed system (closed 25 mL vessel) and changed the media every 24 h [36, 37]. The ethanol exposure lasted for up to 96 h. For proteomics, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected in 200 µL solubilising buffer (7 M urea, 2 M thiourea, 1% C7bZO and 40 mM Tris–HCl, pH 10.4; Sigma). After sonication (3 times for 5 s each) the samples were stored at -80 °C [38, 39].

Estimation of Cell Numbers and Immunofluorescence

Cells on coverslips were fixed with 4% paraformaldehyde for 15 min, followed by washing with PBS and exposure to 0.3% Triton-X100 for 5 min. After washing with PBS, the cells were incubated (blocked) with 5% normal horse serum for 1 h, followed by anti-microtubule-associated-protein 2 antibody (MAP2, mouse, 1:1000; Sigma) for 2 h. The cells were further incubated in mouse IgG FITC labelling reagent (1:1000, Invitrogen) for 45 min and subsequently washed with PBS. Following the application of DAPI (for 10 min) cells were observed under Leica SPE2 confocal microscope (Leica Microsystem). The images were captured and the cells with DAPI-labelled nuclei were counted (about 150 per image) using Image J software (manual option). Whole coverslip contained, typically, 200 to 300 cells or fewer in ethanol treated cultures. (cf. legend of Fig. 1 for further details).

For immuno-histochemistry of neurons and astrocytes, the cells were fixed, treated with Triton X-100 and blocked with horse serum as described above (immunofluorescence) and incubated with a mixture of neuron- or astrocyte-specific marker antibodies. For neurons, anti-microtubule-associated-protein 2 (MAP2, mouse and rabbit, 1:1000; Sigma) was used as a marker while anti-glial fibrillary acidic protein (GFAP, mouse and rabbit, 1:1000; Sigma) was applied to label astrocytes. The cells were further incubated with anti-rabbit nucleophosmin (NPM1, 1:500; Santa Cruz Biotech, Australia) or anti-mouse dead end protein homolog 1 (DND1, 1:500; Santa Cruz Biotech, Australia) for 2 h. Anti- rabbit and/or anti-mouse secondary antibodies (always diluted at 1:1000) conjugated with CF-568 and/or CF488 were applied for 45 min and then washed with PBS. The cells were observed under confocal microscope (Leica SPE2) and the intensity of the fluorescence was quantified by image J software [40, 41].

Lipid Peroxidation Assay

Cells were homogenised in 0.5 mL of 1% KCl solution and supernatant was collected after centrifugation at 16,000 g x 10 min at 4 °C. Aliquots of 0.25 mL of the homogenates were mixed with double volume (0.50 mL) of 15% acetic acid containing thiobarbituric acid (0.5% [42]). The mixture was heated at 95 °C for 15 min and, after cooling, centrifuged at 5000 g x 5 min to remove any precipitated matter. The absorbance of the supernatant was measured at 532 nm. The malondialdehyde concentration was calculated from the following formula: absorbance/1.56×105; (where 1.56×105 $M^{-1}cm^{-1}$ is the malondialdehyde extinction coefficient).

Statistical Analyses and Presentation of Data

Statistical evaluation of differences between alcohol-free (controls) and alcohol-treated cells was done by one way ANOVA followed by post-hoc Tukey multiple comparisons where a significant effect was found. Both the statistics and the graphical representation of data (Figs. 1, 3, 4) was done using GrapPad Prism software version 7.02 except for Fig. 2 where BioRad software was used. For details see the figure legends.

Proteomics

The cells (exposed to ethanol for 96 h; *cf*. the section on "Ethanol treatment and sample collection" above) were trypsinized, extracted with urea buffer (7 M urea, 2 M thiourea and 1% C7bZO and 40 mM tris-HCl) and the proteomics performed as detailed earlier [30, 33, 38, 39, 43, 44]. The extracted samples were pelleted at 16,000 g for 20 min at 15 °C; the supernatant was reduced and alkylated with 5 mM tributyl phosphine and 10 mM acrylamide monomer at room temperature for 2 h. The reaction was quenched by 10 mM dithiothreitol (DTT). The citric acid was used to acidify the samples to approximately pH 5. The acetone-precipitated pellets were air dried and resuspended in 0.3 mL of buffer consisting of 7 M urea, 2 M thiourea and 1% C7bZO. The final extracts were stored at − 80 °C before isoelectric focusing.

The protein was quantified [45] using BSA (Sigma–Aldrich, Castle Hill, NSW, Australia) as a standard. Details of isoelectric focusing, electrophoresis and staining have been published [30, 33, 38, 39, 43, 44]. A total of 12 gels (duplicates for each sample, n = 3) were scanned using a flatbed scanner (UMAX). The images were analysed by Phoretix 2D Expression software (Nonlinear). Averaged gels were created for each alcohol treated/control group and averaging parameters were set at 70%. Protein spots were evaluated as volumes (spot area × optical density) with the

image analysis software and compared between groups with statistical analysis (ANOVA, $P < 0.05$). The false discovery rate (FDR) was calculated as described by Storey [46]. The protein spots of interest were cut from the gel, de-stained using 25 mM NH_4HCO_3/50% (v/v) acetonitrile (ACN) for 3 min × 15 min at 37 °C and digested with 12.5 ng/mL trypsin (Roche, sequencing grade), The peptides were purified using C_{-18} purification tips (Eppendorf). Matrix (5 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 70% (v/v) ACN/0.1% (v/v) TFA) mixed samples were analysed by Qstar XL Excll Hybride MS system (AB Applied Biosystems) in positive reflector mode, with delayed extraction.

The spectra from MALDI-TOF were searched against the Swissprot protein databases using the MASCOT search engine (http://www.matrixscience.com/). Positive protein identification was performed based on a MOWSE score (> 54, rattus database) with matched isoelectric pH (pI) and molecular weight (MW) values (estimated from 2D gels) and sequence coverage.

Western Blot Analysis

Western blot analysis was performed as described previously [29–31]. Proteins separated by SDS–PAGE were transferred to PVDF (polyvinylidene difluoride) membranes [30] blocked with 5% skim milk and incubated with primary antibodies [NPM, DND1, heat shock protein, heterogeneous nuclear ribonucleoproteins-C (hnRNP-C), enolase and synaptosome associated protein-29 (SNAP), all diluted at 1:500] solution. Secondary antibodies (anti-mouse and anti-rabbit IgG, Sigma, diluted at 1:1000) were added and the spots were visualized using an ECL (enhanced chemiluminescence) full spell system (GE Healthcare, Australia). The intensity of the spots was quantified by Biorad software (Bio-Rad, Sydney, Australia).

Results

Exposure (96 h) to the lowest ethanol concentration (25 mM) had no significant ($P > 0.05$) impact on either cell numbers or morphology but the higher concentrations (50 or 100 mM) reduced cell growth (Fig. 1a). MAP-2 positive cells appeared to be reduced in numbers at 50 and 100 mM ethanol relative to control (Fig. 1a). The reduction in cell numbers was quantified by counting the cell nuclei stained by DAPI. DAPI-positive cells were reduced in numbers by 38% and 52% ($P < 0.05$) at 50 and 100 mM ethanol relative to control (Fig. 1b).

Lipid peroxidation analysis (Fig. 1b) revealed that the exposure of the cells to ethanol induced oxidative stress compared to controls (at 50 mM about 1.6-fold and at 100 mM about 2.1-fold).

In the proteomics, typical 2-DE gels displayed 550 spots (six gels from controls and six gels from ethanol-treated samples) and more than 92% of all spots were matched across the two groups. Analysis revealed that 40 protein spots were differentially expressed in ethanol-exposed cells relative to the ethanol-free controls ($P < 0.05$; ANOVA); 17 protein spots (~ 43%) showing an increased expression while the remainder were decreased. From the differentially expressed protein spots, we have positively identified 28 proteins using MALDI-TOF (Table 1) while the remaining ones were not identifiable because of low expression of proteins. Functionally, the identified proteins could be classified as structural (two proteins, ~ 7%), intracellular metabolism (six proteins, ~ 21%), nuclear (seven proteins, ~ 25%), related to oxidative stress (six proteins, ~ 21%), signal transduction (four proteins, ~ 14%) and no specific class (three proteins, ~ 11%). Among the identified proteins, nine were increased (including two out of six metabolic, three out of seven nuclear and three out of six oxidative stress-related proteins) while the remaining ones were decreased (including both structural, four out of six metabolic, four out of seven nuclear, three out of six oxidative stress-related and all four signalling proteins).

Heat shock protein-70 (HSP-70), synaptosomal associated protein-29 (SNAP-29), enolase (α) and heterogeneous nuclear ribonucleoprotein C (hnRNP C) which were altered in ethanol-exposed rNSC (Table 1) were assessed by Western blotting (Fig. 2; all primary antibodies were purchased from Santa Cruz Biotech, Australia). These analyses did not contradict the outcomes of the estimations obtained by the proteomic studies (Table 1) and were not pursued further. Nucleolar phosphoprotein (nucleophosmin, NPM1) and dead end homolog1 (DND1) which were selected for immunocytochemical analyses in cultured neurons and astrocytes were also estimated by Western blotting in those preparations (Figs. 3, 4).

Immunofluorescence indicated somewhat higher expressions of NPM1 at 25 mM ethanol, especially in neurons (cf. also Western blotting in Fig. 3d, f), but a lower expression in astrocytes exposed to 100 mM ethanol (Fig. 3e). The immunofluorescence was detected particularly over the nuclear area though we also noticed the NPM1 staining in cytosolic area at the lowest concentration (25 mM) of ethanol (Fig. 3c).

In the case of DND1, immunocytochemical analysis revealed that the expression significantly increased both in neurons and astrocytes at 25, 50 and 100 mM ethanol (Fig. 4). Western blotting was consistent with the immunocytochemistry, indicating that DND1 protein expression increased in both neurons and astrocytes at the higher (50 and 100 mM) concentration of ethanol (Fig. 4).

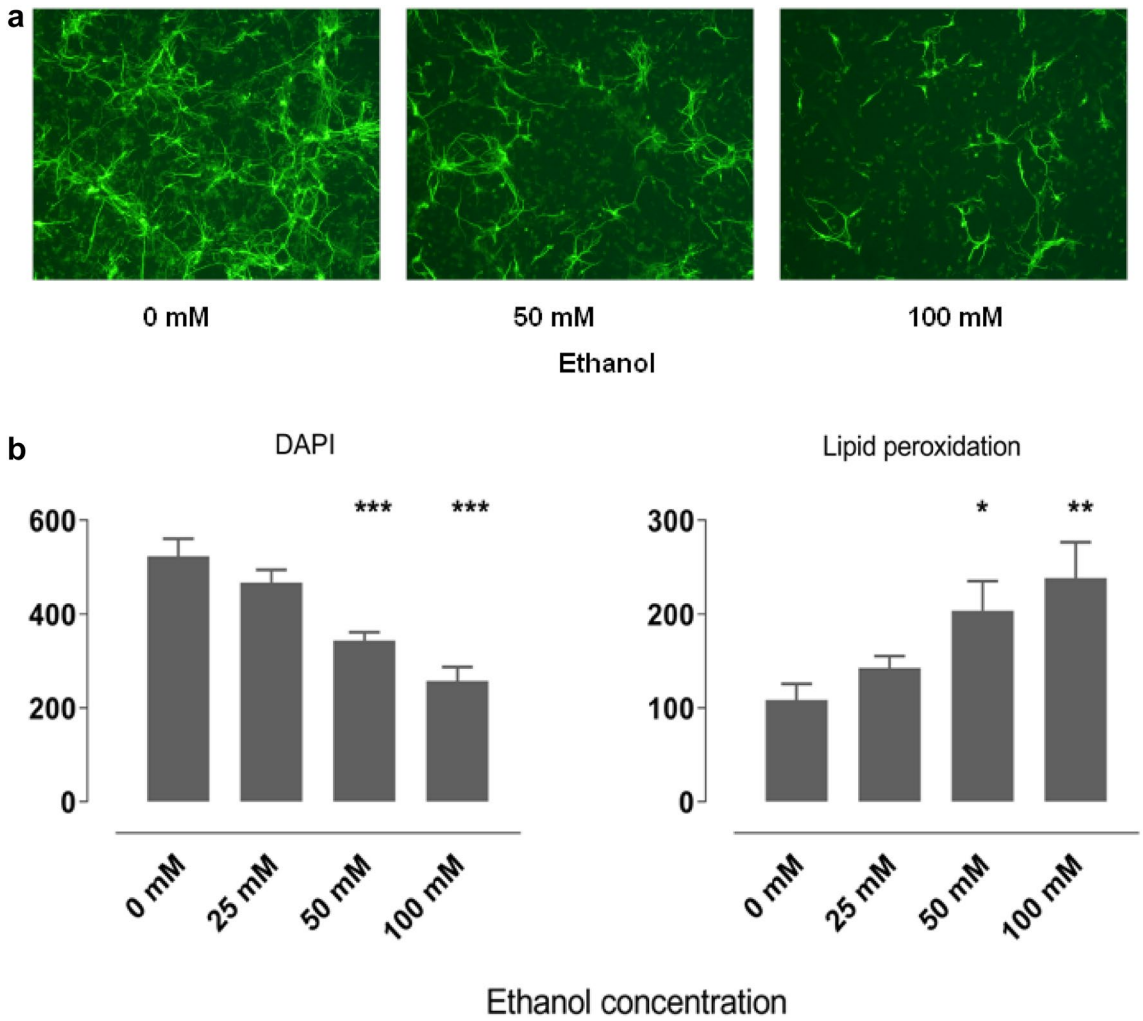

Fig. 1 Immunofluorogenic visualization of rat neural stem cells treated with various concentration of alcohol at 96 h of incubation (**a**). **a** displays MAP-2 positive cells. **b** shows the numbers of DAPI stained nuclei and the extent of lipid peroxidation in the cells after 96 h of ethanol exposure. The scales are in arbitrary units and the columns are means ± SD (n = 3). The data were analysed by one way ANOVA followed by post-hoc Tukey multiple comparisons and found significantly different from control (0 mM ethanol) at *P < 0.05, **P < 0.01 and ***P < 0.001

Discussion

Inhibition of glutamate NMDA receptors [47] and/or activation of a subtype of GABA receptors [48–50] parallel immediate effects of alcohol-drinking and probably account for much of the short-term response and consequences (acute alcohol intoxication followed by "morning after") [51, 52]. In contrast to the receptor-mediated acute effects, longer exposures to ethanol may trigger additional mechanisms leading to profound (and probably irreversible) changes in the living cells and tissues. Apart from resulting in cytotoxicity, chronic alcohol can specifically influence protein synthesis and cell proliferation, particularly in the developing brain [53, 54]. The main finding of the present study is the major changes in the protein composition of cells cultured from developing rat brain following a four-day exposure to high concentrations of ethanol. In addition, we noted reduced cell numbers and found evidence for neurotoxic events in the alcohol-exposed cultures.

As we did not specifically investigate neural stem cell functions and properties; the "rNSC" culture represents, strictly speaking, merely a primary culture of fetal brain tissue harvested at certain stage of development. However, the methodology that we used is known to produce > 95% NSC [33] and the results in Fig. 1a are, therefore, consistent with significant cytotoxic effects of alcohol on the fetal rat brain-derived stem cells. This is further underscored by the increased lipid peroxidation in the presence of alcohol (Fig. 1b). Lipid peroxidation has been used as a marker of oxidative stress associated with cytotoxicity in a range of

Table 1 Alcohol induced differential expression of proteins in rNSC exposed to 50 mM ethanol for 96 h

Protein class	Fold change (p/q values)	Accession no	PI/Mass (kDA)	Protein name	Function
Structural Protein	− 1.68 (0.048/0.095)	P47942	5.95/62	Dihydropyrimidinase related protein-2 (DRP-2)	Involves neural development, polarity, axon growth and guidance, migration and remodelling of the cytoskeleton
	− 1.56 (0.028/0.065)	Q62952	5.96/62	DRP-3	
Metabolic protein	− 2.98 (0.018/0.025)	P04764	5.0/47	Enolase (α)	Glycolytic enzyme works as marker of stress in cellular system
	− 1.98 (0.024/0.045)	Q02589	5.5/40	ADP-ribosylarginine hydrolase	Regulates G1-S phase of cell cycle and regulates DNA stability
	− 1.76 (0.0161/0.027)	Q68FS2	5.4/46	COP9 signalosome complex subunit 4	Involves in various cellular & developmental processes including signalling
	2.88 (0.0032/0.026)	P54921	5.04/40	Soluble NSF attachment protein (α)	Required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus
	5.92 (0.0012/0.016)	Q9JI56	4.66/29	SNAP-29 protein (synaptosomal associated protein-29)	The protein encoded by this gene binds tightly to multiple syntaxins and is localized in intracellular plasma membrane
	− 1.88 (0.0014/0.022)	Q8VHK7	5.2/26	Hepatoma-derived growth factor	Heparin-binding protein, with mitogenic activity for fibroblasts. Acts as a transcriptional repressor and involves in proliferation, angiogenic, and neurotrophic activity
Nuclear protein	− 1.68 (0.0022/0.036)	P13084	5.1/32	Nucleolar phosphoprotein (nucleophosmin, NPM-1 or B-23)	Involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of p21/p53
	1.75 (0.0033/0.046)	Q8IYX4	6.9/38	Dead end homolog protein 1	RNA-binding protein that positively regulates gene expression by prohibiting miRNA-mediated gene suppression
	− 3.74 (0.0024/0.039)	Q8VHV7	5.32/45	Heterogeneous nuclear ribonucleoprotein H	These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. hnRNP C heavily involved in the alternative splicig mechanisms
	− 2.69 (0.0032/0.026)	P07910	5.4/33	Heterogeneous nuclear ribonucleoprotein C	
	− 2.70 (0.0327/0.081)	Q63413	5.42/49	Spliceosome RNA helicase Bat-1	Involved in nuclear export of spliced and unspliced mRNA
	1.80 (0.012/0.047)	Q68FR9	5.02/35	Translation elongation factor-1 (δ)	Regulates induction of heat-shock-responsive genes through association with heat shock transcription factors and direct DNA-binding at heat shock promoter elements
	1.53 (0.042/0.067)	Q3T1J1	5.12/16	Translation initiation factor 5A-1	Involve in cell proliferation and regulation of apoptosis

Table 1 (continued)

Protein class	Fold change (p/q values)	Accession no	PI/Mass (kDA)	Protein name	Function
Oxidative stress	3.5 (0.01/0.04)	P34058	5.37/90	Heat shock protein (HSP-90)	Molecular chaperone, promotes maturation and maintenance the structure of the proteins and control signal transduction
	4.44 (0.012/0.047)	P55063	5.3/70	Heat shock protein (HSP-70)	
	1.88 (0.016/0.037)	P63039	5.23/60	Heat shock protein (HSP-60)	
	− 2.65 (0.0079/0.042)	Q6AXV9	6.14/25	Glutathione S transferase	Exhibits glutathione-dependent thiol transferase activity
	− 1.68 (0.002/0.0205)	P28480	5.8/60	T-complex protein 1 subunit	Molecular chaperone; assists the folding of proteins upon ATP hydrolysis
	1.63 (0.007/0.040)	Q5VLR5		BWK4	Cell redox homeostasis
Signalling protein	− 1.68 (0.0015/0.029)	P62138	4.95/53	Serine/threonine protein phosphatase (PP-1)	PP-1 is essential for cell division, and regulates glycogen metabolism, and protein synthesis. It is associated with over 200 regulatory proteins and dephosphorylates 100 of biological targets
	− 2.2 (0.0012/0.0197)	Q32PX6	6.9/21	Ras homolog gene family, member G	GTPase activity
	− 2.2 (0.0013/ 0.018)	P50399	5.5/50	RAB GDP dissociation inhibitor (β)	Regulates the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from them
	− 1.82 (0.0013/ 0.018)	P50398	5.2/50	RAB GDP dissociation inhibitor (α)	Regulates the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from them
Not classified elsewhere	1.91 (0.001/0.0197)	Q9GZS3	7.0/33	WD repeat-containing protein 61	Component of the polymerase associated factor and is implicated in regulation of development and maintenance of embryonic stem cell pluripotency
	− 1.86 (0.007/ 0.0177)	Q62952	5.95/61	Collapsin response mediator protein 4 (CRMP-4)	Plays a role in axon guidance, neuronal growth cone collapse and cell migration
	− 1.49 (0.0014/0.0247)	Q5RKH6	5.5/75	Protein OS-9	May bind terminally misfolded non-glycosylated proteins

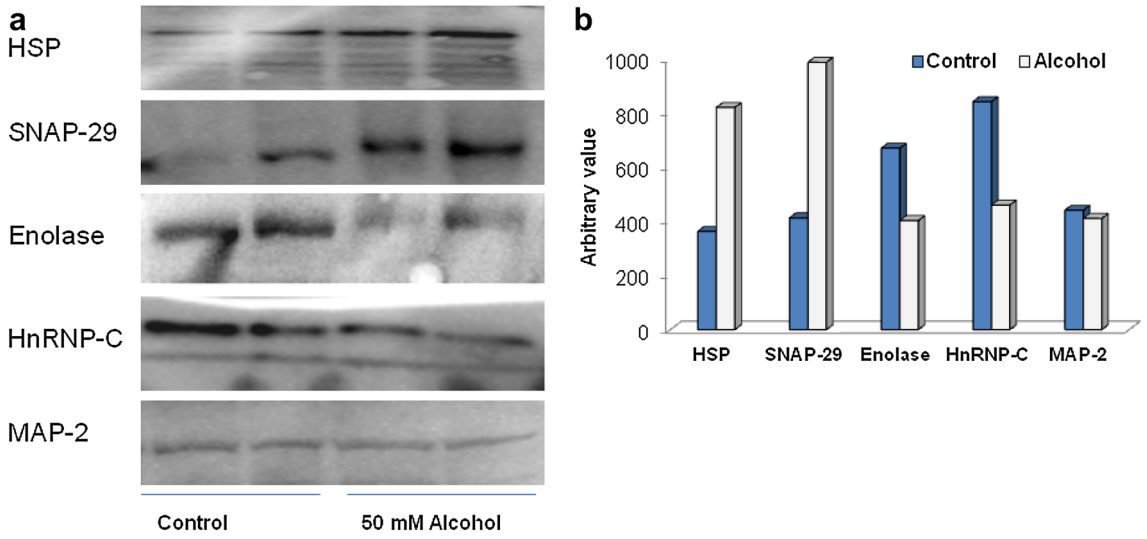

Fig. 2 Total protein was extracted from the neural stem cells from both control and 50 mM alcohol-exposed cells and western blotting was performed on selected proteins identified as altered by alcohol-exposure by proteomics. MAP-2 was used as an internal control. Bands were digitized and evaluated by Bio-Rad software. **b** represents average values of two bands

brain cells and tissues [55–57]. Therefore, the current data suggest that oxidative damage is a potentially significant component of the ethanol-related cytotoxicity leading to the loss of cells cultured from embryonic (E15) rat brain tissue.

The remainder of this section will try to evaluate possible consequences of the detected changes in protein composition following the alcohol exposure; given that ethanol interferes with the NSC proliferation and increases the cell death [15, 16] the focus will be on proteins potentially involved in the cell proliferation/differentiation, oxidative stress and nucleus-associated mechanisms.

Cell Proliferation and Differentiation

Hepatoma-derived growth factor (HDGF) was significantly decreased in ethanol exposed cells (Table 1). HDGF has been linked to hepatocellular carcinoma (HCC) as a multifunctional protein involved in cell proliferation, angiogenesis and anti-apoptosis mechanisms [58]. HDGF overexpression promotes the proliferation of HCC [59] and/or gliomas [60] while the reduction in HDGF expression inhibits the proliferation of HCC cells. Knocking down of nuclear HDGF expression in the cells of human glioblastoma multiformis induces apoptosis [61]. By analogy, the decreased expression of HDGF in the ethanol-exposed rNSC could explain lower proliferation rates of brain cells early in the development.

Two isoforms of dihydropyrimidinase-related protein-2 and 3 (DRP-2 and DRP-3) were reported to be lower in human alcoholic brain tissue [62]. The DRP family of proteins, also called the collapsin response mediator proteins (CRMP), has been implicated in the development of the CNS particularly in axon guidance and cell migration. Lower DRP-2 could, therefore, disturb neural development and plasticity [63] and this is supported by reports of DRP-2 being decreased in other conditions involving abnormal development such as schizophrenia and Down's syndrome [64]. Alternatively, the decreased DRP-2 levels observed in the present study could be a result of proteolysis activated by cytosolic Ca^{2+} increased by the ethanol-exposure ([65]; cf. also the effect of ethanol on Ca^{2+}-permeable NMDA receptors [51]).

ADP-ribosylarginine hydrolase (ADPRH) removes mono-ADP-ribose moieties from arginines in cellular proteins. Mutation in the *ADPRH* gene alters G_1 stage of the cell-cycle and the reduced expression ADPRH (Table 1) could lead to altered G_1-S phase thus decreasing the cell proliferation [66] which could contribute to the lower cell numbers.

NPM1, a nucleolar and nucleoplasma-localized protein [67–69], may bind to unduplicated centrosomes thus altering Cyclin E/CDK2 activity which, in turn, regulates G_1/S transition [70]. NPM1 has been shown to foster survival of proliferating cells [71, 72], therefore the lower cell numbers in the present study may have been, in part, caused by the lower expression of NPM1 induced by ethanol and/or its metabolites (see also below in the section on "Nuclear Activities and Protein Synthesis").

Changes in Oxidative Stress Defence Pathways

Ethanol may generate free radicals (review [65]) in vivo either through its metabolism (e.g. via the oxidation to aldehyde) or by interfering with radical scavenging systems.

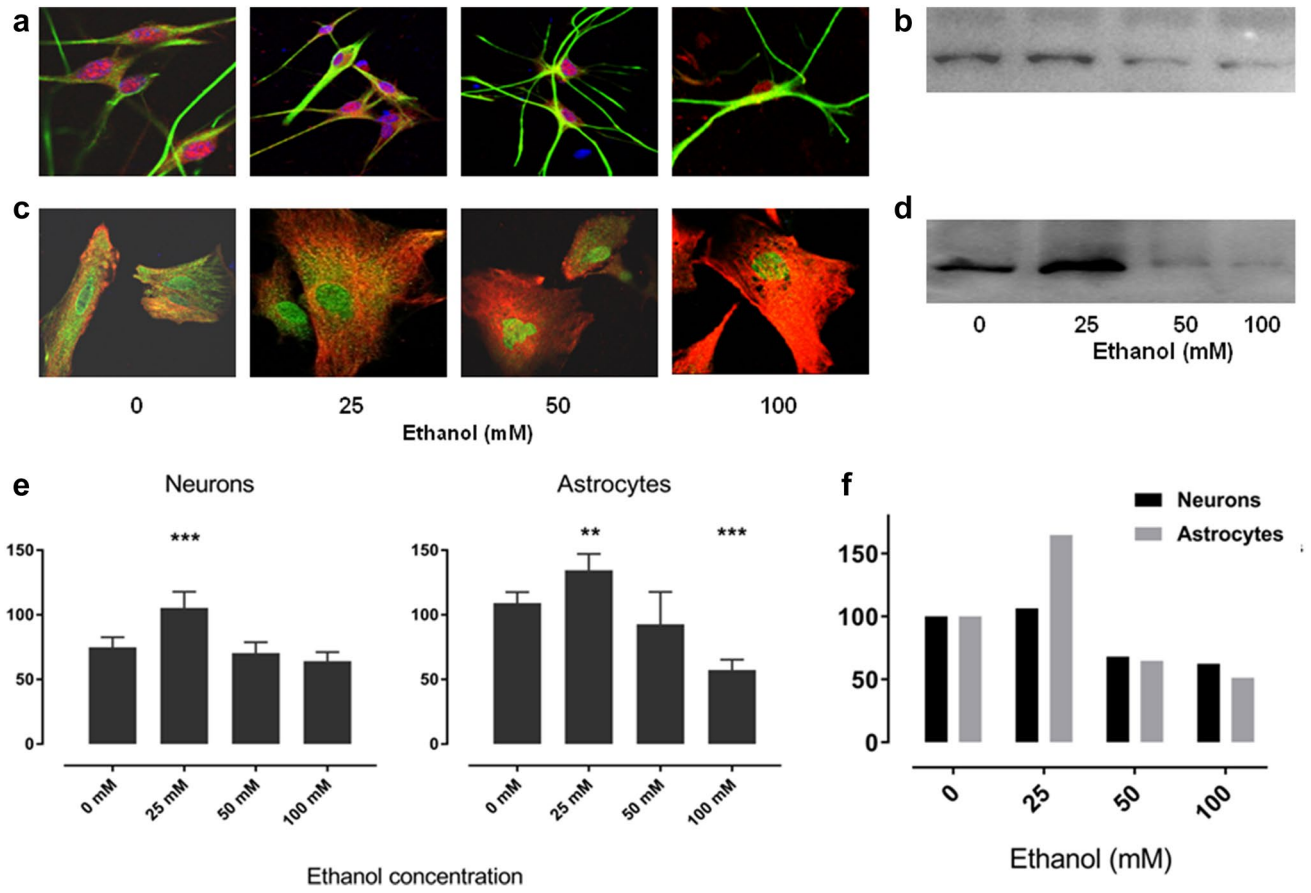

Fig. 3 Immunocytochemical visualization of the expression of NPM1 in neurons (**a**) and astrocytes (**c**) exposed to various concentrations of alcohol for 96 h. The green colour of the Fig. 3a is MAP-2 and red colour is NPM protein; in astrocytes (**c**), green is NPM and red is GFAP. Intensity of fluorescence was estimated for 9 or 10 cells by Image J software (**e**). The scales (**e**) are in arbitrary units and the columns are means ± SD (n = 9–10). The data were analysed by one way ANOVA followed by post-hoc Tukey multiple comparisons; those significantly different from control (0 mM ethanol) are marked by asterisks as $^{**}P < 0.01$ and $^{***}P < 0.001$. Total protein was extracted from cells exposed to 50 mM alcohol for 96 h and subjected to western blotting using an antibody against NPM1 protein (neurons: b, astrocytes: d). Bands were analysed by Bio-rad software; **f** shows average of the two bands shown in (**b**) and (**d**), normalized so as the control (0 mM ethanol) equals 100

Indeed, long-term chronic ethanol induced lipid peroxidation and depleted GSH levels in human striatum [31]. Reduced expression of glutathione transferase (GST) in the present study is also reminiscent of results obtained in human post-mortem brain tissue of heavy drinkers [24, 27, 29–31]. The ethanol-produced reactive oxygen species are eliminated by GST activity [73] and low expression of GST reduces the protection of neurons against oxidative insults.

The glycolytic enzyme α-enolase also acts as a neurotrophic factor [74], a heat-shock protein (HSP48) and a hypoxic stress protein (review: [75]). Glycolytic enzymes including α-enolase are enriched in apoptotic cells and α-enolase deficit (Table 1) could compromise the hypoxia tolerance (which may include nonglycolytic mechanisms [75, 76]).

Changes in SNARE complex have been associated with mental disease [77] and the major increase in the level SNAP-29 (also a part of the SNARE system) may indicate activation of various intracellular mechanisms such as transport of proteins [78], which might have been activated as an adaptation to the effects of ethanol [79].

Heat shock protein 70 (hsp70) expression is regulated by environmental stresses including ethanol exposure [80, 81]. Upregulations of hsp70 mRNA, hsp70 protein and heat shock factor-1 (HSF1) have all been reported in the liver of mice exposed to acute ethanol [79, 82]. Acute and chronic ethanol induces HSP genes such as *hsp70, hsp90*, and glucose-regulated protein 78 (*GRP-78*), via HSF-1 activation in neuronal cells [83]. HSP contributes to cell survival via binding to other proteins thus preventing the protein misfolding [84, 85]. The observed increases in the HSP's expression (Table 1) could reflect activation of such defence mechanisms.

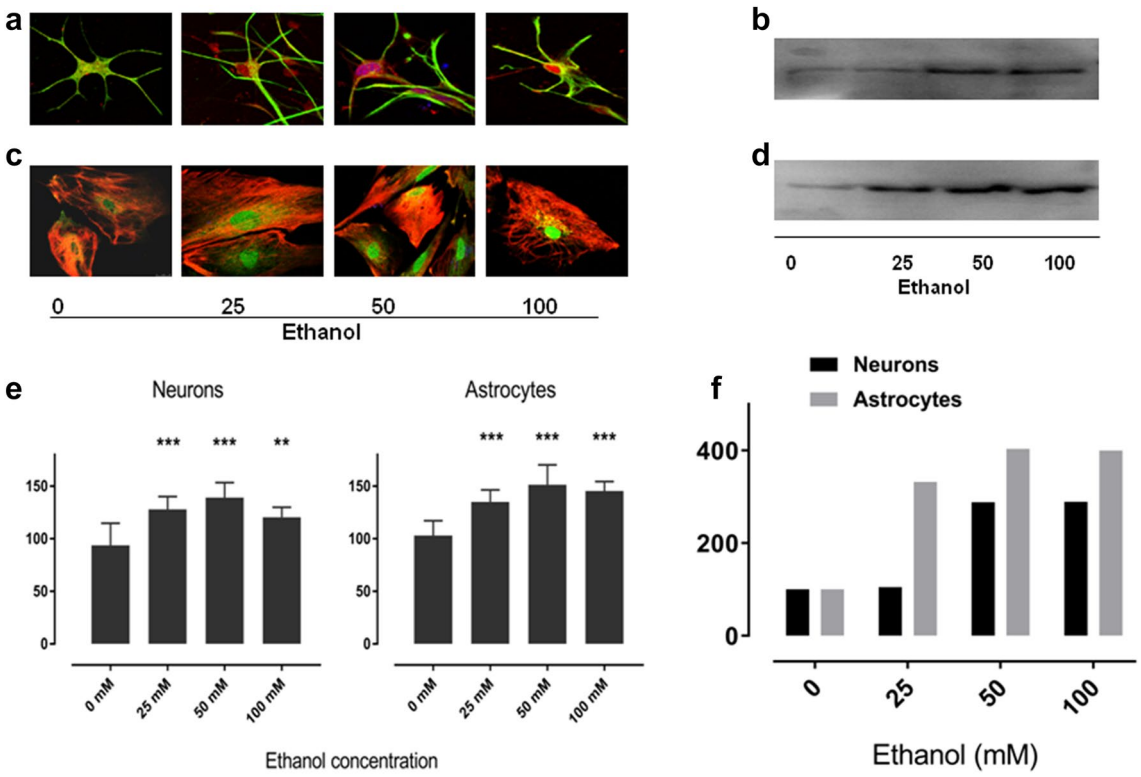

Fig. 4 Immunocytochemical visualization of the expression of DND1 in neurons (**a**) and astrocytes (**c**) exposed to various concentrations of alcohol for 96 h. The green colour of the Fig. 4a is MAP2 and red colour is DND1 protein; in astrocytes, green is DND1 and red is GFAP. Intensity of fluorescence was estimated for 10 or 11 cells by Image J software (**e**). The scales (**e**) are in arbitrary units and the columns are means ± SD (n = 10–11). The data were analysed by one way ANOVA followed by post-hoc Tukey multiple comparisons; those significantly different from control (0 mM ethanol) are marked by asterisks as *P < 0.05, **P < 0.01 and ***P < 0.001. Total protein was extracted from cells exposed to 50 mM alcohol for 96 h and subjected to western blotting using an antibody against DND1 protein (neurons: **b**, astrocytes: **d**). Bands were analysed by Bio-rad software; **f** shows average of the two bands shown in **b** and **d**, normalized so as the control (0 mM ethanol) equals 100

Nuclear Activities and Protein Synthesis

The heterogeneous nuclear protein (hnRNP) corresponds to a family of multifunctional RNA-binding nuclear proteins (review: [86]). Two of these proteins, hnRNP H and hnRNP C were significantly reduced in the ethanol-exposed rNSC (Table 1). The protein hnRNP H may be linked to human cancers while protein C of the hnRNP family is best known for its role in pre-mRNA alternative splicing [86]. Alternative splicing is of interest in alcoholism since the glutamate transporter EAAT1 (GLAST), reported as severely affected by chronic drinking (review: [87] but see [88, 89]), exists in many alternate splicing forms [90]. EAAT1 (GLAST) is a key protein in glutamatergic neurotransmission which is known to be perturbed by ethanol [48]; the conflicting data ([88, 89] v. [91, 92]) could be explained by a changed pattern of its alternate splicing in ethanol-affected tissue. The protein hnRNP C has been linked to breast cancer [93] but, perhaps more importantly in the context of FASD, its abnormal expression has been associated with birth defects in mammals including humans [94, 95].

"Helicase" is a group of enzymes which can unwind double helices of nucleic acids [96]. Spliceosome RNA helicase Ddx39b is involved in the nuclear export of mRNA and its reduced expression in the ethanol-exposed rNSC (Table 1) could have a severe impact on the RNA translation [97].

The DND1 (increased by ethanol in both neurons and astrocytes, Fig. 4) can block microRNA-mediated gene suppression. DND1 plays a key role in the primordial germ cell (PGC) survival and migration [98] and its defects also cause sperm sterility, inducing testicular germ cell tumours in mice [99, 100]. DND1 inactivation in mice leads to sterility at birth stage [101]. DND1 interacts with several mRNAs including mRNAs of both anti- and pro-apoptotic factors, BCLX and BAX, respectively. Transcripts of pluripotency factors, cell cycle regulators and apoptotic factors associated with DND1 have been previously identified using DND1 recombinant study in human embryonic stem cells [100].

Exposure to high concentrations of ethanol decreased NPM1 in rNSC and perturbed its expression in both neurons and astrocytes (Table 1; Fig. 3). This might in part explain the lower cell numbers of the ethanol-exposed cells (Fig. 1) since NPM1 is involved in DNA replication, recombination, transcription and repair [67, 102] as well as elsewhere [103, 104]. This interpretation is also in agreement with reports that the knockout of NPM1 in mice leads to unrestricted centrosome duplication, genomic instability and impaired ribosome biogenesis [67, 105]. In addition, NPM-induced p21 expression activates gene transcription by de-repressing p300–CREBBP (CREB-binding protein; [106, 107] and can regulate p53-mediated apoptosis under the conditions of cellular stress [108]. Thus, the normal level of NPM1 expression could very well be a critical factor in determining the healthy proliferation and differentiation (including the regulation of the normal rate of cell death) of the rNSC (cf. also above under "Cell Proliferation and Differentiation").

Summary and Concluding Statements

The main finding of the study is the large extent of changes in the expression of proteins in cultured embryonic cells (including neural stem cells) exposed to alcohol. Altered expression of so many proteins that are crucial for the normal cell proliferation and survival, should it occur following a similar exposure to alcohol *in vivo*, would not be without profound consequences for brain development.

More specifically, the present study resulted in the first observation of changes in DND1 and NPM1 expressions linked to ethanol exposure. Given the roles of NPM1 in cell proliferation, the lower expression of NPM1 could, in part, account for the reduction in neural stem cell numbers following the exposure to ethanol; the changes in the NPM1 expression may have a lesser impact on the cultured neurons and astrocytes, though, where NPM1 was actually increased at 25 mM ethanol and only moderately decreased at the highest (100 mM) ethanol concentration.

The present observations may help to identify novel mechanisms by which chronic alcohol exposures in utero exert their cytotoxic effects. It should be obvious that the existence of such mechanisms does not rule out the role of GABA(A) and/or NMDA receptors in the actions of alcohol including the neurotoxicity [48–52]. Ethanol would interact with the neurotransmitter receptors regardless of the length of the exposure and, as recent studies with the anaesthetics propofol, sevoflurane and ketamine suggest, such interactions could be cytotoxic for brain stem cells [109–111]. Our data merely suggest that additional mechanisms involving many more protein species (particularly those 28 protein molecules identified by the present study) could contribute to the process.

The findings of the study are of particular significance for the understanding of mechanisms underlying alcoholism-related conditions such as the foetal alcohol spectrum disorder, especially in relation to alcohol interference with the earliest stages of brain development. Given that neurogenesis may occur in adult brains, too, the present results could also be relevant for the understanding of changes in adult alcoholic brains.

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