Published in final edited form as:

Alcohol Clin Exp Res. 2017 October ; 41(10): 1715–1724. doi:10.1111/acer.13474.

Alcohol-mediated missplicing of Mcl-1 pre-mRNA is involved in neurotoxicity

Rahsan Sariyer^{1,a}, Francesca I. De Simone^{1,a}, Martina Donadoni¹, Jan B Hoek², Sulie L. Chang³, and Ilker Kudret Sariyer^{1,*}

¹Department of Neuroscience, Center for Neurovirology, Temple University School of Medicine, 3500 North Broad Street, 7th Floor, Philadelphia, PA, USA

²Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA

³Institute of Neurolimmune Pharmacology and Department of Biological Sciences, Seton Hall University, South Orange, NJ. USA

Abstract

Background—Heavy and chronic ethanol (EtOH) exposure can cause significant structural and functional damage to the adult brain. The most devastating consequence of EtOH exposure is the neurotoxicity associated with the depletion of neurons. Regulation of splice variants in the brain can modulate protein functions, which may ultimately affect behaviors associated with alcohol dependence and EtOH-mediated neurotoxicity. Since alcohol consumption is associated with neurotoxicity, it is possible that altered splicing of survival and pro-survival factors during the development of alcoholism may contribute to the neurotoxicity.

Methods—Primary human neurons and a neuroblastoma cell line were exposed to different concentrations of EtOH for various time periods. Cell viability and neuronal marker expression were analyzed by MTT assay and immunoblotting, respectively. Effect of EtOH exposure on splicing regulatory protein expression and alternative splicing of candidate genes were analyzed by a biochemical approach. Transcriptional activity of SRSF1 gene was determined by reporter gene analysis.

Results—Our results suggest that EtOH exposure to neuronal cells at 25 mM and higher concentrations are detrimental. In addition, EtOH exposure caused a dramatic reduction in serine-arginine rich splicing factor 1 (SRSF1) expression levels. Furthermore, EtOH exposure led to pre-mRNA missplicing of Mcl-1, a pro-survival member of the Bcl-2 family, by downregulating the expression levels of serine/arginine rich splicing factor 1 (SRSF1). Moreover, ectopic expression of both SRSF1 and MCL-1L isoform was able to recover EtOH-mediated neurotoxicity.

*Corresponding author: Ilker Kudret Sariyer, Telephone: 215-707-6337, Fax: 215-707-4888, isariyer@temple.edu.

^aThese authors contributed equally.

DR. ILKER KUDRET SARIYER (Orcid ID : 0000-0002-0641-6141)

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: IKS, JBH, SLC. Performed the experiments: FIDS, RS, and MD. Analyzed the data: IKS, JBH, SLC. Contributed reagents/materials/analysis tools: SLC and IKS. Wrote the paper: IKS.

Conclusions—Our results suggest that ethanol exposure can lead to pre-mRNA missplicing of Mcl-1 in neuronal cells. Our results indicate that ethanol exposure of neurons leads to a decrease in the ratio of Mcl-1L/Mcl-1S by favoring pro-apoptotic Mcl-1S splicing over anti-apoptotic Mcl-1L isoform suggesting that Mcl-1S may play a crucial role in neurotoxicity associated with alcohol consumption.

INTRODUCTION

Heavy and chronic ethanol (EtOH) consumption can cause significant structural and functional damage to the brain. Many studies have shown that heavy alcohol exposure leads to neurodegeneration in the mature brain (Tiwari and Chopra, 2013; Luo 2014; de la Monte et al., 2014). The developing nervous system is even more vulnerable to EtOH exposure. Prenatal exposure to EtOH during pregnancy can cause fetal alcohol spectrum disorders (FASD), characterized by malformation of the nervous system, deficits in craniofacial development, growth deficiencies, and mental retardation (Sampson et al., 2000; May et al., 2009; Riley et al., 2011). FASD incidence in the United States is nearly 5% (May et al., 2009), and estimated lifetime cost of FASD was over \$2 million per case in 2004 (Lupton et al., 2004). The most devastating consequence of developmental exposure to EtOH is the neurotoxicity associated with the depletion of neurons in the developing brain. Therefore, it is crucial to elucidate the mechanisms of neuroapoptosis induced by EtOH exposure in order to develop effective therapeutic strategies to overcome EtOH-induced neurotoxicity.

Alternative pre-mRNA splicing makes a large and significant contribution to proteomic diversity. Utilization of various potential splice sites of the pre-mRNA in various combinations by spliceosome in the guidance of alternative splicing regulatory factors leads to the translation of several functionally distinct protein isoforms. Regulation of splice variants in the brain can modulate protein functions, which may ultimately affect behaviors associated with alcohol dependence and alcohol mediated neurotoxicity. A limited number of studies has shown that the pre-mRNA splicing patterns of genes are potentially altered during the development of alcoholism (Farris and Mayfield, 2014; Sasabe and Ishiura, 2010). EtOH exposure in experimental animals has been reported to alter pre-mRNA splicing of the dopamine D2 receptor (DRD2) (Oomizu et al., 2003), the NR1 subunit of the NMDA receptor (Laurie et al., 1995; Hardy et al., 1999), and the $\gamma 2$ subunit of the GABAA receptor (Petrie et al., 2005). Altered splicing of these receptor units during the development of alcoholism was mainly proposed to be involved in behavior changes, such as alcohol dependence. Many intriguing questions remain to be answered, such as how alcohol affects splicing and splicing regulatory proteins. Since alcohol consumption is associated with neurotoxicity, it is possible that altered splicing of survival and pro-survival factors during the development of alcoholism may contribute to the neurotoxicity associated with EtOH exposure.

Here we investigated the possible impact of EtOH exposure on expression of alternative splicing factors and the alternative splicing of candidate genes in neurons. Our data indicate that the anti-apoptotic Mcl-1L isoform is the major form of Mcl-1 expressed in primary human fetal neurons. Moreover, our data suggest that EtOH exposure of primary neurons suppresses expression levels of SRSF1 and causes a decrease in the ratio of Mcl-1L/Mcl-1S

by favoring the pro-apoptotic Mcl-1S splicing over anti-apoptotic Mcl-1L, suggesting that Mcl-1S may play a crucial role in neurotoxicity associated with alcohol consumption.

MATERIALS & METHODS

Ethics Statement

All primary human cells were obtained and utilized in accordance with Temple University Human Subjects Protections and the approval of the Institutional Review Board.

Plasmids and constructs

pcDNA3.1-MCL-1L plasmid encoding human MCL-1L isoform was reported previously (Morel et al., 2009) and obtained from Addgene (#25375). The luciferase reporter plasmid pLuc-SRSF1 was made by cloning the –1000 to +49 promoter region of SRSF1 gene into the pGL3 vector at the BamHI site and was described previously (Craigie et al., 2015; Sariyer et al., 2016). Human serine and arginine rich splicing factor 1 (SRSF1, NCBI Reference Sequence: NM_006924.4) was cloned into the eukaryotic expression vector pcDNATM6/myc-His at Hind-III and Xho-I restriction enzyme sites and labeled as pcDNATM6/myc-His-SRSF1. The plasmid was created with the following primers: SRSF1-F: 5'-AAGATTAAGCTTATGTCGGGAGGTGGTGTG-3' and SRSF1-R: 5'-AAGATTCTCGAGTGTACGAGAGCGAGATCT-3'.

Antibodies and Reagents

Antibodies were obtained from the following sources; anti-MAP2, anti-beta-tubulin, and anti-synaptophysin from Cell Signaling Inc. (Beverly, MA), β -tubulin from LI-COR, Odyssey (Lincoln, NE), anti-SRSF1 from Invitrogen, anti-SRSF2 from Sigma Aldrich, anti-MCL1, anti-SRSF3, anti-SRSF4, and anti-HNRNP A1 from Santa Cruz Biotech, and anti-Grb2 from BD Biosciences. Mammalian protease inhibitors were obtained from Sigma-Aldrich (St Louis, MO). Bradford reagent was from BioWorld (Dublin, OH). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was obtained from Thermo Fisher Scientific (#M6494).

Cell culture

Primary cultures of human fetal neurons (PHFN) were described previously (Saribas et al., 2017). Briefly, PHFNs were obtained in 6-well plates or 2-well chamber slides from the Comprehensive Neuro-AIDS Center (CNAC) tissue culture core at Temple University Lewis Katz Medical School. Cells were maintained in Neurobasal medium (Gibco) containing B-27 supplement (Gibco), 0.05 % GlutaMAXTM (Gibco) and gentamicin (10 μ g/ml) at 37°C under 5% CO₂. Half of the neuronal growth medium was replaced with fresh medium every 3–4 days. SH-SY5Y neuroblastoma cells were obtained from American Tissue Culture Collection (ATCC® CRL-2266TM). They were plated in the poly-D-lysine coated 6-well dishes and maintained in growth medium [Dulbecco's Modified Eagle Medium (DMEM): F12 medium supplemented with 10% fetal bovine serum (FBS), 10% GlutaMAXTM, insulin and gentamicin (10 μ g/ml)] at 37°C under 5% CO₂ atmosphere. The growth medium was changed every 3–4 days.

EtOH anti-evaporation chambers

To maintain constant ethanol concentrations in cell culture media, we utilized an “anti-evaporation system” adopted from Eysseric, (1997) and Rodriguez et al., (1992) with some modifications. Briefly, primary human neurons were exposed to 50 mM EtOH and placed in a polystyrene box with a lid which allowed the CO₂ transport. In addition, an open pan with 50 mM EtOH was also placed into the polystyrene box in advance to saturate the evaporated EtOH in the polystyrene box that limited ethanol evaporation from the media of cultured neurons.

EtOH assay

EtOH concentrations in culture media were determined using a commercial kit (Sigma-Aldrich #MAK076) according to the manufacturer’s instructions.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for cell viability

PHFN or SH-SY5Y cells were plated in 6-well tissue culture plates (2×10^5), and treated with various concentrations of EtOH. Twenty four hours post-treatments, cells were incubated with 1 ml of MTT working solution (DMEM with 0.5 mg/ml MTT) for 2 hour at 37°C. The converted dye was solubilized with 1 ml acidic isopropanol (0.004 M HCL in isopropanol). The absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 650nm.

Alternative splicing analysis and RT-PCRs

SH-SY5Y and PHFN cells were exposed to 50 mM EtOH in a polystyrene box to prevent EtOH evaporation, adopted from Eysseric H, 1997 and Rodriguez et al., 1992. At various post-exposure time points, cells were harvested, and total RNA was extracted with an RNA extraction kit (RNeasy, Qiagen) according to the manufacturer’s instructions. RT-PCR reactions were performed as described previously (De Simone et al., 2015). After treatment with DNase I, followed by phenol/chloroform extraction and EtOH precipitation, cDNAs were synthesized using M-MuLV reverse transcriptase. RNA templates were removed by RNase H digestion. A total of 100 ng cDNA was used as template for PCR reactions. Alternatively spliced isoforms of BIM, RON, MCL-1, and BIN1 genes were amplified by PCR using specific primers listed in Table 1. β -Actin mRNA was also amplified from the same set of RNA samples by RT-PCR as an internal control. Amplified gene products were resolved on a 2% DNA-agarose gel.

Immunocytochemistry

Primary human fetal neurons (PHFNs) were cultured in 2 well chamber slides (2×10^5 cells / well). PHFNs were either exposed to 50 mM EtOH or left untreated. Immunocytochemistry was performed as described previously (Merabova et al., 2015). Briefly, at 24 h post-exposures, neurons were fixed with cold Acetone:MEtOH (50:50) for one minute, washed three times with PBS, then, blocked with 10% BSA in PBS for one hour, and incubated with rabbit polyclonal anti-MAP-2 and mouse monoclonal anti-Synaptophysin antibodies. Primary antibody dilutions were 1:300 in 5% BSA overnight at 4 °C with gentle rocking. After primary antibody incubation, cells were washed 3 times with

PBS and then incubated with a secondary solution containing 1:500 FITC rabbit secondary and 1:500 rhodamine mouse secondary, in 5% BSA for 2 hours. Wells were then washed 5 times with PBS and mounted with Vectashield mounting solution containing DAPI. Then glass coverslips were added before imaging on a Leica Fluorescence Microscope.

Preparation of the PHFN and SH-SY5Y cell lysates, and SDS-PAGE/Western blotting

PHFN and SH-SY5Y lysates were prepared using TNN buffer with 1% NP40 supplemented as described previously (Saribas et al., 2015 and 2017). Briefly, the cell lysates were prepared as follows. At the end of incubations, cells were washed in PBS and harvested by trypsinization. Cells were then lysed in TNN buffer with 1% NP40 supplemented with mammalian protease and phosphatase inhibitors, 0.4 mM NaF and 2 mM Na₃VO₄. Protein concentrations were determined by Bradford Protein Quantification assay. Protein samples were first denatured in SDS loading dye, heated at 95°C for 5 min, then separated on 10% SDS-PAGE. After electrophoresis, proteins were transferred to 0.45 µm nitrocellulose membranes for 2 h at 250 mA, alternatively overnight at 40 mA in 1 × cold transfer buffer (100 mM TrisHCl, pH 7.4, 150 mM NaCl, 20% ME OH). Membranes were blocked in either 1 × PBST buffer containing 10% milk or 1 × LI-COR blocking buffer for at least 1 h at RT. The primary monoclonal antibodies, anti-SRSF1, anti-SRSF2, anti-SRSF3, anti-SRSF4, anti-HNRNP A1, anti-MCL1, anti-Tubulin, and anti-Grb2 were diluted 1/1000 in buffer containing 5% milk and added to membranes where needed and were incubated overnight at 4°C with gentle shaking. After washing the membranes with 1 X PBST buffer three times, the secondary antibodies [LI-COR Goat against Rabbit 680 for polyclonal, LI-COR Goat against Mouse 800 for monoclonal] were added to the membranes and incubated for 45 min at RT followed by three times (5 min) washing with 1 X PBST buffer and a final wash with 1 X PBS buffer. Washed membranes were stored in 1 X PBS before scanning images on an ODISSEY™ Clx instrument. The intensity of each protein band was calculated using ImageJ (NIH) software and bar graphs were produced in Microsoft Excel.

Stable cell lines

SH-SY5Y cells (1×10^5 cells/35 mm tissue culture dish) were either transfected with pcDNA3.1 vector alone (Invitrogen) or pcDNA3.1-hMCL-1L (2 µg/plate) using lipofectamine 2000 method according to the manufacturer's recommendations (Invitrogen). Stable clonal cell lines were generated as described previously (Sariyer et al., 2011). Briefly, at six hours post-transfection, cells were washed twice with phosphate buffered saline (PBS) and re-fed with DMEM culture media containing 10% FBS. After twenty-four hours, cells from each 35-mm plate were trypsinized and re-plated onto three 100-mm plates. Cells were allowed to attach for six hours and then the medium was replaced with DMEM containing 800 µg/ml G418 and 10% FBS. The medium of the cells was replaced every two-three days until individual colonies formed. The single cell colonies were isolated and screened for MLCL-1L overexpression by Western blot, expanded and frozen in liquid nitrogen.

Luciferase Reporter Assay

PHFN or SH-SY5Y cells were plated in 6-well tissue culture dishes and transiently transfected with the pLuc-SRSF1 (−1000 to +49) luciferase reporter plasmid. Cells were treated with increasing concentrations of EtOH (10, 25, 50, and 100 mM) in compensation

chambers to prevent of EtOH evaporation. At 48 h post-transfection, cells were extracted and lysed using reporter lysis buffer for the luciferase reporter system provided by the manufacturer Promega. After cell lysis, luciferase activities of samples were determined using luciferase assay reagent (LAR). The luciferase activities were then corrected for protein concentration and normalized to the basal levels of transcription, allowing the determination of the fold changes.

Statistical analysis

All of the values presented on the graphs are given as means $\pm$ S.E.M. ANOVA and unpaired Student's t-tests were used to analyze the statistical significance. P-values of less than 0.05 were considered statistically significant.

RESULTS

Ethanol (EtOH) exposure is toxic to neuronal cells

In order to gain insight into the impact of EtOH exposure on neurons, primary human fetal neurons (PHFNs) were plated in 6-well tissue culture dishes until they formed typical neuronal networks. Neurons were then treated with increasing concentrations of EtOH (0, 1, 10, 25, 50, and 100 mM) for 24 h and cell viabilities were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. As seen in Fig. 1A, at low concentrations (1 and 10 mM), EtOH had no significant effect on neuronal viability. Interestingly, there was a mild but significant reduction in neuronal viability when cells were treated with 25 mM EtOH. Larger reductions in neuronal viability were observed with 50 and 100 mM EtOH treatments. These results have suggested that EtOH exposure is toxic to neurons at 25 mM or higher doses. In parallel to the PHFNs, we also analyzed the impact of EtOH exposure on the cellular viability of SH-SY5Y cells, a human neuroblastoma cell line. SH-SY5Y cells were exposed to 50 mM EtOH for 24 h. Cellular viabilities were determined by MTT assay at 0, 8, and 24 h post-exposure. As shown in Fig. 1B, SH-SY5Y cells showed an increasing trend in cellular viability as expected due to the active replication and growth of the cells. On the other hand, SH-SY5Y cells which were exposed to EtOH not only lost their increasing trend of cell growth but also showed a significant reduction in their viability at 24 h post-treatment suggesting that EtOH exposure is also toxic to SH-SY5Y cells. In order to gain more insight into the effects of EtOH exposure on neurons, PHFN cells were also plated in chamber slides and exposed to EtOH for 24 h. Cells were fixed and immunolabeled with neuronal Map2 and Synaptophysin markers. As shown in Fig. 1C, control neurons showed a typical staining of Map2 and Synaptophysin with partial co-localization of both markers. Interestingly, EtOH exposure to PHFN cells caused a significant reduction in Map2 and even more significant decrease in Synaptophysin staining in the majority of the neurons (Fig. 1C, compare control with EtOH). Together, these results suggest that EtOH exposure causes a significant toxicity to neuronal cells in a dose dependent manner.

Ethanol (EtOH)-mediated suppression of serine/arginine rich splicing factor 1 (SRSF1) in primary human neurons

Regulation of splice variants in neurons can modulate protein functions, which may ultimately contribute to alcohol-mediated neurotoxicity. Alternative splicing is mainly regulated by a group of proteins that are classified as serine/arginine-rich proteins (SR proteins). The SR proteins have been extensively studied and are known to play important roles in pre-mRNA splicing of cellular genes (Manley and Krainer, 2010). In order to gain insight into the possible effect of EtOH exposure on SR protein expressions, PHFN cells were plated in 6-well tissue culture dishes and treated with 50 mM EtOH up to 48 h. In order to gain an idea about dose and time dependent impact of EtOH on SR protein expression, EtOH evaporation was allowed during these treatments. At 4, 8, 24, 32, and 48 h post-treatments, whole cell protein extracts were collected and analyzed by Western blot for SRSF1, SRSF2, SRSF3, and HNRNP A1 expression. The culture media was also collected to monitor EtOH concentrations at various time points post-treatment. As shown in, Fig. 2A, there was a significant decrease in the expression levels of all the splicing regulatory proteins at 8 h post-treatments. Interestingly, at 24 h post-treatment, when EtOH concentration in culture media dropped to less than 5 mM (Fig. 2B) due to the evaporation, expression levels of SRSF2, SRSF3, and HNRNP A1 was completely recovered and stayed stable at 32 and 48 h post-treatment. On the other hand, expression levels of SRSF1, which had the most dramatic decrease at 8 h, never reached to the normal expression levels at 24, 32, and 48 h post-treatments even after EtOH concentration in culture media dropped below to 2 mM. In order to further analyze the effect of EtOH exposure on SRSF1 expression, PHFN cells were also exposed to EtOH by using a “compensation system” to prevent EtOH evaporation, adopted from Eysseric (1997) and Rodriguez et al. (1992). As shown in Figs. 2C and 2D, EtOH exposure caused a dramatic decrease in SRSF1 levels over time under constant level of exposure suggesting that EtOH inhibits SRSF1 expression in PHFN cells. Neither SRSF4 nor tubulin levels were altered by EtOH exposure.

EtOH-mediated alternative splicing of candidate genes in neuronal cells

As shown above (Fig. 2), EtOH exposure of PHFNs caused a dramatic decrease in the expression levels of SR family alternative splicing factors, particularly SRSF1. Next we sought to investigate the effect of EtOH exposure on alternative splicing patterns of candidate genes which have been shown to be regulated by SRSF1 and involved in regulation of apoptosis. Among the possible candidate genes, we analyzed the alternative splicing of Bcl2-like protein 11 (BCL2L11 or BIM), macrophage-stimulating protein receptor (MST1R), bridging integrator 1 (BIN1), and myeloid cell leukemia 1 (Mcl-1) (Anczukow et al., 2012; Khan et al., 2014; Karni et al., 2007). SH-SY5Y cells were exposed to 50 mM EtOH for up to 8h. EtOH evaporation during treatment was controlled by “compensation system” as described in Materials and Methods. At 0, 1, 4, and 8 h post-exposure, total RNA was isolated from cells and analyzed by RT-PCR using dedicated primers to amplify all the possible spliced isoforms of the genes. As shown in Fig. 3 (left panel), SH-SY5Y cells express majorly the EL isoform of BIM and full-length isoform of MST1R genes. Interestingly, EtOH caused a shift in BIM splicing to $\gamma 1$ and $\gamma 2$ isoforms by 4 h and delta-11 isoform in MST1R by 8 h post-exposures. More interestingly, EtOH exposure caused a significant switch in MCL-1 alternative splicing from anti-apoptotic

MCL-1L isoform to pro-apoptotic MCL-1S isoform in SH-SY5Y cells with no significant alteration in the alternative splicing pattern of BIN1 gene. In addition to SH-SY5Y cells, we have also analyzed and compared the effect of EtOH exposure on alternative splicing patterns of selected genes in primary human fetal neurons (PHFNs). PHFN cells were also exposed to 50 mM EtOH for up to 36 h. EtOH evaporation during the treatments was controlled by “compensation system” as described in materials and methods. At 0, 1, 4, 8, 24, and 36 h post-exposure, total RNA was isolated from cells and analyzed by RT-PCR using specific primers. As shown in Figure 3 (right panel), PHFN cells express majorly EL isoform of BIM, full-length isoform of MST1R, and +13 isoform of BIN1, and neither their splicing patterns, nor expression profiles of the isoforms were altered during EtOH exposure. On the other hand, consistent with SH-SY5Y cells, EtOH exposure of PHFN cells induced the alternative splicing of MCL-1S isoform over MCL-1L and caused a decrease in the ratio of Mcl-1L/Mcl-1S by favoring pro-apoptotic Mcl-1S splicing over anti-apoptotic Mcl-1L isoform.

Ectopic expression of MCL-1L rescues EtOH-mediated neurotoxicity

As shown above, EtOH exposure causes a shift in alternative splicing of Mcl-1 from Mcl-1L to Mcl-1S isoform suggesting that the reduced levels of anti-apoptotic MCL-1L may play a crucial role in neurotoxicity induced by EtOH exposure. In order to test this, PHFN cells were transfected with an expression plasmid encoding human Mcl-1L isoform (Addgene, plasmid #25375). Cells were then exposed to 50 mM EtOH for 24 h. Cell viabilities were determined by MTT assay. As shown in Fig. 4A, EtOH caused ~ 50% decrease in neuronal viability (lane 2) as expected. Interestingly, ectopic expression of Mcl-1L completely recovered the neuronal viability suppressed by EtOH exposure, suggesting that Mcl-1L isoform can protect primary neurons against EtOH induced neurotoxicity. To gain more insight into the neuroprotection by MCL-1L, we obtained clonal cell line derivatives of SH-SY5Y cells, which stably express the MCL-1L isoform. SH-SY5Y cells were transfected with either pcDNA3.1 vector alone or pcDNA-3.1-MCL-1L. Stable clonal cells were selected by G418 treatment and cloned by limiting dilution. As shown in Fig. 4B, control cells showed a significant reduction in cell viability when exposed to 50 mM EtOH. On the other hand, EtOH exposure did not cause any significant viability loss in MCL-1L stable cells suggesting that stable expression of MCL-1L isoform in neuroblastoma cells is protective against EtOH exposure.

Overexpression of SRSF1 recovers toxicity associated with EtOH exposure

To gain more insight into the EtOH induced suppression of SRSF1 expression, possible impact of EtOH on SRSF1 transcription was analyzed by reporter gene analysis. The SRSF1 promoter region (–1000 to +49) was cloned into a firefly-luciferase reporter plasmid. PHFN cells were transfected with the SRSF1 reporter construct and exposed to 10, 25, 50, and 100 mM EtOH for 24 h. The transcriptional activity of the SRSF1 promoter was analyzed by luciferase assay as described in the Materials and Methods. As shown in Fig. 5A, the basal transcriptional activity of the SRSF1 promoter was decreased significantly in a dose dependent manner when cells were exposed to increasing concentrations of EtOH. These results suggest that EtOH exposure causes transcriptional suppression of SRSF1 gene expression in PHFN cells. In order to determine a possible role for SRSF1 downregulation in

suppression of the MCL-1L isoform, SH-SY5Y cells were transiently transfected with an expression vector encoding SRSF1 in fusion with Myc/His tags. Cells were exposed to EtOH (50 mM) and analyzed by Western blot for MCL-1L. As shown in Figs. 5B and C, EtOH exposure caused a significant reduction in MCL-1L expression in cells either untransfected or transfected with vector alone (compare lane 1 with lanes 2 and 3). Interestingly, MCL-1L expression was significantly recovered by overexpression of SRSF1 (lane 4) suggesting that EtOH-mediated suppression of SRSF1 expression is involved in MCL-1L downregulation that may lead to the EtOH induced toxicity. To gain more insight into the role of SRSF1 downregulation in neuronal toxicity associated with EtOH exposure, the MTT cell viability assay was also performed in cells with SRSF1 overexpression. Interestingly, transient expression of SRSF1 with an expression vector under CMV promoter completely recovered the viability loss associated with EtOH exposure (Fig. 5D) suggesting that EtOH-mediated suppression of SRSF1 was associated with neuronal toxicity.

DISCUSSION

RNA splicing is the editing of pre-mRNA transcripts that involves removal of introns and ligation of exons. Splicing is an essential step for genes that contain introns to create an mRNA molecule that can be processed by ribosomes for translation into the protein. Alternative splicing of pre-mRNAs is a regulated process during gene expression that makes a significant contribution to proteomic diversity. Utilization of different potential splice sites of the pre-mRNA in various combinations by spliceosome in the guidance of alternative splicing regulatory proteins leads to the translation of several functionally distinct protein isoforms. Regulation of splice variants in the brain can modulate protein function, which may ultimately affect behaviors associated with alcohol dependence and alcohol mediated neurotoxicity. In this manuscript, we have shown that EtOH exposure dysregulates expression of serine/arginine rich splicing factor 1 (SRSF1) in primary human fetal neurons. Furthermore, alternative splicing of the candidate genes which are known targets of SRSF1 and involved in apoptosis regulation have shown an alteration in pre-mRNA splicing of myeloid cell leukemia -1 (MCL-1) gene. Mcl-1 gene can mainly express two isoforms (Mcl-1L and Mcl-1S) which are regulated by alternative splicing. The pre-mRNA of Mcl-1 can be alternatively spliced to remove exon 2, which produces shortened form of Mcl-1, named Mcl-1S, which lacks BH domains 1, 2 and transmembrane domain. While the longer gene product Mcl-1L enhances cell survival by inhibiting apoptosis, the alternatively spliced shorter gene product Mcl-1S promotes apoptosis (Thomas LW., et al., 2010; Bae et al., 2000). Our results suggest that EtOH exposure causes a significant decrease in the ratio of Mcl-1L/Mcl-1S by favoring pro-apoptotic Mcl-1S splicing in neuronal cells.

Mcl-1 is a prosurvival member of the Bcl-2 family that was initially identified in myeloid leukemia cells during differentiation (Kozapos et al., 1993). Sequence analysis revealed that Mcl-1 contained 3 putative BH domains and experimentally it was found to protect against apoptosis. Germline deletion of Mcl-1 has been shown to cause peri-implantation lethality at embryonic day 3.5 (E3.5) (Rinkenberger et al., 2000), which is one of the few lethal phenotypes observed among Bcl-2 family members. Mcl-1 is believed to suppress apoptosis by interacting with proapoptotic Bcl-2 family member proteins (Youle and Strasser, 2008). Mcl-1 shows high binding activity for Noxa (a BH3 only member of Bcl-2 family), PUMA

(apoptosis regulator induced by p53), Bim (Bcl-2-like protein 11), and BMF (Bcl-2 modifying factor) (Kuwana et al., 2005 and Chen et al., 2005). In addition to its importance in the regulation of apoptosis, Mcl-1 plays a key role in nervous system development and neuronal cell death. Neuronal precursors within the ventricular zone and newly committed neurons in the cortex are shown to express high levels of Mcl-1 (Arbour et al., 2008). Mcl-1 upregulation has been shown to have a role in Notch1-mediated survival of neuronal precursor cells (Oishi et al., 2004) and in the maintenance of granule cell survival during differentiation (Zhang et al., 2004). Heterozygous germline deletion of Mcl-1 results in increased susceptibility of neurons to seizures induced by pilocarpine (Mori et al., 2004). Moreover, Mcl-1 plays a key role in regulation of neuronal survival after injury (Arbour et al., 2008).

Mcl-1 is essential for the survival of many different cell lineages and its overexpression is associated with tumorigenesis. Therefore it is crucial that its expression is strictly controlled. Indeed, MCL-1 expression is regulated by transcriptional, post-transcriptional and post-translational mechanisms (Mojša et al., 2014). Our data suggest that EtOH exposure does not impact the transcript levels of MCL-1 but leads to altered splicing of the pre-mRNA resulting in a decrease in MCL-1L and increase in MCL-1S isoforms. However, these results did not exclude the possibility that post-translational modifications leading to MCL-1L isoform degradation might also be contributing to the decrease in the levels of the protein. Whether EtOH has any impact on post-translational regulation of MCL-1L or its stability remains to be elucidated.

Alternative splicing of MCL-1 pre-mRNA has been shown to be regulated mainly by SRSF1. Downregulation of SRSF1 expression in breast cancer cell lines was associated with decreased splicing of MCL-1L over MCL-1S isoform and increased susceptibility of the cancer cells to apoptosis (Gautrey et al., 2012). Consistent with these observations, our data suggest that EtOH-mediated downregulation of SRSF1 expression leads to a shift in MCL-1L/MCL-1S ratio in favor of MCL-1S splicing over MCL-1L and may suggest a possible mechanism of neurotoxicity associated with EtOH exposure. Moreover, our data also suggest that either SRSF1 or MCL-1L overexpression in neuronal cells during the course of EtOH exposure can rescue the cellular viability suppressed by EtOH exposure. These observations have suggested novel and key roles of SRSF1 and MCL-1 genes in EtOH-induced neurotoxicity.

Acknowledgments

This study utilized services offered by core facilities of the Comprehensive NeuroAIDS Center (CNAC NIMH Grant Number P30MH092177) at Temple University School of Medicine. This work was made possible by grants awarded by NIH to IKS (AI101192) and SLC (AA023172 and DA036175). We would like to also thank Dr. Martyn White for the critical reading and editing of the manuscript.

The authors declare no conflict of interest.

References

- Anczukow O, Rosenberg AZ, Akerman M, Das S, Zhan L, Karni R, Muthuswamy SK, Krainer AR. The splicing-factor SRSF1 regulates apoptosis and proliferation to promote mammary epithelial cell transformation. *Nat Struct Mol Biol.* 2012; 19:220–8. [PubMed: 22245967]

- Arbour N, Vanderluit JL, Le Grand JN, Jahani-Asl A, Ruzhynsky VA, Cheung EC, Kelly MA, MacKenzie AE, Park DS, Opferman JT, Slack RS. Mcl-1 is a key regulator of apoptosis during CNS development and after DNA damage. *J Neurosci*. 2008 Jun 11; 28(24):6068–78. DOI: 10.1523/JNEUROSCI.4940-07.2008 [PubMed: 18550749]
- Bae J, Leo CP, Hsu SY, Hsueh AJ. MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. *J Biol Chem*. 2000; 275(33):25255–25261. [PubMed: 10837489]
- Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell*. 2005; 17:393–403. [PubMed: 15694340]
- Craigie M, Regan P, Otolara YL, Sariyer IK. Molecular interplay between T-Antigen and splicing factor, arginine/serine-rich 1 (SRSF1) controls JC virus gene expression in glial cells. *Virology*. 2015 Nov 24; 12:196. [PubMed: 26596376]
- de la Monte SM, Kril JJ. Human alcohol-related neuropathology. *Acta Neuropathol*. 2014 Jan; 127(1): 71–90. [PubMed: 24370929]
- De-Simone FI, Sariyer R, Otolara YL, Yarandi S, Craigie M, Gordon J, Sariyer IK. IFN-Gamma Inhibits JC Virus Replication in Glial Cells by Suppressing T-Antigen Expression. *PLoS One*. 2015 Jun 10; 10(6):e0129694. [PubMed: 26061652]
- Eysseric H, Gonthier B, Soubeyran A, Bessard G, Saxod R, Barret L. There is not simple method to maintain a constant EtOH concentration in long-term cell culture: keys to a solution applied to the survey of astrocytic EtOH absorption. *Alcohol*. 1997 Mar-Apr; 14(2):111–5. [PubMed: 9085710]
- Farris SP, Mayfield RD. RNA-Seq reveals novel transcriptional reorganization in human alcoholic brain. *Int Rev Neurobiol*. 2014; 116:275–300. [PubMed: 25172479]
- Gautrey HL, Tyson-Capper AJ. Regulation of Mcl-1 by SRSF1 and SRSF5 in Cancer Cells. *PLoS ONE*. 2012; 7(12):e51497. [PubMed: 23284704]
- Hardy PA, Chen W, Wilce PA. Chronic EtOH exposure and withdrawal influence NMDA receptor subunit and splice variant mRNA expression in the rat cerebral cortex. *Brain Res*. 1999; 819:33–39. [PubMed: 10082858]
- Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat Struct Mol Biol*. 2007; 14:185–93. [PubMed: 17310252]
- Khan DH, Gonzalez C, Cooper C, Sun JM, Chen HY, Healy S, Xu W, Smith KT, Workman JL, Leygue E, Davie JR. RNA-dependent dynamic histone acetylation regulates MCL1 alternative splicing. *Nucleic Acids Res*. 2014 Feb; 42(3):1656–70. [PubMed: 24234443]
- Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci USA*. 1993; 90(8):3516–3520. [PubMed: 7682708]
- Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell*. 2005; 17:525–535. [PubMed: 15721256]
- Laurie DJ, Putzke J, Zieglansberger W, Seeburg PH, Tolle TR. The distribution of splice variants of the NMDAR1 subunit mRNA in adult rat brain. *Brain Res Mol Brain Res*. 1995; 32:94–108. [PubMed: 7494468]
- Luo J. Autophagy and EtOH neurotoxicity. *Autophagy*. 2014; 10(12):2099–108. [PubMed: 25484085]
- Lupton C, Burd L, Harwood R. Cost of fetal alcohol spectrum disorders. *Am J Med Genet C Semin Med Genet*. 2004; 127C(1):42–50. [PubMed: 15095471]
- Manley JL, Krainer AR. A rational nomenclature for serine/arginine-rich protein splicing factors (SR proteins). *Genes Dev*. 2010 Jun 1; 24(11):1073–4. [PubMed: 20516191]
- May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Dev Disabil Res Rev*. 2009; 15(3):176–92. [PubMed: 19731384]
- Merabova N, Sariyer IK, Saribas AS, Knezevic T, Gordon J, Turco MC, Rosati A, Weaver M, Landry J, Khalili K. WW domain of BAG3 is required for the induction of autophagy in glioma cells. *J Cell Physiol*. 2015 Apr; 230(4):831–41. [PubMed: 25204229]

- Mojša B, Lassot I, Desagher S. Mcl-1 ubiquitination: unique regulation of an essential survival protein. *Cells*. 2014 May 8; 3(2):418–37. [PubMed: 24814761]
- Morel C, Carlson SM, White FM, Davis RJ. Mcl-1 integrates the opposing actions of signaling pathways that mediate survival and apoptosis. *Mol Cell Biol*. 2009 Jul; 29(14):3845–52. DOI: 10.1128/MCB.00279-09 [PubMed: 19433446]
- Mori M, Burgess DL, Gefrides LA, Foreman PJ, Opferman JT, Korsmeyer SJ, Cavaliheiro EA, Naffah-Mazzacoratti MG, Noebels JL. Expression of apoptosis inhibitor protein Mcl1 linked to neuroprotection in CNS neurons. *Cell Death Differ*. 2004; 11:1223–1233. [PubMed: 15286683]
- Oishi K, Kamakura S, Isazawa Y, Yoshimatsu T, Kuida K, Nakafuku M, Masuyama N, Gotoh Y. Notch promotes survival of neural precursor cells via mechanisms distinct from those regulating neurogenesis. *Dev Biol*. 2004; 276:172–184. [PubMed: 15531372]
- Oomizu S, Boyadjieva N, Sarkar DK. EtOH and estradiol modulate alternative splicing of dopamine D2 receptor messenger RNA and abolish the inhibitory action of bromocriptine on prolactin release from the pituitary gland. *Alcohol Clin Exp Res*. 2003; 27:975–980. [PubMed: 12824819]
- Petrie J, Sapp DW, Tyndale RF, Park MK, Fanselow M, Olsen RW. Altered gabaa receptor subunit and splice variant expression in rats treated with chronic intermittent EtOH. *Alcohol Clin Exp Res*. 2001; 25:819–828. [PubMed: 11410716]
- Riley EP, Infante MA, Warren KR. Fetal alcohol spectrum disorders: an overview. *Neuropsychol Rev*. 2011; 21(2):73–80. [PubMed: 21499711]
- Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev*. 2000; 14:23–27. [PubMed: 10640272]
- Rodríguez FD, Simonsson P, Alling C. A method for maintaining constant EtOH concentrations in cell culture media. *Alcohol Alcohol*. 1992 May; 27(3):309–13. [PubMed: 1449566]
- Sampson PD, Streissguth AP, Bookstein FL, Barr HM. On categorizations in analyses of alcohol teratogenesis. *Environ Health Perspect*. 2000; 108(Suppl 3):421–8. [PubMed: 10852839]
- Saribas AS, Cicalese S, Ahooyi TM, Khalili K, Amini S, Sariyer IK. HIV-1 Nef is released in extracellular vesicles derived from astrocytes: evidence for Nef-mediated neurotoxicity. *Cell Death Dis*. 2017 Jan 12.8(1):e2542. [PubMed: 28079886]
- Saribas AS, Khalili K, Sariyer IK. Dysregulation of autophagy by HIV-1 Nef in human astrocytes. *Cell Cycle*. 2015; 14(18):2899–904. [PubMed: 26176554]
- Sariyer IK, Saribas AS, White MK, Safak M. Infection by agnoprotein-negative mutants of polyomavirus JC and SV40 results in the release of virions that are mostly deficient in DNA content. *Virology*. 2011 May 24.8:255. [PubMed: 21609431]
- Sariyer R, De-Simone FI, Gordon J, Sariyer IK. Immune suppression of JC virus gene expression is mediated by SRSF1. *J Neurovirol*. 2016 Oct; 22(5):597–606. [PubMed: 26951564]
- Sasabe T, Ishiura S. Alcoholism and alternative splicing of candidate genes. *Int J Environ Res Public Health*. 2010 Apr; 7(4):1448–66. [PubMed: 20617039]
- Thomas LW, Lam C, Edwards SW. Mcl-1; the molecular regulation of protein function. *FEBS Lett*. 2010 Jul 16; 584(14):2981–9. DOI: 10.1016/j.febslet.2010.05.061 [PubMed: 20540941]
- Tiwari V, Chopra K. Resveratrol abrogates alcohol-induced cognitive deficits by attenuating oxidative-nitrosative stress and inflammatory cascade in the adult rat brain. *Neurochem Int*. 2013; 62(6):861–869. [PubMed: 23422878]
- Xie SY, Ren ZR, Zhang JZ, Guo XB, Wang QX, Wang S, Lin D, Gong XL, Li W, Huang SZ, Zeng F, Zeng YT. Restoration of the balanced α/β -globin gene expression in b654-thalassemia mice using combined RNAi and antisense RNA approach. *Human Molecular Genetics*. 2007; 16(21):2616–2625. DOI: 10.1093/hmg/ddm218 [PubMed: 17716993]
- Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*. 2008; 9:47–59. [PubMed: 18097445]
- Zhang J, D'Ercole AJ. Expression of Mcl-1 in cerebellar granule neurons is regulated by IGF-I in a developmentally specific fashion. *Brain Res Dev Brain Res*. 2004; 152:255–263. [PubMed: 15351513]

Figure 1. EtOH exposure is detrimental to the neurons

A. Dose-dependent effect of EtOH on neuronal viability of primary human neurons assessed by MTT cell viability assay. PHFN cells were plated in 6-well tissue culture dishes and exposed to EtOH at indicated concentrations. MTT viability assay was performed at 24 h post-exposures. Bar graph represents three replicates. **B.** Effect of EtOH exposure on cellular viability of neuroblastoma cell line. SH-SY5Y cells were plated in 6-well tissue culture dishes and exposed to 50 mM EtOH. MTT viability assay was performed at 0, 12, and 24 h post-exposures. Bar graph represents three replicates. The Student's t-test was performed to calculate "P" values. **C.** Effect of EtOH exposure on MAP2 and synaptophysin expression in primary human neurons. PHFN cells were plated in 2-well chamber slides and exposed to 50 mM EtOH. The cells were fixed at 24 h post-exposure and processed for ICC for the detection of MAP2 and synaptophysin.

Figure 2. Time and dose dependent effect of EtOH exposure on expression levels of splicing regulatory proteins

A. PHFN cells were exposed to 50 mM EtOH and whole cell protein extracts were prepared at 0, 4, 8, 24, 32, and 48 h post-exposures. Western blots of protein lysates were performed to access the expression levels of SRSF1, SRSF2, SRSF3, and HNRNP A1. Tubulin was probed in the same blots as a loading control. **B.** EtOH concentrations in culture media in parallel to whole cell extracts were measured and shown as bar graph. **C.** PHFN cells were exposed to 50 mM EtOH in evaporation compensation chambers and whole cell protein extracts were prepared at 0, 24, 48 h post-exposures. Western blots of whole cell protein extracts were performed to access the expression levels of SRSF1 and SRSF4. Tubulin was probed in the same blots as a loading control. **D.** EtOH concentrations in culture media in parallel to whole cell extracts from the same studies described in panel C were measured and shown as bar graph.

Figure 3. EtOH-mediated alternative splicing of candidate genes in neurons
PHFN and SH-SY5Y cells were exposed to 50 mM EtOH for up to 36 h. EtOH evaporation during the treatments was controlled by “compensation system”. At each time point, total RNA was isolated from control cells (cont.) and from cells exposed to EtOH by using the RNeasy extraction kit (Qiagen). One microgram of total RNA was used in reverse transcription reactions and cDNA was synthesized. Splicing isoforms of BIM, MST1R, MCL-1, and BIN1 were amplified by PCR using specific primers (Table 1), and separated and monitored on agarose gel by ethidium bromide staining. Alternative splicing isoforms of the proteins are indicated by arrows. The location of primer pairs and map of exon-intron structure of the genes are schematized in the right-hand panels.

Figure 4. MCL-1L isoform can rescue EtOH-induced viability loss in neurons

A. PHFN cells were plated in 6-well tissue culture dishes and transfected with pCDNA3.1-MCL-1L expression plasmid (Addgene#25375). At 24 h post-transfections, cells were exposed to 50 mM EtOH in incubators with “compensation system” to prevent EtOH evaporation. Cellular viability was determined by MTT assay at 24h post-exposure to EtOH. Bar graph represents three independent experiments. **B.** MCL-1L stable cells are resistant to toxicity associated with EtOH exposure. SH-SY5Y cells were transfected with pCDNA3.1 vector and pCDNA3.1-MCL-1L expression plasmid and treated with G418 for the selection

of transfected cells over un-transfected cells. G418 resistant cells were seeded in 96-well tissue culture dishes as 1 cell/5 wells (limiting dilution) for the establishment of sub-cell line clones. All the clones were analyzed by Western blot for stable over-expression of MCL-1L gene. The control cells (originated from pCDNA3.1 control vector) and MCL-1L stable cells (originated from pCDNA3.1-MCL-1L) were plated in 6-well tissue culture dishes and exposed to 50 mM EtOH for 24h. MTT assay was performed to determine cellular viability. Bar graph represents three independent experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. EtOH-mediated neurotoxicity is associated with SRSF1

A. EtOH exposure inhibits transcription of SRSF1 gene. PHFN cells were transfected with SRSF1 reporter construct and exposed to 10, 25, 50, and 100 mM EtOH for 24 hrs. Transcriptional activity of SRSF1 promoter was analyzed by luciferase assay as described in the materials and methods. **B.** SH-SY5Y cells were transiently transfected with an expression vector encoding SRSF1 in fusion with Myc tag. Cells were exposed to EtOH (50 mM) and analyzed by Western blot for MCL-1L, SRSF1, and tubulin. **C.** MCL-1L band intensities from immunoblots shown in panel B were quantified, normalized to tubulin, and shown as bar graph. **D.** PHFN cells were transfected with an expression vector encoding SRSF1 and exposed to 50 mM EtOH at 24 h post-transfections. MTT viability assay was performed at 48 h post-EtOH exposures and shown as bar graph. The data were obtained from three independent experiments.

Table 1

RT-PCR primer sequences.

GENE	SEQUENCE	REFERENCE
BIM	F: 5'-GGCAAAGCAACCTTCTGATG-3' R1: 5'-TAACCATTCGTGGGTGGTCT-3' R2: 5'-ATGGTGGTGGCCATACAAAT-3'	Anczuków et al., 2012
MSTR1 (RON)	F: 5'-CCTGAATATGTGGTCCGAGACC-3' R: 5'-CTAGCTGCTTCCTCCGCCACC-3'	Anczuków et al., 2012
MCL-1	F: 5' - GGACACAAAGCCAATGGGCAGGT-3' R: 5' - GCAAAAGCCAGCAGCACATTCCTGA-3'	Gautrey et al., 2012
BIN1	F: 5'-CCTCCAGATGGCTCCCCTGC-3' R: 5'-CCCGGGGGCAGGTCCAAGCG-3'	Anczuków et al., 2012
B-ACTIN	F: 5' - CTACAATGAGCTGCGTGTGGC - 3' R: 5' - CAGGTCCAGACGCAGGATGGC - 3'	Xie et al., 2007