Effects of Moderate and Heavy Alcohol Exposure on Fetal Neural Stem Cell-Derived Extracellular Vesicles

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Introduction

Prenatal alcohol exposure (PAE) is the leading cause of neurodevelopment disability worldwide, commonly resulting in growth and neurobehavioral deficits. Neural stem cells (NSCs) are an important target of ethanol during the late first through second trimester, during the peak period of fetal neurogenesis. The NSC microenvironment is rich in sub-200 nanometer-sized extracellular vesicles (EVs), that may function as a mode of intercellular communication, transporting proteins, lipids, and RNAs between NSCs and their progeny. Using fetal mouse derived cortical neuroepithelium, cultured ex-vivo as non-adherent neurosphere cultures, we previously found that ethanol exposure resulted in significant elevation of miRNA cargo like miR-140-3p in EVs, which direct NSCs towards an aberrant lineage. EVs may amplifying PAE's temporal and spatial effects in the NSC niche, resulting in an overall decline in neurogenic capacity. For this study, we further investigated the impact of ethanol on the proteome of NSC-EVs by employing quantitative mass-spectrometry to profile the protein expression across alcohol-treated and control NSCs.

Ethanol-exposed NSCs significantly altered the profile of proteins packaged within EVs. Of the 3,617 consistently expressed EV proteins, moderate ethanol exposure (26mM) differentially regulated 65 proteins compared to controls, with >95% being upregulated, while heavy ethanol exposure (70mM) differentially regulated 108 proteins compared to controls, with ~66% being upregulated (Paired t-test, $p<0.05$; effect size, Cohen's $d>0.5$, $\alpha=5\%$, $1-\beta=0.8$). For cells, in contrast, out of 4,698 expressed proteins, moderate ethanol exposure differentially regulated 492 proteins compared to controls, with >92% being downregulated, while heavy ethanol exposure differentially regulated 750 proteins, with >95% again being downregulated. Due to this contrast of protein upregulation in EVs and downregulation in cells, expression of proteins that were significantly altered in EVs by ethanol exposure were compared to the expression of the same proteins in cells. For both moderate and heavy ethanol exposures, the majority of affected proteins were upregulated in EVs but downregulated in cells. Therefore, ethanol exposure results in increased loading of specific proteins into EVs, at the expense of their intracellular levels in NSCs.

Methods

Neurosphere Culture Model:
Gestational day 12.5-derived mouse cortical neurosphere cultures were propagated as non-adherent spheres. Neurospheres were subjected to four ethanol treatment conditions: 0 mg/dL, 60 mg/dl (13 mM),120 mg/dl (26 mM) or 320mg/dl (70 mM). Mitogen withdrawal driven differentiation was achieved by seeding neurospheres onto laminin coated cultureware.

Nanoparticle Tracking Analysis
The concentration and size of extracellular vesicles were measured by nanoparticle tracking analysis (Nanosight LM10; Malvern Panalytical; Westborough MA/USA).

Extracellular Vesicle Isolation:
Extracellular vesicle fractions were isolated from ethanol-treated and control neurospheres and cultures following an established differential ultracentrifugation protocol (Théry et al., 2006)

Western Blot
20ug of protein was size-fractionated on a 4-12% Bis-Tris Gel and blotted to a PVDF membrane. Membranes were subsequently probed with antibodies.

Transmission electron microscope (TEM):
EV samples were immunogold labeled with anti-CD63. TEM sample preparation and imaging was performed at the Texas A&M MIC.

Proteomic Analysis:
EV proteome was assessed by LC-MS/MS, then analyzed using Mascot (Matrix Science, London, UK; v2.6.0). Relative peptide/protein quantification were measured by Data-Independent Acquisition mass spectrometry. MS/MS based peptide/protein identifications were validated by Scaffold (Proteome Software Inc., Portland, OR; v4.8.5).

Statistical Analysis
Statistical analysis, student's t-test or one-way ANOVA with Tukey HSD, was conducted using GraphPad Prism version 6.00 for Windows.

Efficient Isolation Of NSC-Derived Extracellular Vesicles

EV & neurosphere probed for EV markers

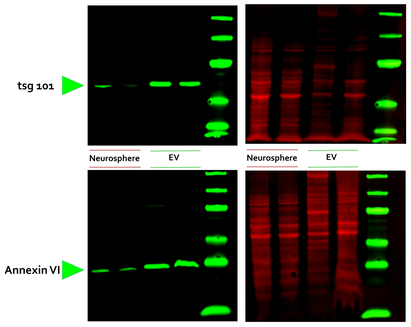
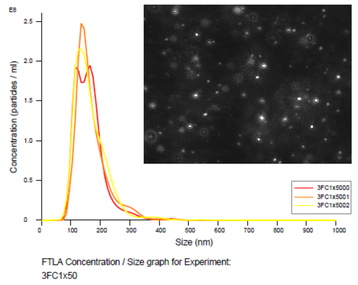
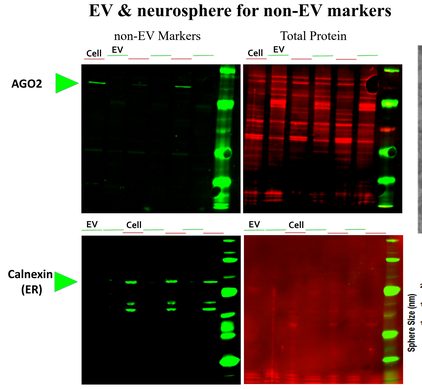


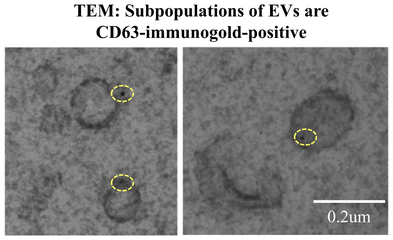
Image of 30-200 nm EVs and particles as captured by the NanoSight LM10 instrument



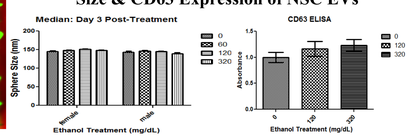
EV & neurosphere for non-EV markers



TEM: Subpopulations of EVs are CD63-immunogold-positive

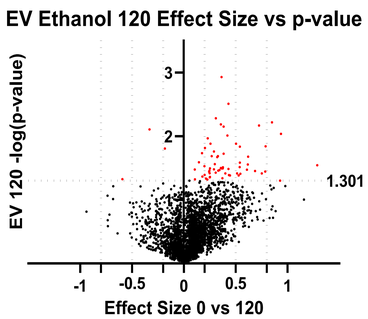


Size & CD63 Expression of NSC EVs

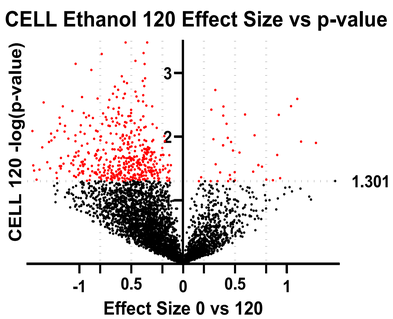


Ethanol Exposure alters the Proteomic Profile of NSC-EVs

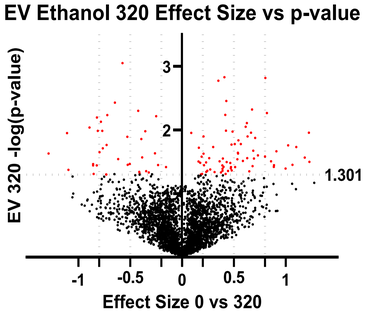
EV Ethanol 120 Effect Size vs p-value



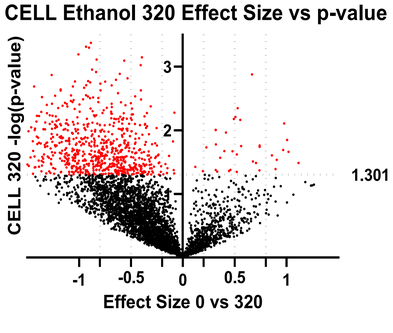
CELL Ethanol 120 Effect Size vs p-value



EV Ethanol 320 Effect Size vs p-value

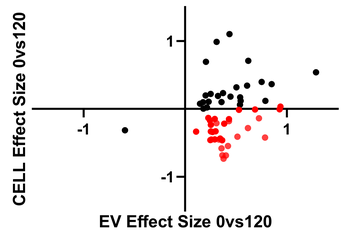


CELL Ethanol 320 Effect Size vs p-value

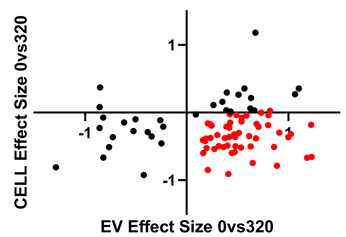


Effect Size Difference due to Ethanol: EV Proteins vs. Cell Proteins

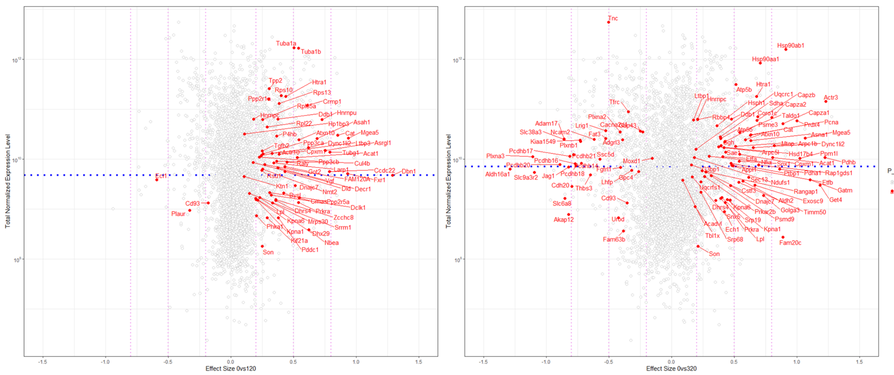
EV 120 Significant p-value Effect size EV vs Cell



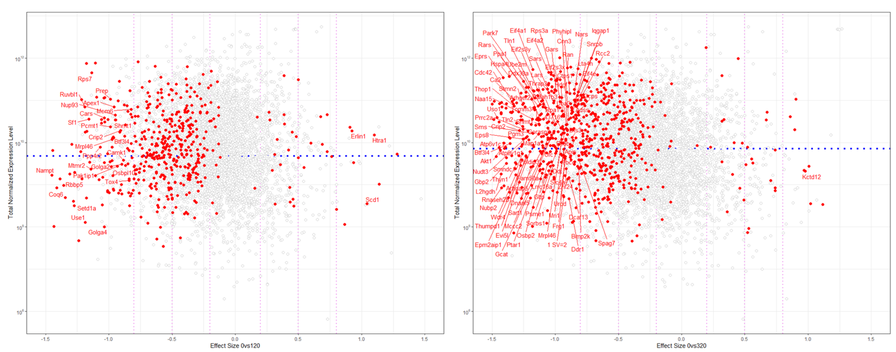
EV 320 Significant p-value Effect size EV vs Cell



Extracellular Vesicle Effect Size vs. Expression Level by Ethanol Dose



Cell Effect Size vs. Expression Level by Ethanol Dose



Discussion

- Neural stem cell-derived EVs contained proteins necessary for mRNA translation and signaling pathways, important biological processes that regulate NSC maturation.
- Ethanol significantly altered the protein content of EVs released by NSCs in location-dependent manner.
  - The ethanol dose increased ethanol-sensitive proteins in EVs
  - The ethanol dose decreased ethanol-sensitive proteins in cells
- At the expense of their intracellular levels in NSCs, ethanol exposure results in increased loading of specific ethanol-sensitive proteins into EVs; cells may be communicating within and outside of its own population group through EVs to influence neural behavior.

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