Evaluating Alcohol-Sensitive Proteins of Extracellular Vesicles Produced by Neural Stem Cells

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Introduction

Prenatal alcohol exposure (PAE) is the leading cause of neurodevelopment disability worldwide that can result in craniofacial deformities and growth deficiency. Neural stem cells (NSCs) are most vulnerable during the late first to second trimester, when they are most extensively involved in neurogenesis—the process where neurons are produced. Extracellular vesicles (EV) are sub-200nm intercellular complexes found amid the rich microenvironment of NSCs, facilitating the transportation of proteins, lipids, and RNA between cells.

Using cortical neuroepithelium derived from fetal mice, cultured non-adherent neurospheres where grown ex-vivo. We found there to be a significant increase in EV-miRNA content (miR-140-3p), associated with abnormal astroglial lineage maturation. To further examine ethanol's (EtOH) impact on NSC-derived EVs, quantitative proteomics was used to investigate protein expression on 18 EV samples and their 18 parent NSC samples.

Mass-spectrometric analysis identified proteins necessary for eukaryotic translation initiation, suggesting that EVs have the capacity to translate chaperoned mRNAs. The analysis of statistical and pathway overrepresentation indicates that moderate (~26mM) EtOH exposure facilitates a significant protein increase in the Nonsense-Mediated Decay (NMD) EV pathway, a surveillance pathway that prevents gene expression error by eliminating premature stop codon-containing mRNA transcripts. Thus, NMD proteins isolated in EV's are hypothesized to traffic neuroprotection to cells with depleted error-correcting protein translation. Furthermore, severe (~70mM) EtOH exposure results in EV overexpression of mitochondrial proteins constituting a Danger-Associated Molecular Pattern (mito-DAMP) pathway. Knowing that eukaryotic cells under stress discharge mitochondrial proteins, activating pattern recognition receptors, and pro-inflammatory responses in target cells, mitoDAMP's in EVs from ethanol-exposed NSCs are anticipated to spread inflammation between NSCs, jeopardizing development and differentiation.

These studies suggest that EVs are a unique means of communicating stress between cells in an ethanol-induced fetal NSC niche. Ongoing studies are focused on validating ethanol-induced alterations in EV's using western immunoblot assays.

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.

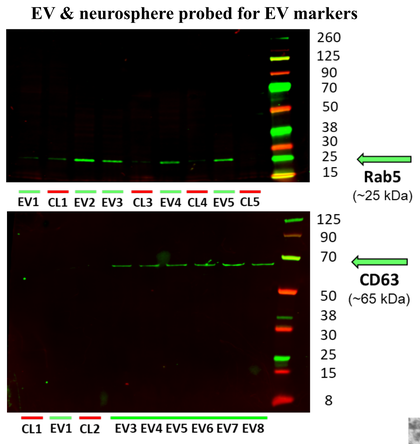
Transmission electron microscope (TEM):
EV samples were immunogold labeled with anti-CD63. TEM sample preparation and imaging was performed at the Texas A&M MIC.

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



EV & neurosphere for non-EV markers

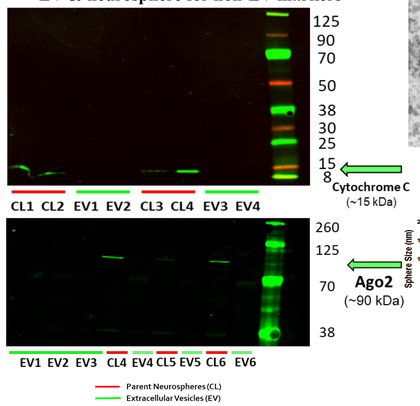
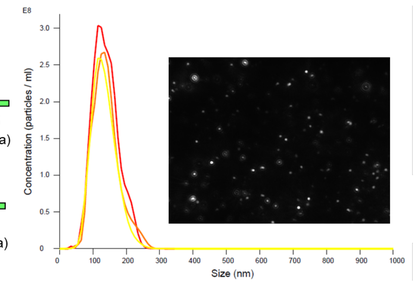
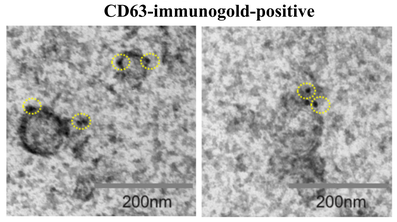


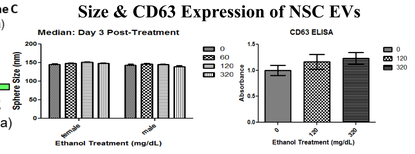
Image of 30-200 nm EVs and particles as captured by the NanoSight LM10 instrument



TEM: Subpopulations of EVs are CD63-immunogold-positive

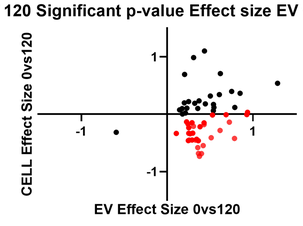


Size & CD63 Expression of NSC EVs

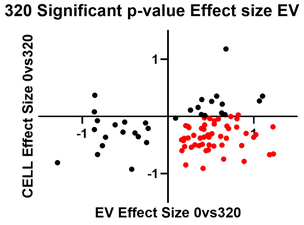


Effect Size Difference due to Ethanol: EV Proteins vs. Cell Proteins

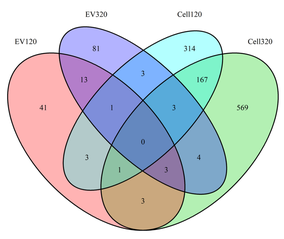
EV 120 Significant p-value Effect size EV vs Cell



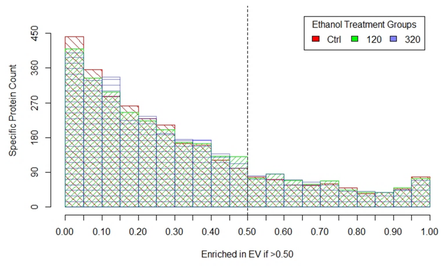
EV 320 Significant p-value Effect size EV vs Cell



Ethanol-Sensitive Proteins in EV and Cell by Treatment



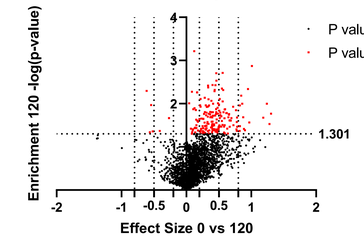
Distribution of EV Enrichment for Treatment Groups



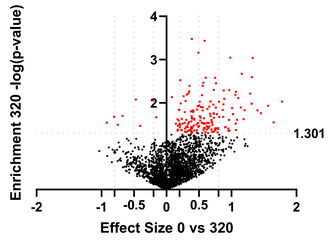
Supported by R01AA024659 and R01HD086765

Protein Enrichment Expression by Treatment

EV Enrichment 120
Effect Size vs p-value



EV Enrichment 320
Effect Size vs p-value



EV vs. Cell: Ethanol-sensitive Proteins

Group	Significant p-value	Upregulated	Downregulated	Negative Correlation EV vs. Cell
EV 0vs120	65	62	3	30
Cell 0vs120	495	37	458	NA
EV 0vs320	109	72	37	53
Cell 0vs320	751	36	715	NA

Sample Ethanol-sensitive EV Proteins
Exosome complex component RRP45
Heterogeneous nuclear ribonucleoprotein A1
60S ribosomal protein L12
60S ribosomal protein L23
Extracellular serine/threonine protein kinase FAM20C
DNA damage-binding protein 1
DNA-directed RNA polymerases I, II, and III subunit RPABC3
mRNA cap guanine-N7 methyltransferase
Fragile X mental retardation syndrome-related protein 2
Heterogeneous nuclear ribonucleoprotein U
Parkinson disease 7 domain-containing protein 1

Enriched Pathways relative to Controls

Group	Cluster Description	Count	p-value	FDR q-value
EV 120	Nonsense-Mediated Decay	5/113	9.67E-04	5.70E-02
EV 120	HSP90 Chaperone Cycle for Steroid Hormone Receptors	4/53	4.53E-04	5.70E-02
EV 320	Cellular Response to Stress	12/410	7.03E-05	8.00E-03
Cell 120	RNA Metabolism	68/567	1.08E-11	8.13E-09
Cell 320	Translation Initiation	30/117	1.86E-08	3.10E-06

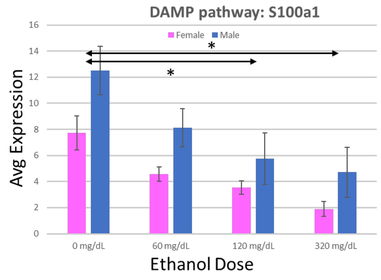
Pathway Overrepresentation Analysis

Tests of Between-Subjects Effects

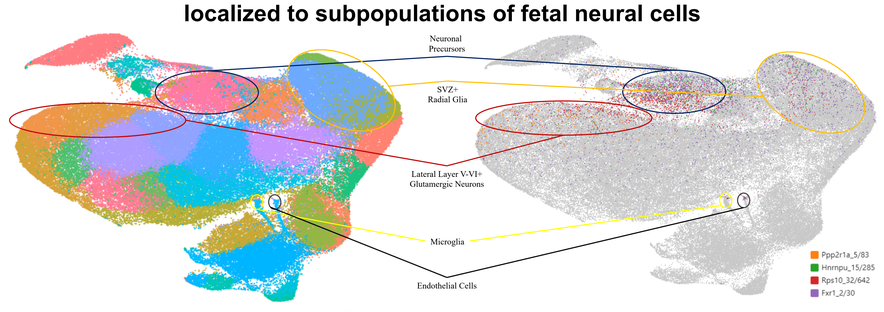
Source		Type III Sum of Squares	df	Mean Square	F	Partial Eta Squared
Treatment	Smg5	327.8153	3	109.2718	2.008058	0.200644
	Smg6	4.964765	3	1.654922	1.189528	0.129444
	Smg7	107.0366	3	35.67886	1.335498	0.143056
	Xrn1	72.86888	3	24.28963	0.555476	0.064926
	Upf2	42.85502	3	14.28501	0.911651	0.102299
	Upf3a	16.5129	3	5.504301	0.630393	0.073043
	Upf3b	81.56613	3	27.18871	0.277962	0.033579
	Upf1	20.25562	3	6.751873	1.163292	0.126951
	Smg1	16.70366	3	5.567887	0.524646	0.061545
	Etf1	1595.407	3	531.8025	0.803079	0.091227
	Gspt1	4586.861	3	1528.954	1.111923	0.122029
	Gspt2	1.140124	3	0.380041	0.571076	0.066628

Nonsense-Mediated Decay Pathway MANOVA on NSC RNAseq data

DAMP pathway: S100a1



scRNA seq: Ethanol-sensitive EV proteins localized to subpopulations of fetal neural cells



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 a dose-dependent manner.
  - The low ethanol dose increased EV proteins associated with 'nonsense-mediated decay'
  - The high ethanol dose increased EV proteins associated with 'response to stress'
- Ethanol-sensitive EV proteins' mRNA transcripts localized to subpopulation of cells in the developing cortex; cells communicating within and outside of its own population group through EVs to influence neural behavior.