Research article

Dose-related shifts in proteome and function of extracellular vesicles secreted by fetal neural stem cells following chronic alcohol exposure

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ABSTRACT

Accumulating evidence indicates that extracellular vesicles (EVs) mediate endocrine functions and also pathogenic effects of neurodevelopmental perturbagens like ethanol. We performed mass-spectrometry on EVs secreted by fetal murine cerebral cortical neural stem cells (NSCs), cultured ex-vivo as sex-specific neurosphere cultures, to identify overrepresented proteins and signaling pathways in EVs relative to parental NSCs in controls, and following exposure of parental NSCs to a dose range of ethanol. EV proteomes differ substantially from parental NSCs, and though EVs sequester proteins across sub-cellular compartments, they are enriched for distinct morphogenetic signals including the planar cell polarity pathway. Ethanol exposure favored selective protein sequestration in EVs and depletion in parental NSCs, and also resulted in dose-independent overrepresentation of cell-cycle and DNA replication pathways in EVs as well as dose-dependent overrepresentation of RNA processing and mTor stress pathways. Transfer of untreated EVs to naïve cells resulted in decreased oxidative metabolism and S-phase, while EVs derived from ethanol-treated NSCs exhibited diminished effect. Collectively, these data show that NSCs secrete EVs with a distinct proteome that may have a general growth-inhibitory effect on recipient cells. Moreover, while ethanol results in selective transfer of proteins from NSCs to EVs, the efficacy of these exposure-derived EVs is diminished.

1. Introduction

The developing cerebral cortical ventricular (VZ) and subventricular (SVZ) zones generate most of the projection neurons of the adult cerebral cortex during a restricted developmental window, corresponding to the latter half of gestation in mouse, and to the mid-first through second trimester in humans [1]. The generation of neurons from neural stem cells (NSCs) during this critical period can be disrupted by the presence of chemical perturbagens in the maternal-fetal environment, and may lead to, for example, diminished neuron number, microencephaly, and intellectual disability. Alcohol (ethanol) exposure during pregnancy is an example of an important, health-relevant perturbagen, since ~9.8 % or more of all pregnancies globally are exposed to alcohol [2]. Ethanol is the causal agent in a spectrum of brain and craniofacial anomalies and growth deficits that are collectively termed Fetal Alcohol Spectrum Disorders (FASD) [3], which are prevalent with between 3.1 and 9.9 % of school-aged children in the US [4]. Studies in human populations [5, 6, 7, 8] and in animal models [9, 10, 11] have shown that developmental alcohol/ethanol exposure can result in decreased brain size or microencephaly. Moreover, previous studies document that fetal NSCs in developing regions like the cerebral cortex do not die following ethanol exposure, but rather undergo a proliferative burst of rapid transit amplification and premature maturation resulting in the depletion of NSCs [12, 13, 14, 15, 16, 17, 18, 19, 20, 21].

Recent studies from our group suggest that some of the aberrant prematuration effects of ethanol may be mediated by microRNAs (miRNAs) secreted by NSCs within a class of small membrane-bound extracellular vesicles (EVs) [22, 23]. EVs are secreted by most cell types and are thought to participate in paracrine and endocrine transfer of proteins, lipids, DNAs, and RNAs to other cells, without any direct cell-to-cell contact [24, 25, 26, 27, 28, 29, 30]. The existence and functions of EVs were first discovered in the mid-20th century as procoagulant platelet-derived particles that could be isolated by ultracentrifugation from plasma [31] and as lipid-enriched particles [32]. Interest in EVs was amplified by discoveries in 2006–2007 that these particles could transfer RNAs between cells as a paracrine or endocrine signal [33, 34]. EVs have
significant value as biomarkers, but may also be therapeutic vehicle for disease management [35]. For instance, EVs have been identified as intercellular signaling mediators in osteosarcoma and mesenchymal stromal cell niches, where they modulate a variety of regulatory pathways [36, 37, 38]. Several studies have found that the miRNA and protein contents of EVs can be taken up, and influence a recipient cell’s physiology [39, 40, 41, 42, 43, 44].

In our previous studies, we reported that ethanol-induced EV miRNAs such as miR-140-3p could mediate aberrant astroglial differentiation, while suppressing neuronal and oligodendroglial lineage markers [23], and retroviral-like proteins present in EVs, like PEG10 and FNM2A, could lead to apoptosis-resistance [22]. Moreover, EV reprogramming has been implicated in microglial activation and inflammation-mediated loss of developing hypothalamic neurons [45], and in the pathogenesis of alcohol-associated liver disease [46, 47], suggesting that EVs are a component of a broader cellular and tissue sensitivity to this common perturbagen. These observations advance a novel possibility that EVs secreted by NSCs may reflect NSC reprogramming by the environment and, consequently, transfer the effects of a perturbagen between cells, thereby contributing to the spread and persistence of deleterious effects within the neurogenic niche.

In our studies reported here and previously [23], we focus on EVs secreted by neural stem cells dissected from sex-specific fetal mouse iso-cortex and maintained ex vivo, as neurosphere cultures to model the early period of cortical plate neurogenesis. Assessed EVs are less than 200 nm in diameter, with a median size of 150 nm, a population that may include both exosomes and microvesicles, and express known markers for exosomes. We previously reported that ethanol exposure did not significantly change the concentration and size of EVs secreted by NSCs, nor did exposure influence the expression of at least one key EV marker, CD63 [23]. In the current study, we therefore assessed whether ethanol exposure altered the protein cargo of EVs relative to their parental NSCs. We used mass spectrometric analysis of proteins from EVs and their parental NSCs, from both male and female mouse fetuses, to assess the contribution of both genetic sex and ethanol dose on protein abundance in EVs. Exploratory weighted gene co-expression network analysis (WGCNA) identified candidate proteins and gene networks that were drivers of alterations to the proteomes. These analyses showed that the EV proteome is substantially different from the proteome of parental cells, with ethanol exposure and genetic sex as additional contributory factors to modulation of the EV proteome. EVs were also found to sequester peptides for proteins from every parental cell sub-compartment, from nucleus to cytoplasm to membrane. Surprisingly, we identified proteins, from every cellular sub-compartment, that were enriched in EVs compared to their cell of origin, suggesting that these proteins, collectively serving developmental programs, were preferentially sequestered into EVs. Ethanol exposure, in a dose-dependent manner, resulted in a preferential enrichment of specific proteins into EVs, as these proteins were decreased intracellularly and increased in EVs. This suggests a perturbagen-dependent trafficking of proteins from cells to EVs. A moderate dose of ethanol resulted in preferential enrichment of proteins in EVs related to ribosomal RNA processing and cell cycle, whereas a high dose of ethanol resulted in EV enrichment of proteins associated with mTOR activation due to amino acid deprivation and DNA replication. These data suggest the presence of a systemic cell stress response in NSCs that results in the transfer of networks of related proteins into EVs in a dose- and sex-dependent manner.

2. Results

2.1. Fetal mouse neural stem cells secrete an abundant amount of extracellular vesicles

The heterogeneity of EVs reported in the literature, likely reflecting the molecular diversity of their parental cells, as well as the heterogeneity in technical approaches for EV isolation [48], caused us to define EVs by their size, density, biochemical composition, and cell of origin type. Therefore, in this study, we ultra centrifuged EV population was defined as female or male mouse fetal NSC-derived, obtained under either control or ethanol-exposed conditions (Figure 1A,B). Transmission electron microscopy showed that the NSC-derived EVs were round to oval in shape and were CD63+ as indicated by immuno-gold labeling localized to the EV surface (Figure 2A). Nanoparticle tracking analysis (NTA) showed that after 72 h, culture-conditioned media contained approximately ~10^5 EV per million seeded cells, or a net release of ~10–14 EVs/hour/seeded cell, ranging in diameter from 50–200 nm with a median diameter of 150 nm, consistent with the known size range for exosomes (Figure 2B) [29, 49]. Immunoblot analyses demonstrated that, compared to parental cells, the EV fraction from our NSC supernatant was enriched in transmembrane and cytosolic proteins commonly used as EV markers: CD9, CD63, Tsg101, annexin VI, and Rab5 (Figure 2C,E and Supplementary Figure 1) [49]. In contrast, proteins such as calnexin, cytomega, and Ago2 which are negative EV markers were present in our parental NSCs but absent or detected at low levels in our EVs (Figure 2D,F and Supplementary Figure 2). A third group of markers, PBP2B1A, VCA01M, TIM50, and NduS1, was selected based on our mass spectrometric evidence for their presence in both NSCs and their secreted EVs. In all cases, immuno-reactive bands of identical molecular weight were observed in both EV and cell samples (Supplementary Figure 3), suggesting that full-length proteins from cells are packaged into EVs. These data also collectively show that our isolation method is efficient in collecting CD63+/CD9+/TSG101+ EVs from NSCs, while eliminating non-EV fractions and other cell contaminants.

2.2. Baseline differences in the proteome of EVs and their parent cell-of-origin NSCs

Using data-independent acquisition mass spectrometry for global protein identification and relative quantification, there were 4703 unique proteins in EVs and 6262 unique proteins in cells (Supplementary Table 1–3). Of these, 2765 proteins were identified in all 18 EV samples and were used for further bioinformatic analysis (Supplementary Table 4). Abundance values were imputed for proteins that were present in EVs but undetectable in parental cells in order to compare protein levels in EVs to the parent cell-of-origin. Abundance values for each protein were z-score transformed across samples (Supplementary Table 5).

2.2.1. Weighted gene co-expression network analysis to compare the proteome of EVs to parental cells

We matched protein names (UniProt ID) to their corresponding gene IDs (ENTREZ ID), used WGCNA to construct gene co-expression networks to identify clusters (modules) of interconnected genes and the hub genes within each module, and performed hierarchical clustering using Pearson correlation. All 36 samples including EV and parental NSC samples were included in this analysis. Five distinct modules were identified; the brown, yellow, blue, green, and turquoise modules (colors for module labeling purpose) contained 170, 120, 545, 82, and 1698 correlated genes (ME; 1st principal component of a given module) with traits (Location: EV vs. cell, pregnancy biological replicates denoted as set numbers, sex, and ethanol treatment), we identified modules that are significantly associated with sample traits by correlating module eigengenes (ME; 1st principal component of a given module) with traits (Figure 3C). We observed that there was a significant correlation between all MEs and sample trait of Location (EV_Cell), with eigengenes of

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blue \( (r = 0.93, p = 5 \times 10^{-16}) \), green \( (r = 0.91, p = 3 \times 10^{-14}) \), yellow \( (r = 0.65, p = 2 \times 10^{-05}) \), and brown \( (r = 0.51, p = 0.002) \) modules having significant positive correlations with Location (i.e., higher in EVs relative to parental NSCs) while eigengene of turquoise \( (r = -0.98, p = 5 \times 10^{-25}) \) module had a significant inverse correlation with Location (i.e., higher in parental NSCs relative to EVs). For sample trait of sex, only eigengenes of the brown module had a significant positive correlation \( (r = 0.52, p = 0.001) \).

In preparation for enrichment analysis, to confirm the significant correlation between modules and the sample trait Location, we examined the correlation of MEs with Location (Supplementary Figure 4A), Location trait-based average protein/gene significance (GS) across modules (Supplementary Figure 4B), and the correlation between GS and module membership (Supplementary Figure 4C–G). GS for EV correlated with module membership in the blue module \( (r = 0.72, p = 3.4 \times 10^{-08}) \), green module \( (r = 0.89, p = 5.2 \times 10^{-29}) \), yellow module \( (r = 0.64, \ldots) \).
EV and Cell, suggesting proteins loaded into EVs have a different profile of protein localization and transport, respectively. Collectively, through WGCNA of protein localization and transport, Ras protein signal transduction, immune response regulation, and proteins related to negative regulation of cell development and differentiation, blue, green, yellow, and brown, were highly enriched for proteins (Supplementary Table 7). These analyses indicated that the EV-enriched proteome is significantly more important than the parental proteome in terms of module identity, with the turquoise module identifying cells, and the yellow, brown, blue and green modules identifying EVs.

2.2.2. EVs are enriched for proteins from each sub-cellular compartment of parental NSCs

Using a reference proteome database (UniProt Knowledgebase) [50], proteins were characterized by their preferential localization within the cell to six subcellular compartments: the nucleus, cytoplasm, mitochondria, lysosomes, endosomes, and membrane. We found that proteins from each of these subcellular compartments were also localized to EVs. Moreover, we observed a number of proteins from each sub-cellular compartment (Nucleus (Figure 4A), Cytoplasm (Figure 5A), Mitochondria (Figure 6A), Lysosome (Figure 7A), Endosome (Figure 8A), Membrane (Figure 9A)) that were, surprisingly, enriched in EVs relative to their parental NSCs in Nucleus (Figure 4C vs. 4D), Cytoplasm (Figure 5C vs. 5D), Mitochondria (Figure 6C vs. 6D), Lysosome (Figure 7C vs. 7D), Endosome (Figure 8C vs. 8D), Membrane (Figure 9C vs. 9D). For protein list associated with each subcellular compartment, see Supplementary Table 8.

Two-way ANOVA with FDR correction (a = 0.05) followed by a Sidak’s Test for post-hoc multiple comparisons was conducted to examine global differences in average z-score transformed relative protein abundance in EV compared to parental NSCs. We found a significant interaction effect between EV vs. cell samples and female vs. male samples for many subcellular compartments (Nucleus: F(1, 32) = 15.09, p.adj = 0.002; Cytoplasm: F(1, 32) = 8.325, p.adj = 0.010; Mitochondria: F(1, 32) = 4.934, p.adj = 0.034; Lysosomes: F(1, 32) = 13.969, p.adj = 0.002; Endosomes: F(1, 32) = 7.527, p.adj = 0.012; Membrane: F(1, 32) = 8.587, p.adj = 0.010) (Supplementary Table 9). A Sidak’s post-hoc test revealed significant pairwise mean differences (Diff) in average z-score between female-derived NSCs vs. EVs and male-derived NSCs vs. EVs, with a positive Diff indicating overall enrichment in EVs compared to cell (Nucleus: female: Diff = 0.56, p.adj < 0.0001, male: Diff = 0.95, p.adj < 0.0001

Figure 3. WGCNA-based Comparisons of the Protein Content of EVs Relative to Parental NSCs. WGCNA of proteins expressed in all 36 samples, shows that EVs and their parent cells have distinct protein networks and correlations with the trait of “location” (Cell vs. EV) contributing to the majority of the difference between clusters. Other traits including “pregnancy” (cells derived from three separate pregnancies), fetal sex (female or male), and “alcohol” (ethanol exposure at 0, 120 or 320 mg/dL) were smaller contributors to the overall composition of identified networks. 3A) Topological overlap matrix (TOM) plot for visualizing the weighted gene (protein) co-expression network, where the topological overlap considers each pair of proteins’ similarity in relation to all other proteins in the network. Modules are defined by hierarchical clustering, visualized by dendrograms and module color notation. Each row or column corresponds to a single protein, where lighter red denotes low topological overlap and darker red denotes high topological overlap. 3B) Multidimensional scaling (MDS) plot showing high gene overlap and similarity across brown, yellow, blue, and green modules, while the turquoise module, containing a majority of proteins, did not exhibit overlap or similarity to other modules. 3C) Heatmap of WGCNA module significance (MS) correlations with sample traits. In the rows, modules are shown named by their corresponding colors and across the columns are the sample traits of interest. Numbers in the table correspond to the correlation coefficients between the module eigengene (ME) and the specific trait, with p-value in parentheses. The degree of correlation is illustrated with the color legend; the more intense the box color, the more positively (red) or negatively (blue) correlated is the module with the trait. The results of this analysis show that location (cell vs. EV) is the most important determinant of module identity, with the turquoise module identifying cells, and the yellow, brown, blue and green modules identifying EVs.

p = 3.6 × 10^{-18}, and brown module (r = 0.22, p = 0.0039), while GS for Cell correlated with module membership (MM) in the turquoise module (r = 0.98, p < 1 × 10^{-200}). This analysis suggests that proteins/genes highly significantly associated with EV samples were the most important element of the blue, green, and yellow modules, while those highly significantly associated with cell samples were the most important element of the turquoise module. Each protein/gene’s module color, GS to Location trait, and MM can be found in supplementary materials (Supplementary Table 6). Finally, enrichment analysis was performed for proteins/genes in all five modules to study biological mechanisms (Supplementary Table 7). These analyses indicated that the EV-enriched modules, blue, green, yellow, and brown, were highly enriched for proteins related to negative regulation of cell development and differentiation, Ras protein signal transduction, immune response regulation, and protein localization and transport, respectively. Collectively, through WGCNA, we observed that proteins grouped strongly by sample type of EV and Cell, suggesting proteins loaded into EVs have a different profile compared to protein constituents of the parental cells and may support distinct biological processes.

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Figure 4. Nucleus Subcellular Located Proteins in EVs and Cells. 4A) Volcano plot depicting relative abundance of nucleus-localized proteins (based on UniProt Knowledgebase), with the expression of individual proteins z-score transformed across all samples. 4B) Graph showing changes in average protein content as represented by the average z-score calculated for each sex and sample type (EV or cell). Pairwise analysis showed that, as predicted, cells contain more total nucleus-localized protein compared to EVs, ****p < 0.0001; n = 9 for each group. 4C) Top EV-enriched nucleus-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples. 4D) Top cell-enriched nucleus-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples.

Figure 5. Cytoplasm Subcellular Located Proteins in EVs and Cells. 5A) Volcano plot depicting relative expression of cytoplasm-localized proteins (based on UniProt Knowledgebase), with the expression of individual proteins z-score transformed across samples. 5B) Graph showing changes in average protein content as represented by the average z-score calculated for each sex and sample type (EV or cell). Pairwise analysis showed that, as predicted, cells contain more total cytoplasm-localized protein compared to EVs, ****p < 0.0001; n = 9 for each group. 5C) Top EV-enriched cytoplasm-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples. 5D) Top cell-enriched cytoplasm-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples.

(Figure 4B): Cytoplasm: female: Diff = 0.45, p.adj < 0.0001, male: Diff = 0.80, p.adj < 0.0001 (Figure 5B); Mitochondria: female: Diff = 1.11, p.adj < 0.0001, male: Diff = 1.34, p.adj < 0.0001 (Figure 6B); Lysosome: female: Diff = -0.14, p.adj = 0.63, male: Diff = 0.71, p.adj = 0.0002 (Figure 7B); Endosome: female: Diff = -0.37, p.adj = 0.063, male: Diff = 0.27, p.adj = 0.21 (Figure 8B); Membrane: female: Diff = -0.33, p.adj =
Figure 6. Mitochondria Subcellular Located Proteins in EVs and Cells 6A) Volcano plot depicting relative expression of mitochondria-localized proteins (based on UniProt Knowledgebase), with the expression of individual proteins z-score transformed across samples. 6B) Graph showing changes in average protein content as represented by the average z-score calculated for each sex and sample type (EV or cell). Pairwise analysis showed that, as predicted, cells contain more total protein compared to EVs; ****p < 0.0001; n = 9 for each group. 6C) Top EV enriched mitochondria-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples. 6D) Top cell enriched mitochondria-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples.

Figure 7. Lysosome Subcellular Located Proteins in EVs and Cells 7A) Volcano plot depicting relative expression of lysosome-localized proteins (based on UniProt Knowledgebase), with the expression of individual proteins z-score transformed across samples. 7B) Graph showing changes in average protein content as represented by the average z-score calculated for each sex and sample type (EV or cell). Pairwise analysis showed that, contrary to predictions, female fetal NSC-derived EV's expressed equivalent levels of lysosome-localized proteins compared to parental NSCs, whereas male NSC-derived EVs contained a smaller quantity of lysosome-localized proteins compared to parental NSCs. ***p < 0.001; ns, not significant; n = 9 for each group. 7C) Top EV enriched lysosome-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples. 7D) Top cell enriched lysosome-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples.
proteome. Differences in the average z-score between EV and cell samples by subcellular location suggest that the EV proteome varies from the cell proteome. The expression of individual proteins z-score transformed across samples. 8B) Graph showing changes in average protein content as represented by the average z-score calculated for each sex and sample type (EV or cell). Pairwise analysis showed that, contrary to predictions, male fetal NSC-derived EV's are involved in planar cell polarity for cell migration [53, 54] and in the regulation of RUNX3 expression and activity pathway, both involved in intracellular and intercellular communication as signal transducers [51, 52], were also significantly overrepresented. Overall, our data show that, in addition to proteins that are important for EV biogenesis, proteins enriched in EVs may serve as cell-to-cell signal transducers to regulate cell fate, proliferation, and migration during fetal development.

2.3. Ethanol exposure differentially alters protein networks in EVs compared to parental cells

Ethanol exposure differentially alters protein networks in EVs compared to parental cells. For the expressed equivalent levels of endosome-localized proteins compared to parental NSCs, whereas female NSC-derived EVs exhibited a trend (p = 0.06) towards a larger quantity of endosome-localized proteins compared to parental NSCs (n = 9 for each group). 8C) Top EV enriched endosome-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples. 8D) Top cell enriched endosome-localized proteins with nonoverlapping 95% confidence estimates between EV and cell samples.

Figure 8. Endosome Subcellular Located Proteins in EVs and Cells 8A) Volcano plot depicting relative expression of endosome-localized proteins (based on UniProt Knowledgebase), with the expression of individual proteins z-score transformed across samples. 8B) Graph showing changes in average protein content as represented by the average z-score calculated for each sex and sample type (EV or cell). Pairwise analysis showed that, contrary to predictions, male fetal NSC-derived EV's are involved in planar cell polarity for cell migration [53, 54] and in the regulation of RUNX3 expression and activity pathway, both involved in intracellular and intercellular communication as signal transducers [51, 52], were also significantly overrepresented. Additionally, the planar cell polarity/convergent extension (PCP/CE) pathway and the regulation of RUNX3 expression and activity pathway, are involved in planar cell polarity for cell migration [53, 54] and in neural stem cell proliferation and differentiation [55], respectively, were significantly overrepresented. Our previous transcriptomic studies also suggested that developmental ethanol exposure can disrupt entire gene networks [12, 59]. We therefore used WGCNA to assess the contribution of ethanol exposure to alteration of protein networks in both parental NSCs and their secreted EVs. We classified samples by location (EV vs. cell) and then sub-classified by ethanol treatment (0, 120, and 320 mg/dL), and constructed WGCNA analyses for each of the resulting 6 groups (Figure 11A). The TOM heatmap plots and module counts can be viewed as an index of biological complexity. A chi-square test was performed on the ratio of modules in EVs relative to cells to examine the relationship between ethanol treatment and number of modules across locations (EV vs. cell), using the control values as percent expected (Figure 11B). The number of modules in EVs relative to cells was significantly increased in the high ethanol-exposure group compared to controls ($X^2 (1) = 18.316, p < 0.0001$) and trended towards a significant increase for the medium exposure group relative to controls ($X^2 (1) = 3.247, p = 0.072$). Though EV samples exhibited less overall complexity compared to parental cell samples, ethanol treatment resulted in a dose-related increase in the complexity of EV samples, without overall changes in the protein network complexity in parental NSCs.

2.4. Ethanol effects on proteins and on the enrichment of proteins in EVs relative to parental cells

We next examined the effects of ethanol, a salient developmental perturbagen, on the proteome of NSCs and their EVs. NSCs exposed to moderate (120 mg/dL) and heavy (320 mg/dL) ethanol exhibited a significantly altered profile of proteins packaged within EVs. For the...
2765 proteins expressed in all 18 EV samples, moderate and heavy ethanol exposure altered the relative abundance of 68 and 110 proteins (paired t-test, p < 0.05), respectively, not accounting for FDR correction of multiple comparisons (Figure 12A,B and Supplementary Figure 6A,B and Supplementary Table 12). We observed that, for the proteins in EVs whose relative abundances were altered, 44 proteins (~65%) following heavy EtOH exposure and 47 proteins (~43%) following moderate EtOH exposure whose relative abundances were altered, 44 proteins (~65%) following moderate EtOH exposure and 284 proteins (~83%) in the heavy exposure group and 284 proteins (~83%) in the heavy exposure group, respectively, and the majority of them, 175 proteins (~78%) in the control group, were significantly increased relative to controls. In contrast, for cells, moderate and heavy EtOH exposure altered 223 and 343 proteins, respectively, and the majority of them, 175 proteins (~78%) in the moderate exposure group and 284 proteins (~83%) in the heavy exposure group, were at significantly lower abundance (Figure 12C,D and Supplementary Table 13).

In assessing the contrasting effect of ethanol, i.e., proteins at relatively higher abundance in EVs and lower in cells, we hypothesized that ethanol may have increased the transfer of specific proteins into EVs. To test this possibility, we assessed the effects of ethanol on the enrichment of proteins in EVs by calculating the EV to cell protein expression ratio (EV/cell) for each treatment group, followed by a paired comparison of the EV/cell ratio between the control group and each treatment group.

For moderate ethanol exposure, the EV/cell ratio was significantly increased for ~77% of the differentially altered proteins (44 proteins out of 57) relative to controls (Figure 12E and Supplementary Table 14). Likewise, for heavy ethanol exposure, the EV/cell ratio was significantly altered for 58 proteins, with 51 (~88%) significantly increased relative to control (Figure 12F and Supplementary Table 15). We observed that both doses of ethanol consistently increased the EV/cell ratio for a number of specific proteins. Volcano plots of EV/cell enrichment effect size, g, vs. p-value (Figure 12E,F) further support our observations that ethanol exposure results in EV enrichment and/or cellular depletion of a selected group of proteins. These data imply that ethanol results in the transfer of specific proteins from parental NSC to secreted EVs.

2.5. Pathway analyses of ethanol-sensitive proteins

To determine whether proteins whose relative abundances were enriched in EVs but decreased in cells, due to ethanol exposure, collectively served shared biological functions, we subjected the enriched proteins to pathway analysis. This analysis of proteins that reached criteria for EV/Cell enrichment (paired t-test, p < 0.05; Hedges’ g > 0.4, with non-zero containing 95% confidence estimate) identified significant biological pathways (Figure 13A, B and Supplementary Table 16–19). For moderate ethanol exposure, pathways associated with rRNA processing were over-represented. Cell cycle-related pathways including DNA replication and G1/S phases and transition, aligning with previously published literature on the developmental effects of ethanol exposure on cell cycle [19, 60], were also significantly overrepresented (Supplementary Table 16,17). Nonsense mediated decay (NMD), an error surveillance pathway that eliminates premature stop codon-containing mRNA transcripts among other functions [61, 62, 63], was also significantly overrepresented. In the case of heavy ethanol exposure, surprisingly, a criterion cutoff of Hedges’ g > 0.4 did not result in the identification of enriched pathways. However, increasing stringency to Hedges’ g > 0.5 did result in the identification of stress and amino-acid deprivation pathways related to mTOR signaling, that have previously been implicated in the pathogenesis of prenatal ethanol exposure [64, 65], as well as RHO GTPase signaling, an important inhibitor of NSC differentiation [66] (Supplementary Table 18). Additional network analyses (Figure 13C,D) document the contribution of individual proteins to core pathways.
overrepresented pathways. Overall, our data show that medium and high levels of ethanol exposure result in a dose-dependent preferential transfer of distinctly different classes of protein, that are a part of largely nonoverlapping biological pathways, from parental NSCs to their secreted EVs.

2.6. scRNA-seq analysis of fetal ventricular zone cells and ethanol-sensitive EV proteins

We next wanted to determine the identity of cell-types, in vivo, within the developing cerebral cortex, that were likely contributors to the increased ethanol-dependent sequestration of proteins within EVs. We reasoned that the cell subtype identity would also include mRNA transcripts for ethanol-sensitive EV proteins. Using our previously published scRNAseq dataset (GSE158747) of GD14.5 fetal mouse cerebral cortical cells (Figure 14) [12], we investigated the transcript expression of upregulated ethanol-sensitive EV enriched proteins with \( g > 0.4 \) effect size for moderate (120 mg/dL) (Figure 14C) and heavy (320 mg/dL) (Figure 14D) ethanol exposures to identify potential parental cells-of-origin within the fetal neurogenic niche (Supplementary Table 13,14). We extracted cells belonging to previously identified VZ, SVZ, and transit progenitor cells (TPC) clusters (Figure 14A,B) [22], since these were the most similar cell-types to those contained in our neurosphere model. We found that the selected mRNA transcripts for EV-enriched proteins were not globally or uniformly expressed throughout the neurogenic niche, but that, collectively, transcript expression was most abundant in the fetal mouse cortical VZ (Figure 14E,F). These data indicate that, among the different cell type subpopulations in VZ, SVZ, and TPC clusters, stem cells in the fetal VZ are the principal cells-of-origin for proteins that are transferred to EVs following ethanol exposure.

2.7. Assessment of function of EVs derived from NSCs

Since EVs are hypothesized to serve as an endocrine signal, we next examined whether NSC-derived EVs are actively taken up by cells. We found that purified EVs, directly labeled with the fluorescence reporter, MemBrite, and re-purified to eliminate free dye, were taken up by naive NSCs and could be detected within the cytoplasm of recipient NSCs, suggesting that they were internalized by endocytosis (Figure 15A). We compared the number of labeled cells by EV uptake to a positive control, directly labeled NSCs (Supplementary Figure 7), and negative control, NSC exposure to culture medium that was mixed with MemBrite and then processed through the same purification steps as used for labeling EVs (Supplementary Figure 8). In the latter instance, no residual fluorescence was observed in recipient cells, supporting the specificity of the labeling process for EVs. Flow cytometric analysis of label-positive NSCs indicates that ~31% of NSCs incorporate labeled EVs following incubation (Figure 15B) and that the amount of directly labeled cells was comparable to that seen with cells that uptake labeled EVs, indicating robust uptake of EVs in our neurosphere cultures.

We next assessed the effects of inhibiting the biogenesis and/or release of EVs on oxidative metabolic activity and glycolysis in NSCs (Supplementary Figure 9), using three separate EV inhibitors, GW4869 (10 \( \mu M \)), Manumycin-A (0.5 \( \mu M \)), and Calpeptin (90 \( \mu M \) [67]). Repeated-measures one-way ANOVA with Geisser-Greenhouse correction, followed by a Dunnett's Test for post-hoc comparisons, indicated that there was a statistically significant effect of EV inhibition on
the appearance of neuronal markers and loss of NSC markers, as we have morphological transformation of NSCs into migratory bi-polar cells, and extracellular matrix adhesion. Over 72 h, this paradigm results in the withdrawal model with the provision of laminin to promote cell-study, NSCs were transformed into EDCs using a standard mitogen-differentiating neural cells (EDCs) (Supplementary Figure 10). For this effects of adding EVs, derived from naïve NSCs, to naïve recipient early in neural progenitors, the possibility exists that EVs secreted by NSCs may drugs to inhibit EV biogenesis (Figure S9B).

Finally, since the fetal VZ/SVZ includes both NSCs and maturing neural progenitors, the possibility exists that EVs secreted by NSCs may influence the biology of more mature cells. We therefore examined the effects of adding EVs, derived from naïve NSCs, to naïve recipient early differentiating neural cells (EDCs) (Supplementary Figure 10). For this study, NSCs were transformed into EDCs using a standard mitogen-withdrawal model with the provision of laminin to promote cell-extracellular matrix adhesion. Over 72 h, this paradigm results in the morphological transformation of NSCs into migratory bi-polar cells, and the appearance of neuronal markers and loss of NSC markers, as we have previously published [14, 17]. Paired samples t-tests were conducted to examine the effect of NSC-derived donor EVs on recipient naïve EDCs oxidative metabolic activity and glycolysis, while unpaired samples t-tests were used for gene expression of neuronal markers. NSC-EV exposure significantly decreased oxidative metabolic rate in naïve recipient EDCs (Diff = -0.1942, p = 0.0002, compared to the control EDCs that received no exogenous EVs (Figure S10A). In contrast, NSC-EV exposure significantly increased the rate of glycolysis in naïve recipient EDCs (GW4869: Diff = -0.3106, p = 0.0035; Calpeptin: Diff = -0.3958, p = 0.0587), compared to the control NSCs that received no drugs to inhibit EV biogenesis (Figure S9B).

2.8. Effects of exposing naïve NSCs to EVs secreted by ethanol-treated donor NSCs

REDOX, glycolysis, and apoptosis: We next assessed the biological functionality of EVs derived from ethanol-treated NSCs (0, 120, 320 mg/dL), by transferring these purified EVs to naïve recipient NSCs (Figure 16). The sex of the donor-derived EVs and naïve recipient NSCs was matched. Repeated-measures two-way ANOVA with Geisser-Greenhouse correction, followed by a Dunnett’s or Tukey’s Test for post-hoc comparisons, was conducted to examine the effect of both treatment and sex on recipient naïve NSCs’ oxidative metabolic activity, glycolysis, and apoptosis (Figure 16A-C). There was a statistically significant main effect of EV addition on oxidative metabolic activity (F(1,1788) = 8.451, p = 0.0016), while there was no significant mean difference (Diff) for sex or an interaction effect between EV addition and sex. The Dunnett post-hoc test showed that purified EVs, regardless of the donor source, significantly decreased oxidative metabolic rate in recipient NSCs (EV 0: Diff = -0.3660, p = 0.0001; EV 120: Diff = -0.2417, p = 0.0005; EV 320: Diff = -0.2426, p = 0.0007), compared to the control NSCs that received no exogenous EVs (Figure 16A). Moreover, there was no significant difference between the two control groups, i.e., NSCs that received filtered culture-conditioned media and NSCs that received neither purified EVs nor filtered culture-conditioned media (Diff = 0.0404, p = 0.9811, ns). A secondary analysis, comparing the
effects of ethanol treatment of donor EVs, did show that recipient NSCs exhibited a statistically significant and ethanol dose-related main effect on oxidative metabolism ($F_{(1,905,40,02)} = 4.400$, $p = 0.0202$), with a Tukey post-hoc test showing that the EV 320 group (exposed to EVs from donor cells treated with 320 mg/dL ethanol), had a significantly increased oxidative metabolic rate compared to the EV 0 group (exposed to EVs from control, untreated donors; Diff = -0.1234, $p_{adj} = 0.0345$), with an intermediate effect that trended towards significance for the EV 120 group (Diff = -0.1243, $p_{adj} = 0.0651$). In contrast to oxidative metabolism, there was no significant difference in the rate of glycolysis, either due to the introduction of EVs or recipient cells’ sex (Figure 16B), or caspase 3/7 activity due to EV addition (Figure 16C).

As a comparison, repeated-measures two-way ANOVA with Geisser-Greenhouse correction followed by Tukey’s post-hoc test was used to assess the effects of ethanol (0, 120, 320 mg/dL) on cellular oxidative metabolic activity, glycolysis, and apoptosis of NSCs (Figure 16D-F). There was a significant interaction between the effects of ethanol and sex on the oxidative metabolic rate ($F_{(2,36)} = 4.598$, $p = 0.0167$). A follow-up, sex-regressed analysis of the main effects of treatment showed that the high dose (320 mg/dL) of ethanol exposure significantly increased oxidative metabolic rate in female NSC samples compared to controls (Tukey’s post-hoc test, $p_{adj} = 0.0270$) and to the moderate dose (120 mg/dL) group ($p_{adj} = 0.0075$), but no effect of ethanol in NSCs derived from male fetal tissue (Figure 16D). For glycolysis, there was a statistically significant interaction between the effects of ethanol and sex on the cellular oxidative metabolic activity ($F_{(2,34)} = 3.705$, $p = 0.0350$). A post-hoc sex segregated analysis showed that medium and high dose of ethanol exposure significantly increased glycolysis rate in male NSC samples compared to controls (120 mg/dL: Tukey’s post-hoc test, Diff = 0.4059, $p_{adj} = 0.0283$; 320 mg/dL: Diff = 0.5647, $p_{adj} = 0.0056$), but not in female samples (Figure 16E). Ethanol exposure did not significantly alter caspase 3/7 activity, suggesting that exposure had no significant effect on apoptosis (Figure 16F). Therefore, changes in cellular oxidative metabolism and glycolysis were not due to apoptosis and neither EV addition nor ethanol exposure significantly affected programmed cell death.

Cell cycle: We further investigated the effects of EV transfer on cell cycle using flow cytometry. Using a two-way ANOVA, we found a significant main effect of EV addition ($F_{(3,66)} = 4.181$, $p = 0.0090$) and sex ($F_{(1,66)} = 8.122$, $p = 0.0058$) on the S-phase of cell cycle, compared to the control NSCs that were not exposed to purified EVs (Figure 16G,H). Dunnett post-hoc test showed that adding purified EVs to NSCs resulted in a significantly increased S-phase compared to control group (EV 0 group; $p_{adj} = 0.0075$).
in a significant decrease in the percentage of cells in S-phase (EV 0: Diff = -0.4 for moderate ethanol exposure (13A) and >-0.5 for heavy ethanol exposure (13B), a non-zero containing 95% confidence estimate and a significance of p < 0.05 by paired t-test. The size of each data point represents the number of genes/proteins in a pathway that were within this subset of proteins while color of data point denotes the FDR-corrected p-value for pathway overrepresentation. 13C,D) Plots of key enriched pathways and constituent proteins involved in these pathways, for moderate (13C) and heavy (13D) ethanol exposure. The size of each pathway element denotes the number of proteins in a pathway that were within that subset of pathway proteins, while the color of each element represents the effect size for that pathway component.

Figure 13. Pathway Analysis of Ethanol-Sensitive EV-Enriched Proteins 13A,B) Pathway overrepresentation plots for proteins that met the criteria for an effect size, >+0.4 for moderate ethanol exposure (13A) and >+0.5 for heavy ethanol exposure (13B), a non-zero containing 95% confidence estimate and a significance of p < 0.05 by paired t-test. The size of each data point represents the number of genes/proteins in a pathway that were within this subset of proteins while color of data point denotes the FDR-corrected p-value for pathway overrepresentation. 13C,D) Plots of key enriched pathways and constituent proteins involved in these pathways, for moderate (13C) and heavy (13D) ethanol exposure. The size of each pathway element denotes the number of proteins in a pathway that were within that subset of pathway proteins, while the color of each element represents the effect size for that pathway component.

3. Discussion

EVs have gained significant attention as a novel and potential class of endocrine mediators of information transfer between cells and tissues. While EVs have been studied most extensively in tumor microenvironments and in cancer progression [68, 69, 70, 71], they may also contribute to the physiology and pathogenesis of developmental disorders, including FASD. This study showed that NSCs that were micro-dissected from fetal mouse cerebral cortex and maintained ex vivo, as neurosphere cultures, are remarkably active and have a net release of ~10–14 EVs/hour/seeded parent cell. Moreover, our evidence shows that these EVs can be taken up by NSCs where they in contribute to the physiology and pathogenesis of developmental disorders, including FASD. To our knowledge, this is the first study to assess the global effects of developmental ethanol exposure on the proteome of EVs secreted by NSCs, and to show that ethanol exposure resulted in selective enrichment of specific proteins from each sub-cellular compartment into EVs at the expense of donor parent cells. This study is also to our knowledge, the first to explicitly assess the contribution of biological sex to the proteome of EVs, and to show, using WGCNA, that fetal sex was a significant contributor to at least one network of proteins expressed in EVs, specifically the brown module, though most protein networks were sex-independent.

Our data also show that the EV proteome is complex. We identified peptides for over 4,700 unique proteins in EVs representing ~75% of the parental NSC proteome. Moreover, the proteome of EVs is broadly conserved, since peptides from ~59% of EV-containing proteins were present in all analyzed samples. Among the proteins that were tested, Western blot analyses indicated they correspond to full-length proteins of the expected molecular weights (Supplementary Figure 1–3). In addition, EV samples had increased relative abundance for proteins involved in ESCRT endosome sorting mechanism, which plays a key role in exosome biogenesis [72]. Therefore, the evidence indicates that EVs sequester full-length, and potentially functional, proteins, although the possibility exists that EVs also contain misfolded proteins or peptide products of intracellular proteolytic cleavage. The substantial number of proteins identified in the EVs suggests that the EV population is likely to be heterogeneous, potentially with different, as-yet-undefined subclasses of EVs, and to show, using WGCNA, that fetal sex was a significant contributor to at least one network of proteins expressed in EVs, specifically the brown module, though most protein networks were sex-independent.
female fetuses were generally similar. However, we did observe increased sequestration of lysosome-localized proteins in female EVs compared to male EVs.

This study uniquely compared the proteome of EVs to their paired parent-of-origin NSCs. With this comparative analysis, we identified proteins within every subcellular location, as assigned by the UniProt Knowledgebase [50, 73], that were in greater relative abundance in EVs compared to their parental NSCs, suggesting specific enrichment in EVs. In addition to proteins like TSG101, CD63, and ESCRT-related vacuolar protein sorting proteins (VPSs) that have previously been documented to be enriched in EVs and were also enriched in our NSC-derived EVs, we observed a number of proteins assigned to the subcellular compartments that were present at higher relative abundance in EVs to suggest selective loading of these proteins into EVs (Figures 4, 5, 6, 7, 8, and 9). It was surprising to find proteins enriched in EVs relative to parental NSCs that were categorized as multi-compartmental and having a nuclear location, i.e., categorized for cytoplasm and membrane compartments but also nuclear compartment. For example, LRP1 and members of the Notch family, which are multifunctional receptors and regulators of neural development [74, 75], were among the most highly enriched proteins in EV samples compared to parental cells. Interestingly, proteolytic cleavage of both LRP1 and Notch can generate both intracellular and extracellular fragments which serve as transcription regulators and decoy receptors, respectively. EV samples contained peptides for LRP1 as well as for Notch1, 2, and 3, across the length of these proteins, suggesting that their multifunctional potential is retained by EVs. Moreover, EVs were enriched for Jagged-1, a cell surface ligand that binds to Notch receptors to activate Notch signaling pathway and by that mechanism [76], controls neurogenesis. This suggests that EVs have the capacity to transmit a functional ligand-receptor entity to recipient cells. EVs were also enriched for tyrosine kinase receptors like epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR), which play essential roles in the regulation of embryonic development, cell proliferation, migration, and differentiation [77, 78]. Other neurodevelopmental-related proteins, like Wdr82, associated with Kleeofstra Syndrome 2, an autosomal dominant neurodevelopmental disorder [79], and Gpc6, associated with Omodysplasia 1, an autosomal recessive skeletal dysplasia syndrome, that is also associated with intellectual disability [80], were also examples of proteins that were heavily enriched in EVs. Finally, pathway overrepresentation analysis indicated that EVs sequester proteins that control nervous system development broadly. These include, interestingly, non-canonical Wnt signaling and

![Figure 14.](https://example.com/image14.png)
Figure 15. Fluorescent-Labeled EVs from Donor NSCs are Sequestered by Recipient NSCs 15A) Confocal photomicrograph showing the presence of MemBrite-labeled EVs (red fluorescence) purified from donor NSCs within the cytoplasm of naïve NSCs counterstained with DAPI (blue) to visualize nuclei. 15B) Flow cytometric analysis shows that compared to unstained cells, MemBrite readily stains NSCs. Purified EVs stained with MemBrite are also readily taken up by naïve recipient cells, whereas no stain was observed in cells exposed to purified culture medium with MemBrite dye that underwent the same labeling and filtration process as isolated EVs; n = 6 to 13 samples per group; Kruskal-Wallis test for nonparametric one-way ANOVA, p < 0.0001; Dunn’s multiple comparisons post-hoc test, *p < 0.05.

Figure 16. Functional Analysis of the Effects of Purified Control EVs, and EVs Purified from Ethanol-treated NSCs on the Behavior of Naïve Recipient NSCs. Following EV administration, oxidative metabolism was measured by the alamarBlue assay (16A), glycolysis was measured by the Lactate-Glo assay (16B), and apoptosis was measured by the activation of Caspase3/7 (16C) in naïve recipient NSCs; n = 5 to 23 samples per group; repeated-measures two-way ANOVA with Geisser-Greenhouse correction; Tukey’s or Dunnett’s multiple comparisons post-hoc test, *p < 0.05, #p < 0.08. 16D,E,F) Quantification in donor, ethanol-treated and control NSCs, of oxidative metabolic activity (16D), glycolysis activity (16E), and Caspase 3/7 apoptosis activity (16F) following moderate (120 mg/dL) or high (320 mg/dL) ethanol exposure in NSCs; n = 5 to 23 samples per group; repeated-measures two-way ANOVA with Geisser-Greenhouse correction; Tukey’s multiple comparisons post-hoc test, *p < 0.05, 16G) Flow cytometry analysis of the proportion of cells in S-phase of the cell cycle, following addition of EVs derived from ethanol-treated or control NSCs, to naïve recipient NSCs; n = 19 samples per group; repeated-measures two-way ANOVA with Geisser-Greenhouse correction; Dunnett’s multiple comparisons post-hoc test, *p < 0.05, #p < 0.08. 16H) Representative flow cytometry image.
the planar cell polarity/convergent extension (PCP/CE) pathway which controls brain morphogenesis, including the planar orientation of neural progenitors and their directional growth to form the laminar structure of the developing cerebral cortex [81]. These and our additional findings from a scRNAseq re-analysis that mRNA transcripts for upregulated ethanol-sensitive EV-enriched proteins were unevenly expressed in the neurogenic niche, localized to VZ-type cells rather than SVZ and TPC clusters, suggest that EVs have the potential to transfer important and developmentally relevant signaling mediators among cells. These findings support a hypothesis that EVs homogenize and coordinate some behaviors of cells within the neurogenic niche by transferring proteins derived from NSCs to other cells.

An important question that needs to be further explored is whether maternal-fetal perturbagens like ethanol reprogram EVs. Although ethanol inhibits fetal growth, it surprisingly does not kill fetal NSCs, but rather causes a loss of NSC renewal potential and aberrant premature maturation [9, 12, 13, 14, 17, 19, 56, 59, 82, 83]. Consequently, the effects of ethanol on EVs, a novel endocrine mediator, need to be better understood to determine if the reprogramming of EVs contributes to pathogenic processes. In the current study, we again observed that ethanol exposure did not result in an increase in apoptosis in parental NSCs, consistent with our previous observations [12, 19, 23, 83]. Because there was no change in apoptosis, it is unlikely that the release of apoptotic bodies contributed to the EV proteome signature following ethanol exposure. Our dose-response analysis showed that ethanol does result in some dose-independent effects on NSC-derived EVs, including the selective enrichment of proteins in EVs that were also depleted in parental NSCs. For example, the heat-shock protein chaperone DNAJC7 isoform 1, which regulates protein folding and protein transport [84], was significantly enriched in EVs relative to NSCs following NSC exposure to both medium and high doses of ethanol, compared to controls. Similarly, the heterogeneous nuclear ribonucleoprotein HNRNPY, which binds to pre-mRNAs and controls nuclear pre-mRNA processing and transport [85], was significantly enriched in EVs relative to cells for both ethanol-treated groups, but not controls. Pathway enrichment analysis, showed that across treatment groups, ethanol-sensitive proteins were enriched for RNA-binding and chaperone proteins involved in ribosome and translation pathways (Supplementary Table 16–19). This coordinated outcome suggests that ethanol exposure results in transfer of proteins from cells to EVs. Surprisingly, this effect is not generalized to all EV proteins, and therefore, suggests the presence of a potentially novel and selective ethanol-dependent sequestration mechanism that controls protein sorting into EVs.

An intriguing finding from WGCNA was that the ethanol-dependent sequestration of proteins into EVs was accompanied by an increase in the number of networks of tightly interconnected proteins. This outcome suggests that ethanol exposure resulted in a dose-related increase in the biological complexity of EVs, without a corresponding increase in the proteomic network complexity in parental NSCs. Not surprisingly, pathway overrepresentation analysis of proteins that were enriched in EVs relative to parental NSCs due to ethanol exposure, uncovered evidence for dose-specific pathways. For example, moderate ethanol exposure favored overrepresentation of proteins related to mitosis and ribosomal RNA processing. We also observed increased sequestration of proteins associated with nonsense mediated decay (NMD) into EVs at this lower dose, an interesting finding, given that NMD is necessary for early embryo and fetal development, for stem cell renewal and maturation [56], and that disruption of NMD is embryonically lethal [49]. In contrast, in response to the high dose of ethanol, there was increased sequestration of proteins associated with stress response to amino-acid deprivation, inhibition of DNA replication, and pathways that may be expected to impair neurogenesis [88, 89]. We also found that the high dose of ethanol, like the medium dose, resulted in sequestration of proteins related to DNA replication into EVs. However, there were also significant differences. For example, the dominant pathway overrepresentation with heavy exposure, was the mechanistic target of rapamycin (mTOR) cellular response amino acid starvation, an established stress response in animal models of prenatal ethanol exposure [90, 91], that is associated with inflammation and fetal brain injury [92] and disorganized cortical laminarization [93].

A secondary analysis of our previously published in vivo single cell RNAseq data of the developing cerebral cortex [12] showed that the translated mRNAs that contributed to the EV response to ethanol were predominantly localized to cells of the fetal ventricular zone and were not localized to the subventricular zone or transient progenitor cells. This analysis outcome is important because it supports the hypothesis that fetal NSCs that populate the VZ in vivo and give rise to neurospheres ex vivo are the dominant source of this ethanol response in EVs, rather than their more mature progeny. In the current study, we did find evidence for biological activity of EVs. Our data show that fluorescently-labeled EVs added to culture medium are taken up avidly by NSCs, and inhibiting the formation and release of EVs results in metabolic inhibition of NSCs. Moreover, purified EVs added to culture medium reduced the proportion of recipient NSCs in S-phase without influencing cell death. Since stem cell differentiation is coupled with cell-cycle progression (reviewed in [94]), it is possible that exogenously added EVs, from neurospheres ex vivo and NSCs from the VZ in vivo, serve to inhibit differentiation. Added support comes from our observations that exogenously added EVs derived from NSCs also decreased the rates of oxidative metabolism in both NSCs and EDCs, while increasing the rates of glycolysis and decreasing mRNA transcripts for the oligodendrocyte marker Olig2, in EDCs. Since the maturation of stem cells is accompanied by increased oxidative phosphorylation and decreased glycolysis (reviewed in [95, 96]), these data argue for a basal role for un-treated NSC-derived EVs in maintaining the stem cell niche. However, it remains to be determined whether EVs secreted by NSCs influence the biology of other spatially proximate cell populations such as those of the SVZ, or even possibly, the early neurons of the developing cortical plate.

Interestingly, exogenously added EVs derived from ethanol-treated NSCs did result in additional effects in NSCs, beyond those discussed for control EVs obtained from treatment-naive NSCs. For instance, we observed that while EVs obtained from naïve donor controls resulted in decreased oxidative metabolism and S-phase in recipient NSCs, EVs from ethanol treated donors were not as effective. One caveat that needs to be acknowledged is that endogenous EVs secreted by recipient NSCs may have competed with donor EVs from ethanol-exposed NSCs, to diminish the overall effect of ethanol exposure. While future analyses will be needed to determine whether EVs secreted by ethanol-treated NSCs confer changes in physiology and gene expression in recipient cells, these data suggest that underlying shifts in the sequestered proteome may be part of an overall picture of diminished EV efficacy due to ethanol exposure. However, these outcomes do not minimize a different possibility, i.e., that proteomic shifts in EVs due to an environmental perturbation are more consequential for parental NSCs than for recipient NSCs. It is feasible that the transfer and sequestration of proteins into EVs represent diminished/decreased functionality by secreting cells, more than a transfer of functionality to recipient cells.

In conclusion, mouse-derived fetal NSCs secreted an abundance of EVs. These data collected in this exploratory study showed that the proteome of these EVs is complex and likely reflects the presence of multiple EV subpopulations. Moreover, because selected proteins from each subcellular compartment, from nuclear to cytoplasmic and membrane-bound proteins, were preferentially enriched in EVs relative to parental cells, it is likely that an active sorting mechanism operates among distinct EV proteomes. Our evidence also suggests that secreted EVs are biologically functional and may play a role in maintaining stem cell identity, though future studies in whole-animal models will be needed, to ascertain both spatial and temporal effects of EVs on neurogenesis and stem cell identity. Importantly, our data also show for the first time, that a developmental perturbagen like ethanol has a significant, yet specific impact on the proteome of EVs secreted by NSCs. Ethanol exposure resulted in a dose-dependent and selective enrichment of proteins in EVs.
and concurrent decrease of those proteins from parental NSCs, implying the activation of a re-sorting process in loading specific proteins into EVs. Re-sorted proteins appeared to serve specific functions, from the control of cell cycle and protein misfolding specifically at a medium dose, to resorting of stress response, DNA replication, and Rho-GTPase pathway components at a high dose of ethanol exposure. Moreover, EVs derived from ethanol-exposed NSCs did have effects beyond that observed with EVs from ethanol treatment-naïve NSCs. While further confirmatory studies in whole animal models are needed, these data do suggest that the sequestration of proteins into EVs following ethanol exposure represents a loss of function for the parental NSCs, as well as possible gain of function for recipient NSCs, that may collectively constitute a stress-adaptive response to a perturbagen like ethanol.

4. Materials and methods

4.1. Ex vivo fetal mouse neurosphere culture model

C57BL/6J (Ai14) mice (Jackson Laboratories; Catalog # 007914) were bred in-house and time-mated overnight, from the start of the dark phase of the light-dark cycle, with the following morning defined as gestational day (GD) 0.5. Ex vivo neurosphere cultures were created from NSCs obtained from three separate pregnancies, from acutely dissociated GD 12.5 fetal mouse dorsal telencephalic neuroepithelium, which corresponds to the future isocortex, and used to model early neuronal development [17, 19]. At the time of collection, fetal sex was determined as we have previously reported [12]. Briefly, genomic DNA was obtained from fetal tissue samples using alkaline lysis and fetal sex ascertainment by a rapid qPCR protocol using primers to detect repetitive sequences on X and Y chromosomes [97, 98]. Cortical neuroepithelial tissues within a single pregnancy were pooled by sex, to generate male- and female-specific cultures. Sex-specific cultures were obtained from three separate pregnancies, to generate three male and three female biological replicates. Sex-specific cultures were maintained as non-adherent neurospheres in serum-free mitogenic media and passages 7 to 10 were used in this study, as previously published [17, 99, 100] (Figure 1A). All animal procedures were performed in accordance with the Texas A&M University Institutional Animal Care and Use Committee guidelines and approval.

4.2. Ethanol treatment

Dispersed single NSCs were seeded at a density of 4 × 10^5 cells in 10 mL of culture media per T75 flask, with four flasks being defined as a single sample. Cell count and viability (~75–85% viability) were measured using the Invitrogen Countess Automated Cell Counter (Invitrogen; Catalog #C10227; Carlsbad, CA/USA). Ethanol, unlike other psychotropic agents, is consumed, and exhibits its psychological effects in the millimolar range, and can be tolerated by humans at doses in the range, from moderate (100–134 mg/dL; 22 mM–29 mM) to high (250–380 mg/dL; 54 mM–82 mM), is consistent with our previously published studies [13, 17, 101, 102]. Neurospheres and culture-conditioned medium (for the concentration of EVs) were collected on day 5.

4.3. EV separation and concentration

EV fractions were separated and concentrated from culture-conditioned medium following an established ultracentrifugation protocol (Théry et al., 2006) with a few added steps [103]. Briefly, culture-conditioned medium was separated from the cell fraction by centrifugation at 200 x g for 5 min, and the cell pellet isolated for separate protein analysis. The culture media supernatant was next centrifuged at 2,000 x g for 10 min at 4°C to eliminate dead cells and other debris (Figure 1B), then passed through a 0.2 μm sterile filter with a polyethersulfone membrane (VWR; Catalog # 28145-501; Radnor, PA/USA) to exclude debris with diameters greater than 200 nm. The filtered supernatant containing particles <200 nm was centrifuged through a 100 kDa molecular weight cutoff (MWCO) polyethersulfone membrane (PALL; Catalog # MAP100C37; Port Washington, NY/USA) at 4,000 x g for 30 min to concentrate the EV-enriched supernatant while filtering out any particles below 100 kDa. The material that was collected on the membrane was transferred to polypropylene thick-walled centrifuge tubes (Beckman; Catalog # 355646; Brea, CA/USA), adding chilled (4°C) 1 x PBS buffer (Thermo Fisher; Catalog # 14190144; Waltham, MA/USA) to a total volume of 7 mL per tube. This PBS-suspended material was centrifuged at 100,000 x g for 90 min at 4°C in a Type 70 Ti fixed-angle titanium rotor (Beckman; Catalog # 337922; Brea, CA/USA). Pellets were washed, by decanting the supernatant and resuspending the EV-enriched pellet in 1 mL chilled PBS, followed by the addition of 6 mL chilled PBS and centrifugation at 100,000 x g for 90 min at 4°C.

4.4. Nanoparticle tracking analysis

The concentration and size of EVs were measured by nanoparticle tracking analysis (Nanosight LM10; Malvern Panalytical; Westborough, MA/USA), as we have previously published [23]. 1 x PBS buffer (Gibco®; Thermo Fisher; Catalog # 14190144) was used for the dilution of isolated EV samples.

4.5. Transmission electron microscopy (TEM) and immunogold labeling of EVs

TEM and immunogold labeling were used for the validation of EV isolation. TEM sample preparation and imaging were performed at the Texas A&M Microscopy and Imaging Center, following a protocol we have previously published [23]. For immunogold labeling of the EV surface marker CD63, polyclonal rabbit anti-CD63 antibody diluted 1:100 (System Biosciences; Catalog # EXOAB-CD63A-1; Palo Alto, CA/USA) was used as the primary antibody, and binding detected incubation with 12 nm goat anti-rabbit antibody conjugated with colloidal gold (Jackson ImmunoResearch Lab; Catalog # 111-205-144; West Grove, PA/USA).

4.6. Western blot analysis

Protein was extracted from neurospheres and their corresponding EVs using 1 x RIPA lysis buffer (EMD Millipore; Catalog # 20–188; Burlington, MA/USA) with addition of Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher; Catalog # 78442). Extracted protein concentration was determined using Pierce™ BCA protein assay kit (Thermo Fisher Scientific; Catalog # 23225). Protein (20 μg) was size-fractionated on a 4–12 % Bis-Tris Gel, run at 150 V for 60 min, and transferred to a PVDF membrane using the iBlot transfer system (Thermo Fisher; Catalog # IB301001). The membrane was briefly washed in deionized water and
then dried overnight at room temperature in the dark. Subsequently, the membrane was blocked for 1 h with Odyssey Blocking Buffer in Tris-buffered saline containing 0.1 % sodium azide (Licor; Catalog # 927–50000; Lincoln, NE/USA) and then incubated overnight at 4 °C in the dark with primary antibodies (Supplementary Table 20). The immunoblot was washed and incubated with an IRDye-conjugated goat anti-mouse or anti-rabbit IgG (LI-COR; Catalog # 926–68070 and # 926–32350) at dilution 1:15,000 for 1 h and then imaged using a LI-COR Odyssey CLX Imager.

4.7. Protein sample preparation and mass spectrometry analysis

The retained EV pellet from ultracentrifugation was resuspended in 50 μL of Laemmli lysis buffer (Amresco; Catalog #M337-25ML; Solon, OH/USA), with addition of Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher; Catalog # 78442). Samples of the matched cell-origin NCV pellets were similarly resuspended. EV and cell samples were frozen at -80 °C, placed on dry ice, and shipped overnight to the University of Texas Health Science Center at San Antonio Mass Spectrometry Instrumentation Core Laboratory. Protein quantities were assessed using the EZQ™ Protein Quantitation Kit (Thermo Fisher; Catalog #R33200). Aliquots between 70–100 μg (cell samples) and 2–10 μg (EV samples) were reduced with tris(2-carboxyethyl)phosphine hydrochloride (TCEP), alkylated in the dark with iodoacetamide and applied to S-Trap mini columns (ProtiFi; Farmingdale, NY/USA) for tryptic digestion with sequencing grade modified trypsin (Promega; Catalog #V5111) in 50 mM TEAB. Peptides were eluted from the S-Traps with 0.2 % formic acid in 50 % aqueous acetonitrile and quantified using Pierce™ Quantitative Fluorometric Peptide Assay (Thermo Fisher; Catalog # 23290).

Data-independent acquisition mass spectrometry was conducted on an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher). On-line HPLC separation was accomplished with an RSLciano HPLC system (Thermo Scientific/Dyonex): column, PicoFrit™ (75 μm internal diameter; New Object; Littleton, MA/USA) packed to 15 cm with C18 adsorbent (218MS 5 μm, 300 Å; Vyodac/Grace; Columbia, MD/USA); mobile phase A, 0.5 % acetic acid (HAc)/0.005 % trifluoroacetic acid (TFA) in water; mobile phase B, 90 % acetonitrile/0.5 % HAc/0.005 % TFA/9.5 % water; gradient 3–42 % B in 120 min; flow rate, 0.4 μL/min. For a reference library, a pool was made of all samples in each experiment, and aliquots of the digests [0.67 μg (male EVs), 0.75 μg (female EVs, or 1 μg cells)] were analyzed using gas-phase fractionation and 4-m/z windows (120k resolution for precursor scans, 30k for product ion scans, all in the orbitrap) to create an empirically corrected DIA chromatogram library [104, 105] by searching against a Proist-generated predicted spectral library [106] based on the Uniprot Knowledgebase [50, 73]. As defined in this knowledgebase, a protein may be present non-exclusively in multiple sub-cellular compartments; therefore, for further analysis, non-exclusive proteins were assigned to all associate compartments. After classifying proteins by subcellular localization, an average z-score was computed for all proteins within each subcellular location for each sample. And a 2-way ANOVA analyses (EV or cell) x sex with Tukey's HSD test as the post-hoc tests were computed to assess the contribution of proteins from each sub-cellular location to differences in the overall composition of EVs relative to cells (e.g., are nuclear proteins as a group more highly expressed in cell samples than EVs). Next, the subcellular location of proteins that were specifically enriched in EVs compared to cell samples was determined, and for each protein, it's average z-score and 95% confidence estimate for each sample group (EV vs cell) was computed, and proteins with non-overlapping confidence estimates were classified as statistically significantly enriched in EVs relative to parental cells.

Weighted correlation network analyses (WGCNA) were conducted using R-based “WGCNA” package [107, 108]. Heat maps from the WGCNA processed data were generated using the R-based “gplots” package [109]. Using a topological overlap matrix (TOM) created under the WGCNA method, we constructed gene networks and identified modules with hierarchical clustering dendrograms (trees). Distinct modules were identified from the expression levels of 2615 gene equivalents; gene equivalents were proteins whose expression levels varied between samples, signifying expression level above “0”, and detected in at least 27 of the 36 samples to limit numbers of missing entries in genes and samples. Weighted networks were visualized by heatmaps, with the gene dendrograms and module colors displayed along the top and left side; each row or column corresponds to a single gene/protein, where light colors denote low topological overlap and dark colors denote high topological overlap. While this protocol was initially designed to examine gene expression, we are applying it to protein expression. When referring to protein expression, we will refer to the proteins; when

Proteomic data were analyzed to identify significant differences in: protein abundance between EV and cell groups, regardless of sex or ethanol dose; ethanol- and sex-dependent protein abundance between EV and cell groups; and pathways enriched in proteins that showed differential protein abundance. All computation and analysis involving protein expression were conducted in “Rstudio” software (RStudio, Inc.; Boston, MA/USA), v.1.2.1335 for Windows.

Only proteins detected in all 18 EV samples (2765 specific proteins) out of 36 total samples, 18 EV and 18 cell samples, were used for analysis. Each protein within a sample was given a normalized value (Normalized Value Protein X from Sample A = N(X) from the ratio derived by the total exclusive peptide intensity of the protein of a sample (Total Exclusive Peptide Intensity Protein X = P(X)) divided by the sum of the same sample's total exclusive peptide intensity (Sum of Total Exclusive Peptide Intensity sample A = S(X)).

After normalization, any missing value for a protein in cell samples was imputed by a randomized number (Imputed Value Protein X from Sample A = I(X)), within a range of minimum, defined as the protein mean across samples subtracting three standard deviations of the protein expression value across samples (μ<sub>Protein X from all samples</sub>−3*σ<sub>Protein X from all samples</sub> = μ<sub>X</sub>−3*σ<sub>X</sub>) and of maximum being the protein mean across samples subtracted by two standard deviations of the protein expression across samples (μ<sub>Protein X from all samples</sub>−2*σ<sub>Protein X from all samples</sub> = μ<sub>X</sub>−2*σ<sub>X</sub>):

\[ I(X) = (\mu_X - 3\sigma_X, \mu_X - 2\sigma_X) \]

The rationale for imputing undetectable values in cells stems from the hypothesis that, because EVs are derived from a cell, any protein detected in EVs must be present in their parent cells. Therefore, any protein that is detected in EVs but not in cell samples is hypothesized to be present in cells, but below the limit of detection. Any imputation that resulted in a negative number was replaced by a value of “0”. After imputation, a z-score for each specific protein was calculated across all 36 samples. Each protein was then categorized by subcellular location (Nucleus, Cytoplasm, Mitochondria, Lysosome, Endosome, Membrane), based on the Uniprot Knowledgebase [50, 73]. As defined in this knowledgebase, a protein may be present non-exclusively in multiple sub-cellular compartments; therefore, for further analysis, non-exclusive proteins were assigned to all associate compartments. After classifying proteins by subcellular location, an average z-score was computed for all proteins within each subcellular location for each sample. And a 2-way ANOVA analyses (EV or cell) x sex with Tukey’s HSD test as the post-hoc tests were computed to assess the contribution of proteins from each sub-cellular location to differences in the overall composition of EVs relative to cells (e.g., are nuclear proteins as a group more highly expressed in cell samples than EVs). Next, the subcellular location of proteins that were specifically enriched in EVs compared to cell samples was determined, and for each protein, it’s average z-score and 95% confidence estimate for each sample group (EV vs cell) was computed, and proteins with non-overlapping confidence estimates were classified as statistically significantly enriched in EVs relative to parental cells.
referring to an identified hub, we will use gene as it is a measure from the WGCNA. A chi-square test of independence was performed on WGCNA EV/cell module counts to examine the relationship between ethanol treatment and number of modules across locations (EV vs. cell), using the control values as percent expected.

Protein enrichment in EVs relative to parent cells was next compared between control and treatment groups. The distribution of a specific protein was assessed by comparing its value (ValueX Protein X from Sample A = VX_Cell) of EV sample (ValueX Protein X of EV Sample by Treatment per Pregnancy = VX_EV) to Cell sample (ValueX Protein X of Cell Sample by Treatment per Pregnancy = VX_Cell) in each pregnancy (3 pregnancies) by ethanol treatment groups (0, 120, 320 mg/dL), resulting in an enrichment value ((R Protein X from EV sample to Cell sample by Treatment per Pregnancy = RX):

\[ RX = \frac{VX_{EV}}{VX_{EV} + VX_{Cell}} \]

Interestingly, principal component analysis showed that proteins that contributed to both the first and second principal components separated cell samples by pregnancy (Supplementary Figure 12) suggesting that protein content of EVs can vary substantially from one pregnancy to the next. Therefore, to reduce variability due to individual differences in pregnancies/mouse litters and to account for the fact that cells from each pregnancy were partitioned to three treatment groups at the initiation of ethanol exposure, we used paired samples for t-test and repeated-measures analyses for ANOVA. To identify proteins where EV enrichment was significantly altered by treatment, we used paired samples t-tests, a Hedges’ g (g) effect size and a 95% confidence estimate for g that were non-zero, containing, were assessed. Enrichment pathway analysis was conducted using the R-based “ReactomePA” package, v.1.36.0 [110], and “clusterProfiler” package, v.4.0.5 [111]. Repeated-measures two-way ANOVA with Geisser-Greenhouse correction, followed by a Dunnett’s or Tukey’s Test for post-hoc comparisons, were used to assess the biological functionality of EVs derived from ethanol-treated NSCs (0, 120, 320 mg/dL) on naïve recipient NSCs. All other statistical analyses were conducted using the “R” software (R Foundation for Statistical Computing; Vienna/Austria), v3.6.1 for Windows and the “GraphPad Prism” software (GraphPad Software; San Diego, CA/USA), v8.4.2 for Windows.

4.9. Data analysis of scRNA sequencing

To further understand the identity of the parental NSCs that were the source of ethanol responsive EV proteins, we analyzed our previously published single cell RNAseq (scRNAseq) data from GD 14.5 mouse cerebral cortex (NCBI GEO accession number GSE158747) [112], focusing on cell clusters that resemble the ventricular zone (VZ), the subventricular zone (SVZ), and transit progenitor cells (TPC), the three cell populations of a developing fetal mouse brain that most closely resemble cells present in our neurosphere model [12]. After cell populations with VZ, SVZ and TPC identity were visualized as previously published [22], the cell-types expressing the complete transcriptomic signature of the differentially regulated proteins in EVs (p < 0.05; g > 0.5 with a non-zero containing 95% confidence estimate), from both the 120 and 320 mg/dL exposure conditions, were shown.

4.10. Fluorescent labeling of EVs and delivery of EVs to NSCs

To visualize the uptake of EVs to NSCs in vitro, we labeled EVs, purified as described earlier, with cyanine-based, membrane-localized, MemBrite Fix 568/580 (Biotium; Catalog # 30095-T; Fremont, CA/USA) according to the manufacturer’s instructions. Briefly, isolated EVs (~10^9) were incubated with MemBrite at final concentration of 200 nM in PBS (Thermo Fisher; Catalog # 14190144) for 30 min in the dark, at room temperature. PBS was then added to the EV supernatant for a total of 15 mL volume and passed through a 0.2 um sterile filter with polyethersulfone membrane (VWR; Catalog #: 28145-501) to exclude any possible aggregates with diameters greater than 200 nm. The filtered supernatant containing particles <200 nm was subjected to centrifugation through a 100 kDa molecular weight cutoff (MWCO) polyethersulfone membrane filter tube (PALL; Catalog #: MAP100C37) at 4,000 x g for 30 min to concentrate the small EV-enriched supernatant while filtering out unbound dye. PBS (15 mL) was added to the remaining EV supernatant and centrifuged once more at 4,000 x g for 30 min. The labeled EV supernatant was collected into a new 100 kDa MWCO filter tube in 10 mL of fresh culture media and centrifuged at 4,000 x g for 30 min, with this step being repeated with new 10 mL of fresh culture media. Finally, naïve NSCs were exposed to labeled EVs for 24 h before the cells were processed for flow cytometry or confocal fluorescence microscopy analyses.

As a negative control, fresh culture media was processed for labeling using the same procedure as that used for EVs, and the resulting supernatant introduced to NSCs as described above. To directly label cells, as a positive control, MemBrite Fix 568/580 dye was diluted in 1 mL of fresh culture media for 200 nM final concentration, and ~500,000 NSCs were resuspended in the resulting dye-containing media and incubated for 30 min in the dark at room temperature. To eliminate unbound dye, cells were rinsed three times with 5 mL of fresh culture media and centrifuged at 300 x g for 5 min. Finally, labeled cells were processed for flow cytometry or confocal fluorescence microscopy analyses.

4.11. Confocal imaging of neurospheres

Following addition of labeled EVs or filtered culture media, cells were briefly fixed (2% PFA, 15 min). NSC nuclei were counterstained with 300 nM DAPI (Thermo Fisher; Catalog #D1306), mounted onto glass slides (Vetashield, Vector Laboratories; Catalog # H-1200-10; Burlingame, CA/USA), coverslipped, and imaged using a confocal-laser scanning microscope (FluoView 1200, Olympus Corporation of the Americas; Center Valley, PA/USA) equipped with a 405 nm laser to excite DAPI and a 559 nm laser to excite MemBrite 568/580. Micrographs were acquired using a 60x magnification objective (UPlanApo 60X Oil, Olympus) with 2x zoom through image spatial resolution adjustment.

4.12. AlamarBlue™ REDOX assay

Cellular metabolic activity was assessed using alamarBlue™ HS (Thermo Fisher; Catalog # A50100) as a fluorometric/colorimetric indicator according to manufacturer’s instructions. Briefly, 10 μL alamarBlue™ HS was added to each well with ~30,000 cells in 100 μL media in a 96 well flat bottom clear plate, incubated at 37°C for 4 h, then the fluorescence read at 570 nm and 600 nm wavelengths using Cytation 5 cell imaging multi-mode reader (Agilent Technologies, Inc.; Santa Clara, CA/USA). For studies on the effects of EV addition, ~10^5 EV isolated from ethanol-treated (0, 120, 320 mg/dL) sex-specific NSC cultures were added to 10^5 naïve recipient, sex-matched NSCs (female NSC-derived EVs were delivered to female naïve recipient NSCs and male NSC-derived EVs to male recipient NSCs). Recipient cultures were incubated for 72 h before being processed for the alamarBlue assay. For studies on the effects of naïve NSC-derived EV addition to EDCs, ~10^5 EV isolated from naïve sex-specific NSC cultures were added to 10^5 naïve recipient, sex-matched EDCs. Recipient cultures were incubated for 72 h before being processed for the alamarBlue assay.
4.13. Lactate-Glo glycolysis assay

The Lactate-Glo Assay (Promega; Catalog #G8091; Madison, WI/USA) was used according to the manufacturer’s instructions to measure the glycolysis rate of viable cells. The assay detects lactate produced by glycolysis. Briefly, 50 μL of Lactate Detection Reagent was added to 50 μL of 1:50 diluted sample in a 96 well flat bottom white plate, incubated at room temperature for 1 h, then luminescence read using the Cytation 5 cell imaging multi-mode reader. For studies on the effects of EV addition, ~10^9 purified EVs from ethanol-exposed (0, 120, 320 mg/dL) male and female NSC cultures were added to 10^6 sex-matched but naïve recipient NSCs. Recipient cultures were incubated for 72 h before being processed for glycolysis assay. For studies on the effects of EV inhibition, one of three EV inhibitors, GW4869 (10 μM), Manumycin-A (0.5 μM), or Calpeptin (90 μM), were added to 10^6 naïve recipient, sex-matched NSCs. Recipient cultures were incubated for 48 h before being processed for the glycolysis assay. For studies on the effects of naïve NSC-derived EV addition to EDCs, ~10^9 EV isolated from naïve sex-specific NSC cultures were added to 10^6 naïve recipient, sex-matched EDCs. Recipient cultures were incubated for 72 h before being processed for the glycolysis assay.

4.14. Caspase apoptosis analysis

The Promega Caspase-Glo® 3/7 Assay Systems (Promega; Catalog #G8091) was used according to the manufacturer’s instructions to quantify apoptotic cell death at day 5 of ethanol exposure and at 48 h following exposure of naïve cells to EVs. Briefly, 100 μL Caspase-Glo® 3/7 Reagent was added to each well with ~30,000 cells in 100 μL media in a 96 well flat bottom white plate, incubated at 37 °C for 2 h, then luminescence read using the Cytation 5 cell imaging multi-mode reader. For studies on the effects of EV addition, ~10^9 purified EVs from ethanol-exposed (0, 120, 320 mg/dL) male and female NSC cultures were added to 10^6 sex-matched but naïve recipient NSCs, and incubated for 48 h, before being processed for caspase assay.

4.15. Flow cytometry and cell cycle analysis

For cell cycle analysis, after addition of ~10^9 EV to ~10^6 cells for 72 h, with ethanol-treated (0, 120, 320 mg/dL) sex-specific NSC culture-derived EVs added to respective sex-specific recipient NSCs, DNA synthesis was assessed by pulse-labeling cells with 10 μM EdU (5-ethyl-2’-deoxyuridine) for 1 h at 37 °C. Immediately after, cells were collected and labelled using the Click-IT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Thermo Fisher; Catalog #C10425), in conjunction with 7-amino-actinomycin D (Thermo Fisher; Catalog # 00–6993-50), according to the manufacturer’s instructions. Cell cycle analysis was performed with the BD LSR Fortessa X-20 Cell Analyzer flow cytometer (Becton, Dickinson and Company; Franklin Lakes, NJ/USA). Data were analyzed using FCS Express software v.7.12.0007 (De Novo Software; Pasadena, CA/USA).

For detection of labeled EVs in NSCs, after addition of EVs, cells were briefly fixed (2% PFA, 15 min) before undergoing flow cytometry using the BD LSR Fortessa X-20 Cell Analyzer. Data were analyzed using FCS Express software v.7.12.0007 (De Novo Software).

4.16. RNA isolation and mRNA qPCR

RNA from early differentiating cells was isolated using the miR-Neasy™ mini kit (Qiagen; Catalog # 217004; Germantown, MD/USA). cDNA synthesis was performed using the qScript™ cDNA SuperMix kit (Qiagen; Catalog # 95048). qPCR analysis was done on an Applied Biosystem ViiA 7 Real-time PCR system (ABI/Life Technologies; Grand Island, NY/USA). For mRNA transcript quantification, presented data correspond to ΔΔCT after being normalized to β-actin. Primers for neuronal lineage markers were same primer design we have previously published [23]. Briefly described, we designed primers to span exon-exon junctions. Each primer pair’s thermal stability curves were assessed for evidence of a single amplicon, with each amplicon’s length being verified using agarose gel electrophoresis, and amplicon identity being verified by Sanger sequencing. A list of primers and their sequences is presented in Supplementary Table 21.

Declarations

Author contribution statement

Dae D Chung: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Rajesh C Miranda: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Susan T Weintraub and Amanda H Mahnke: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Nihal A Salem: Performed the experiments; Analyzed and interpreted the data.

Khang T Le, Elizabeth A Payne and Tenley E Lehman: Performed the experiments.

Marisa R Pinson: Analyzed and interpreted the data.

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Data availability statement

Data associated with this study has been deposited at https://massive.ucsd.edu.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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