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# A Comparative Proteomic Analysis of Human and Rat Embryonic Cerebrospinal Fluid

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During vertebrate central nervous system development, the apical neuroepithelium is bathed with embryonic Cerebrospinal Fluid (e-CSF) which plays regulatory roles in cortical cell proliferation and maintenance. Here, we report the first proteomic analysis of human e-CSF and compare it to an extensive proteomic analysis of rat e-CSF. As expected, we identified a large collection of protease inhibitors, extracellular matrix proteins, and transport proteins in CSF. However, we also found a surprising suite of signaling and intracellular proteins not predicted by previous proteomic analysis. Some of the intracellular proteins are likely to represent the contents of microvesicles recently described within the CSF (Marzesco, A. M., et al. *J. Cell Sci.* **2005**, *118* (Pt. 13), 2849−2858). Defining the rich composition of e-CSF will enable a greater understanding of its concerted actions during critical stages of brain development.

**Keywords:** embryonic CSF (e-CSF) • human CSF • rat CSF • brain development • cerebrospinal fluid • mass spectrometry • proteomics

## Introduction

During the process of neurulation, the neural groove forms and the neural folds fuse to form the neural tube. Once the neural tube is fused, the fluid within the lumen is considered cerebrospinal fluid (CSF), whereas before fusion is complete, the neuroepithelium lining the inside of the neural tube is still in contact with amniotic fluid.[2] During the early stages of neural tube growth and development, groups of specialized neuroepithelial cells lining the neural tube are believed to secrete fluid into the neural tube space in order to support growth and development of the embryo. As the neural tube continues to elongate and develop, specific highly vascularized epithelial cell types begin to invaginate at specific locations within the neural tube to form the specialized choroid plexus.

The choroid plexus is a highly vascularized epithelial cell structure that during development is believed to be involved in the specific intracellular transfer of proteins into the CSF from the blood.[3] The choroid plexus develops in the lateral ventricles, and in the third and fourth ventricles of the brain. In rats, the choroid plexus can be first identified in the fourth ventricle at embryonic day 12 (E12) and in the lateral ventricle at E13 as a midline structure, and by E15, it represents paired structures protruding into the lateral ventricles.[4,5] In the human embryo, the choroid plexus begins to develop in the lateral and fourth ventricle at Carnegie Stage (CS) 18, approximately 44 days post-ovulation.[6] The first appearance of cerebral cortical neurons in the human embryo occurs at CS 21, shortly following the appearance of the choroid plexus,[6] and a similar temporal sequence is seen in mice and rats.

In adults, CSF has been described to have many functions. It has been described as an intermediary between blood and brain for the transport of nutrients and growth factors, and as a fluid buffer for the brain to protect both the brain and the large vessels that supply blood to the brain.[7,8] It has also been proposed to be involved in the elimination of toxins and other metabolic byproducts.[8,9] A mathematical analysis taking into account the pulsatile nature of CSF flow proposed that the CSF pulsations buffer the capillary bed from the effects of arterial pulsations that might otherwise prevent linear blood flow due to the mechanics of the brain being enclosed in the skull.[9] CSF has been reported to contain nerve growth factor (NGF) and transforming growth factor alpha (TGF-alpha), and levels of these are altered in neurological and developmental disorders,[9−13] but potential functions of these factors have not been demonstrated. Recently, it was shown that the ciliary action of CSF in the lateral ventricle of adult rats creates a gradient of SLIT2 protein, a chemorepulsive factor for neuronal olfactory bulb

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migration, within the CSF,[14] suggesting that CSF factors might have instructive roles for developing neurons or neural progenitors.

Although the role of the CSF during embryogenesis is just starting to be studied, several recent papers suggest an important role for CSF in brain development.[9,15−19] Miyan et al. have shown that rat cortical cells are viable and proliferate in embryonic CSF (e-CSF),[18] and recent studies have begun to test discrete signaling factors that may regulate neurogenesis. Gato et al. and Martin et al. have studied the role of chick e-CSF in regulating survival, proliferation, and neurogenesis of neuroepithelial cells, and identified FGF-2 in the chick CSF as a vital trophic factor.[15,16] Intriguingly, in mutant animals, CSF factors that may inhibit proliferation have been suggested. In studies of the hydrocephalic Texas (H-Tx) rat, it has been found that cell proliferation in the ventricular zone decreases, and although cell migration still occurs, there is a decrease in the number of migrating cells.[17,18] In addition, CSF from the lateral ventricles of affected H-Tx fetuses can completely inhibit in vitro proliferation of neuronal progenitors isolated from a normal fetus at 10% CSF addition to the media, suggesting that factors intrinsic to the CSF of the H-Tx fetuses are present that inhibit proliferation.[17−19]

The identification of other CSF factors that may play developmental roles has been impeded by our limited understanding of the components of the CSF. However, recent reports have provided our first glimpse of the protein composition of e-CSF.[20,21] Chick and rat e-CSF have been analyzed in proteomic studies and revealed many similarities, with both containing proteins of the extracellular matrix, regulators of osmotic pressure, ion carriers, hormone binding proteins, regulators of lipid metabolism, and various enzymes and enzyme regulators. One of the most striking differences between rat and chicken CSF as noted by Parada et al. was the increased number and complexity of apolipoproteins found in the rat which may be related to neuronal complexity.[20,21] The studies by Parada et al. are the first attempts at analyzing the proteome of the chick and rat e-CSF and have so far identified only 31 proteins within the rat e-CSF.

Here, we undertake a systematic and unbiased proteomic analysis of human e-CSF from Carnegie Stage 19−20 (approximately 48−53 days post-ovulation). We also report an extensive proteome analysis of rat e-CSF from three different time points E12.5, E14.5, and E17.5 during cortical development and list all the proteins that are common among the three time points as well as those proteins that are different.

We report a list of the common proteins found between the human and rat e-CSF. Furthermore, using various gene ontology programs, we categorize the proteins in the e-CSF and compare the subcellular localization, molecular function, and biological process of embryonic human and rat CSF. We find 135 proteins shared between the human and rat e-CSF and that there are many similarities in the categories of proteins found within the CSF based on molecular function and biological process. This systematic analysis of proteins common to many ages lays the groundwork for analysis of changing CSF components that may have more specific developmental roles.

## Experimental Procedures

**Isolation of CSF from Human Embryos.** Human embryos were collected through the joint MRC-Wellcome Trust Human Developmental Biology Resource at the University of Newcastle, Institute of Human Genetics. Embryos were donated for research anonymously and with consent by women undergoing elective termination of pregnancy for non-pathological reasons. Embryos were karyotyped and carefully examined to determine developmental stage and to detect any morphological abnormalities. Only samples with a normal karyotype and morphology were used in this study. One CS 19 and one CS 20 embryo were washed with ice-cold, sterile phosphate-buffered saline (PBS) solution and carefully placed on the dissection platform under the microscope, and all extra-embryonic membranes and tissues were removed. A Hamilton syringe was placed carefully into the fourth ventricle, and the CSF was collected, paying close attention not to make contact with the neuroepithelium lining of the fourth ventricle. The time from passing of the embryo to collection of the CSF was less than 3 h. The samples used for analysis had no microscopically visible contaminating neuroepithelial cells or red blood cells. Nonetheless, the CSF samples were centrifuged at 10 000*g* at 4 °C for 10 min to remove any intact contaminating cells and then were frozen at −80 °C until further analysis.

**Isolation of CSF from Rat Embryos.** Rat embryos (Sprague Dawley) at stages E12.5, E14.5, and E17.5 were removed from extra-embryonic membranes and tissues and placed in sterile Hanks balanced salt solution (HBSS). Each embryo was handled individually and washed in HBSS, gently patted dry, and placed on a microdissection tray. The CSF was carefully aspirated from each rat embryo under the microscope with a pulled tip glass microcapillary pipet (Drummond Scientific Company, 20 $\mu$L). The needle was steadily held within the inside of the ventricle so as to prevent major contact with the neuroepithelial wall, and the CSF was slowly aspirated. For E17.5, the embryo was placed on its back, and the glass needle was inserted into the left lateral ventricle and then into the right lateral ventricle to collect the maximum amount of CSF from the lateral ventricles (see Supporting Information Movie 1 for CSF sample collection technique from an E17.5 rat embryo). For E12.5, the embryo was placed on its side, and the glass needle was inserted directly into the lateral ventricle. Because of the patency of the neural tube at this stage, the CSF was collected from the developing lateral, third and fourth ventricle. For E14.5, the embryo was also placed on its side, and the glass needle was either inserted into the lateral ventricle or into the fourth ventricle, and the CSF was collected from each location separately. Figure 1A is a diagram depicting CSF isolation from E14.5 rat. CSF for each analysis was collected from two entire litters (approximately 20−24 rat embryos) and pooled as one sample. To minimize protein degradation, CSF samples were kept at 4 °C during collection. CSF samples were centrifuged at 10 000*g* at 4 °C for 10 min to remove any contaminating cells. The samples that we used for analysis had no visible sign of contaminating neuroepithelium cells or red blood cells as we could detect under the microscope. Samples were frozen at −80 °C until further analysis.

**In-Gel Digestion and Mass Spectrometry.** Frozen CSF samples were thawed on ice. Sample buffer was added, and the samples were boiled for 5 min and subjected to SDS-PAGE using either 10% or 7.5% polyacrylamide (37.5:1 acrylamide/bis-acrylamide) gels as indicated in Figure 1B,C. Each gel lane (which included the 4.2% polyacrylamide stacking gel) was cut into 10 regions, and each region was diced and subjected to in-gel digestion with sequencing grade modified trypsin (Promega, 6 ng/$\mu$L) in 50 mM ammonium bicarbonate overnight at 37 °C. Peptides were extracted with 50% acetonitrile (MeCN) and 2.5% formic acid (FA) and then dried. Peptides were then

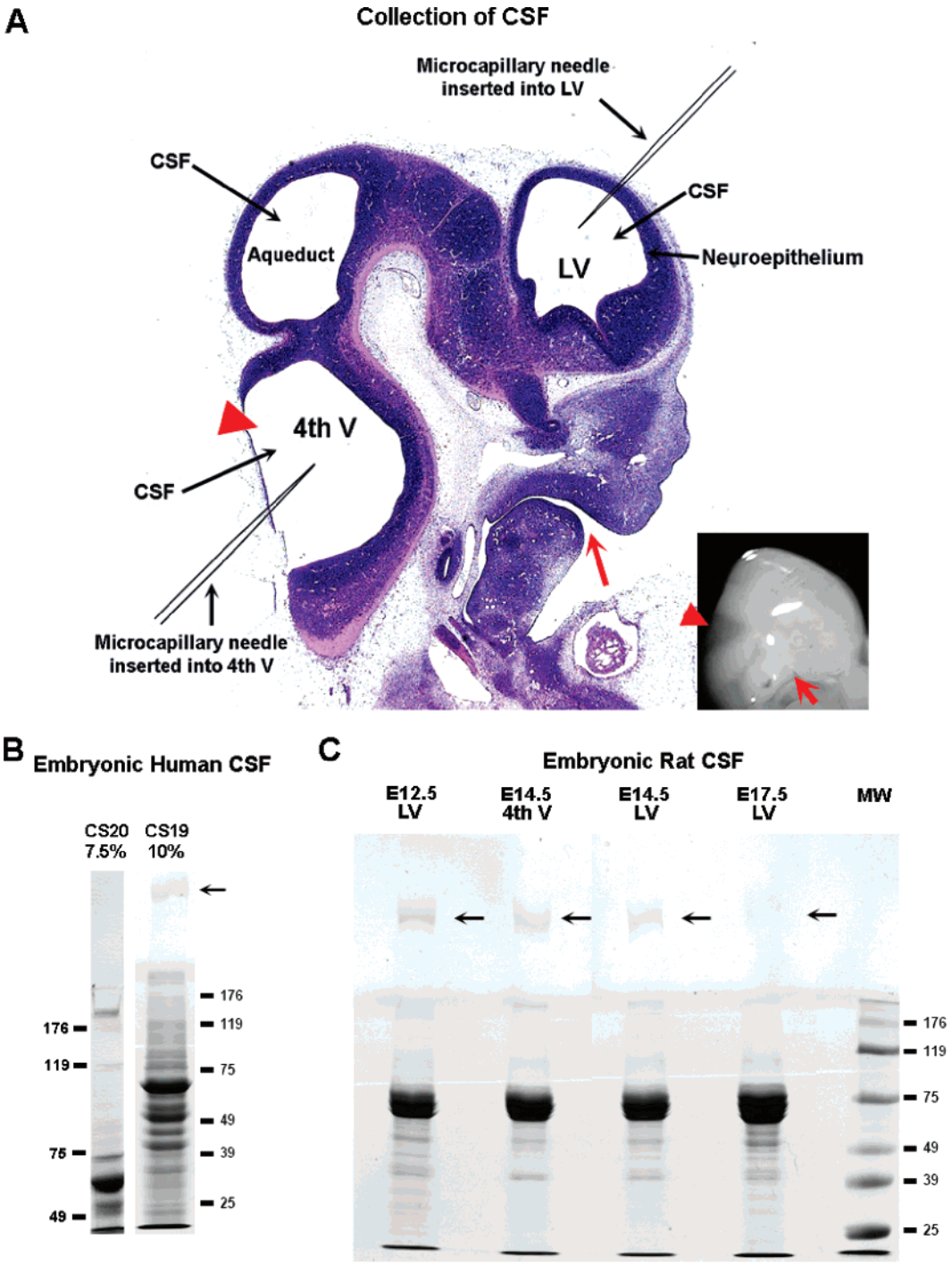


**Figure 1.** Extraction and SDS-PAGE analysis of human and rat embryonic CSF. (A) Image of hematoxylin and eosin sagittal section of E14.5 rat showing CSF aspiration technique and the position of the syringe needle relative to surrounding tissues in the lateral ventricle (LV) and the fourth ventricle (fourth V). Inset image of E14.5 rat embryo provides orientation. Red arrowhead is fourth V and red arrow is the mouth/chin. (B) CSF aspirated from the fourth ventricle of a CS20 human embryo (CS20) and a CS19 human embryo (CS19) was separated by size by SDS-PAGE on a 7.5% or 10% polyacrylamide gel, respectively. For clarity, the CS20 sample shows one-seventh of the sample used in the final analysis. (C) CSF aspirated from the lateral ventricles (LV) of E12.5, E14.5, and E17.5 and from fourth ventricle (fourth V) of E14.5 rat. During the procedure to collect CSF from E12.5, the micro-thin capillary needle is placed into the lateral ventricle of the E12.5 rat, and due to the patency of the neural tube at that point in development, the CSF is acquired from the lateral ventricle, the third ventricle, and the fourth ventricle. This is clearly visible as a sequential collapse of ventricles as the CSF is being aspirated. CSF from E14.5 rat can be collected from both the lateral ventricles (LV) and from the fourth ventricle (fourth V). The arrow in all samples represents Apolipoprotein-B.

resuspended in 2.5% MeCN and 2.5% FA and loaded using an autosampler onto a microcapillary column packed with 12 cm of reversed-phase MagicC18 material (5 $\mu$m, 200 Å, Michrom Bioresources, Inc.). Elution was performed with a 5−35% MeCN (0.1% FA) gradient over 60 min, after a 15 min isocratic loading at 2.5% MeCN and 0.5% FA. Mass spectra were acquired in

LTQ and LTQ-XL linear ion trap mass spectrometers (Termo Electron) over the entire 75 min using 10 MS/MS scans following each survey scan. Raw data were searched against either the human or rat IPI forward and reverse concatenated databases[22] using Sequest software requiring tryptic peptide matches with a 2 Da mass tolerance. Cysteine residues were required to have a static increase in 71.0 Da for acrylamide adduction, and differential modification of 16.0 Da on methionine residues was permitted. The resultant top matches for all analyses of each gel lane were compiled. Each list was then filtered independently using a dCn2 score of 0.2 and Xcorr scores of 1.8, 2.0, and 2.5 for singly, doubly, and triply charged ions, respectively. Proteins on these filtered lists that had two or more peptides were retained. However, keratin proteins were removed as they are known contaminants in most gel-based proteomic analyses. On the basis of the number of reverse database false-positives that were also retained following these filtering criteria, we estimate the following false-positive rates for the proteins in each sample: rat E12.5 lateral ventricle (LV), 0.45%; rat E14.5LV, 0.30%; rat E17.5LV, 0.50%; rat E14.5 fourth ventricle, <0.00%; and human CS 20, <0.00%. For the human CS 19 sample, the estimated false-positive rate for proteins identified by more than three peptides is <0.00%. The dataset of proteins for the embryonic mouse brain was extracted from LC−MS/MS data collected from 16 strong cation exchange (SCX) fractions generated during our previous study of the forebrain and midbrain extracts of E16.5 mouse embryos, where our methodology is described in detail.[23] Briefly, embryonic day 16.5 murine forebrains and midbrains were lysed and subjected to preparative SDS-PAGE. The gel was separated into four regions, and proteins were digested in-gel with trypsin. Tryptic peptides were subjected to SCX chromatography, and more than 40 fractions were collected for each region. Fractions were subjected to LC−MS/MS analysis, and fractions in the middle of the SCX runs were found to contain diverse proteins all with a solution charge state of 2+. For the analysis here, we compiled the LC−MS/MS data from four SCX fractions in the middle of the SCX gradients from each of the four regions of the gel, and the top 200 identified proteins were used for comparisons to e-CSF.

## Results and Discussion

**Human Embryonic Proteome.** Human CSF was collected from the fourth ventricle, as mentioned above, from two independent embryos at Carnegie Stage (CS) 19−20. From the first embryo (CS19), a total of 15 $\mu$L was collected, and from the second embryo (CS20), a total of 70 $\mu$L was collected. The CSF from these two independent samples was separated by 1-D SDS-PAGE, and Figure 1B shows the Coomassie stained protein pattern of the CSF from CS20 and CS19 embryos run on 7.5% and 10% polyacrylamide gels, respectively. The two human e-CSF samples were analyzed separately. Supporting Information Table 1 shows the proteomic analysis of the CSF collected from the CS20 embryo and lists 188 proteins with 2 or more peptides identified. Using a number of protein analysis programs such as UniProt, Gene Ontology (GO), and the PANTHER (Protein Analysis Through Evolutionary Relationships) classification system, we categorized the proteins found from the mass spectrometry data and listed subcellular localization, protein function, tissue specificity, and relevant notes pertaining to each protein (Supporting Information Table 1).[24] Analysis of the CSF from the CS19 human sample revealed 772 proteins with more than three peptides identified. The search results from this analysis suggested the presence of a number of non-CSF contaminants including 7 different mitochondrial specific precursor proteins such as the mitochondrial precursors for 4-Aminobutyrate Aminotransferase, Fumarate Hydratase, and Isoform Dut-M of Deoxyuridine 5′-Triphosphate Nucleotidohydrolase, whereas no mitochondrial precursor proteins were identified in the rat CSF or in the CS20 human CSF sample. Therefore, the CS19 list was not further considered in the comparison to rat CSF. However, the proteins from this analysis are presented in Supporting Information Table 2 as this list is certainly enriched for human e-CSF proteins. The substantial differences between this sample and the other human and rat samples suggest that this sample contained multiple impurities, likely from lysed blood and/or neuroepithelial cells. The impurities may be accounted for by differences between the time of death and the passing of the embryo; however, the difference was less than 3 h. Nonetheless, the differences also highlight that the MS analysis is highly sensitive to contaminants, and that the absence of mitochondrial proteins in other samples indicates that they are probably quite pure.

**Rat Embryonic Proteome.** CSF was collected from the lateral ventricle of E12.5, E14.5, and E17.5 rat embryos and from the fourth ventricle of E14.5 rat embryos. We chose to study three time points during rat development. We chose two time points (E12.5 and E14.5) that correspond to periods of neuronal development in which the neuroepithelial cells are highly proliferative. Both of these time points occur before the formation of the cortical plate which correspond closely to similar neuronal time points of CS 19−20 human embryos. In addition, we found that E12.5 is the first time point that CSF can be consistently aspirated from the lateral ventricle through a microcapillary pipet. At this time point, the ventricle is relatively thin and is primarily composed of proliferating neuroepithelial cells along the ventricular surface. E12.5 is approximately the same time period that the first post-mitotic neurons appear in the preplate just below the pial surface.[25,26] As the post-mitotic neurons accumulate in the preplate, they differentiate to become the subplate and the marginal zone during E14, with the first appearance of the cortical plate between the subplate and marginal zone at E15.[25,26] At E17.5, a time period post-cortical plate formation, the lateral ventricles are still large enough to collect pure samples of CSF, and all layers of the ventricle from the ventricular zone, the subventricular zone, the intermediate zone, the subplate, the cortical plate, and the marginal zone are clearly visible.[25,26] CSF from two litters (approximately 20−24 rat embryos) was pooled for each time point and was separated by 1-D SDS-PAGE, and the proteins were visualized with Coomassie blue stain. Figure 1C shows the Coomassie stained protein pattern of CSF collected from all three time points. Mass spectrometry analysis of the rat CSF was performed separately for E12.5, E14.5, E17.5 lateral ventricle, and E14.5 fourth ventricle and is presented as Supporting Information Table 4. There were 423 proteins identified in E12.5 LV CSF, 318 proteins in E14.5 LV, 249 proteins in E14.5 fourth V, and 382 proteins in E17.5 LV. There are 137 proteins common to E12.5, E14.5, and E17.5 rat CSF samples that are presented in Supporting Information Table 3 which includes the name of the protein, its molecular weight, subcellular localization, function, and tissue specificity. Also included are any relevant notes about each protein obtained from UniProt and Gene Ontology (GO) databases. Interestingly, there are 61 proteins identified in E12.5 LV, E14.5 LV, and E17.5 LV that were not identified in E14.5 fourth V and only 5 proteins

identified in E12.5 LV, E14.5 fourth V, and E17.5 LV that were not identified in E14.5 LV. This does not appear to be simply due to an overall reduction in E14.5 fourth V protein concentration as similar numbers of peptides were identified for the proteins found in common with LV CSF samples. Instead, the difference suggests potential differences in the protein composition of CSF between the lateral and fourth ventricles, though further studies would be needed to confirm this and to assess its significance.

Parada et al. identified 31 proteins within the rat e-CSF finding an abundance of extracellular matrix proteins, enzymes, and enzyme regulators, which is consistent with our study.[21] We identified in this study a much larger number of proteins within the CSF while identifying 24 of the 31 previously identified proteins. The 7 proteins that we did not find are the following: calreticulin, DJ-1, EEf1 g, laminin receptor 1, malate dehydrogenase 1, set beta isoform, and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta polypeptide. The differences between our study and the study by Parada et al. appear to be more a consequence of methodology rather than true sample differences. Parada et al. chose for mass spectrometry the most prominent silver-stained e-CSF proteins resolved by two-dimensional electrophoresis, whereas we performed an analysis of the entire e-CSF separated by one-dimensional electrophoresis. Although our one-dimensional approach enabled a more comprehensive analysis which would be unwieldy for an entire two-dimensional gel, the study by Parada et al. is complementary with ours as some proteins resolved in two dimensions would have a reduced likelihood of becoming suppressed due to comigration in one dimension with abundant protein species such as albumin.

Although our analyses are only semiquantitative, we found interesting differences between our various rat e-CSF samples. Apolipoprotein M is found in both E14.5 LV and E14.5 fourth V, but our analysis did not identify it in either E12.5 LV or E17.5 LV; phosphatidylethanolamine binding protein was found only in the E17.5 LV; collagen alpha 1 (XI) was identified in E14.5 and E17.5 LV, and phosphatase 2 (alpha isoform of regulatory subunit A) was found in E12.5 LV. Also, apolipoprotein D, an apolipoprotein that was not identified by Parada et al., was identified only in the E14.5 fourth V.

**Comparison of Human and Rat CSF.** In a comparison of proteins found in the human e-CSF with those proteins found in the rat e-CSF, we found that of the 188 proteins identified in the human e-CSF, 135 human proteins were identified in any one of the four samples of embryonic rat CSF. Eighty-three of those proteins were present in all four samples of embryonic rat CSF. Supporting Information Table 1 includes the human proteins found common to rat CSF. We have indicated the specific rat samples in which each protein was identified. Out of the top 50 proteins found in the human CSF, 45 were also found in the rat CSF.

In an attempt to match cortical stages of development between CS 19−20 humans to embryonic rats, we chose to isolate CSF from E12.5 and E14.5 rats. The first appearance of the cortical plate in rats is at E15 and as previously mentioned in humans at CS 21.[25,26] Therefore, to match cortical stages of embryonic human and rat CSF, we chose to isolate the CSF from E12.5 and E14.5 rats, two time periods preceding cortical plate formation and post choroid plexus formation. In addition, an analysis to predict neuronal development across species indicates that the neural development corresponding to human cortex, limbic, and other neural tissues from day 48 to day 53 (CS 19−20) translate to a range in rat embryonic days 11−14 with the majority of the overlap occurring at rat embryonic days 12.5−13.[27] Therefore, the analysis of rat embryonic CSF at E12.5 and E14.5 attempts to match the stage of human development at CS 19−20 from a neural development perspective.

Proteins common to human and rat CSF presumably represent proteins related to fundamental CSF functions. For example, e-CSF contains many transport and carrier proteins including transferrin, albumin, alpha-fetoprotein, transthyretin, ceruloplasmin, and plasma retinol-binding protein that are all involved in either metal ion or vitamin transport through fluid or across cell membranes. There are a number of apolipoproteins involved in the transport and metabolism of lipids and fatty acids in the CSF as reported in this paper and by Parada et al.[21] There are also a large number of enzymes and protease inhibitors in the CSF that are involved in regulating immune response and maintaining homeostasis.

Other proteins common to rat and human CSF may play more specific roles in neurogenesis. One factor in the e-CSF is Amyloid Beta A4 Protein Precursor (APP), which we identified in rat CSF at E12.5, E14.5, and E17.5 and human CSF at CS20. This protein is normally present in brain, and a soluble form is known to circulate in adult CSF.[28] The soluble form of APP has been shown to stimulate proliferation of embryonic neural stem cells as well as adult neural progenitor cells from the subventricular zone.[29−31] APP may play a role during neurogenesis not only within the cell, but may be released in the extracellular space and taken up in the CSF in order to diffuse throughout the CSF and play a function at more distant sites. Similarly, Tenascin, which we found in all CSF samples from rat and human from CS 20, is a secreted extracellular matrix glycoprotein implicated in axon guidance during development and regeneration,[32] which was recently shown to be expressed in progenitor cells in the ventricular zone of the developing brain.[46] CSF contains multiple critical extracellular matrix factors including fibronectin, laminin, tenascin, fibulin, versican, and neurocan core protein. Since many of these factors can support or orient neuronal migration, it raises the possibility that they may also be acting in the CSF as external cues for proliferating and differentiating neuronal progenitor cells.

Few proteins were identified that may be exclusive to rat or human e-CSF. The protein Pigment Epithelium Derived Factor (PEDF) was only found in the human e-CSF, is known to circulate in the adult CSF, and is significantly decreased in CSF of patients with frontotemporal dementia.[33] This secreted serpin family member, known to be released by retinal pigment cell into the matrix, is a known neurotrophic protein involved in survival and potentially differentiation of specific neurons.[34] PEDF is known to act on photoreceptor cells but also may play a role in spinal motor neuronal survival. PEDF has also been shown to be secreted by adult murine subventricular zone cells and maintains a niche for adult neuronal stem cells.[35] It is likely that PEDF is released by the photoreceptor cells or proliferating neuronal progenitor cells into the matrix and taken up by the CSF and may act on cell types and neurons by diffusion through the CSF. Similarly, the Neuronal Cell Adhesion Molecule L1-Like Protein, also found only in the human e-CSF, is known to play important roles in neurite outgrowth and neuronal survival.[36−38]

Conversely, only in rat e-CSF did we find the Extracellular Superoxide Dismutase, a protein known to remove free radicals that can be toxic to cells. One of the functions of the e-CSF may be the removal of toxins and toxin metabolic byproducts,

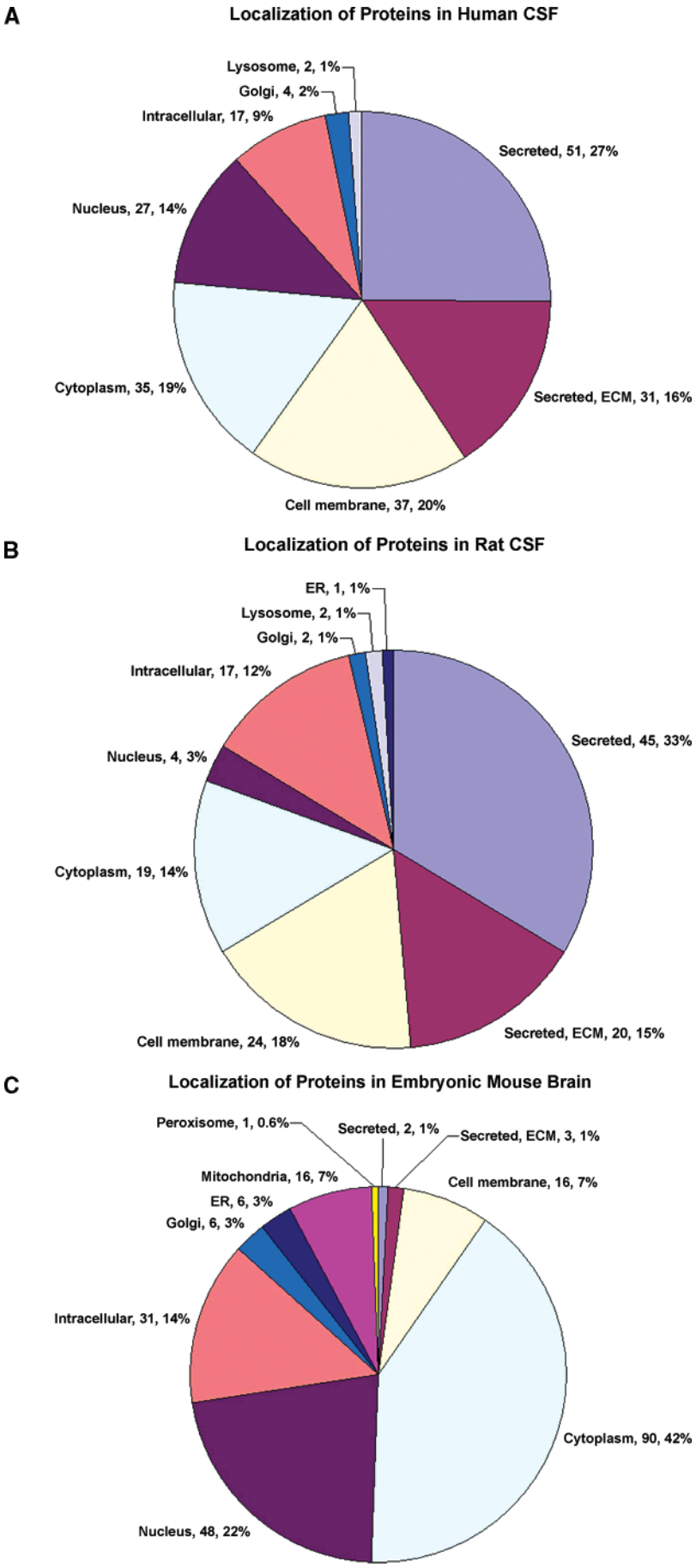


**Figure 2.** (Continued)

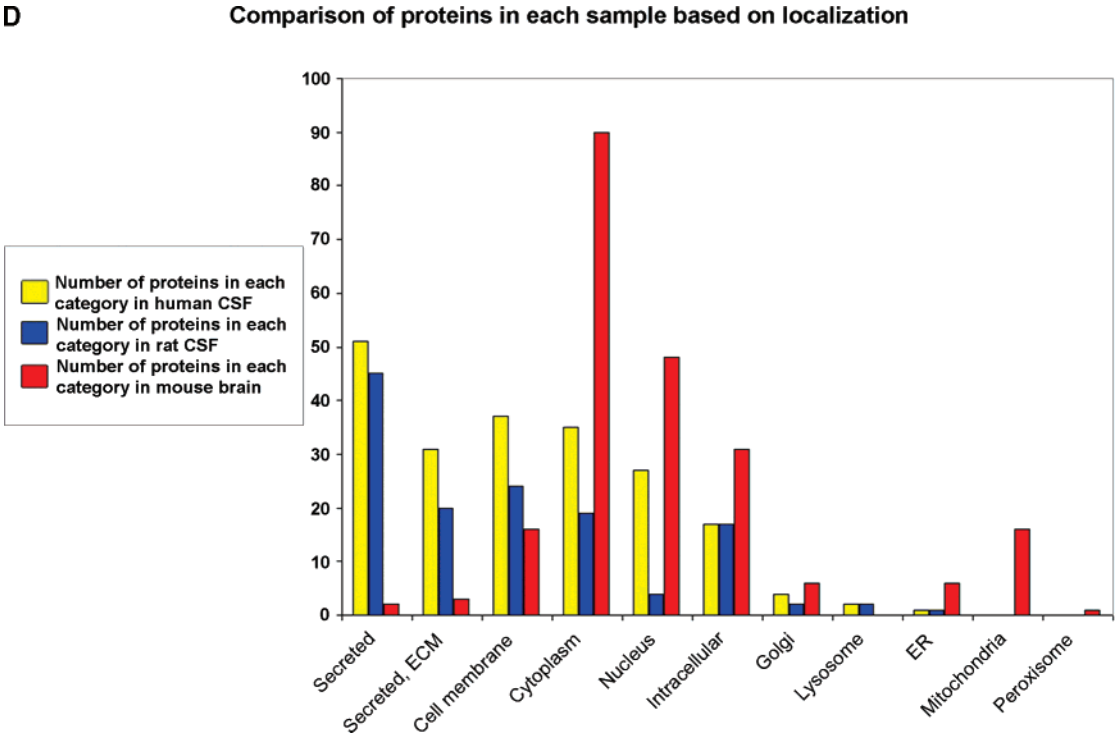


**Figure 2.** Classification and comparison of proteins based on subcellular localization. Graphic representation of the subcellular localization of proteins in CS 20 embryonic human CSF (A), embryonic rat CSF (B), and E16.5 mouse brain (C). The percentage of protein localization is calculated based on the total number of proteins localized to each space divided by the total number of proteins in the CSF that we were able to assign localization (human CSF-187 proteins, rat CSF-137 proteins, and mouse brain-179 proteins). Some proteins were localized to multiple compartments within the cell. (D) A comparison between human CSF, rat CSF, and mouse brain of the number of proteins from each category based on localization.

and therefore, it would be important to have proteins within the CSF that help neutralize some of the toxic products released into the CSF. Also of note we found in the rat e-CSF Mannose 6-phosphate/Insulin-like Growth Factor II Receptor (IGF2R). A soluble form of the receptor has been found in the serum, amniotic fluid, and urine of both rodents and humans, affecting organ size based on its interaction with IGF2 and other factors.[39−43]

**Subcellular Localization.** To compare the e-CSF of human and rat further, we analyzed the 188 proteins found in the human e-CSF and the 137 proteins in the rat e-CSF present in all samples based on subcellular localization, molecular function, and biological process. The subcellular localization of each protein in the CSF is shown in Supporting Information Tables 1 and 3. Figure 2 shows that a majority of proteins found in the human (A) and rat (B) e-CSF are secreted proteins which compose 27% and 33% of the total proteins found within the CSF, respectively. The second most common localization of proteins found in the e-CSF of both humans and rats are cell membrane proteins, composing 20% and 18%, respectively. The relatively high percentage of membrane proteins is consistent with the recent discovery of membrane bound particles in the CSF.[1] Out of 188 proteins found in the human e-CSF, 19% are cytoplasmic proteins, 16% are secreted proteins found in the extracellular space or extracellular matrix (ECM), 14% are nuclear proteins, and 9% are intracellular proteins that could not be specifically localized to one compartment. Out of 137 proteins present in all rat e-CSF samples, 14% are cytoplasmic proteins, 15% are ECM proteins, 3% are nuclear proteins, and 12% are intracellular proteins. As a control to assess subcellular localization in a protein population of embryonic brain, we chose to analyze the top 200 proteins identified from E16.5 mouse forebrain and midbrain in a previous study.[23] Figure 2C shows that 42% of these proteins are cytoplasmic, 22% nuclear, 14% intracellular, 7% at the cell membrane, and 7% mitochondrial. Strikingly, no mitochondrial proteins were found in the CSF. Out of the 200 proteins analyzed from embryonic mouse brain, two are secreted and three are found in the extracellular space or matrix. Figure 2D shows a graphical representation of the comparison of embryonic human CSF, rat CSF, and mouse brain based on localization. The e-CSF as compared to brain tissue clearly has an abundant number of secreted proteins, extracellular matrix proteins, and cell membrane proteins as opposed to an overwhelming majority of cytoplasmic, nuclear, and mitochondrial proteins found in the brain tissue.

**Molecular Function and Biological Process.** For a more comprehensive understanding of the classes of proteins found in the embryonic human and rat CSF, we used the PANTHER protein ontology database to classify the proteins into distinct categories of molecular function and biological process. Panther identified 180 out of 188 proteins with a total number of 237 functional hits for the human e-CSF, 119 out of 137 proteins with a total number of 155 functional hits for the rat e-CSF, and 191 out of 200 proteins with a total number of 234 functional hits for embryonic mouse brain. Table 1 shows the percentage of proteins assigned to each functional category in the embryonic human CSF, embryonic rat CSF, and E16.5 embryonic mouse brain. Supporting Information Figure 1 represents functional classification of the samples as individual pie charts including the absolute number of proteins assigned to each function group.

**Table 1.** List of Protein Categories Based on Molecular Function for Embryonic Human CSF, Rat CSF, and Mouse Brain

| human CSF | percent proteins in each category | rat CSF | percent proteins in each category | mouse brain | percent proteins in each category |
|---|---|---|---|---|---|
| Cell adhesion | 11.1% | Cell adhesion | 12.6% | Cell adhesion | 2.60% |
| Chaperone | 5.0% | Chaperone | 5.0% | Chaperone | 8.40% |
| Cytoskeletal | 7.2% | Cytoskeletal | 8.4% | Cytoskeletal | 11.50% |
| Defense/Immunity | 8.3% | Defense/Immunity | 6.7% | Defense/Immunity | 0.00% |
| Extracellular matrix | 15.6% | Extracellular matrix | 10.9% | Extracellular matrix | 0.50% |
| Hydrolase | 2.2% | Hydrolase | 1.7% | Hydrolase | 6.30% |
| Kinase | 1.1% | Kinase | 2.5% | Kinase | 2.60% |
| Ligase | 0.6% | Ligase | 0.8% | Ligase | 3.70% |
| Membrane traffic | 1.1% | Membrane traffic | 0.8% | Membrane traffic | 2.60% |
| Miscellaneous | 4.4% | Miscellaneous | 3.4% | Miscellaneous | 2.60% |
| Unclassified | 7.2% | Unclassified | 5.9% | Unclassified | 14.70% |
| Nucleic acid binding | 10.0% | Nucleic acid binding | 5.0% | Nucleic acid binding | 18.30% |
| Oxidoreductase | 2.8% | Oxidoreductase | 5.0% | Oxidoreductase | 4.70% |
| Phosphatase | 1.1% | Phosphatase | 2.5% | Phosphatase | 1.60% |
| Protease | 7.2% | Protease | 6.0% | Protease | 1.60% |
| Receptor | 7.8% | Receptor | 10.1% | Receptor | 2.10% |
| Calcium binding | 2.8% | Calcium binding | 4.2% | Calcium binding | 3.70% |
| Regulatory molecule | 13.3% | Regulatory molecule | 12.6% | Regulatory molecule | 8.40% |
| Signaling molecule | 6.1% | Signaling molecule | 6.0% | Signaling molecule | 1.60% |
| Synthase and synthetase | 0.6% | Synthase and synthetase | 1.0% | Synthase and synthetase | 2.60% |
| Transcription factor | 1.1% | Transcription factor | 1.0% | Transcription factor | 3.70% |
| Transfer/Carrier | 8.3% | Transfer/Carrier | 12.6% | Transfer/Carrier | 3.70% |
| Transferase | 1.7% | Transferase | 1.0% | Transferase | 4.70% |
| Transporter | 3.9% | Transporter | 3.4% | Transporter | 4.70% |
| Cell junction protein | 1.1% | Cell junction protein | 0% | Cell junction protein | 0% |
| Lyase | 0% | Lyase | 0% | Lyase | 1.60% |
| Ion channel | 0% | Ion channel | 0% | Ion channel | 1.60% |
| Isomerase | 0% | Isomerase | 1.7% | Isomerase | 1.00% |

Panther analysis of molecular function reveals that the majority of proteins found within the human and rat CSF share similar functional categories (Table 1, Figure 3, and Supporting Information Figure 1). Proteins involved in extracellular matrix function make up, respectively, 16% and 11% of the majority of proteins found in the e-CSF of humans and rats. Other

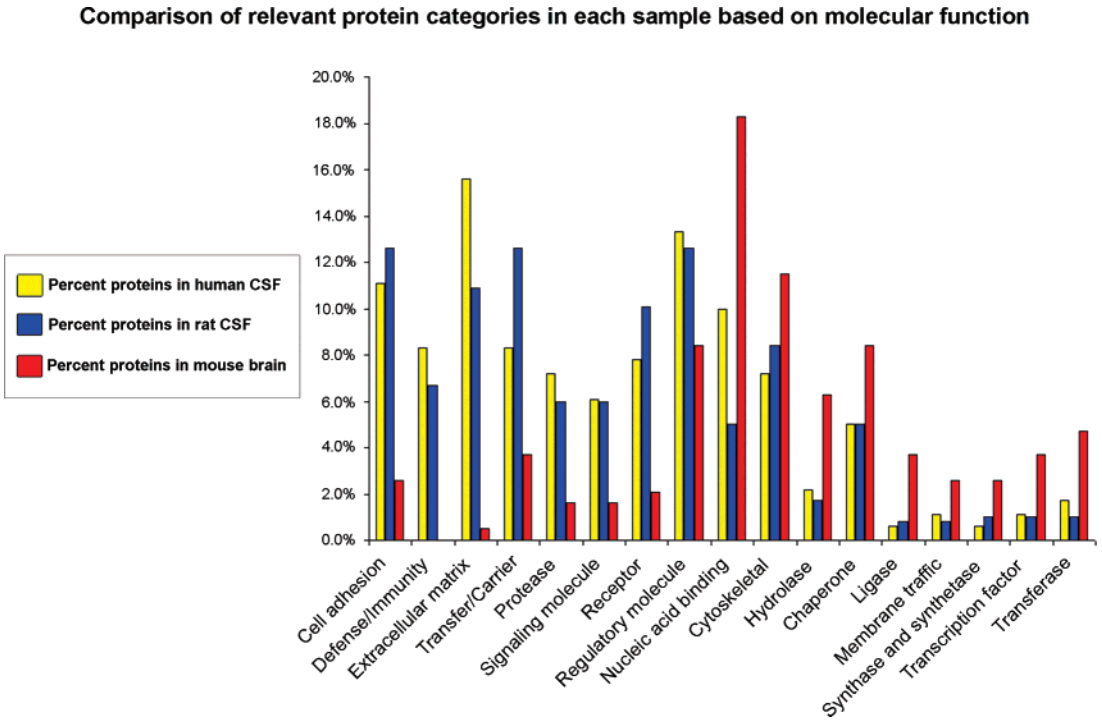


**Figure 3.** Comparison of proteins based on molecular function. Proteins present in embryonic human CSF, embryonic rat CSF, and embryonic mouse brain were analyzed using the Panther gene ontology database and classified according to molecular function. Chart includes protein category name, and percentage is calculated from number of proteins assigned to each category over total number of proteins analyzed. We show a comparison between human CSF, rat CSF, and mouse brain of the relative percentages from relevant categories based on molecular function.

**Table 2.** List of Protein Categories Based on Biological Process for Embryonic Human CSF, Rat CSF, and Mouse Brain

| human CSF | percent proteins in each category | rat CSF | percent proteins in each category | mouse brain | percent proteins in each category |
|---|---|---|---|---|---|
| Neuronal activities | 0.6% | Neuronal activities | 0.8% | Neuronal activities | 1.60% |
| Signal transduction | 25.0% | Signal transduction | 26.1% | Signal transduction | 8.90% |
| Developmental processes | 16.1% | Developmental processes | 16.8% | Developmental processes | 7.30% |
| Cell proliferation and differentiation | 4.4% | Cell proliferation and differentiation | 6.7% | Cell proliferation and differentiation | 3.70% |
| Coenzyme and prosthetic group metabolism | 0.6% | Coenzyme and prosthetic group metabolism | 1.7% | Coenzyme and prosthetic group metabolism | 1.60% |
| Cell structure and motility | 13.9% | Cell structure and motility | 16.0% | Cell structure and motility | 10.50% |
| Immunity and defense | 22.2% | Immunity and defense | 18.5% | Immunity and defense | 4.20% |
| Apoptosis | 2.8% | Apoptosis | 2.5% | Apoptosis | 3.10% |
| Oncogenesis | 2.2% | Oncogenesis | 3.4% | Oncogenesis | 2.10% |
| Muscle contraction | 0.6% | Muscle contraction | 0.8% | Muscle contraction | 0.50% |
| Transport | 8.9% | Transport | 15.1% | Transport | 9.40% |
| Blood circulation and gas exchange | 5.0% | Blood circulation and gas exchange | 5.9% | Blood circulation and gas exchange | 0.50% |
| Carbohydrate metabolism | 1.1% | Carbohydrate metabolism | 1.7% | Carbohydrate metabolism | 3.70% |
| Nucleoside, nucleotide and nucleic acid metabolism | 10.6% | Nucleoside, nucleotide and nucleic acid metabolism | 5.0% | Nucleoside, nucleotide and nucleic acid metabolism | 18.80% |
| Homeostasis | 0.6% | Homeostasis | 2.5% | Homeostasis | 1.60% |
| Protein metabolism and modification | 27.8% | Protein metabolism and modification | 27.7% | Protein metabolism and modification | 24.60% |
| Cell cycle | 6.7% | Cell cycle | 7.6% | Cell cycle | 11.00% |
| Intracellular protein traffic | 9.4% | Intracellular protein traffic | 11.8% | Intracellular protein traffic | 13.10% |
| Cell adhesion | 20.0% | Cell adhesion | 17.6% | Cell adhesion | 1.60% |
| Lipid, fatty acid and steroid metabolism | 3.3% | Lipid, fatty acid and steroid metabolism | 5.9% | Lipid, fatty acid and steroid metabolism | 3.10% |
| Sensory perception | 1.1% | Sensory perception | 1.7% | Sensory perception | 0.50% |
| Electron transport | 0.6% | Electron transport | 0.8% | Electron transport | 1.00% |
| Amino acid metabolism | 0.6% | Amino acid metabolism | 0.8% | Amino acid metabolism | 1.00% |
| Biological process unclassified | 5.0% | Biological process unclassified | 5.0% | Biological process unclassified | 15.20% |
| Protein targeting and localization | 2.2% | Protein targeting and localization | 2.5% | Protein targeting and localization | 4.20% |
| Miscellaneous | 1.1% | Miscellaneous | 0.8% | Miscellaneous | 1.60% |
| Phosphate metabolism | 0.0% | Phosphate metabolism | 0.0% | Phosphate metabolism | 0.50% |
| Other metabolism | 0.0% | Other metabolism | 0.0% | Other metabolism | 1.00% |

abundant categories of proteins found in the e-CSF include regulatory molecules such as protease inhibitors (human-13%, rat-13%), cell adhesion proteins (human-11%, rat-13%), nucleic acid binding proteins (human-10%, rat-5%), transfer/carrier proteins (human-8%, rat-13%), immune defense proteins (human-8%, rat-7%), and receptors (human-8%, rat-10%). The total number of enzymes also is a large component of the CSF. The embryonic human CSF has a total of 28 different functional enzymes (16%), and embryonic rat CSF has a total of 23 different functional enzymes (19%). Furthermore, the e-CSF is composed of a large number of different enzyme classes, and is particularly high in proteases (human-7%, rat-6%), and oxidoreductases (human-3%, rat-5%).

Panther analysis reveals distinct functional groups of proteins present in the CSF as compared to embryonic tissue. Protein categories in the embryonic human and rat CSF are quite similar, and to control for random similarity in categorization based on molecular function, we compared the CSF protein samples to a sample of 200 most abundant proteins in embryonic E16.5 mouse brain (Table 1). The comparison of relevant protein categories in each sample is shown in Figure 3. The two largest categories of proteins in the embryonic mouse brain include nucleic acid binding proteins (18.3%) and cytoskeletal proteins (11.5%). Interestingly, proteins involved in defense and immunity which comprised 7−8% of e-CSF were completely absent from the top 200 proteins in the embryonic mouse brain sample. One category of proteins that appears to be similar in all three comparisons is regulatory molecules (13.3% in human CSF, 12.6% in rat CSF, and 8.4% in mouse brain). We further classified the regulatory molecules into smaller categories, and although the larger classification shows similar percentages of regulatory molecules, the subclassification clearly distinguishes the e-CSF samples from the embryonic brain sample (Supporting Information Figure 3). The majority of proteins in the e-CSF within the regulatory molecule class are subclassified as protease inhibitors comprising 75% and 87% of proteins within the class in human and rat CSF, respectively, as compared to 0% in the mouse brain (Supporting Information Figure 3). On the basis of molecular function, the most abundant classes of protein present in the e-CSF are found to be proteins of the extracellular matrix, regulatory molecules, transfer/carrier proteins, cell adhesion proteins, and proteins involved in immunity and defense.

Panther analysis of proteins based on biological process reveals strong similarity between the embryonic human and rat CSF and differences between the CSF and the embryonic brain (Table 2, Figure 4, and Supporting Information Figure 2). The five most abundant classes in both embryonic human and rat CSF are protein metabolism and modification, signal transduction, immunity and defense, cell adhesion, and developmental processes. The majority of proteins in the analysis of the embryonic mouse brain are involved in protein metabolism and modification, nucleic acid metabolism, intracellular protein traffic, cell cycle, and cell structure and motility. Comparing the analysis of the mouse brain with the e-CSF shows that the CSF samples contain proteins that are enriched

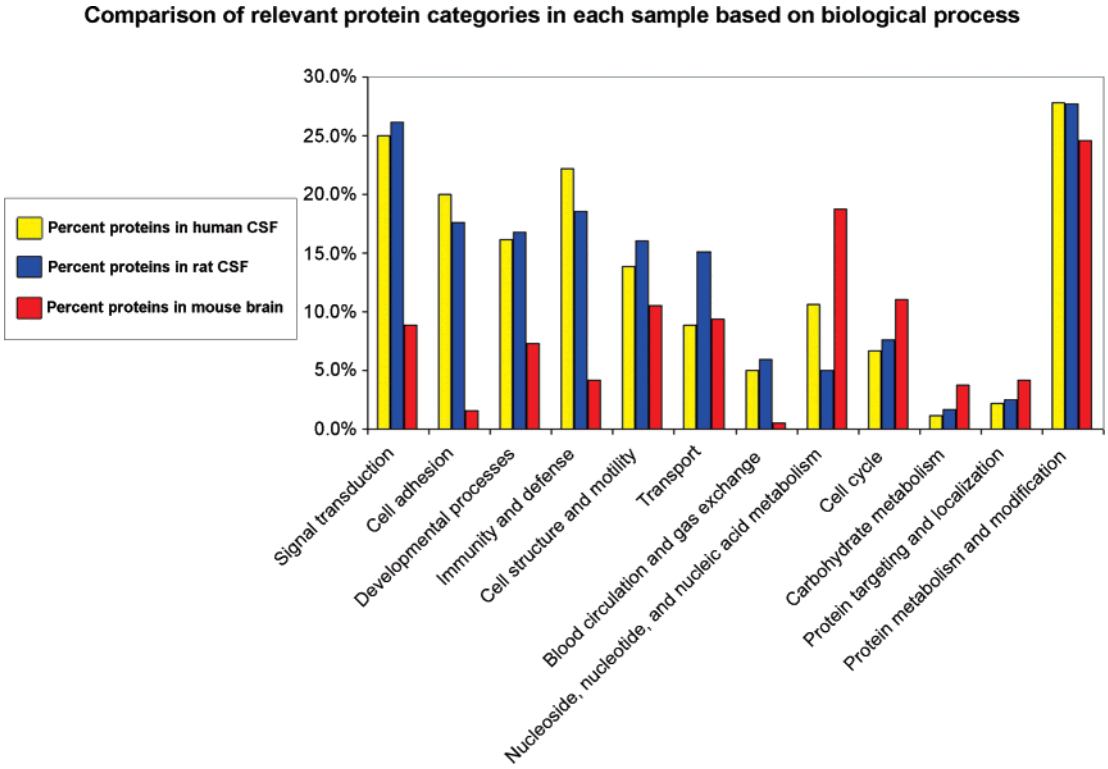


**Figure 4.** Comparison of proteins based on biological process. Proteins present in embryonic human CSF, embryonic rat CSF, and embryonic mouse brain were analyzed using the Panther gene ontology database and classified according to the biological process the proteins are involved with. Chart includes protein category name, and percentage is calculated from number of proteins assigned to each category over total number of proteins analyzed. We show a comparison between human CSF, rat CSF, and mouse brain of the relative percentages from relevant categories based on biological process.

for a number of various biological processes that are distinct from that of embryonic brain tissue (Figure 4). Interestingly, all three samples are most abundant in proteins involved in protein metabolism and modification (Figure 4). However, Panther analysis shows that CSF and brain show different types of proteins even among the same overall class (Supporting Information Figure 4). Subclassification of this category reveals the majority of proteins in the mouse brain involved in protein biosynthesis (30%) and protein modification (28%) with only 19% of proteins involved in proteolysis (Supporting Information Figure 4). However, in both the human and rat e-CSF, the overwhelming majority of proteins in both samples is involved in proteolysis comprising 58% in humans and 54% in rats (Supporting Information Figure 4). This class of biological processes includes the large number of protease inhibitors and proteases found within the CSF.

The similarities between the embryonic human and rat CSF are quite apparent when the proteins are classified into groups and analyzed on the basis of subcellular localization, molecular function, and biological process. On the basis of the analysis of the functional characteristics of the proteins found in the e-CSF, it is clear that the CSF is a very heterogeneous mixture of many classes of proteins with varying functions. It is also becoming apparent that the e-CSF is much more complex than previously thought. This may be due to active secretion from the choroid plexus into the CSF, or from the contents within the extracellular membrane bound particles that are present in the rodent CSF during development, or potentially to aposomes budding from the choroid plexus and floating within the CSF that have been shown previously to support protein translation.[3,44,45] Whether these particles or aposomes have any function during development still needs to be determined.

Although we did not find the growth factor FGF-2 as reported by Martin et al.,[16] many growth factors are in low abundance and are of smaller molecular weight making them more challenging to identify by multiple peptide assignments using mass spectrometry on a complex mixture. Therefore, a further proteomic exploration of the CSF may involve approaches to remove some of the very abundant proteins prior to analysis. In addition, targeted Western blots may be used for the determination of the presence of specific growth factors in the CSF. Nevertheless, we did identify a number of protein factors with signaling capacity such as PEDF, APP, apolipoproteins, tenascin, and soluble IGF2R as discussed above.

## Conclusion

An in-depth analysis of the composition of the CSF bathing the developing neuroepithelium of the vertebrate central nervous system is important to an understanding of the optimal fluid environment promoting and maintaining embryonic neurogenesis. Here, we present an extensive proteomic analysis of e-CSF, and present the first large-scale analysis of human e-CSF. We have found that e-CSF is a complex fluid harboring a large number of functionally diverse proteins. Through side-by-side comparisons, we have found great similarity in the composition and biological function of proteins present in the e-CSF of humans and rats. We anticipate this wealth of molecular information will set the groundwork for more targeted analyses into how these proteins might function

individually and in concert to stimulate neuronal proliferation and differentiation to effectuate proper brain development.

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**Supporting Information Available:** Movie showing CSF sample collection technique from an E17.5 rat embryo; figure showing the classification of proteins based on molecular function, biological process, subclassification of regulatory molecules based on molecular function, subclassification of protein metabolism and modification based on biological process; tables listing the mass spectrometry analysis of CS20 and CS19 human e-CSF, common proteins of embryonic rat CSF from E12.5 LV, E14.5 LV and fourth V, and E17.5 LV. This material is available free of charge via the Internet at http://pubs.acs.org.

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