RESEARCH ARTICLE

Proteomic analysis of cerebrospinal fluid in California sea lions (*Zalophus californianus*) with domoic acid toxicosis identifies proteins associated with neurodegeneration

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Proteomic studies including marine mammals are rare, largely due to the lack of fully sequenced genomes. This has hampered the application of these techniques toward biomarker discovery efforts for monitoring of health and disease in these animals. We conducted a pilot label-free LC-MS/MS study to profile and compare the cerebrospinal fluid from California sea lions with domoic acid toxicosis (DAT) and without DAT. Across 11 samples, a total of 206 proteins were identified (FDR<0.1) using a composite mammalian database. Several peptide identifications were validated using stable isotope labeled peptides. Comparison of spectral counts revealed seven proteins that were elevated in the cerebrospinal fluid from sea lions with DAT: complement C3, complement factor B, dickkopf-3, malate dehydrogenase 1, neuron cell adhesion molecule 1, gelsolin, and neuronal cell adhesion molecule. Immunoblot analysis found reelin to be depressed in the cerebrospinal fluid from California sea lions with DAT. Mice administered domoic acid also had lower hippocampal reelin protein levels suggesting that domoic acid depresses reelin similar to kainic acid. In summary, proteomic analysis of cerebrospinal fluid in marine mammals is a useful tool to characterize the underlying molecular pathology of neurodegenerative disease. All MS data have been deposited in the ProteomeXchange with identifier PXD002105 (http://proteomecentral.proteomexchange.org/dataset/PXD002105).

Keywords:
Animal proteomics / Label-free / Marine mammal / Neuron cell adhesion molecule / Reelin / Spectral count

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Abbreviations: **AMPA**, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; **C3**, complement C3; **CDS**, coding sequence; **CFB**, complement factor B; **CSF**, cerebrospinal fluid; **DA**, domoic acid; **DAT**, domoic acid toxicosis; **DKK**, dickkopf; **GSN**, gelsolin; **HGNC**, HUGO gene nomenclature committee; **MDH1**, malate dehydrogenase, cytoplasmic; **NCAM1**, neural cell adhesion molecule 1; **NMDA**, N-methyl-D-aspartate; **NRCAM**, neuronal cell adhesion molecule; **SIS**, stable isotope standard

Statement of significance

This study presents the first proteomic characterization of cerebrospinal fluid from a marine mammal. The use of a composite mammalian proteomic database together with optimized search parameters to limit the local FDR provides a method by which proteomic analysis of marine mammal samples can be accomplished. Peptide level identifications were confirmed using stable isotope standard peptides, improving the confidence and potential of this workflow. When applied to the problem of domoic acid toxicosis in sea lions, proteomic characterization revealed several interesting targets that may prove useful as biomarkers for diagnosis or staging.

1 Introduction

Mass strandings of California sea lions (*Zalophus californianus*) have been occurring seasonally since the mid-1990s, coinciding with blooms of algae that produce the potent neurotoxin domoic acid [1–3]. Ingestion of domoic acid by sea lions causes neurotoxicity and is characterized by seizures (*status epilepticus*), disorientation, and lethargy. Domoic acid resembles the neurotransmitter glutamate in structure, but is a highly potent agonist of kainate and high-affinity AMPA receptors [4] which leads to excessive activation and neuronal cell death [5]. Sea lions with chronic domoic acid toxicosis (DAT) exhibit significant neuron loss in all regions of the hippocampus, but notably more so in the right lobe [6]. The presence of characteristic epileptiform waves measured by electroencephalogram, pathological similarities between temporal lobe epilepsy in humans, and the fact that kainic acid is utilized to develop models of epilepsy in rodents, all support the conclusion that domoic acid toxicosis is an epileptic disease [6–8].

Diagnosis of DAT is problematic because domoic acid is rapidly cleared from the body [9, 10] and may not be directly detected when stranded sea lions are ultimately found. Biomarker studies have focused on providing support for the diagnosis of DAT when the toxin is not detected, using less-invasive samples such as serum, plasma, or whole blood [11–15] or behavioral criteria [16], but have not yet been validated in large independent trials. Biomarkers of prognosis or predictors of response to therapy are not known because follow-up collections are difficult to obtain from wild animals. Although other organs are reported to be affected or injured by domoic acid [10, 14, 17], the most profound effects are localized in the brain [6, 18, 19], but techniques such as magnetic resonance imaging to diagnose DAT are impractical for wildlife rehabilitation on a large scale.

Cerebrospinal fluid (CSF) surrounds the brain and central nervous system, the majority of which is produced at the choroid plexus in the lateral ventricles of the brain [20] which are adjacent to the hippocampus. The CSF has been routinely investigated as a source of biomarkers for the identification and treatment of neurological disorders [21–25] and the study of kainate neurotoxicity [26]. Because CSF is separated from the blood compartment, problems related to biomarker discovery due to high abundance proteins found in serum and plasma [27] are less imposing, and the number of proteins contained within the CSF more closely reflects neuropathology than those found in blood [28].

Aside from the fact that sampling CSF is more invasive than sampling blood, the use of proteomics to investigate this relevant biofluid in marine mammals is confounded by the fact that proteomics relies on sequenced and annotated genomes for protein identification. For marine mammals, only a few genomes at low sequence coverage have been assembled [29], and only the annotated dolphin genome (http://www.ensembl.org/Tursiops_truncatus/Info/Index; 2.59X coverage) is publicly available for proteomic query at this time. Fortunately, sea lions are closely related to terrestrial mammals (order Carniovora, suborder Caniformia) that do have fully sequenced genomes [30, 31]. By combining databases for mammals, we demonstrate that conserved peptides across multiple taxa can provide suitable information to make peptide-level and protein-level identifications in CSF from sea lions. Further, we present validation for the peptide level identifications using synthetic stable isotope labeled peptides. A pilot proteomic analysis of CSF was conducted for sea lions with DAT and without DAT to implicate proteins as candidate biomarkers for the diagnosis of DAT and for future qualification as prognostic and predictive biomarkers to guide clinical trials.

2 Materials and methods

2.1 Samples

Samples were collected by The Marine Mammal Center (TMMC; Sausalito, CA, USA) under the National Fisheries Service permit number 932-1489-00 (MA-009526) as part of a standard clinical care regime. CSF (2 to 4 mL) was collected aseptically via the atlanto-occipital joint, as described for domestic dogs [32], using a 3.5 in long 18G needle and immediately transferred to a cryovial and stored at –70°C. Clinical laboratory data or notes and detailed sampling methods are included in Supporting Information and Supporting Information Table 1.

2.2 Protein digestion and LC-MS/MS

CSF proteins were precipitated with acetone and resuspended proteins were digested with trypsin prior to analysis by

LC-MS/MS according to detailed protocols included in Supporting Information.

2.3 Protein identification

Raw files were converted to peak lists in the mgf format using the AB Sciex MS Data Converter (v. 1.1 beta, July 2011). These data were searched using the Mascot algorithm (v. 2.4.1; Matrix Science) against a mammalian database. This database was created using the 2015_01 releases of the SwissProt, SwissProt varsplic, and TrEMBL databases from UniProt. Within Mascot, the mammalian taxon ID, 40674, was specified, as well as the common Repository of Adventitious Proteins database (cRAP; 2012.01.01; the Global Proteome Machine), resulting in 1 319 830 sequences. The following search parameters were used: trypsin was specified as the enzyme allowing for two mis-cleavages; carbamidomethyl (C) was fixed and deamidated (NQ) and oxidation (M) were variable modifications; 30 ppm precursor mass tolerance and 0.5 Da fragment ion tolerance; instrument type was specified as ESI-QUAD-TOF; a decoy database was used within Mascot to provide local FDR. These database and precursor tolerances were confirmed as optimal since they provided the highest number of identified proteins and the lowest local FDR when compared to searches that did not use TrEMBL and/or used a species subset as was previously utilized [12] (Supporting Information Table 3). Mascot results were confirmed using Scaffold (v. 3.6.5; Proteome Software, Portland, OR, USA), with a minimum protein confidence of 99%, two peptides and 80% peptide confidence. Experiment wide grouping was used which resulted in 206 proteins identified at >0.1% FDR (as determined by Mascot's local FDR). Unweighted spectrum counts were exported from Scaffold for statistical analysis in R (v. 3.1.2). The MS proteomics data have been deposited to the ProteomeXchange Consortium [33] via the PRIDE partner repository with the dataset identifier PXD002105 and 10.6019/PXD002105.

2.4 Data analysis

Because samples were interfaced to the tandem mass spectrometer based on original volume, spectral count data was not normalized by TIC because this would have removed the concentration dependent nature of the analysis. Count data were $\log_2$ transformed, and if no counts were observed the minimum observed value was used (i.e., 1). A moderated *t*-test was used to detect significantly different proteins using the *limma* package [34]. Using the linear model, the plot of residual standard deviation versus average log counts was used to determine an appropriate independent filter based on abundance, which was 1. Using this filter, of the 206 protein families with count data, 117 were evaluated with the moderated *t*-test. Once the linear model was fit to the data, the empirical Bayes statistics for differential expression was performed with the following parameters: trend = TRUE, robust = TRUE. Proteins were considered significant at $p < 0.05$.

In order to evaluate the composition of the CSF proteome, each of the 206 protein families were converted to a human equivalent protein. This was either based on the original species assignment or sequence similarity search results via UniProt's BLAST (Basic Local Alignment Search Tool). Once assignments were made, spectral counts from the same proteins were combined (e.g., seven entries that aligned with albumin were merged), resulting in a dereplicated list of 172 proteins.

2.5 Validation of peptide level identifications

Peptide identifications of four proteins were validated using stable isotope standard (SIS) peptides synthesized by New England Peptide (Gardner, MA, USA) with isotopically labeled ('^'; ^{13}C and ^{15}N) c-terminus amino acids: QELENLER^ (dickkopf-3), TGAQELLR^ (gelsolin), NAPTPQEFR^ (NCAM1), and IISVELPDDAR^ (reelin). Concentration was determined by amino acid analysis and collision energy was optimized as previously described [35]. An aliquot of CSF (60 μL) was digested the same as described above except that a ratio of 1:10 trypsin:protein was used. After 16 h, the digestion was halted with 200 μL 1% formic acid, and 5 μL of a 100 fmol/μL stock of the four SIS peptides was added (i.e., 500 fmoles each). Next the sample was desalted as described above, and brought up in 100 μL MPA prior to analysis. Tryptic peptides (1 μL) were separated by reverse phase at 300 nL/min with a 37 min gradient as follows: 3% to 30% MPB over 12 min, 30 to 85% MPB over 8 min, held at 85% MPB for 5 min then returned to 3% MPB. PRM was performed by setting the TripleTOF 5600 in positive ion mode and TOF-MS data were collected in a window of 400 to 1250 *m/z* for 150 ms, followed by each parent ion MS/MS for 100 ms, collecting data from 100 to 1600 *m/z*. The MS data have been deposited to the PeptideAtlas SRM Experiment Library (PASSEL; http://www.peptideatlas.org/passel/) with the dataset identifier PASS00677.

3 Results

3.1 Study population

Samples of CSF were taken from 11 California sea lions with and without domoic acid toxicosis between 2003 and 2009 after admission to The Marine Mammal Center. Out of the eight individuals exhibiting signs of DAT, six were diagnosed with chronic DAT and two with acute DAT. Due to the small sample size all eight were broadly grouped together as DAT (Supporting Information Table 2). Out of the three non-DAT individuals, two had encephalopathy and were released, and all three were confirmed as not having DAT. Since samples were not acquired prospectively, the two populations are different based on gender and age. Specifically the non-DAT group was all male and younger than the DAT group that was mostly adult/subadult females (6 of 8). Protein concentration

tended to be higher in the DAT group, 0.32 ± 0.15 mg/mL (± SD), than the non-DAT group, 0.19 ± 0.07 mg/mL, though this difference was not significant (equal *t*-test, $p = 0.181$).

3.2 Sea lion CSF proteomics

Shotgun proteomics was used to characterize the CSF proteome and compare protein abundance between samples. Using a mammalian proteome database, 206 proteins were identified across the 11 samples (Supporting Information Table 4). More than half of these proteins were assigned to entries from the panda, dog, and ferret databases (46, 31, and 30, respectively), with only two from sea lions (Supporting Information Table 5). Protein identifications were assigned HGNC gene symbols and concatenated into 172 homologous gene groups to evaluate relative protein abundance within the CSF. The ten most abundant proteins based on concatenated spectral counts, comprising half of the proteome, were albumin, serotransferrin, complement C3, fibronectin, prostaglandin-H2 D-isomerase, apolipoprotein E, clusterin, gelsolin, IGHV3-23, and beta-2-micoglobulin, while the top 33 proteins comprised 75% of the proteome (Fig. 1 and Supporting Information Table 6). The protein composition was compared to studies of CSF from other species, human [36], mouse [37], and rat [38]. Comparisons were made only for proteins with HGNC symbols and not complete datasets. Based on presence/absence, protein composition was compared using hierarchical clustering and the sea lion dataset clustered closest to human and rat (Supporting Information Fig. 1). These three datasets were compared further and identified a high degree of overlap between the sea lion and rat (33 proteins, 75%) and human (64 proteins, 75%), while 78 proteins (51%) were unique to the sea lion dataset. Similarly, the presence of the seven differentially abundant proteins in CSF from sea lions diagnosed with DAT was determined in the other studies and showed a similar degree of overlap with the human and rat studies (six of seven in the human study, and three of seven in rat study; Supporting Information Table 7).

To detect which proteins were differentially abundant between samples, log-transformed spectral counts were used to perform a moderated *t*-test (Supporting Information Table 4). Seven proteins were identified as differentially abundant (moderated *t*-test, $p < 0.05$) and all were increased in the DAT group: neuronal cell adhesion molecule (NRCAM), neural cell adhesion molecule 1 (NCAM1), dickkopf-3 (DKK3), gelsolin (GSN), malate dehydrogenase, cytoplasmic (MDH1), complement C3 (C3), and complement factor B (CFB). The spectral count distribution for each of these proteins is shown in Fig. 2 as well as within Supporting Information Table 1. A Benjamini–Hochberg procedure was also utilized to correct for multiple testing, though this yielded no proteins at an FDR < 10%. Depending on the severity of abundance based independent filtering prior to testing, it was possible to identify NRCAM and NCAM1 as different at FDR < 10% (e.g., using a mean abundance cutoff of four spectral counts, both have a Benjamini–Hochberg adjusted $p = 0.089$). In addition to identifying proteins by MS/MS, peptide level identifications were validated using SIS peptides created for prototypic peptides identified belonging to DKK3, gelsolin, NCAM1, and reelin. Based on retention time and product ions generated by CID, the native and synthetic peptides matched almost identically (Fig. 3).

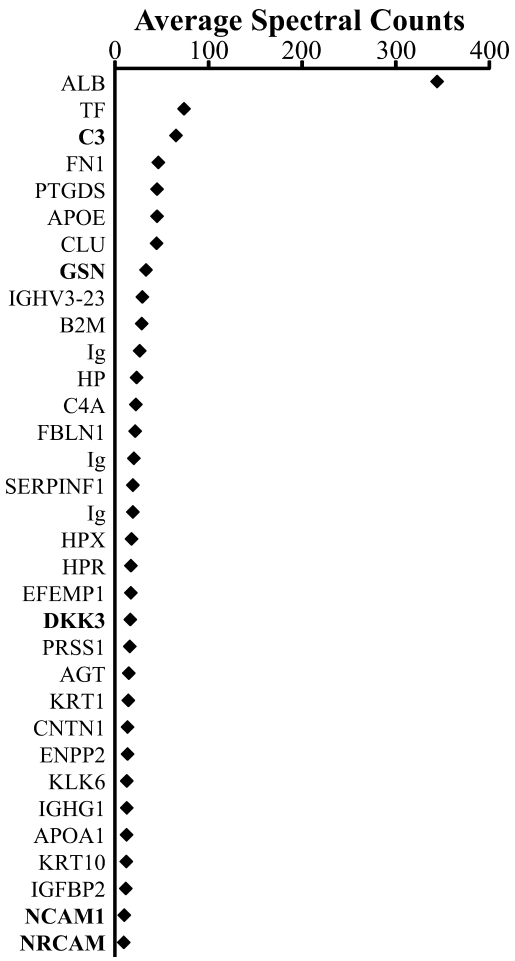

Figure 1. Thirty three of the most abundant proteins identified in CSF. Average spectral abundance was used to rank protein abundance, and the top 33 (representing 75% of the proteome) are shown. HGNC gene symbols are given, and 'Ig' is generically used in place of immunoglobulin without an assigned symbol. Bold symbols indicate significantly different protein abundances (moderated *t*-test, $p < 0.05$).

3.3 Attempted secondary confirmation of proteomic changes

Due to the low statistical power of our study design, we employed immunoblot analysis to determine whether the directionality of these changes was similar to the spectral count data (Supporting Information Fig. 2). Only one of

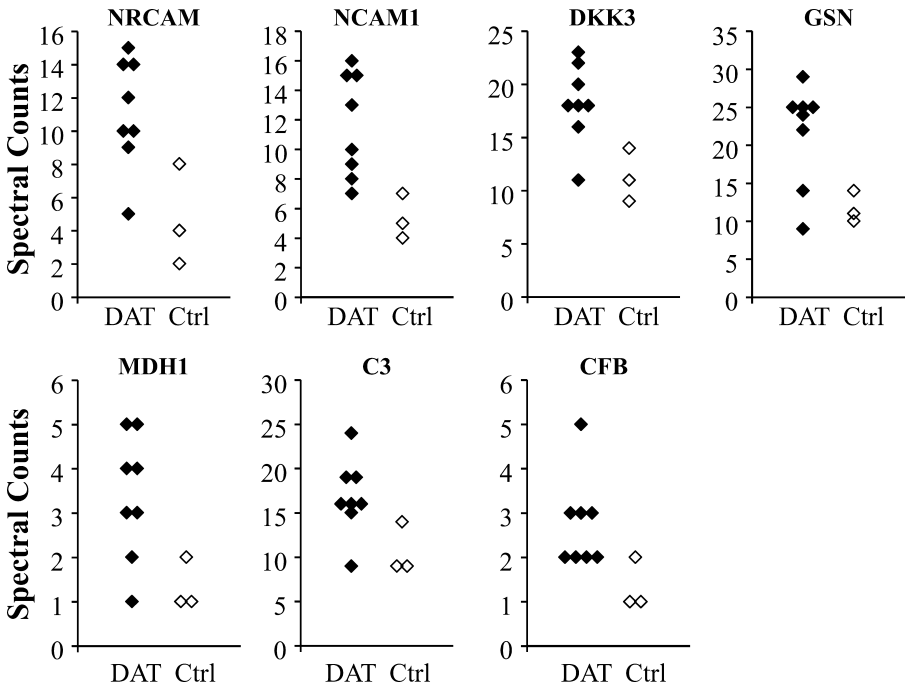

Figure 2. Spectral count data for the seven differentially abundant proteins. Data are shown for DAT (solid diamonds; $n = 8$) and non-DAT controls (hollow diamonds; $n = 3$). All seven were significantly increased in the DAT group (moderated *t*-test, $p < 0.05$).

the seven identified differentially abundant proteins, DKK3, could be detected by a commercial antibody with sufficient cross-reactivity to sea lion DKK3. The mean densitometric values for the DAT group were numerically higher than that of the non-DAT group (Supporting Information Fig. S2; 1.5-fold), but this was not significantly different. Reelin was also evaluated based on the relationship between reelin and NCAM1 in excitoxin-induced epilepsy [39] and because it is known to exist in different oligomeric states that are not differentiated by proteomic methods. Mean reelin spectral counts were numerically lower in DAT individuals (1.6-fold), but again not statistically different. We utilized a reelin antibody that had cross-reactivity to an approximately 160 kDa form of sea lion reelin (n-term cleaved). Immunoblot analysis results showed that 160 kDa reelin was similarly decreased 1.5-fold in DAT (Supporting Information Fig. S2; equal *t*-test, $p = 0.001$).

3.4 Hippocampal reelin changes in DA exposed mice

Since reelin was decreased in CSF from sea lions with DAT, we measured whether DA exposure resulted in a similar decrease in mouse hippocampus (Supporting Information Fig. 3). The 460 kDa form of reelin was not significantly different while the 160 kDa form of reelin was decreased 1.4-fold in DA exposed mice (equal *t*-test, $p = 0.03$).

4 Discussion

Despite several reports of candidate biomarkers in sea lions with DAT, there are no reports of cerebrospinal fluid biomarkers for the diagnosis or prognosis of domoic acid induced injury, or are there any reports describing the CSF proteome in any marine mammal. The intention of this study was twofold: (1) demonstrate that proteomics could provide relevant information in a mammalian species that does not have a sequenced genome, and (2) demonstrate that the CSF proteome is a resource for relevant biomarker discovery in sea lions with toxin-induced epilepsy. To address the first aim of this study, we utilized a combined mammalian database containing proteins that should be both homologous, and in some sequences, identical to sea lion proteins. Typically a database of confirmed protein sequences (e.g., UniProt Swiss-Prot), or translated coding sequences (CDS) are utilized. Even low coverage genome sequencing can be used to search marine mammal data with success [35]. Currently, there is no California sea lion genome, and only 86 protein sequences are in the UniProt Knowledge Base. Using the combined mammalian database 206 protein families were identified with high confidence belonging to 172 homologous human proteins. Moreover, four SIS peptides were utilized to validate four peptide identifications by PRM (dickkopf-3, gelsolin, NCAM1, and reelin). Due to the precise nature of PRM (i.e., separation in time, accurate mass selectivity, and fragmentation pattern), this suggests a high degree of confidence in the accuracy of these protein assignments. The accuracy of the protein identifications is further supported by taxonomic relationships where most of the 206 sea lion protein identifications were from entries belonging to three species in the suborder Caniformia. In the future this taxon identifier may be useful in proteomic studies of other pinniped species and these results demonstrate that confident proteomic assignments can be made when a genome sequence is unavailable.

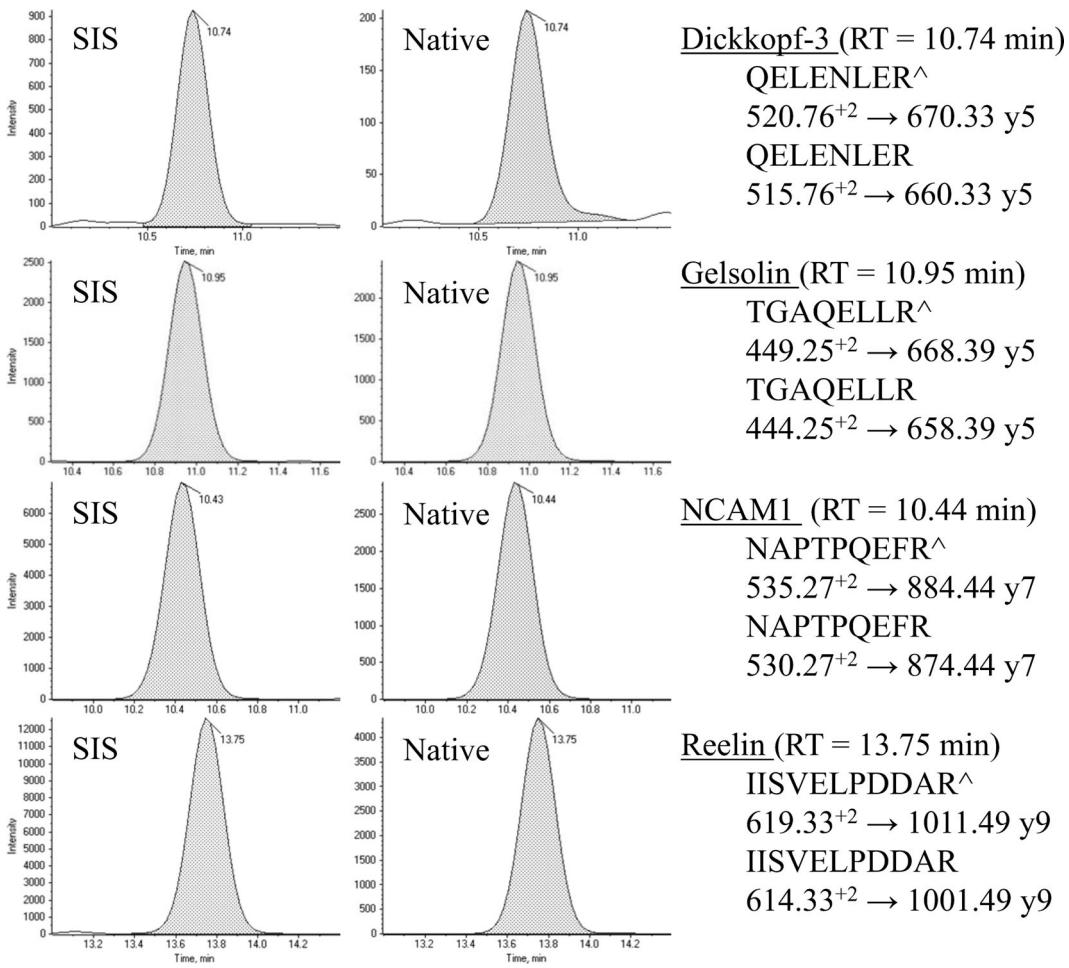

Figure 3. Validation of tryptic peptide identification for dickkopf-3, gelsolin, NCAM1, and reelin in sea lion CSF. Synthetic stable isotope standard (SIS) peptides were spiked into a single CSF sample and analyzed by LC-MS/MS. The c-terminal arginine was isotopically labeled (^) and the on column amount of each SIS peptide was 5 fmoles (equivalent to 8.3 fmol/μL CSF). Extracted ion chromatograms of select transition ions for each parent ion are presented with retention time.

Proteomic analysis of CSF is not as common as other biofluids or tissues due to the technically difficult nature of acquiring CSF and the low protein concentration of this fluid. There have been high quality characterizations of CSF from healthy model species: human [36], mouse [37], and rat [38]. These studies identified between 48 and 128 proteins, while a study of mice with Alexander disease identified 289 proteins [40], and are similar to the number of identified proteins in sea lion CSF. Based on relative amounts, four of the ten most abundant proteins in sea lions (albumin, serotransferrin, prostaglandin-H2 D-isomerase, and apolipoprotein E) were also the most abundant in rat CSF, with albumin being the most abundant [38]. To make broader comparisons between the datasets, presence/absence was used to compare protein composition. The sea lion CSF protein composition was most closely related to the human and rat datasets (Supporting Information Fig. 1). Specifically 75% (64 proteins) of the proteins identified in human CSF were also identified in sea lions. This high degree of similarity further lends confidence in the protein identifications made, despite using a multi-species database. Moreover, this homology indicates that mechanistic understanding of each system may provide a level of translation between species.

4.1 Candidate CSF biomarkers of DAT

The second aim of this study was to demonstrate that the sea lion CSF proteome is dynamic and should be considered a resource for biomarker discovery in sea lions with toxin-induced epilepsy. Because the CSF bathes the central nervous system, this fluid has been the focus of numerous biomarker studies for epilepsy and excitotoxicity [22–24, 26, 41–48]. Although sampling CSF is more invasive and carries additional risk, the CSF proteome contains mechanistic information that can be used to better understand the pathophysiological continuum of neurological disease.

Several proteins were found to be elevated in the CSF of sea lions with DAT (Fig. 2) and are discussed individually below. In comparison with gene expression studies, published for other model animals, these CSF proteins were not identified as potential biomarkers, nor were they considered indicative of DAT. Gene expression studies investigating the effect of DA toxicity on brain profiles of zebrafish [49, 50], brain profiles of mice [51], and blood profiles in sea lions [15], do not report differences in any of the gene products that were statistically elevated in this study. One possible explanation for this discrepancy lies in the fact that an elevation or reduction in gene expression at a single tissue or organ may not contribute significantly to a change in protein concentration, especially in circulating body fluids where multiple tissues contribute to the overall composition. Case in point, the induction of BDNF gene expression does not correspond to a measureable elevation in plasma or CSF BDNF levels in rats [15]. However, the fact that increased permeability of the blood–brain barrier can cause dilution of the CSF by the blood cannot be discounted in our study. Although the integrity of the blood–brain barrier was not measured in this study, DAT sea lions did have higher protein concentrations on average suggesting the possibility of a more permeable or less selective blood–brain barrier.

4.2 Complement factor B and C3

Complement proteins are produced in several cell types in the brain and play a role in immunity, but have also been described as mediators of injury and inflammation in the central nervous system [52]. Elevations in two CSF complement proteins (complement C3 and complement factor B) were observed for sea lions with DAT. Interestingly, these proteins belong to the alternative complement pathway and together with properidin form a C5 convertase complex responsible for the cleavage of C5 into C5a and C5b. Excitoxic injury by kainate results in an elevation in complement 5a receptors through a FosB-dependent mechanism [53] and inhibition of the complement 5a receptor type 1 reduced seizure activity and hippocampal degeneration [54]. Although we did not find C5 in our proteomic dataset, it is possible that an elevation in C3 and factor B could lead to the formation of C5 during the time at which excitotoxins are elevating C5 receptors, thereby leading to a positive feedback loop. However, it should not be discounted that an elevation in C3 may also lead to elevated C3a and could be part of a protective mechanism by which the brain reduces cell death via overstimulation of NMDA receptors [55]. Kainate-induced excitotoxic injury is not limited to alternative complement pathways. Complement protein C1q was increased in CA3 pyrimidal cells of the hippocampus following kainate administration [56] suggesting classical complement pathway components and alternative complement pathway components may be overly activated or present in the context of excitotoxic brain injury.

Complement proteins have been studied as candidate biomarkers for several major neurodegenerative diseases [57–59]. Studies have implicated a role for complement in epilepsy, but to date no study has been conducted to assess whether complement proteins have value as biomarkers for epilepsy. Expression and/or deposition of classical complement proteins is higher in rats and humans with temporal lobe epilepsy suggesting localized inflammation leads to neuronal network destabilization [60]. Severity of status epilepticus, induced by pilocarpine in mice, was also marked by an increase in C3 activation in the hippocampus [61]. In sea lions with DAT the role of complement remains an interesting target for biomarker qualification and may, in part, underlie some of the chronic changes described for this disease.

4.3 DKK3

Dickkopf proteins are a family of Wnt signaling antagonists. DKK3 was elevated in CSF of sea lions with DAT based on spectral counting, but was not statistically different when analyzed by immunoblotting. The semi-quantitative validation of biomarkers using antibody-based techniques does not hold in the case of DKK3 and highlights a problem when attempting to validate protein results in nonmodel species when commercial antibodies are largely absent. A single antibody to DKK3 was able to detect a protein that migrated to the expected size by SDS-PAGE, but the identification of proteins in this band are not known. If we assume that immunoreactivity equals identity, then five out of eight sea lions with DAT have DKK3 levels that are above the highest immunoreactive level in non-DAT sea lions, but given that only three non-DAT sea lions were included in the analysis, the resulting low statistical power requires a cautious negative interpretation of the data.

If the observed elevation of DKK3 in sea lions with DAT is true, this implicates Wnt signaling pathways in the progression of this toxicosis, but mechanistic rationale for elevated DKK3 remains obscure. DKK3 is an atypical family member in that it does not inhibit Wnt signaling through LRP proteins. Rather DKK3 binds to and apparently sequesters Kremen2 proteins, which can also oppose Wnt signaling, resulting in the propagation of signal [62]. Based on the specific cell-type, DKK3 may be neuroprotective, because it promotes Wnt signaling and reduces caspase activity in retinal glial cells [63]. In cancer cell lines the opposite is true because DKK3 appears to function as a Wnt inhibitor and is considered a tumor suppressor [64]. An elevation in DKK3 protein has been reported for CSF and plasma of Alzheimer's patients [65] and in CSF from patients with relapsing-remitting multiple sclerosis [25]. Whether the elevation of DKK3 in sea lion CSF is indicative of an antiapoptotic response or suppressor of Wnt signaling requires validation in a larger study.

4.4 Gelsolin

Gelsolin is an actin-severing and calcium-binding protein that has been implicated in neuroprotection. Gelsolin is expressed

in myelin producing cells, localized to the myelin sheath, and increases during Schwann cell regeneration [66]. Gelsolin regulates the influx of calcium through voltage-dependent calcium channels and NMDA receptors, and counters the toxicity of glutamate in mouse hippocampal neurons [67]. Exogenously administered plasma gelsolin was found to reduce infarct volume in an experimental rat model of ischemic stroke [68], attenuate caspase-3 activity in the brain of mice following burn injury [69], and reduce neuronal injury due to HIV gp-120 [70]. Exogenous administration of gelsolin was also found to increase the integrity of the blood-cerebrospinal fluid barrier at the choroid [71].

In this study, we observed that sea lions with DAT had an elevation in spectral counts related to gelsolin in the CSF, which is in contrast with ELISA results from human patients with epilepsy, where lumbar CSF gelsolin levels and levels in the temporal neocortex were statistically lower than controls [44]. Only in humans with secondary progressive multiple sclerosis compared with "other" neurological disorders has gelsolin been reported as elevated [38]. Because the plasma form of gelsolin, rather than cytosolic form, has been reported to be the dominant gelsolin form in CSF [72], it is plausible that elevated levels indicate that the blood–brain barrier integrity is compromised.

4.5 Malate dehydrogenase, cytoplasmic (MDH1)

Cytosolic malate dehydrogenase is a component of the malate-aspartate shuttle that creates malate at the expense of oxaloacetate and NADH. MDH1 was elevated in the CSF of sea lions with DAT, but no other enzymes linked to cellular respiration were differentially abundant. It stands to reason that MDH1 could be upregulated to counter the reduction in mitochondrial oxidative phosphorylation efficacy due to mitochondria damage as has been reported for cardiac mitochondria exposed to domoic acid [73] and brain mitochondria from picrotoxin-treated rats [74]. Elevations in skeletal muscle malate dehydrogenase activity in pilocarpine-treated rats suggests this enzyme may derive from seizure activity which is consistent with the phenotype of DAT, but we cannot exclude that increased MDH1 in sea lions with DAT may also be derived from the brain due to neurodegeneration. The mechanistic role of MDH1 in DAT is speculative at this point, and due to the low number of spectral counts assigned to this protein, less confidence should be given to this protein as a putative biomarker of DAT.

4.6 NRCAM

Neuronal cell adhesion molecule (NRCAM) is an ankyrin-binding protein that is expressed in the central and peripheral nervous system and is involved in synapse formation, axon growth, formation of myelin, and neuron differentiation [75, 76]. Mice lacking NRCAM are less prone to anxiety, novelty seeking, and addiction vulnerability [77]. Studies describing the effects of NRCAM overexpression, as suggested by the elevation in CSF from DAT sea lions, have not been conducted. NRCAM has been implicated as a CSF biomarker for staging of Alzheimer's disease where lower levels of this protein are associated with more severe dementia [78]. In a recent study, the validity of NRCAM alone as a staging marker of dementia has been contested [79], but in combination with Tau and YKL-40, NRCAM was able to predict patients with a positive clinical dementia rating (AUC = 0.9) [78]. The role of elevated NRCAM in DAT is difficult to resolve, largely because anxiety and novelty seeking is not a characteristic of DAT. In fact domoic acid has been considered an anxiolytic in mice [80]. It is possible that elevation in NRCAM reflects neuroregeneration following an excitotoxic insult.

4.7 NCAM 1

The NCAM1 protein is involved in neuritogenesis, synaptogenesis, and neurogenesis [81]. NCAM1 is expressed in multiple tissues, and is considered a circulating protein of the CSF and plasma [82], and is likely shed to enhance neurite outgrowth [83]. Consistent with our findings that NCAM1 is elevated in DAT, NCAM1 is also elevated in CSF of patients with schizophrenia [84] and the cleaved form of NCAM was found to be positively associated with higher ventricular volume in schizophrenic patients [85]. A small proteomic study of CSF proteins from patients with Alzheimer's disease or Parkinson's disease reported levels of NCAM1 (120 kDa form) are about twofold higher in both neurodegenerative groups [86]. However, other studies have reported that low levels of NCAM1 are CSF biomarkers of epilepsy in humans [24] and reduced levels were associated with multiple sclerosis, Alzheimer's disease, and meningitis [87]. The dichotomy related to NCAM1 abundance appears to be linked to chronicity based on studies in humans and rats. Hippocampal NCAM1 has been shown to be elevated in patients with temporal lobe epilepsy and minor neuronal loss, but is reduced in patients with major neuronal loss [88]. Children with frequent seizure activity also have fewer NCAM1 positive staining cells in the hippocampus compared to children with less frequent seizure activity [89]. Rats with induced *status epilepticus* for only 14 days had elevated NCAM1 expression in the CA-1 pyramidal layer and dentate gyrus of the hippocampus [90] which is suggestive of acute upregulation of the NCAM1 gene. These studies are consistent with the idea that NCAM1 abundance is elevated acutely and decreases with loss of NCAM1-producing neurons as the disease progresses to a chronic state. The sea lions with DAT were a mix of acute and chronic cases and it is not possible to know the history of the toxicosis or frequency of seizure activity in these cases. The fact that NCAM1 is observed to be elevated suggests minor neuronal loss and a less chronicity, but a prospective study is required to confirm this suspicion.

Because strict LC-MS/MS proteomic quantification approaches do not include all modification states of a peptide, polysialylated proteins such as NCAM1may appear falsely elevated simply due to the fact that fewer residues are sialylated leading to more unmodified peptides being counted in the DAT group. Polysialylation is an important modification that promotes activity of NCAM1. NCAM1 was shown to mediate glial-derived neurotrophic factor signaling and inactivation of NCAM1 with endoneuraminidase expedited hippocampal neurodegeneration in kainate-treated mice [91]. Given that the observed elevation in NCAM1spectral counts can be influenced by degree of polysialylation, it is possible that the proteomic data imply the inactive form of NCAM1 is elevated and that differential glycosylation underlies the predictive ability of this candidate marker. It also stands to reason that the lack of polysialylation of this otherwise neuroprotective protein could be an underlying mechanism that promotes seizure susceptibility [92].

Interestingly, administration of kainate to adult mice results in the loss of neurons expressing NCAM1 and also depresses the expression of an extracellular matrix protein known as reelin [39]. Reelin functions to promote proper migration of progenitor dentate granule cells of the hippocampus and loss of reelin is associated with improper neuroblast migration in temporal lobe epilepsy [93]. Additional studies have shown that reelin is depressed in models of schizophrenia [94, 95], Alzheimer's disease [96], and epilepsy [97], but have not been measured in studies of domoic acid-induced neurotoxicity. Reelin serves as a ligand to the ApoE receptor 2 and very low density lipoprotein receptors and has been shown to inhibit granular cell dispersion in the hippocampus of mice dosed with kainate [98, 99]. In this study, reelin was one of the more abundant and consistently discovered proteins in the CSF, and although it was not statistically different between DAT and non-DAT sea lions, levels tended to be lower in sea lions with DAT.

Because of the association between kainate-induced loss of reelin and epilepsy in mice and the fact that reelin is known to be a heavily glycosylated protein, which can cause a discrepant measurement by spectral counts, we utilized an immunoblotting technique that is less affected by changes in protein glycosylation to determine whether reelin abundance in CSF was lower in sea lions with DAT. For sea lions with DAT, a single reelin protein migrating at approximately 160 kDa was detected and was significantly lower compared to sea lions without DAT. Therefore, we utilized brains from mice administered domoic acid to help address whether domoic acid results in a depression of reelin abundance in the hippocampus. Immunoblot analysis of hippocampal lysates from mice administered domoic acid displayed a reduction in the lower molecular 160 kDa protein form of reelin suggesting that, like kainate, domoic acid has the same depressive effect on reelin levels. The question still remains as to whether lower reelin levels in CSF of sea lions with DAT are reduced due to depressed expression in the hippocampus in sea lions, but circumstantial evidence based on previous studies [39, 93] and those conducted within, imply that domoic acid toxicosis may also lead to similar phenotypes in part mediated through lower reelin expression.

In conclusion, the CSF proteome of sea lions is very similar to that found in humans, rats, and mice. This fluid is a rich resource for the discovery of neurological disease biomarkers in marine mammals and despite no associated proteome, the use of homologous protein databases can reveal changes in CSF proteins for future health monitoring. Based on these studies, the association of NCAM1 and reelin with domoic acid toxicosis may be an underlying mechanism that promotes the chronicity of this disease. Large-scale biomarker studies of CSF proteins in sea lions should consider using MS as a method for protein discovery and quantification.

4.8 Limitations

One of the major limitations impeding generalization of the differential protein abundance in CSF is the fact there are no female sea lions in the non-DAT group. Further, only young animals were assayed in the non-DAT group whereas the DAT group included both young and old individuals. The differences in age and sex were a product of sample availability rather than choice. Given these differences, the CSF protein abundance data must be interpreted with caution until a larger prospective study is conducted to substantiate differences. Due to the small sample size and lack of females in the control groups, it is possible that differential protein abundance could be an artifact of sex and age; however, some of the differences are difficult to explain solely on group bias. For example, reelin RNA abundance is lower for interstitial white matter level I neurons in schizophrenic human males compared to females [100] Another example is that NCAM expression is a marker of immature neurons [101, 102] and does not exhibit sex differences [103]. Based on the groups in this study, NCAM levels should be lower in the DAT group that was comprised of older females, but instead were elevated. At the least, differential abundance should allow for prioritization of target proteins for follow-up studies.

The MS proteomics data in this paper have been deposited in the ProteomeXchange Consortium (http://proteomecentral .proteomexchange.org) via the PRIDE partner repository [104]: dataset identifier PXD002105. We thank the PRIDE Team and PeptideAtlas project for hosting the mass spectrometry proteomic data.

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