

RESEARCH PAPER



## 14-3-3 and enolase abundances in the CSF of Prion diseased rats

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### ABSTRACT

Creutzfeldt-Jakob disease (CJD) is characterized by an extended asymptomatic preclinical phase followed by rapid neurodegeneration. There are no effective treatments. CJD diagnosis is initially suspected based upon the clinical presentation of the disease and the exclusion of other etiologies. Neurologic symptoms are assessed in combination with results from cerebrospinal fluid (CSF) biomarker abundances, electroencephalography (EEG), magnetic resonance imaging (MRI), and in some countries, real-time quaking-induced conversion (RT-QuIC). Inconsistencies in sensitivities and specificities of prion disease biomarker abundance in CSF have been described, which can affect diagnostic certainty, but the utility of biomarkers for prognosis has not been fully explored. The clinical presentation of CJD is variable, and factors such as prion protein polymorphic variants, prion strain, and other genetic or environmental contributions may affect the disease progression, confounding the appearance or abundance of biomarkers in the CSF. These same factors may also affect the appearance or abundance of biomarkers, further confounding diagnosis. In this study, we controlled for many of these variables through the analysis of serial samples of CSF from prion-infected and control rats. Prion disease in laboratory rodents follows a defined disease course as the infection route and time, prion strain, genotype, and environmental conditions are all controlled. We measured the relative abundance of 14-3-3 and neuron-specific enolase (NSE) in CSF during the course of prion infection in rats. Even when disease-related, environmental and genetic variables were controlled, CSF 14-3-3 and NSE abundances were variable. Our study emphasizes the considerable diagnostic and prognostic limitations of these prion biomarkers.

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CJD; preclinical; CSF; 14-3-3; NSE; Prion disease diagnosis



## Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are neurodegenerative disorders that affect humans and other mammals including sheep, cattle, and cervids. Prion diseases are always fatal and are characterized by an extended preclinical, asymptomatic period, followed by a rapid clinical phase. Prion diseases arise when the normal cellular prion protein (PrP<sup>C</sup>) is converted into infectious, protease-resistant prions, PrP<sup>CJD</sup>, through a process where the  $\alpha$ -helical coil structure is refolded into  $\beta$ -sheet [1,2]. Definitive diagnosis of human prion disease involves direct detection of PrP<sup>CJD</sup>, typically from post-mortem brain samples. Ante-mortem methods such as CSF protein biomarkers, EEG, MRI, and RT-QuIC are used in combination with clinical symptoms to diagnose probable CJD [3–5]. The inclusion of MRI in the diagnostic criteria and development of direct methods for detecting PrP<sup>CJD</sup>, such as RT-QuIC, has reduced the dependence on


indirect markers for diagnosis, however, 14-3-3 and NSE remains testable with the potential for prognostic utility.

Elevated 14-3-3 proteins in the CSF was added to the World Health Organization (WHO) diagnostic criteria for sporadic CJD (sCJD) in 1998 [3,6,7]. 14-3-3s are 28-30 kDa proteins present in all eukaryotic cells, and comprise nearly 1% of soluble brain protein [8–10]. Of the seven 14-3-3 proteins, the beta ( $\beta$ ) and gamma ( $\gamma$ ) family members have been most associated with human prion disease [11,12]. Another biomarker upregulated in the CSF of CJD patients is NSE [13,14]. Increased concentrations of 14-3-3 and NSE in CSF have been suggested as a means to move patients from the possible to probable category of CJD [15,16]. NSE, or gamma-enolase, is a 47 kDa protein specific to neurons and neuroendocrine tissues [17–19].

A wide range of sensitivities and specificities have been reported for CSF biomarkers of CJD. For example,

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14-3-3 detection ranged from 53–97% in sensitivity and 40–100% in specificity, depending on the study as reviewed by Forner et al, 2015 [20]. The presence of 14-3-3 and NSE in CSF of patients with prion disease is not specific to prion disease; these proteins are present in CSF from patients with other neuronal injury e.g. brain trauma, brain tumors, subarachnoid hemorrhage, stroke, hypoxemia, and encephalitis [21–28]. This lack of specificity may result in false positives if applied to a diverse patient population, therefore, CSF biomarker testing is restricted to patients with clinical symptoms indicative of CJD [29,30]. As patients are assessed for CSF biomarkers after clinical onset, the positive predictive values in CJD studies are often overstated [31]. It has been suggested that many factors impact prion disease biomarker appearance including disease strain, subtype, duration, age at onset, and timing of lumbar puncture [7,32–40]. For example, when 70 sCJD cases with distinct molecular subtypes were evaluated for 14-3-3 using ELISA, the most elevated levels of 14-3-3 were observed in classical molecular subtypes (MM1), and lower levels were observed with less common subtypes (MV2, MM2) with longer durations and atypical presentations of CJD [41]. CSF biomarkers in sCJD have been tracked over the clinical course of disease [42,43] but have not been examined during the preclinical asymptomatic phase of the disease.

The progressive neurodegenerative nature of clinical prion disease suggests that biomarker abundances should also change as disease advances, providing a means for prognosis and determination of disease course when assessed over time. The objective of this study was to evaluate the performance of 14-3-3 and NSE in the diagnosis and prognosis of prion disease when time of infection, prion strain and dose, and other variables (age, genetic background, and environmental conditions) were controlled. We found that, even in a controlled study of prion infection, 14-3-3

and NSE abundances were variable, suggesting that dose, age of infection, environment, and *Prnp* genetics do not confound the abundances of these biomarkers.

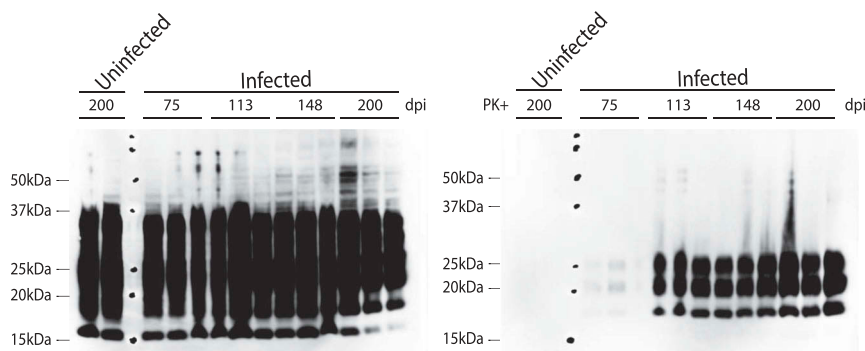
## Results

### Development of rat-adapted scrapie

The RML strain of mouse-adapted scrapie was transmitted to laboratory rats by intracranial inoculation [44]. Upon subsequent serial passage, the incubation period stabilized at 200 days. Clinical symptoms of rat-adapted scrapie (RAS) include ataxia, lethargy, weight loss, kyphosis, myoclonus and an increase in secretions from the Harderian glands. PrP<sup>Sc</sup> is widely deposited in the cerebral cortex, hippocampus, thalamus, inferior colliculus and granular layer of the cerebellum [44]. A significant increase in Proteinase K resistant PrP is observed between 75 and 113 days post infection with amounts plateauing thereafter (Figure 1).

### Time course analysis of 14-3-3s and NSE CSF abundance

CSF from control uninfected rats and rats infected with RAS was collected at preclinical and clinical disease time points. To determine differences between infected and uninfected and refine the timeline of detection, samples were pooled (equal volumes) and analyzed by western blot for 14-3-3s and NSE abundances. Samples were standardized by loading equal volumes of CSF. Two monoclonal antibodies, 14-3-3 pan-specific (beta, eta, epsilon, gamma, and zeta proteins) and 14-3-3 gamma-specific, were used for comparison. The pan-specific antibody is currently approved for diagnostic use [3] although some studies have suggested that 14-3-3 gamma has better performance [45]. An elevated 14-3-3 abundance was observed in the CSF of prion-infected rats (Figure 2).



**Figure 1.** Accumulation of Proteinase K-resistant PrP in the brains of RAS-infected rats through the time course of infection. Individual rat brain homogenates (panel (a)) and PK treated (panel (b)) with  $n = 3$  per time point (75, 113, 148, and 200 days post inoculation (dpi)). Antibody SAF83 1: 20,000 dilution.

14-3-3 gamma showed more robust diagnostic sensitivity at earlier time points. 14-3-3s were also detected at the early time points (75 and 113 days) in the uninfected samples. NSE abundance was also elevated in the pooled CSF samples from infected rats at later time points, however, detection at early time points in both infected and control CSF samples suggests a lack of specificity (Figure 2). 14-3-3 and NSE abundances were variable in both infected and uninfected rats.

An advantage of using rat prion disease to model CJD is the ability to analyze CSF samples at defined preclinical time points. Equal volumes of CSF samples from a pre-clinical time point (148 dpi), and age-matched controls were analyzed by western blot. Considerable variability in both 14-3-3 and NSE abundances was observed in infected individuals (Figure 3). AUC values were 0.58, 0.61, and 0.69 for 14-3-3 pan, gamma, and NSE, respectively when samples were loaded by volume. When normalizing for protein concentration, AUC values were 0.78, 0.58, and 0.78 for 14-3-3 pan, gamma, and NSE, respectively (Figure 4). These markers were also detected in CSF from age-matched control samples. This approach was not able to distinguish between infected and control rats.

#### Individual variability in clinically-affected rats (193 days)

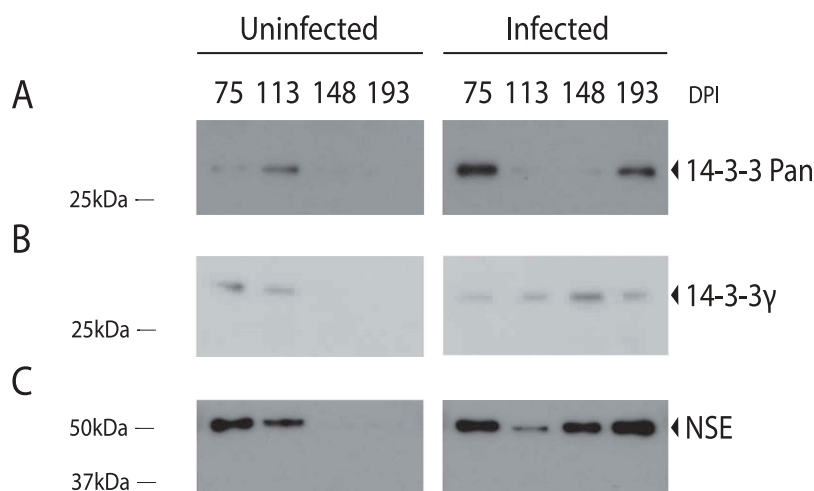
Two approaches were used to determine whether the biomarker abundances differed between individual animals at the clinical phase of disease: i) analysis by loading equal volumes of CSF and ii) analysis by standardizing protein concentrations of the individual CSF samples. Equal volumes (10  $\mu$ l) of CSF samples from clinically

infected (193 dpi) and from age-matched controls were analyzed by western blot (Figure 5). 14-3-3s and NSE abundances were variable in the infected CSF samples and these biomarkers were also detected in some uninfected control samples. The diagnostic trade-off between specificity and sensitivity was analyzed by measuring the area under the receiver operating characteristic (ROC) curve. Values of 0.83, 0.81 and 0.66 were determined for 14-3-3 pan, 14-3-3 gamma and NSE, respectively. There was no significant difference in the abundances of the biomarkers between the infected and uninfected samples when loading was based on volume.

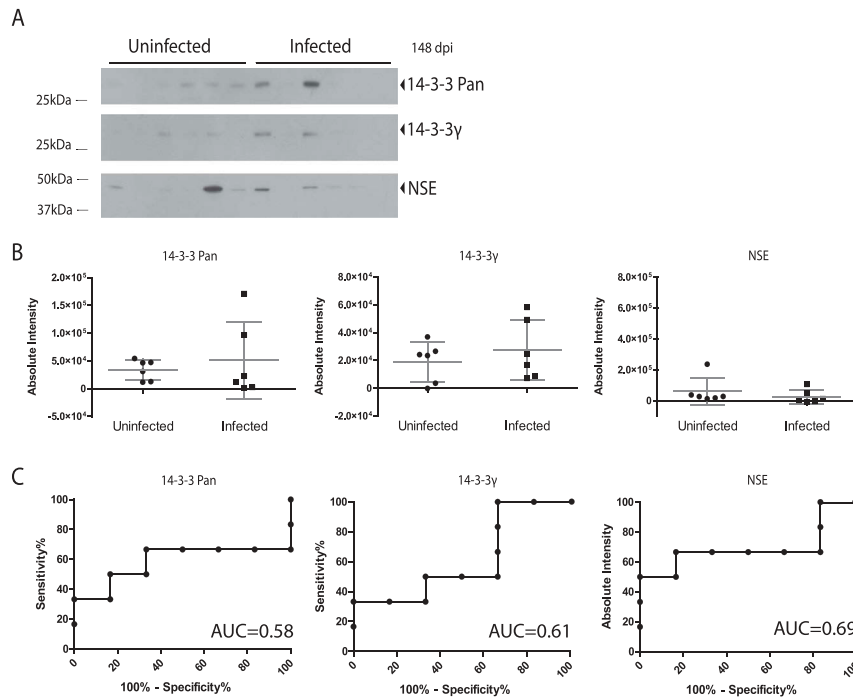
We subsequently investigated whether standardizing total protein concentration of CSF influenced the diagnostic sensitivity and specificity (Figure 6). Protein content prior to normalization in CSF samples was variable (infected  $0.97 \pm 0.80$   $\mu$ g/ $\mu$ l; uninfected  $1.02 \pm 0.65$   $\mu$ g/ $\mu$ l). Following analysis by western blot (0.4  $\mu$ g of total CSF protein per lane), the AUC values for 14-3-3s and NSE increased compared to standardization by volume. 14-3-3 pan and gamma increased to 0.99 and 0.97, respectively ( $p < 0.05$ ), demonstrating that these tests could accurately differentiate infected from uninfected samples in clinically affected animals. The AUC (0.75) for NSE increased as a result of standardization by protein, however this was not significant.

#### Discussion

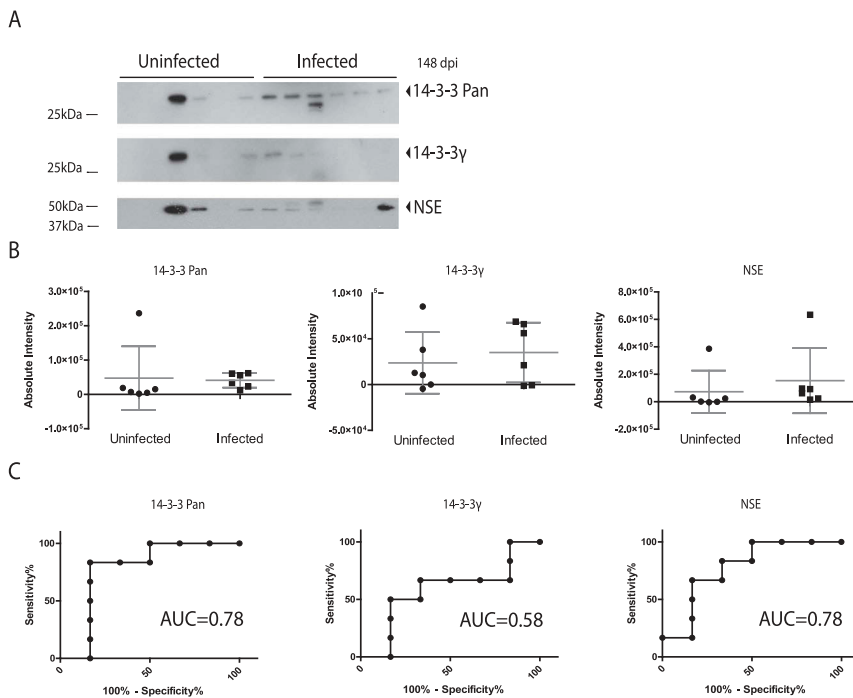
As 14-3-3 is a CJD biomarker in the WHO diagnostic criteria, we investigated the utility of this biomarker in rat prion disease where we could control genetics, environment, prion strain, route, titre and stage of



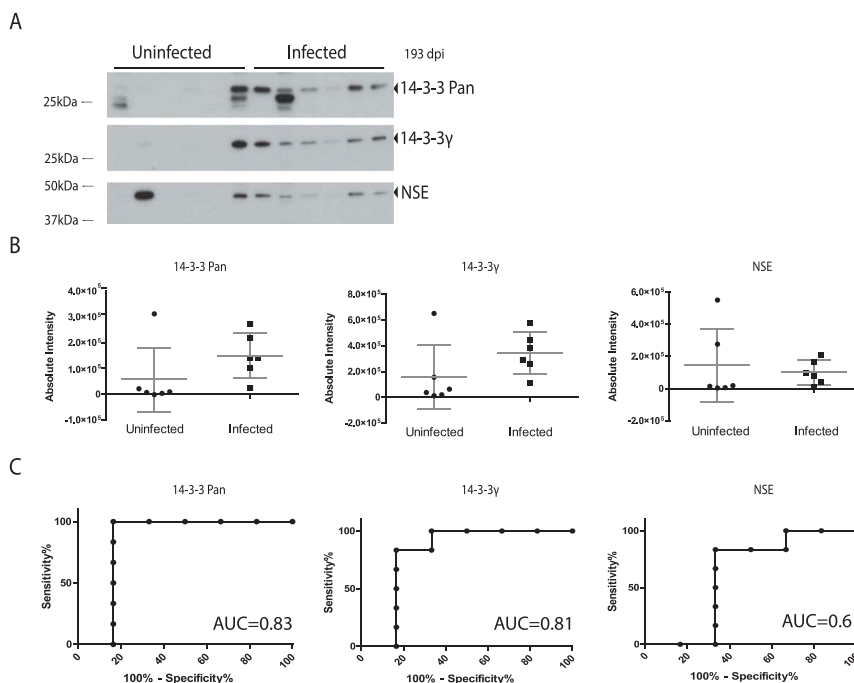
**Figure 2.** Abundances of 14-3-3s and NSE in pooled CSF samples of prion-infected rats and age-matched controls. Pooled CSF ( $n = 4$ ) collected from RAS-infected Sprague-Dawley rats and uninfected age-matched controls was analyzed by western blot. CSF from three preclinical time points (75 dpi, 113 dpi, and 148 dpi), as well as a clinical time point (193 dpi), were probed using (a) pan-specific 14-3-3, (b) gamma-specific 14-3-3 and (c) NSE antibodies.



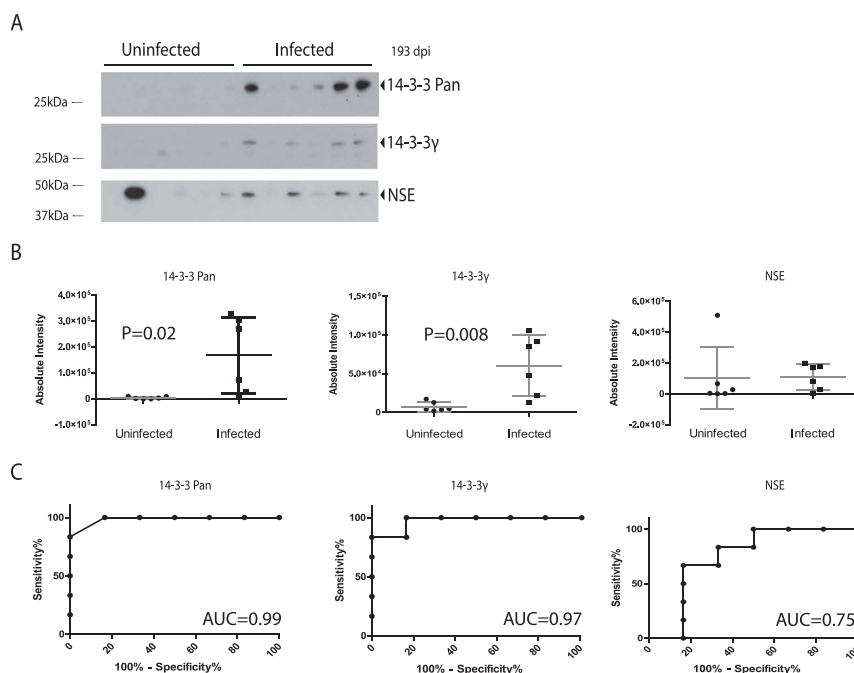
**Figure 3.** Individual CSF variability in abundance of 14-3-3s and NSE in pre-clinical RAS-infected and age-matched controls; CSF standardized by volume. CSF from 6 RAS-infected rats at preclinical time point (148 days post inoculation) were compared to 6 age-matched controls loaded by total volume. (a) Representative western blot analysis of infected and uninfected CSF samples screened for 14-3-3s and NSE. (b) Quantitation of protein abundances by densitometry of western blots. (c) Receiver operating characteristic (ROC) curve. Area under the curve (AUC) indicates diagnostic utility of these markers at this preclinical time point.



**Figure 4.** Individual CSF variability in abundances of 14-3-3s and NSE in pre-clinical RAS-infected and age-matched controls; CSF standardized by protein concentration. CSF from 6 preclinical RAS infected rats (148 days post inoculation) were compared to 6 age-matched controls loaded by protein concentration (0.4ug protein/well). (a) Representative western blot analysis of infected and uninfected CSF samples screened for 14-3-3s and NSE. (b) Quantitation of protein abundances by densitometry of western blots. (c) Receiver operating characteristic (ROC) curve. Area under the curve (AUC) indicates diagnostic utility of these markers at this preclinical time point.



**Figure 5.** Variability in 14-3-3 and NSE abundance in CSF of prion-infected and age-matched rats; CSF standardized by volume. CSF was collected from 6 RAS-infected rats at clinical stage and compared to 6 age-matched controls using 14-3-3 pan, 14-3-3 gamma, and NSE antibodies. (a) Representative western blot. (b) Quantitation of protein abundances by densitometry of western blots. (c) Receiver operating characteristic (ROC) curve. Area under the curve (AUC) indicates diagnostic utility of standardization by volume.



**Figure 6.** Variability in 14-3-3 and NSE abundance in CSF of prion-infected and age-matched rats; CSF standardized by protein concentration. CSF was collected from 6 RAS infected rats at clinical stage and compared to 6 age-matched controls using 14-3-3 pan, 14-3-3 gamma, and NSE antibodies. (a) Representative western blot loaded by standard protein concentration (4  $\mu$ g/well). (b) Quantitation of protein abundances by densitometry of western blots. (c) Receiver operating characteristic (ROC) curve. Area under the curve (AUC) indicates diagnostic utility of these markers when standardized by protein concentration.

disease. NSE was also analyzed, as it is another marker that has been used to diagnose CJD. We hypothesized that by controlling for these variables the diagnostic accuracy of 14-3-3 and NSE abundances would be improved. Absolute band intensity for 14-3-3 and NSE protein levels were measured from immunoblots at both preclinical and clinical stages of rat prion infection. Samples were standardized by either volume or protein concentration. 14-3-3 CSF protein abundance was significantly higher on average in clinically affected rats when standardized for protein content. A number of individual infected samples could not be distinguished from uninfected. The diagnostic sensitivity of 14-3-3 was low. Neither 14-3-3 or NSE protein abundance distinguished infected from uninfected at the pre-clinical stage of disease, regardless of standardization method. 14-3-3 or NSE CSF abundances did not consistently diagnose infected and uninfected animals in this controlled study.

We used the rat model as a method of evaluating biomarkers of prion disease. The rat allows for evaluation of biomarkers at both preclinical and clinical stages of infection in a controlled environment. We found that 14-3-3 and NSE abundances did not track with the disease course and were highly variable in individual CSF samples standardized by either volume or protein concentration. These findings are similar to findings by Torres et al (2012) where 14-3-3 protein levels were inconsistent in longitudinal samples and did not track with advancing disease in CJD patients [43]. Furthermore, use of 14-3-3 detection in the diagnosis of CJD has led to an increase in over diagnosis of sporadic CJD and misdiagnosis of potentially treatable diseases [46,47]. Our data indicates a lack of prognostic utility for 14-3-3 and NSE for prion infection and questions the utility of these biomarkers for clinical diagnosis in the context of improved imaging and development of direct detection of PrP<sup>CJD</sup> by RT QuIC.

## Materials and methods

### Ethics statement

This study was carried out in accordance with the guidelines of the Canadian Council on Animal Care. The protocols used were approved by the Institutional Animal Care and Use Committee at the University of Alberta. All animals were anesthetized using isoflurane prior to CSF collection and then euthanized for remaining sample collection. All animals were anesthetized using isoflurane for rapid induction and easy depth control of anesthesia.

## Animals

Weanling female Sprague Dawley rats were used in this study. These animals were housed 2 per cage in Tecniplast Green Line IVC Sealsafe PLUS Rat cages on Lab Aspen Chip bedding. They were fed Picolab Rodent 20 5053C3N. They were kept on a 12 hour light/dark cycle at approximately 21°C. Rats used in this study were inoculated with rat-adapted scrapie (RAS) prion agent that had been adapted to the rat through multiple passages; incubation period had stabilized at approximately 200 days. Previous characterization of this agent in the rat allowed us to predict three preclinical time periods for sample collection and determine a humane endpoint when clinical signs were present [44]. Mortality did not occur outside of planned euthanasia and humane endpoints. Animals were randomly assigned to infected and uninfected groups.

### Prion infection and CSF collection

Weanling female Sprague Dawley rats were inoculated through the fontanelle to a depth of ~1 cm using a 25 gauge needle with 50 µl of 10% rat brain homogenate prepared from uninfected or RAS-affected rats [44]. CSF and brain samples from RAS-infected and age-matched control rats were collected at preclinical (75, 113, and 148 (dpi)) and clinical time points (193 dpi) and compared with uninfected, age-matched control samples. Clinical signs of prion disease were observed at ~180 days post-inoculation and included porphyrin staining, myoclonus, kyphosis, ataxia, and weight loss. Infected animals at clinical stage weighed approximately 260g. Uninfected age matched controls weighed approximately 330 g. At specified time points, rats were anesthetized with isoflurane and CSF collected via puncture of the cisterna magna with a 25 gauge neonatal spinal needle (BD Biosciences). CSF volumes of 100-200 µL were routinely obtained and scored for blood contamination (Supplementary Table 1). CSF was centrifuged for 30s at 2,000xg on a benchtop 'nano' centrifuge to assess blood contamination (presence of a red cell pellet) and supernatant was collected. Samples that scored level 3 or higher for blood contamination were discarded. CSF was frozen and stored at -80°C. In some experiments, equal volumes of CSF from each time point were pooled to ensure homogeneity before adding sample buffer and being heated to 100°C.

### Immunoblotting

Individual rat brains were homogenized to 10% (w/vol) in PBS. PK (Roche) digested samples were treated at a ratio of 3.5 µg PK/100 µg protein at 50 µg/ml for 30 min at 37°C. Rat

prion proteins were detected with the SAF83 antibody at a 1: 20,000 dilution and the secondary antibody, goat anti-mouse AP, was used at 1:10,000 dilution. Membranes were developed using AttoPhos AP Fluorescent substrate (Promega) and imaged with the Image Quant.

Total protein concentration of CSF samples was determined, in duplicate, using the Pierce bicinchoninic acid protein assay kit. CSF proteins were fractionated on 12% NuPage SDS-PAGE gels and transferred to PVDF membrane. 14-3-3 proteins were detected using two different antibodies: 14-3-3 pan and 14-3-3 gamma. 14-3-3 pan (Santa Cruz SC-629) is recommended by the WHO for prion diagnostics and binds a common epitope in the beta, eta, epsilon, gamma, and zeta 14-3-3 proteins (3,45). 14-3-3 gamma (Santa Cruz SC-731) monoclonal antibodies are specific. Both antibodies were used at a 1:1,000 dilution. NSE antibody (Abcam) was used at 1:2,000. The secondary antibody, goat anti-rabbit HRP, was used at 1:10,000. Membranes were developed using Pierce ECL western blotting substrate.

### Statistical analysis

Densitometry analysis was performed on western blot data for semi-quantification using Adobe Photoshop CS6 Extended version to determine absolute band intensity. Protein biomarker abundances from infected and uninfected samples were always analyzed together from the same western blot. A two-tailed unpaired t-test was performed; the means (and standard error of the mean) between infected and uninfected were compared. This data was used to generate a receiver operating characteristic (ROC) curve to plot sensitivity vs 1-specificity. The area under the curve (AUC) was calculated to determine the diagnostic accuracy of the biomarkers. AUC values were calculated to determine the ability of each marker to accurately distinguish between healthy and prion infected rats. ROC curves and AUC values were generated using Graphpad Prism 5 Software.

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### Disclosure statement

No potential conflict of interest was reported by the authors.

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