Activation of Pro-survival CaMK4β/CREB and Pro-death MST1 signaling at early and late times during a mouse model of prion disease

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Abstract

Background: The signaling pathways most critical to prion disease pathogenesis are as yet incompletely characterized. We have developed a kinomics approach to identify signaling pathways that are dysregulated during prion pathogenesis. The approach is sensitive and specific enough to detect signaling pathways dysregulated in a simple in vitro model of prion pathogenesis. Here, we used this approach to identify signaling pathways dysregulated during prion pathogenesis in vivo.

Methods: Mice intraperitoneally infected with scrapie (strain RML) were euthanized at 70, 90, 110, 130 days post-infection (dpi) or at terminal stages of disease (155–190 dpi). The levels of 139 protein kinases in brainstem-cerebellum homogenates were analyzed by multiplex Western blots, followed by hierarchical clustering and analyses of activation states.

Results: Hierarchical and functional clustering identified CaMK4β and MST1 signaling pathways as potentially dysregulated. Targeted analyses revealed that CaMK4β and its downstream substrate CREB, which promotes neuronal survival, were activated at 70 and 90 dpi in cortical, subcortical and brainstem-cerebellum homogenates from scrapie-infected mice. The activation levels of CaMK4β/CREB signaling returned to those in mock-infected mice at 110 dpi, whereas MST1, which promotes neuronal death, became activated at 130 dpi.

Conclusion: Pro-survival CaMK4β/CREB signaling is activated in mouse scrapie at earlier times and later inhibited, whereas pro-death MST1 signaling is activated at these later times.

Keywords: Prion disease, Kinomics, Protein kinase, Multiplex Western blots, CaMK4β, CREB, MST1

Background

Prion diseases are a family of invariably lethal chronic neurodegenerative diseases that affect humans (kuru; Creutzfeldt-Jakob disease, CJD; Gerstmann-Sträussler-Scheinker disease, GSS; fatal familial insomnia, FFI), and other species such as cattle (bovine spongiform encephalopathy, BSE), goat, sheep (scrapie), deer, elk and moose (chronic wasting disease, CWD) [1,2]. Human prion diseases can be infectious (acquired), inherited (genetic), or sporadic. The latter are the most common, accounting for approximately 85% of cases [3]. Whereas the inherited and acquired cases may be suspected, from the risk facts, before the onset of clinical symptom, the sporadic cases can only be diagnosed after the onset of clinical symptoms [4].

The neuropathology of prion diseases is characterized by gliosis, spongiform degeneration, and neuronal death. The conversion of the cellular prion protein (PrP) into its pathological conformation (PrPSc) is essential for pathogenesis. Neuronal death and disease progression were prevented in scrapie-infected mice by conditional ablation of PrPSc at the time when PrPSc was first detected [5]. Inhibition of PrPSc conversion to PrPSc is thus a validated target for therapeutic intervention. Many compounds have been identified to inhibit PrP conversion or the accumulation of PrPSc in vitro [6,7]. Only one, however, the diphenyl pyrazole
derivative anle138b [3-(1,3-benzodioxol-5-yl)-5-(3-bromophenyl)-1H-pyrazole], prolonged survival of scrapie-infected mice when treatment was started after the onset of clinical signs of disease [8]. Unfortunately, none affected survival of patients with CJD, GSS, or FFI [9-18]. Another compound that prolonged survival of scrapie-infected mice after the onset of clinical disease is the calcineurin/protein phosphatase 3 inhibitor FK506. FK506, however, did not affect the levels of PrPSc or the accumulation of PrPSc [19], indicating that FK506 acts downstream from the accumulation of PrPSc. As FK506 is a known (calcineurin) signaling inhibitor, these results suggest that dysregulated signaling downstream of PrP conversion is an alternative therapeutic target against prion diseases.

Protein kinases (and phosphatases) modulate by reversible phosphorylation the function, localization, or activities of approximately one-third of cellular proteins [20]. Protein kinases are therefore critical regulators of signal transduction. Their dysregulation is implicated in the pathogenesis of many chronic diseases, including neurodegenerative diseases such as Alzheimer’s and Parkinson’s [21-23]. Consequently, protein kinases are major therapeutic targets. It is estimated that up to 30% of the research and development budget of the pharmaceutical industry is invested in protein kinase inhibitors [24,25]. For example, protein kinase inhibitors are the largest group of new cancer therapeutics [26]. Thirty-one protein kinase inhibitors are in clinical use, over 500 are involved in approximately 2,700 clinical trials, and thousands more are in various stages of preclinical development ([26-28] and summary of [29]). Protein kinase inhibitors therefore constitute a rapidly growing group of clinical drugs that have the potential to considerably impact treatment of chronic diseases.

Considering the critical roles that protein kinases play in the pathogenesis of other chronic neurodegenerative diseases, it is not surprising that they also participate in the pathogenesis of prion diseases. For example, the activation of vascular endothelial growth factor receptor (VEGFR) inhibited death of cultured neurons treated with the neurotoxic prion peptide PrP106-126 [30]. Death of PrP106-126-treated cultured neurons was also inhibited by Abelson leukemia oncogene cellular homolog (c-Abl) knockdown [31] and treatment with the glycogen synthase kinase 3 (GSK3) inhibitor lithium [32]. Scrapie-infected mice treated with the protein kinase R-like endoplasmic reticulum kinase (PERK) inhibitor GSK2606414 survived longer than vehicle-treated mice [33]. The phosphoinositide-dependent kinase 1 (PDK1) inhibitor BIX912 also prolonged survival of scrapie-infected mice [34]. Protein kinase inhibitors may have good potential in prion disease therapeutics. Unfortunately, the signaling pathways most critical to prion disease pathogenesis have yet to be fully identified.

We have developed a kinomics approach to identify signaling pathways dysregulated during prion disease pathogenesis (Shott et al., companion paper). We initially tested the approach in a simplified in vitro model of prion disease pathogenesis (Shott et al., companion paper). Here, we applied the approach to mice infected with mouse-adapted scrapie. We identified two signaling pathways dysregulated during scrapie pathogenesis. The calcium/calmodulin-dependent protein kinase, beta isoform (CaMK4β)/cAMP response element-binding protein (CREB) signaling pathway, which promotes neuronal survival, was activated at earlier times but its activation state returned to that in mock-infected mice later on. Mammalian STE20-like protein kinase 1 (MST1) signaling, which promotes neuronal death, was, in contrast, activated at these later times. The dysregulation of CaMK4β/CREB and MST1 signaling pathways may therefore be critical to the neurodegeneration in scrapie infected mice.

**Results**

PrPRES is first detected in scrapie-infected mice at 130 dpi Mock-infected mice or mice infected intraperitoneally with scrapie (mouse-adapted strain RML) were euthanized at 70, 90, 110, 130 days post-infection (dpi), or at terminal stages of disease (155–190 dpi). Brains were dissected into (i) cortical (cerebrum), (ii) subcortical (including thalamus, hypothalamus and hippocampus) and (iii) brainstem-cerebellum as described [35].

We first analyzed the levels of protease-resistant PrPSc (PrPres) and total glial fibrillary acidic protein (GFAP) by Western blot. PrPres was only detected in scrapie-infected mice, and its levels increased with time of infection (Figure 1A). PrPres was first detected in all regions at 130 dpi, and increased coordinately with the levels of GFAP from 130 dpi to terminal stages of disease (unpaired two-tail t-test; GFAP brainstem-cerebellum, \( P = 0.0388 \); subcortical region, \( P = 0.0008 \); cortical region, \( P = 0.0414 \) (Figure 1B), as expected [36,37]. The levels of GFAP were also higher in the brainstem-cerebellum of scrapie-infected mice prior to PrPres accumulation at 70 dpi (\( P = 0.0041 \) and showed a tendency to higher levels in the cortical region at 90 dpi, as observed previously [38]. The lower molecular weight form of GFAP has also been observed previously [39] and is likely the result of degradation.

Primary kinomic screens identified two signaling pathways of potential interest, which are involved in neuronal death and survival

After intraperitoneal infection, prion disease spreads in the brain caudal to rostral [40-42]. We therefore selected the brainstem-cerebellum for the primary screens, for the greatest window of opportunity to identify signaling pathways dysregulated during pathogenesis. Primary multiplex Western blots analyzed the expression levels
of 139 protein kinases (Additional file 1: Table S1) in brainstem-cerebellum homogenates of three mock-infected and three scrapie-infected mice euthanized at 70, 90, 110, 130 dpi or at terminal stages (155–190 dpi). The antibodies included in our analyses were optimized and validated as described (Shott et al., companion paper). We detected 109 protein kinases (78% of the 139 tested), most of which were differentially expressed in scrapie- as compared to mock-infected mice. For example, CaMK4β was expressed to higher levels in scrapie- than in mock-infected mice at
70 dpi (Figure 2A), and dual leucine zipper kinase (DLK) to lower levels at 130 dpi (Figure 2B). Ten protein kinases were not detected in one set of mock- and scrapie-infected mice at one time point (tropomyosin-related kinase B [TrkB], Set 1 at 70 dpi; membrane-associated tyrosine/threonine-specific cdc2-inhibitory kinase [Myt1], Set 2 at 110 dpi; v-Raf murine sarcoma viral oncogene homolog B1 [B-Raf], ribosomal protein S6 kinase, 70 kdalton, poly-peptide 1 [p70S6K], serine/threonine-protein kinase D3 [PKD3], protein kinase R [PKR], serine/threonine-protein kinase N2 [PRK2], STE20-like serine/threonine-protein kinase [SLK], TRAF2 and NCK-interacting protein kinase [TNIK], and tropomyosin-related kinase C [TrkC], Set 3 at 130 dpi). Five other proteins (calcium/calmodulin-dependent protein kinase 1 alpha [CaMK1α], cyclin D1, cyclin G1, p25 and p35) were not resolved in one set at 70, 90, 110, and 130 dpi. Mitogen-activated protein kinase kinase 5 (ASK1) and mitogen-activated protein kinase kinase kinase 1 (MEKK1) were not quantitated in one set at 90 dpi, or two at terminal stages, due to transfer or assay kinase kinase kinase 12 (p38γ), which clustered together because they were expressed to similarly lower levels in scrapie- than in mock-infected mice at 130 dpi. Contrary to expectation, the expression levels of involved protein kinases were most affected (decreased) at later times.

Primary screens of brainstem-cerebellum homogenates from scrapie-infected mice therefore identified two signaling pathways which may be dysregulated during pathogenesis. The NMDAR-regulated CaMK4β signaling pathway promotes neuronal survival and the expression levels of the protein kinases involved changed (increased) the most at earlier times. Conversely, the MST1 signaling pathway promotes neuronal death, and the expression levels of involved protein kinases were most affected (decreased) at later times.

CaMK4β/CREB signaling is activated at early stages of scrapie in mice
The NMDAR-regulated CaMK4β signaling pathway promotes neuronal survival though the activation of CREB [43]. We therefore analyzed the expression levels of CREB. We also analyzed the expression levels of neuronal nitric oxide synthase (nNOS) and the scaffold protein postsynaptic density protein 95 (PSD-95), which associate with, or are regulated by, RSK1, Lyn and p38γ at NMDARs [44-46]. CaMK4β, CREB, p38γ, RSK1, and PSD-95 levels in the brainstem-cerebellum of scrapie-infected mice were different from those in mock-infected mice (nonlinear regression analysis; CaMK4β, P = 0.0404; CREB, P = 0.0372; p38γ, P = 0.0369; RSK1, P = 0.0471; PSD-95, P = 0.0147) (Figure 5). Consistent with the higher levels of RSK1, Lyn, p38γ, and CaMK4β in the brainstem-cerebellum of scrapie-infected mice at 70 dpi, the levels of nNOS and CREB were also higher at this time (two-tailed paired ratio t-test; nNOS, P = 0.0434; CREB, P = 0.0341) (Figure 5). We expanded our analyses to the subcortical and cortical regions. CREB levels were also significantly higher in the subcortical and cortical regions of scrapie-infected mice, but at 90 dpi (subcortical region, P = 0.0196; cortical region, P = 0.0481) (Figure 5). CaMK4β and CREB levels changed coordinately in both the brainstem-cerebellum and subcortical regions (Figure 5).

We next performed targeted tertiary (phosphorylation state-specific) analyses to characterize the activation state of (NMDAR-regulated) CaMK4β/CREB signaling in scrapie-infected mice. The activation of Lyn and RSK1 involves autophosphorylation on Y396 or S380, respectively [47-50]. Activated RSK1 (P-S380) inhibits nNOS by phosphorylation...
Figure 2 (See legend on next page.)
on S847 [45]. Activated p38 (P-T180/Y182) phosphorylates PSD-95 on S290 [46]. Activated CaMK4β (P-T196; corresponding to human T200) phosphorylates CREB on S133 [51,52]. Phosphorylated CREB promotes transcription of genes that encode proteins involved in neuronal survival [53,54]. There is no antibody specific for T180/Y182 phosphorylation on only p38y. We therefore used an antibody that detects T180/Y182 phosphorylation on all p38 isoforms (p38α, p38β, p38γ, p38δ). In summary, we analyzed the levels of activating phosphorylation of p38 (P-T180/Y182), Lyn (P-Y396), RSK1 (P-S380), CaMK4β (P-T196), and CREB (P-S133), and the levels of inhibitory phosphorylation of nNOS (P-S847). No antibody specific for p38-phosphorylated PSD-95 (P-S290) was available.

As their total levels, the levels of activated CaMK4β (P-T196) and CREB (P-S133) changed coordinately. Their levels were significantly higher in the brainstem-cerebellum of scrapie-infected than of mock-infected mice at 70 and 90 dpi (two-tail paired ratio t-test; CaMK4β P = 0.0256 [70 dpi], 0.0248 [90 dpi]; CREB P = 0.0197 [70 dpi], 0.0086 [90 dpi]) (Figure 6). The levels of phosphorylated CREB in the subcortical and cortical regions were also significantly higher (subcortical region P = 0.0412 [90 dpi]; cortical region P = 0.0093 [70 dpi], 0.0376 [90 dpi]), or at least there was a trend to higher levels in scrapie-infected than in mock-infected mice (subcortical region P = 0.0936 [70 dpi]) (Figure 6). The levels of activated CaMK4β in the subcortical and cortical regions at 70 and 90 dpi were similar in scrapie- and mock-infected mice. The levels of activated p38 (P-T180/T182) were significantly higher in scrapie-infected than in mock-infected mice at 70 and 90 dpi (brainstem-cerebellum P = 0.0225 [90 dpi]; subcortical region P = 0.0106 [70 dpi]; cortical region P = 0.0014 [70 dpi]) but also at 110 and 130 dpi (brainstem-cerebellum P = 0.0465 [110 dpi]; cortical region P = 0.0116 [130 dpi]) (Figure 6). There was no correlation between the levels of activated RSK1 (P-S380) and nNOS (P-S847) in the different brain regions (Additional file 2: Figure S1). The levels activated Lyn (P-Y396) were mostly unchanged in scrapie-infected mice relative to mock-infected mice.

In summary, CaMK4β/CREB signaling was activated in scrapie-infected mice at early times. The expression levels and the levels of phosphorylated CREB (P-S133) and activated CaMK4β (P-T196) were higher in scrapie-than in mock-infected mice at 70 and 90 dpi.

MST1 is activated at late stages of mouse scrapie

The MST1 signaling pathway mediates neuronal death by activating forkhead box protein O3 (FOXO3) [55]. We therefore analyzed the expression levels of FOXO3 in targeted secondary Western blots of brainstem-cerebellum homogenates from scrapie-infected mice at 70, 90, 110, 130 dpi or at terminal stages of disease. Consistent with the lower expression levels of DLK (two-tailed paired ratio
Figure 4 (See legend on next page.)
Figure 4: Identification of two signaling pathways of potential interest involved in neuronal survival and death. (A) Hierarchical clustering the normalized and log transformed densitometric data of the expression levels of 109 protein kinases detected in primary multiplex Western blots of brainstem-cerebellums of scrapie-infected mice at 70, 90, 110, 130 dpi or at terminal stage of disease (TER). Red, higher expression level; green, lower expression level; grey, no data (protein kinases that were not resolved, not detected, or not quantitated due to transfer or blotting artifacts). Cluster (i) consists of protein kinases involved in the NMDAR-regulated CaMK4β signaling pathway. Cluster (ii) consists of protein kinases involved in the MST1 signaling pathway. The protein kinases highlighted in Figure 2, CaMK4β and DLK, are indicated by (●), and MKK7 is indicated by (●). (B) Expression levels of protein kinases involved in the CaMK4β and MST1 signaling pathways, and included in the primary multiplex Western blots, at 70 dpi (top panel) and 130 dpi (bottom panel), respectively. Color bars indicate the levels in each of the three scrapie-infected mice normalized to those in mock-infected mice.

Discussion

We describe the application of a new kinomics approach to an in vivo model of prion disease pathogenesis, mice intraperitoneally infected with scrapie strain RML. The primary screens identified CaMK4β and MST1 signaling pathways as of potential interest. Targeted analyses then tested the activation state of these pathways. CaMK4β/CREB signaling, which promotes neuronal survival, was activated at earlier times in scrapie-infected mice, but returned to the levels of mock-infected mice at later times. At these later times, MST1 signaling, which promotes neuronal death, was activated (Figure 9).

The activation of CaMK4β/CREB signaling at preclinical stages of prion disease had not been described. CREB is critical to neuronal survival. CREB/cAMP response element modulator (CREM) double knockout mice, or mice in which CREB is inhibited by overexpression of a dominant negative mutant or inhibitory peptides, suffer extensive neuronal loss [62-64]. Active CREB promotes neuronal survival by regulating transcription of “activity-regulated inhibitor of death” (AID) genes, including gadd45β, gadd45y, bg2, npas4, nr4a1, inhba, atf3, ifi202b, serpinb2 [53,54]. CREB also regulates the transcription of...
Figure 5 (See legend on next page.)
miR132-3p [65,66], which modulates synapse morphology [67]. High-throughput analyses have identified changes in many miRNAs and miRNAs, including miR132-3p, in scrapie-infected mice prior to the accumulation of PrP\textsuperscript{res} or the onset of clinical disease [68-73]. Elevated levels of miR132-3p and AID genes (gadd45β, gadd45γ, bg2, npsa4, nr4a1) were observed at 70–110 dpi after infection with the same scrapie strain and by the same route of inoculation as in the current experiments [72]. Activated CaMK4β/CREB signaling may well promote neuronal survival early in prion infection by upregulating the expression of miR132-3p and AID genes.

CaMK4β/CREB signaling returned to the activation levels of mock-infected mice at 110 dpi. Calcineurin regulates the activity of CaMK4 [74] and CREB [75], and elevated calcineurin activity was observed in scrapie-infected mice at clinical stages of disease [19]. Moreover, the calcineurin inhibitor FK506 prolonged survival of scrapie-infected mice and inhibited neuronal death in cultured neurons treated with PrP106-126 [19,76,77]. CaMK4β/CREB signaling is therefore most likely neuroprotective in scrapie-infected mice. Decreased levels of activated CaMK4β (P-T196) and phosphorylated CREB (P-S133) were associated with decreased expression levels, suggesting that degradation may be involved in downregulation of this pathway. CaMK4 and CREB are degraded by calpain, a calcium-dependent protease [78,79]. Active calpain degraded CaMK4 and CREB in cultured neurons treated with hydrogen peroxide after CREB (P-S133) dephosphorylation [80]. Increased levels of calpain have been observed in vitro and in vivo models of prion disease [81,82], and calpain inhibition limited neuronal death in prion-infected cultured organotypic cerebellar slices or cultured neurons treated with PrP106-126 [81,83,84]. CaMK4β/CREB signaling activation may be inhibited in scrapie-infected mice after 90 days by dephosphorylation and degradation mediated by calpain. CaMK4 and CREB are also dephosphorylated by protein phosphatase 2A (PP2A) [85-87], the activity of which has yet to be evaluated during scrapie infection.

MST1 signaling was activated at 130 dpi, which had not been described in prion disease. MST1 has been shown to be involved in other neurodegenerative diseases. Genetically modified mice that model amyotrophic lateral sclerosis (ALS) lose fewer neurons and survive longer if they are knocked out of MST1 [88]. Active MST1 (P-T183) is cleaved by active (cleaved) caspase-3 [57-60], the levels of which are elevated in scrapie-infected mice (before the accumulation of PrP\textsuperscript{res}) and in cultured neurons or neuroblastoma cells exposed to PrP106-126 or PrP\textsuperscript{Sc} [89-92]. We observed elevated levels of cleaved MST1 at 130 dpi, suggesting that it is also cleaved by caspase-3 in vivo. Although cleaved MST1 retains the T183 phosphorylation site, T183 phosphorylation is not required for cleavage [56,93], and we did not detect cleaved MST1 with the phosphorylation-specific antibody. Caspase-cleaved MST1 translocates to the nucleus and induces chromatin condensation by inducing phosphorylation of histone H2B on S14 [94]. Chromatin condensation has been observed previously in scrapie-infected GT1 cells and mice [95-98]. However, caspase-3 activation is not required for MST1 activation [56] or neuronal death in prion disease [92,99,100]. MST1 activates FOXO3, which is otherwise maintained in the cytosol in an inactive state by interaction with 14-3-3 proteins [101]. Cleaved MST1 predominately localizes to the nucleus and is therefore unable to phosphorylate FOXO3 [102,103]. The levels of phosphorylated FOXO3 (P-S208) might have been higher (but were not statistically different) after the levels of cleaved MST1 had returned to those in mock-infected mice. Active FOXO3 upregulates transcription of genes encoding pro-apoptotic proteins including Bim (bcl-2 interacting mediator of cell death; bcl2I1), Puma (Bcl-2-binding component 3; bbc3) and Noxa (phorbol-12-myristate-13-acetate-induced protein 1; pmaip1) [104-106]. The levels of Bim, Puma, bbc3 and pmaip1 are elevated in scrapie-infected mice at late stages of disease progression [72,107,108], suggesting that neuronal death mediated by MST1 signaling could involve FOXO3.

The CaMK4β/CREB and MST1 signaling pathways were identified in our screens because the expression levels of the involved protein kinases changed coordinately during prion disease progression (Figures 5 and 7). The CaMK4β/CREB signaling pathway promotes neuronal survival. CaMK4β and CREB were expressed to higher levels and activated at earlier stages of disease. Neurons may activate this pathway to protect themselves from prion-mediated...
Figure 6 (See legend on next page.)
death. Later (at 110 dpi), the neuroprotective CaMK4β/CREB signaling was lost and MST1 signaling was activated. Activation of MST1 signaling was associated with lower levels of full length MST1 and FOXO3. The decrease in full length MST1 was two-fold greater than the increase in the cleaved form (detected in the same blots with the same antibody). Cleaved MST1 may be less stable than full length MST1 or full length MST1 may be processed by caspase-dependent and independent pathways.

The opposing changes in expression and activation state suggest an attempt to prevent the neuronal death that would result from activated MST1 signaling. The levels of upstream kinases DLK, MKK7, and JNK2 were also lower in scrapie-infected mice at 130 dpi, albeit only in the brainstem-cerebellum (Figure 7). The cerebellum (in the brainstem-cerebellum) may contain ~50% of all neurons in an adult mouse brain [109] and loses the most neurons in RML-infected mice, as indicated by nuclear DNA fragmentation [96]. The changes in the total levels of proteins involved in the MST1 signaling pathway in each brain region may reflect the differences in the number of affected neurons in each brain region.

The different numbers of affected neurons in each region may also be reflected in the higher levels of total and phosphorylated CaMK4β in the brainstem-cerebellum than in the subcortical or cortical regions. Regardless of absolute numbers, higher levels of total and phosphorylated CREB were expressed in all brain regions. Although CaMK4 (P-T196) could also be responsible for CREB (P-S133) phosphorylation, there were no differences in the levels of activated CaMK4 (P-T196) in scrapie-versus mock-infected mice (data not shown). CREB is also phosphorylated on S133 by other protein kinases, including RSK, cAMP-dependent protein kinase (PKA), and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) (for a review, see [110]). There was no correlation between the levels of active RSK1 (P-S380) and phosphorylated CREB (P-S133). We did not evaluate other protein kinases upstream of CREB because they were not identified in the primary screening. However, CREB may well be activated independently of CaMK4β in the cortical region or subcortical region of scrapie-infected mice.

The data presented cannot discriminate whether the observed changes in signaling pathways are a cause or a consequence of the pathology. Neither can discriminate whether they result from a loss of PrPC function, a gain of PrPSc function, or an altered signaling function resulting from the progressive PrPC misfolding into PrPSc. Moreover, the dysregulation could have been directly triggered by PrPSc or PrPC acting on the neurons, or mediated by the glial alterations that occur during disease progression. The data presented does not resolve either whether the observed signaling changes are specific for prion diseases, or common to other neurodegenerative diseases. Future work will have to address these possibilities.

Conclusion

We used a kinomics approach to identify two dysregulated signaling pathways, involved in neuronal survival/death, in scrapie-infected mice. Their dysregulation at different times during disease is temporally consistent with the neuronal loss during prion disease. Our findings identified novel signaling involved in prion-mediated neuronal death in vivo and identify potential targets for intervention. It is possible to test now the roles of these signaling pathways in prion disease, as well-characterized inhibitors of several of these proteins are available.

Materials and methods

Ethics statement

All of the procedures involving live animals were approved by the Canadian Science Centre for Human and Animal Health – Animal Care Committee (CSCHAH-ACC) or the University of British Columbia Animal Care Committee according to the guidelines set by the Canadian Council on Animal Care. The approval identifications for this study were animal use document (AUD)#H-08-009 and AUD#H-11-020.

Animals and sample collection

CD1 mice (Charles River Laboratories) between 4–6 weeks of age were infected intraperitoneally with the Rocky Mountain Laboratory (RML) mouse-adapted strain of scrapie. The inoculums consisted of 200 μl of 1% brain homogenate in PBS from either clinically ill or normal control mice. Animals were sacrificed at 70, 90, 110, 130 days post infection (dpi) and terminal disease (155–190 dpi). Clinical signs depicting terminal disease
Figure 7 (See legend on next page.)
consisted of kyphosis, dull ruffled coat, weight loss of 20% or more and ataxia. Brain samples were collected and macrodissected into three sections, (i) cortical, (ii) subcortical (including thalamus, hypothalamus and hippocampus) and (iii) brainstem-cerebellum. Each section was flash frozen using a dry ice/methanol mixture and stored at −80°C until processing. A total of 3 scrapie- and 3 mock-infected samples were collected per time point.

Brain homogenization

All procedures were performed at 4°C or on ice. Weighed frozen brainstem-cerebellum, subcortical, and cortical regions from mock- or scrapie-infected mice were homogenized in 3 mL of freshly prepared lysis buffer (20 mM MOPS [pH 7.0], 2 mM EGTA, 5 mM EDTA, 1% Nonidet P-40, 0.01% phosphatase inhibitor cocktail [Pierce, Rockford, Illinois, USA], 0.02% protease inhibitor cocktail [Sigma-Aldrich, St. Louis, Missouri, USA], 10 mM DTT, pH 7.2) [111] per 250 mg of brain, using a tissue homogenizer with disposable tips (TH and hard tissue OMNI tip, respectively; OMNI International, Kennesaw, Georgia, USA). Brain homogenates were passed twice through a 21 gauge needle, sonicated five times for 20 s intervals at 88 W output (431C1 cup horn probe, S-4000 sonicator; Qsonica, Newtown, Connecticut, USA), and pre-cleared by centrifugation for 30 min at 14,000 × g (JLA 16.250 BC rotor, Avanti J-E centrifuge; Beckman/ Coulter, Brea, California, USA). Approximately 200 μL aliquots of supernatant were aliquoted into 1.5 mL tubes, snap frozen in liquid nitrogen and stored at −80°C.

Protein quantitation

Protein concentration was determined by Bradford’s assay (Bio-Rad Laboratories, Hercules, California, USA). Protein concentration and equal sample loading was re-tested in preliminary sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Brain homogenates were mixed with an equal volume of 2X SDS loading buffer (125 mM Tris-Cl [pH 6.8], 20% glycerol, 4% SDS, 0.005% bromophenol blue, 260 mM DTT) and denatured by incubation at 100°C for 10 min. Afterward, 15-well 8% SDS-PAGE gels (Mini-PROTEAN; Bio-Rad Laboratories) were loaded with 40 μg of denatured protein per linear cm of well (running buffer; 190 mM glycine, 24.8 mM Tris, 0.1% SDS, pH 8.3). Proteins were run through the stacking gel at 50 V, and then resolved for 90 min at 100 V, always at room temperature. Proteins were stained with Coomassie blue G-250 (Bio-Safe Coomassie; Bio-Rad Laboratories) according to the manufacturer’s instructions. Signal from Coomassie-stained protein was detected using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, Nebraska, USA) in the 700 nm channel and quantitated using Odyssey 3.0 software (LI-COR Biosciences). Protein amounts were calculated relative to a pre-quantitated standard brain homogenate.

Sodium phosphotungstic acid precipitation

Sodium phosphotungstic acid (NaPTA) precipitation was adapted from [112,113]. One milligram of mouse brain homogenate was mixed with an equal volume of 4% sodium lauroylsarcosine (sarkosyl) in PBS. Samples were incubated for 10 min at 37°C with constant agitation (1000 rpm, Thermomixer; Eppendorf, Hamburg, Germany). A 37°C NaPTA solution (PBS, 4% NaPTA, 170 mM MgCl₂, pH 7.4) was then added to a final concentration of 0.3% NaPTA. Samples were incubated for 60 min at 37°C with constant agitation (1000 rpm, Thermomixer; Eppendorf), then centrifuged at 37°C for 30 min at 16,000 × g (FA-45-18-11 rotor, 5418 microfuge; Eppendorf). Pellets were resuspended in 5 μL of 0.1% sarkosyl in PBS and digested with 20 μg of proteinase K (PK; in 0.01 M CaCl₂) (Roche, Indianapolis, Indiana, USA) for 30 min at 37°C with a brief vortex after 15 min [114]. Digestion was stopped and PK-resistant protein was denatured by quickly adding an approximately equal volume 5X SDS loading buffer (300 mM Tris-Cl [pH 6.8], 50% glycerol, 25% β-mercaptoethanol, 10% SDS, 1% bromophenol blue), and immediately incubating at 100°C for 10 min. Samples were resolved by SDS-PAGE and analyzed by Western blot.

Western blot

All procedures were performed at room temperature and all washes were performed using gentle rocking, unless otherwise indicated.

PrP⁰⁰ was analyzed in 1.0 mg of NaPTA-enriched, PK-treated mouse brain homogenate using 15-well 12% SDS-PAGE gels (NuPAGE Novex Bis-Tris; Life Technologies Inc., Carlsbad, California, USA). The running buffer (50 mM MOPS, 50 mM Tris, 1 mM EDTA, 0.1% SDS, pH 7.7) within the upper (cathode) chamber contained...
Figure 8 (See legend on next page.)
5 mM sodium bisulfite. Proteins were run through the stacking gel at 60 V, and then resolved for 2.5 h at 120 V. Afterward, polyvinylidene fluoride (PVDF) membranes (ImmuNo-Blot, 0.2 μm; Bio-Rad Laboratories) were soaked in methanol for 2 min, then equilibrated in transfer buffer (190 mM glycine, 24.5 mM Tris, 10% methanol) for 20 min. Filter paper (2 sheets/membrane) were equilibrated in transfer buffer for 5 min. Proteins were transferred at 4°C for 2 h at 30 V. After transfer, membranes were dried, soaked in methanol for 2 min and washed twice for 10 min each in Tris-buffered saline (TBS; 140 mM NaCl, 3 mM KCl, 25 mM Tris, pH 7.6). Membranes were blocked for 1 h in TBST (TBS/0.1% Tween-20) with 5% milk, then probed with PrP primary antibody (clone SAF83; a kind gift from Dr. Deborah McKenzie, University of Alberta) diluted to 1:10,000 in TBST with 5% milk for 18 h at 4°C. Afterward, membranes were washed in TBST once for 5 min and thrice for 10 min each. Membranes were incubated with goat anti-mouse horseradish peroxidase (HRP)-labeled secondary antibody (Bio-Rad Laboratories) diluted to 1:40,000 in TBS with 5% milk for 1 h. Membranes were washed in TBST once for 5 min and thrice for 15 min each, incubated for 5 min with enhanced chemiluminescent substrate (SuperSignal West Femto; Pierce) and then exposed to film (Super RX; Fujifilm, Tokyo, Japan). Exposed film was developed and scanned (CanoScan LiDE 200; Canon, Tokyo, Japan). Signal was quantitated using ImageJ (Version 1.47c; National Institutes of Health, Bethesda, Maryland, USA).

To analyze GFAP and total PrP, brain homogenates were mixed with a one-fourth volume of 5X SDS loading buffer. Then, 100 μg of denatured protein was loaded per linear cm onto 15-well 12% SDS-PAGE gels (Mini-PROTEAN; Bio-Rad Laboratories). Proteins were run through the stacking gel at 50 V, and then resolved for 100 min at 100 V. Afterward, gels were equilibrated in transfer buffer (384 mM glycine, 49.6 mM Tris, 20% methanol, 0.01% SDS) for 30 min. PVDF membranes and filter paper (2 sheets/membrane) were also equilibrated in transfer buffer for 20 and 5 min, respectively. Proteins were transferred for 23 h at 4°C; 1 h at 54 mA, 4 h at 189 mA, 8 h at 270 mA and 10 h at 378 mA. Membranes were blocked with 10% blocking buffer (Sigma-Aldrich) for 1 h and probed simultaneously with primary antibodies specific for PrP (clone SAF83) and GFAP (Abcam Inc., Cambridge, Massachusetts, USA) diluted to 1:10,000 and 1:20,000 in 10% blocking buffer with 0.1% Tween-20, respectively. Proteins were washed with TBST once for 5 min and thrice for 10 min each. Membranes were incubated with donkey anti-mouse IRDye 680- (LI-COR Biosciences) and donkey anti-rabbit IRDye 800- (LI-COR Biosciences) labeled secondary

Figure 8: Activation of MST1 in scrapie-infected mice at late stages of disease. Targeted tertiary analyses of MST1 signaling in brainstem-cerebellum, subcortical region, and cortical region of scrapie-infected mice at 70, 90, 110, 130 dpi or at terminal stage of disease (TER; 155–190 dpi).

A) Levels of phosphorylated JNK (T183/Y185), MST1 (T183), FOXO3 (S208), and cleaved MST1, in the brainstem-cerebellums of each of the three scrapie-infected mice at each time point, normalized to those in the mock-infected mice, shown by color bars. The proteins in dashed lines were not analyzed.

B) Phosphorylation levels in all brains regions, normalized to those in the mock-infected mice, shown as time series. Mean ± SD; n = 3 (Mean ± range for MST1 (T183) at 110 dpi [subcortical region] and TER [brainstem-cerebellum], and FOXO3 (S208) at 130 dpi [brainstem-cerebellum] and TER [cortical region]; n = 2). Error bars on the x-axes, range in time of onset of terminal disease. The differences in the phosphorylation levels, or levels of cleaved MST1, in scrapie- versus mock-infected mice were analyzed by two-tailed paired ratio t-test. *(#, cleaved MST1), P < 0.05.

Figure 9: A model for the activation of signaling pathways involved in neuronal survival and death during scrapie pathogenesis.

Relative activation states of CaMK4β/CREB (red line) and MST1 (green line) signaling in scrapie-infected mice during disease progression, indicated in grey. Between 90 and 130 dpi, there is a switch from signaling involved in neuronal survival (activated CaMK4β/CREB) to that involved in neuronal death (activated MST1). Adapted from [72] (Figure 7).
antibodies diluted to 1:20,000 in 10% blocking buffer with 0.1% Tween-20 and 0.01% SDS. Afterward, membranes were washed in TBST thrice for 10 min each, and once with TBS for 5 min. Signal from pre-stained protein standards and IRDye 680-labeled secondary antibody was detected at 700 nm using an Odyssey infrared imaging system (LI-COR Biosciences). Signal from IRDye 800-labeled secondary antibody was detected at 800 nm. Signal was quantitated using Odyssey 3.0 software (LI-COR Biosciences). Membranes were then stained with Coomassie blue R-250 (Bio-Rad Laboratories) for 10 min before destaining with 40% methanol in 10% glacial acetic acid thrice for 10 min each, or until excess stain was removed. Signal from Coomassie-stained protein was detected at 700 nm using the Odyssey and quantitated using Odyssey 3.0 software.

For multiplex Western blots, brain homogenates were mixed with an equal volume of 2X, or one-fifth volume of 6X (375 mM Tris-Cl [pH 6.8], 60% glycerol, 12% SDS, 0.015% bromophenol blue, 780 mM DTT), SDS-PAGE loading buffer, and then 200 μg of denatured protein was loaded per linear cm of single-well 8% SDS-PAGE gels (Mini-PROTEAN; Bio-Rad Laboratories). Proteins were resolved as described for protein quantitation and transferred as described for Western blots of GFAP and total PrP. Dried membranes were probed immediately or stored at ~30°C. All multiplex Western blots were performed in three sets, each composed of one membrane from a mock- and one from a scrapie-infected mouse euthanized at 70, 90, 110, 130 dpi, or at terminal stage of disease. Membranes were blocked for 1 h with 10% blocking buffer (Sigma-Aldrich) for evaluation of total protein levels, or in 3% BSA (Rockland, Gilbertsville, Pennsylvania, USA) for evaluation of phosphorylation levels. Membranes were rinsed briefly with TBS and positioned within a 24-lane multi-screen apparatus (MPX; LI-COR Biosciences). Combinations of primary antibodies were diluted in 10% blocking buffer or 3% BSA, as appropriate, with 0.1% Tween-20. One hundred sixty microliters of each antibody dilution was loaded in each lane of the multi-screen apparatus. After incubation for 18 h at 4°C, membranes were briefly washed once with TBST within the multi-screen apparatus then removed from the apparatus and further washed in TBST, once for 5 min and four times for 15 min each. Membranes were incubated with secondary antibody diluted to 1:20,000 in 10% blocking buffer or 3% BSA, as appropriate, with 0.1% Tween-20 and 0.01% SDS for 1 h. Mouse monoclonal primary antibodies were detected with donkey anti-mouse IRDye 680-labeled secondary antibody. Rabbit or goat primary polyclonal primary antibodies were detected with donkey anti-rabbit or donkey anti-goat IRDye 800-labeled secondary antibody (LI-COR Biosciences), respectively. Membranes were washed in TBST four times for 15 min each and once with TBS for 5 min. Signal from IRDye 680- and IRDye 800-labeled secondary antibody was detected at 700 and 800 nm, respectively, using the Odyssey system. Signal was quantitated using Odyssey 3.0 software.

Membranes from scrapie-infected mice were stripped (only once) together and in parallel with the membranes from the mock-infected mice from the same set, under conditions to minimize protein loss [116]. Membranes were stripped with mild stripping buffer (25 mM glycine, 1% SDS, pH 2.0) once for 5 min and four times for 15 min each, then with harsh stripping buffer (50 mM Tris-Cl [pH 7.0], 2% SDS, 50 mM DTT) [117] once for 15 min at 37°C. Afterward, stripped membranes were washed with TBST once and TBS once for 5 min each, then blocked and reprobed with a second set of primary antibodies as described.

Hierarchical cluster analysis
For each set, the densitometric data from the primary multiplex Western blots of brainstem-cerebellum homogenates of scrapie-infected mice was normalized to that from the mock-infected mice. Relative protein kinase expression levels were then log transformed and analyzed with Gene Cluster 3.0 [118] using Euclidean distance and complete linkage. Java Treeview was used to present the clusters [119].

Statistical analyses
All data was analyzed using Prism (Version 5.0f; GraphPad Software Inc., La Jolla, California, USA). The targeted Western blots were performed in three experimental sets on different days. Each set encompassed one scrapie- and one mock-infected mouse from each time point. The ratios from each set were thus analyzed by paired t-test. The ratios were log transformed before analyses to transform the increased and decreased ratios (ratios greater or smaller than 1, respectively) to proportional values. For nonlinear regression analyses, curves of the normalized total and phosphorylation protein levels in scrapie-infected mice were compared to no changes (a line with intercept = 1, slope = 0), representing the levels in mock-infected mice, using a replicates test for lack-of-fit.

Additional files

Additional file 1: Table S1. Accession numbers for the 130 protein kinases and 9 regulatory subunits analyzed in the primary multiplex Western blots. One hundred and twenty-four protein kinases or regulatory subunits included in our multiplex Western blots were detected in 200 μg of mouse brain homogenate per linear well cm. The other 15 (indicated by the asterisks) were detected in multiplex Western blots using an equivalent amount of lysate from cycling 3T3 mouse fibroblasts. The human accession number for each protein is indicated.

Additional file 2: Figure S1. CREB is expressed and phosphorylated to higher levels in the subcortical and cortical regions of scrapie- than

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http://www.virologyj.com/content/11/1/160
mock-infected mice at 70 and 90 dpi. The normalized expression levels of p38, Lyn, JNK1, CaMK4B, nNOS, CREB, and PSD-95 (A) or levels of phosphorylated p38 (T180/Y182), Lyn (Y396), Src (S348), nNOS (S847), CaMK4B (T196), and CREB (S133) (B) in the subcortical and cortical regions of each of the three scrapie-infected mice at each time point showed by color bars. The proteins boxed in dashed lines were not analyzed.

Additional file 3: Figure S2. Lower levels of MST1 and FOXO3 are phosphorylated to higher levels in the subcortical and cortical regions of scrapie-infected mice at 130 dpi. The normalized expression levels of DLK, MKK7, JNK2, MST1 and FOXO3 (A) or levels of phosphorylated JNK (T183/Y185), MST1 (T183), FOXO3 (S208), and cleaved MST1 (B) in the subcortical and cortical regions of each of the three scrapie-infected mice at each time point shown by color bars. The proteins boxed in dashed lines were not analyzed.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RHS prepared homogenates, performed the kinomic analyses and critically evaluated the results. KLF and AM performed the infections, collected and dissected the brains. LMS designed the study, critically evaluated the results, and co-wrote the manuscript with RHS. SAB and AM critically revised the manuscript. All authors have read and approved the final version of the manuscript.

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