Comparative proteomic analysis of the synaptoneurosome reveals synaptic proteins linked to prion infection

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Overview

Purpose: Synapses appear to be the location of the earliest detectable neuropathological alterations in prion diseases. We aim to determine which components of the synaptic proteome/miRNome are specifically altered during disease as a basis for increasing our understanding of the changes that lead to clinical prion disease.

Methods: Total protein from synaptoneurosomes was prepared from the forebrain region of adult mice. Proteins were digested with trypsin and analyzed by the Thermo Scientific Orbitrap Elite mass spectrometer. miRNA were purified from the same synaptoneurosomes and expression levels were determined using miRNA microarray profiling.

Results: Synaptoneurosome enriched miRNAs appear to very specifically target proteins involved in biological processes involved in the neuropathology of prion disease.

Introduction

Prior to the onset of clinical symptoms in prion diseases, alterations include synaptic vesicle-mediated transport. Synaptic vesicles may represent the means of the synaptic proteome/miRNome are specifically altered during disease as a crude synaptoneurosome-containing fraction. The fraction was subsequently homogenized buffer (0.32 M sucrose, 0.1 mM EDTA, 0.25 mM DTT, 2 mM Hepes, pH 7.4) and disrupted with a Teflon-coated Dounce-Potter homogenizer (Wheaton) by eight up-and-down strokes.

The causative agent is a prion, a transmissible protein that is able to induce self-templated conformational changes resulting in disease progression and transmission. Prion replication in the brain ultimately leads to neuronal necrosis and apoptosis, but the up-stream pathways which trigger the damage and dysfunction of nerve cells are as yet unidentified.

In a previous study we determined very early, pre-clinical alterations in mRNA expression specifically within neurons coinciding with dendritic spine and synapse abnormalities. Gene expression alterations at this time also indicate striking deregulation of genes coding for synaptic proteins and those involved in synaptic vesicle-mediated transport. Synaptic vesicles may represent the means by which replicating prions are transported from neuron to neuron in the brain.

Methods

Preparation of Synaptoneurosomes

Synaptoneurosomes were prepared from the forebrain region of adult Sprague-Dawley rats. Briefly, the forebrain region was macrodissected in 50 ml of homogenization buffer (0.32 M sucrose, 0.1 mM EDTA, 0.25 mM DTT, 2 mM Hepes, pH 7.4) and disrupted with a Teflon-coated Dounce-Potter homogenizer (Wheaton) by eight up-and-down strokes.

Nuclei and cell debris were pelleted by a 2 min centrifugation at 2000 x g. The supernatant was centrifuged for an additional 10 min at 14, 000 x g to pellet a crude synaptoneurosome-containing fraction. The fraction was subsequently layered over 5 - 13% discontinuous Ficoll gradient that has been equilibrated at 4 °C for 1 hour. Synaptoneurosomes were collected from the gradient interface after centrifugation at 46, 000 x g for 45 min at 4 °C. Samples were obtained from every step of the procedure for Western Blot analysis to ensure quality and purity of the preparations. Proteins were extracted and digested using Trypsin for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

MRNAs were purified from the same synaptoneurosome preparations and their expression levels determined by miRNA microarray profiling. miRNA targets were predicted using the Diana microT 4.0 software, a program for miRNA target prediction that is based on Artificial Neural Networks (http://diana.cslab.ece.ntua.gr) We used computational tools (DIANA-miPath, DAVID (http://david.abcc.ncifcrf.gov) and IPA (http://www.ingenuity.com)) to identify molecular pathways potentially altered by the expression of microRNAs that were specifically enriched amongst the proteins present in synaptoneurosomes. Some of these are shown in Table 1.

LCMS

The Thermo Scientific EasyE+LC nano-HPLC system and Microm Mass Magic™ C18 spray tip 15 cm x 75 μm i.d. column (Microm BioResources) were used. Gradient elution was performed from 5 - 45% ACN in 0.1% formic acid over 60 min at a flow rate of 300 nL/min. The samples were analyzed with an Orbitrap Elite™ mass spectrometer. The following MS and MS/MS settings were used: 1T; MsinAgIC Target = 5e4; Msin/MS = 5 μs; 200 ms max ion time; MS = 400-1600 m/z, 60000 resolution at m/z2401, MS Target = 1e6; MS/MS/MS = Top 10 Data-Dependent® acquisition CID Dynamic Exclusion = repeat count 1, Duration 30 sec, Exclusion duration 180 sec; CID Parameters: Collision Energy = 30%. Thermo Scientific Proteome Discoverer software version 1.3 was used for data processing.

Results

Figure 1 shows the overall workflow undertaken for these sets of experiments. Although not exosomes or extracellular microvesicles, synaptoneurosomes contain presynaptic vesicles and elements of the postsynaptic density, which are the site of disease related changes. They are also the location of the exocytosis and endocytosis of vesicles that may act as prion transporters and thus provide the perfect target to conduct our study. Synaptoneurosomes prepared from the forebrain region resulted in the identification of:

- Proteome: Identified 1352 unique proteins by mass spectrometry
- MiRNome: Identified 42 enriched miRNAs

FIGURE 1. Schematic representation of the workflow used in this study.

TABLE 1. Some of the molecular pathways potentially altered by the expression of microRNAs (shown in Figure 3) that were specifically enriched amongst the proteins present in synaptoneurosomes.

<table>
<thead>
<tr>
<th>Category</th>
<th># Genes</th>
<th>P-Value</th>
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<tbody>
<tr>
<td>Eiendsophalpy</td>
<td>102</td>
<td>4.7x10^-16</td>
</tr>
<tr>
<td>Neurodegeneration</td>
<td>48</td>
<td>1.3x10^-9</td>
</tr>
<tr>
<td>Intracellular protein transport</td>
<td>76</td>
<td>1.6x10^-12</td>
</tr>
<tr>
<td>Vesicle transport</td>
<td>29</td>
<td>2.8x10^-4</td>
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<tr>
<td>Synaptic transmission</td>
<td>29</td>
<td>1.5x10^-3</td>
</tr>
<tr>
<td>Stress response</td>
<td>16</td>
<td>4.1x10^-5</td>
</tr>
<tr>
<td>Voltage gated calcium channel</td>
<td>12</td>
<td>5.9x10^-5</td>
</tr>
<tr>
<td>Cytoskeletal protein</td>
<td>55</td>
<td>1.2x10^-7</td>
</tr>
</tbody>
</table>

Interestingly, one protein, GSK-3beta, is predicted to be targeted by all 5 miRNAs. GSK-3beta is aberrantly activated in Alzheimer’s and linked to synapse integrity, amyloid production and the inhibition of neuroprotective transcription.

Conclusion

- Prior to the onset of clinical symptoms in prion diseases, alterations include deregulation of processes involved in synapse integrity, synaptic vesicle production, activation of calcium channels and the stress response.
- Synaptoneurosome enriched miRNAs are similarly deregulated.
- Synaptoneurosome enriched miRNAs appear to very specifically target proteins involved in biological processes involved in the neuropathology of prion disease.
- Key regulatory proteins found in synaptoneurosomes are targeted by these miRNAs, such as GSK-3beta.
- Correlation of synaptoneurosome enriched miRNAs that are deregulated in disease with their putative protein targets found in the same preparations appears to be a basis for further study into the mechanisms of neuronal dysfunction in prion disease.

References

2. Majer et al., Novel Early Mechanisms of Pathobiology are Revealed by Transcriptional Temporal Dynamics in Hippocampal CA1 Neurons of Prion Infected Mice. Accepted with minor revisions in PLoS Pathogens

We previously determined that the miRNAs highlighted in blue were significantly upregulated in prion-infected mouse neurons very early in disease.