Prion protein-induced proteome alterations in sporadic Creutzfeldt-Jakob disease and in SH-SY5Y cell culture model

PhD Thesis

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I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute of higher education, except where due acknowledgement has been made in the text.

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LIST OF ABBREVIATIONS

AD – Alzheimer’s disease
ALDOA – aldolase A
ANXA5 – annexin A5
CJD – Creutzfeldt-Jakob disease
CSF – cerebrospinal fluid
ER – endoplasmic reticulum
GRB2 – growth factor receptor-bound protein 2
G6PI – glucose-6-phosphote isomerase
LDH – lactate dehydrogenase
MM – (methionine/methionine) codon 129 genotype in human PrP gene
NDC – non-demented neurological control
PDIA1 – disulfide isomerase precursor
PPID – 40 kDa peptidyl-prolyl cis-trans isomerase
PRNP – gene encoding human PrP
Prnp – gene encoding murine PrP
PrP – prion protein
PrP<sup>C</sup> – cellular prion protein
PrP<sup>Sc</sup> – scrapie prion protein
PSWC – periodic sharp wave complexes
sCJD – sporadic Creutzfeldt-Jakob disease
TAGL2 – transgelin-2
TCTP – translationally-controlled tumor protein
TSE – transmissible spongiform encephalopathie
VD – vascular dementia
VV – (valine/valine) codon 129 genotype in human PrP gene
1433G – 14-3-3 protein gamma isoform
2D-DIGE – 2D Fluorescence Difference Gel Electrophoresis
ABSTRACT

Cellular prion protein (PrP\textsuperscript{C}) is a glycosylated membrane glycoprotein mainly expressed in the central nervous system. Some still undefined molecular events can lead to the conversion of PrP\textsuperscript{C} into an abnormal conformer called scrapie prion protein (PrP\textsuperscript{Sc}). PrP\textsuperscript{Sc} is characterized by increased β-sheet content, detergent insolubility, partial resistance to protease digestion and tendency to aggregate in the brain tissue.

The conversion and subsequent aggregation of PrP\textsuperscript{Sc} in the brain tissue underlay pathogenesis of transmissible spongiform encephalopathies. In turn, sporadic Creutzfeldt-Jakob disease is most common form of human transmissible spongiform encephalopathie. The heterogeneous disease phenotype is mainly influenced by the methionine/valine (M/V) polymorphism at codon 129 in the human prion protein gene (PRNP gene) and by the presence of two major types of pathological, protease-resistant forms of the prion protein (PrP\textsuperscript{Sc}) leading to 2 different profiles in Western blot (type 1 and type 2).

This thesis includes 2 original publications that investigate the physiology and the pathology of the human prion protein.

In our first study, CSF proteome alterations in living, symptomatic sCJD patients with two different codon 129 genotypes (MM and VV) were analyzed using a proteomic approach consisted of 2D Fluorescence Difference Gel Electrophoresis (2D-DIGE) and mass spectrometry analysis.

CSF proteome profiling revealed up-regulation of 27 and down-regulation of 3 proteins in the MM-sCJD as well as the up-regulation of 24 proteins in the VV-sCJD when compared to control. Beside proteins showing common regulation for both codon 129 genotypes in sCJD, some proteins seem to be specifically regulated in certain genotype.

Almost 40% of sCJD specifically regulated proteins in CSF are involved in glucose metabolism, regardless of codon 129 polymorphism. The validation phase of selected glycolytic enzymes using Western blot technique confirmed up-regulation of ALDOA, LDH and G6PI when compared to three different control groups (NDC, AD and VD).

Furthermore, the immunolabeling showed that G6PI is present in reactive astrocytes in sCJD affected brain while it is predominantly localized in neurons in age-matched control brain. Additionally, decreased level of G6PI was found in the brain of MM1-sCJD subtype.

With these data, for the first time the implication of G6PI in prion-induced pathology was demonstrated.
The identification of sCJD-regulated proteins in CSF proteome alterations in living, symptomatic sCJD- patients will broaden our knowledge about pathological processes occurring in sCJD, as they are still not fully understood. Moreover, they could serve as protein source to identify novel biomarkers for differential sCJD diagnosis.

In our second study, a proteomic approach was applied in order to reveal proteins, and thereby biological processes, affected by stable overexpression of human PrP\textsuperscript{C} in human neuroblastoma SH-SY5Y cell line.

Densitometric analysis of silver stained 2D gels showed 18 differentially regulated proteins in SH-SY5Y cells overexpressing human PrP\textsuperscript{C}. Between them, 13 proteins were up- and 5 down-regulated. The PrP\textsuperscript{C} overexpression in SH-SY5Y cells affected mostly few biological processes such as signal transduction, cytoskeleton organization and protein folding.

Proteomics gives a unique opportunity to analyze both physiological and pathological processes at the protein level on a global scale. Proteome analysis of SH-SY5Y human neuroblastoma cells stably overexpressing PrP\textsuperscript{C} revealed proteins whose expression is directly modulated through PrP\textsuperscript{C} and consequently physiological processes which are influenced by PrP\textsuperscript{C} level in cells. On the other hand side, identification of CSF proteome alterations in sCJD provides more information about pathological processes occurring in the brain and caused by presence of PrP\textsuperscript{Sc}. Both these studies broaden our knowledge about still not fully understood pathobiology of PrP.
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1.1. Cellular prion protein (PrP\textsuperscript{C})

Cellular prion protein (PrP\textsuperscript{C}) is a membrane glycoprotein, which has been found in all vertebrates and it is highly conserved in mammals (Wopfner et al., 1999). In humans, PrP\textsuperscript{C} is encoded by a single-copy gene (PRNP) on chromosome 20 (Sparkes et al., 1986). It is predominantly expressed in the brain, but also found in the heart and skeletal muscle, in follicular dentritic cells, and in some lymphocytes (Bendheim et al., 1992; Ford et al., 2002; McBride et al., 1992).

PrP\textsuperscript{C} is synthesized in the rough endoplasmic reticulum (ER) and transited trough the Golgi apparatus on its way to the plasma membrane. The maturation of the primary translation product consists of the removal of 22 amino acids N-terminal signal sequence, the replacement of 23 amino acids at the C-terminus by a glycosylphosphatidyl inositol (GPI) anchor and the glycolysation of two asparagine residues (181N and 197N). The glycolysation sites are of variable occupancy and PrP exists in di-, mono- and unglycosylated forms, as shown by Western blot analysis (Figure 1). The structure of PrP\textsuperscript{C} comprises a globular domain containing three α-helices, one short anti-parallel β-sheet and a single disulfide bond. The N-terminus contains five octapeptide repeats, which have high affinity for copper (II) ions. Moreover, it is highly flexible, thus does not adopt any stable tertiary structure (Riek et al., 1996; Brown et al., 1997a; Riek et al., 1997) (Figure 1). PrP\textsuperscript{C} is attached by the GPI anchor to lipid rafts on the outer cell surface, but it is also localized inside the cells (Knopman et al., 2003). PrP\textsuperscript{C} undergoes constitutively internalization and endocytosis which is reversibly stimulated by copper (II) ions (Pauly and Harris, 1998; Prado et al., 2004).

1.2. Biological function of PrP\textsuperscript{C}

The exact biological function of PrP\textsuperscript{C} still remains obscure. However, several physiological roles have been proposed, in particular cell adhesion, signaling, regulation of cell death, neuroprotection, protection against oxidative stress and involvement in the metabolic functions related to its copper-binding properties.
The first evidence that PrP\textsuperscript{C} protects against cell death was obtained in murine hippocampal neurons exposed to serum deprivation. Under these conditions, cell death in Prnp\textsuperscript{0/0} cells was more prominent than in the control counterpart cells. Moreover, restoring of PrP\textsuperscript{C} expression in Prnp\textsuperscript{0/0} hippocampal cells followed by transfection with a Prnp gene protected these cells from serum deprivation-induced cell death in a similar manner to the well-known anti-apoptotic protein Bcl-2 (Kuwahara et al., 1999). Moreover, apoptosis induced by microinjection of Bax into human fetal neurons was prevented by co-injection of PRNP gene (Bounhar et al., 2001). In sharp contrast to the data supporting a protective role of PrP\textsuperscript{C} is observation that overexpression of PrP\textsuperscript{C} leads to a gene dose-dependent unprovoked neurodegenerative genotype of transgenic mice (Westaway et al., 1994). Furthermore, ectopic PrP\textsuperscript{C} expression potentiated staurosporine-stimulated caspase 3-dependent apoptosis in both HEK 293 cells and inducible PrP\textsuperscript{C}-transfected rabbit kidney epithelial cells (Paitel et al., 2002). On the other hand side, increased cellular content of PrP\textsuperscript{C} in breast carcinoma MCF7 cells did not affect staurosporine-induced cell death (Roucou et al., 2003). It is very likely that the role of PrP\textsuperscript{C} in cellular sensitivity to the cell death may differ among cell lines and depend on the availability of its interaction partners.

The localization of PrP\textsuperscript{C} at the plasma membrane implies its function as a receptor triggering signals. In fact, PrP\textsuperscript{C} mediates activation of the cAMP/ protein kinase A (PKA) pathway.
leading to neuroprotection in the retinal tissue (Chiarini et al., 2002; Martins et al., 1997). Furthermore, the activity of PI 3-kinase (PI3-K) was found to be higher in the brain of wild type than PrP-null mice and in neural cell lines transfected with Prnp gene when compared to parental cell lines. In Prnp-transfected cells, the activity of PI3-K as well as PrPC-induced cytoprotection against oxidative stress was revoked by either copper chelation or deletion of the octarepeat domain. This suggests a major role that PrPC copper-binding domain in PrPC-mediated/induced upregulation of PI3-K (Vassallo et al., 2005). Moreover, PrPC-mediation of other signal transduction pathways involving mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK), Fyn and Src kinases has been also reported (Chiarini et al., 2002; Schneider et al., 2003; Stuermer et al., 2004). An increasing number of studies support PrPC role in protection cells from oxidative stress. Certainly, the most compelling observation confirming this is the fact that cerebellar granular and neocortical cultured neurons from PrP-null mice are more susceptible than wild type counterpart neurons to treatment with oxidative stress inducing agents such as hydrogen peroxide, xanthine oxidase and copper ions (Brown et al., 1997b; Brown et al., 2002). In line with these cell culture results are in vivo studies showing that brain tissue from PrP-null mice exhibits biochemical changes indicating presence of oxidative stress (Wong et al., 2001). Moreover, brain lesions induced by hypoxia and ischemia are significantly larger in PrP-null mice when compared to wild type mice (Mclennan et al., 2004; Sakurai-Yamashita et al., 2005; Spudich et al., 2005). Since both these pathological conditions probably cause neuronal cell death via oxidative damage, it can tie PrPC to a neuroprotective function against oxidative stress.

1.3. Scrapie prion protein (PrPSc)

1.3.1. Conversion of PrPC into PrPSc

The precise molecular events leading to the conversion of PrPC into the scrapie agent (PrPSc) are still not well defined. It is known that this conversion involves a conformational change in which the α-helical content diminishes and β-sheet content increases (Pan et al., 1993). The most coherent and general model to date proposes that PrP fluctuates between a dominant native state, PrPC, and a series of minor conformations, one or a set of which can self-associate in an ordered manner to produce a stable structure, PrPSc, composed of misfolded PrP.
monomers. Once a stable “seed” structure is formed, PrP is then recruited leading to explosive autocatalytic PrP\textsuperscript{Sc} formation (Collinge et al., 2001) (Figure 2). This model would be extremely sensitive to three factors: 1) the total PrP concentration, 2) the equilibrium of the distribution between the native and self-associating conformation, and 3) the complementarity between conformers in aggregation step. These three theoretical predictions are manifest in the etiology of prion diseases. First, an inversely proportional relationship between PrP\textsuperscript{C} expression and disease incubation period in transgenic mice was described in several studies (Prusiner et al., 1990; Büeler et al., 1993; Collinge et al., 1995; Telling et al., 1995). Second, the predisposition of PrP to adopt a misfolded form induced by rather subtle mutations in the protein sequence was found (Collinge et al., 1997). Finally, molecular homogeneity is required for efficient PrP\textsuperscript{Sc} propagation (Prusiner et al., 1990; Palmer et al., 1991).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Schematic representation of PrP\textsuperscript{C} conversion into PrP\textsuperscript{Sc}}
\end{figure}

\subsection*{1.3.2. Properties of PrP\textsuperscript{Sc}}

PrP\textsuperscript{Sc} is a β-sheet- rich protease resistant aggregating and infectious form of PrP\textsuperscript{C}. Its tertiary conformation is still unresolved, mainly because of its tendency to form large heterogeneous aggregates which are recalcitrant to analysis by high-resolution techniques. The greatest infectivity of PrP\textsuperscript{Sc} is associated with 300-600 kDa particles consisting of 14-28 PrP molecules. Much less specific infectivity was detected for monomeric PrP or large fibrils (Silveira et al., 2005). Many evidences suggest that the infectious form(s) of PrP may not be the proximate cause of neuronal dysfunction and degeneration in prion diseases. It has been shown that mutant forms of PrP (nine octapeptide insertion or GGS mutations) associated with familial prion disease are pathogenic, but not infectious (Chiesa et al., 2003; Nazor et al., 2005).
2005). Furthermore, heterozygous transgenic mice expressing anchorless PrP inoculated with scrapie prions failed to develop typical clinical signs of scrapie infection. However, they were shown to replicate prion infectivity, albeit on lower level than wild type mice. Moreover, histopathological examination of brain tissue revealed a striking accumulation of extracellular protease-resistant PrP deposits with the characteristics of amyloid (Chesebro et al., 2005). Then again, homozygous transgenic mice, which express 2-fold more anchorless PrP than heterozygous transgenic mice, developed a fatal clinical disease upon scrapie infection. However, this disease differed markedly in incubation time, clinical signs and neuropathology from typical scrapie disease observed in wild type mice (Chesebro et al., 2010).

Cellular mechanism which underlies prion neurotoxicity still remains obscure. Different hypotheses are postulated: 1) loss, 2) subversion or 3) gain of PrP function. If the main function of PrP<sup>C</sup> is neuroprotection then loss of it upon conversion into or with PrP<sup>Sc</sup> would lead to prion-induced neurodegeneration. Incompatible with a loss-of-function mechanism of PrP toxicity is the observation that genetic ablation of PrP<sup>C</sup> expression has relatively little phenotypic effect and does not contribute to the development of any prion diseases features (Büeler et al., 1992; Manson et al., 1994; Mallucci et al., 2002). On the other hand, a dispensable biological activity of PrP<sup>C</sup> under physiological conditions may become essential in pathological state due to cellular or organismal stress. The subversion-of-function hypothesis presumes that interaction with PrP<sup>Sc</sup> converts PrP<sup>C</sup> from a neuroprotective signal transducer into a neurotoxic signal transducer. Consistent with this hypothesis is the observation that cross-linking of PrP<sup>C</sup> with anti-PrP antibodies induces apoptotic processes in neurons in vivo (Solforosi et al., 2004). Alternatively, PrP<sup>Sc</sup> may bind to and block specific regions of PrP<sup>C</sup>, thereby altering the neuroprotective signaling properties. The neurodegenerative phenotype of transgenic mice expressing PrPΔ32-121/134 suggests that specific domains of PrP are essential for its protective role and that deletion of these domains unmasks a neurotoxic activity, perhaps by altering interaction with critical signaling molecules (Behrens et al., 2002). These two above mentioned hypothesis stand in contrast to a toxic gain-of-function mechanism, which is usually evoked to explain dominantly inherited neurodegenerative disorders including Alzheimer’s, Huntington’s, and Parkinson’s disease. In these cases, the protein aggregates accumulating in the brain are presumed to possess a novel neurotoxic activity that is independent of the normal physiological function of the parent
protein. Similarly, PrP\textsuperscript{Sc} aggregates may have a neurotoxic effect by blocking axonal transport or interfering with synaptic transmission. Moreover, PrP\textsuperscript{Sc} may disturb function of lysosomes and proteasome, where it accumulates, and/or increased ER stress.

1.4. Prion diseases

Prion diseases or transmissible spongiform encephalopathies (TSEs) are unique fatal neurodegenerative disorders of diverse phenotypes and forms affecting both humans and animals. The prototypic prion disease is scrapie, a naturally occurring disease affecting sheep and goats. More recently defined animal prion diseases include transmissible mink encephalopathy, chronic wasting diseases and bovine spongiform encephalopathy. Human prion diseases have been classified in three forms sporadic (sporadic Creutzfeldt-Jakob disease), familial (genetic Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia and genetic TSEs) and acquired by infection (iatrogenic CJD, variant CJD and Kuru).

1.4.1. Animal prion diseases

Scrapie occurring in sheep and goats was the first prion disease being recognized in Europe for over 200 years ago. However, only little is known about its natural routes of transmission. Scrapie is also the first TSE experimentally transmitted to primates, rodents, and other species (Wharton et al., 2005).

To date chronic wasting disease (CWD) and transmissible mink encephalopathy of wild as well as captive deer and mink appear only in North America. Some evidences suggest that these prion diseases can spread through contamination of feed and water sources with urine, saliva and faeces. Moreover, soil and soil minerals can serve as a reservoir of TSE infectivity (Petersen et al., 2006). This brings a potential risk to cattle and also to humans although no evidence of natural transmission of CWD to non-cervids has been reported so far (Belay et al., 2004).

The first case of bovine spongiform encephalopathy (BSE) was recognized in 1986 in UK and spread next decade as a massive epidemic infecting about 1 million cows (Anderson et al., 1996). Export of cattle and feed scattered BSE to countries around the world. The source of disease outbreak was evidently the contamination of meat-and-bone meal supplements with
scrapie-infected sheep carcasses. Thanks to the prohibition of feeding cattle with ruminant carcasses, the incidence of BSE diminished.

1.4.2. Human prion diseases

Sporadic Creutzfeldt-Jakob disease (CJD) is the most common form of human TSEs and accounts for about 85% of all cases. The annual incidence rate is 1-2 cases per million people worldwide. Unfortunately, the etiology of sporadic CJD remains unclear. The hypothesis favours either a spontaneous change of PrP structure or a somatic mutation in the PRNP gene leading to an abnormal form of the protein. Familial prion diseases are associated with autosomal dominant inheritance of mutations in the PRNP gene (Knight et al., 2006). Over 30 different mutations in the PRNP gene have been already reported, but only four point mutations, at codon 102, 178, 200 and 210, as well as insertions of five or six octapeptide repeats account for 95% of all familial cases (Mead et al., 2006) (Figure 3).

![Figure 3. The most common polymorphism and mutation sides in human PRNP gene](image)

In general, familial prion diseases are characterized by earlier age of onset and longer clinical course when compared to sporadic CJD. Patients affected by Gerstmann-Sträussler-Scheinker (GSS) syndrome, caused by the point mutation either at codon 102 (P102L) or at codon 105 (P105L), developed progressive cerebellar ataxia and spastic paraparesis between 20-40th year of life. Moreover, the presence of PrP-amyloid plaques in the brain is unique for this syndrome (Bugiani et al., 2000). Both, genetic CJD forms linked to E200K-129M and V210I-
129M mutations show similar clinical and pathological features like sporadic CJD (Gambetti et al., 2003). The disease phenotype associated with the mutation at codon 178 is determined by the polymorphism at codon 129; D178N-129M causes fatal familial insomnia (FFI), while D178N-129V causes typical genetic CJD (Goldfarb et al., 1992). The clinical course of FFI is dominated by progressive insomnia, autonomic dysfunction and dementia.

To the acquired forms of human prion diseases belong iatrogenic CJD (iCJD), variant CJD (vCJD) and Kuru. Transmission of iCJD from one person to another has occurred via medical or surgical treatment such as corneal transplants, dural grafts, growth hormone extracted from human pituitary glands and contaminated neurosurgical instruments (Gibbs et al., 1994; Lang et al., 1998; Will, 2003). Moreover, the increased susceptibility to iCJD has been found for methionine or valine homozygosity at codon 129 in the PRNP gene (Brown et al., 2000). vCJD is considered to be a result of BSE infectious agent entering human food chain. Biochemical analysis and transmission studies confirmed that vCJD and BSE are caused by the same scrapie prion strain (Collinge et al., 1996; Hill et al., 1997). The risk period in the UK fell approximately on 1980-1996, when the most of cases appeared (Knight et al., 1999). The young age at onset, the prominence of psychiatric and sensory symptoms and the long disease duration distinguish vCJD from sporadic form. Furthermore, all vCJD cases tested to date have been homozygous for methionine at codon 129 (Will et al., 2000). Kuru had been transmitted through a ritual cannibalism. However, after abolition of a bereavement ceremony, in which highly infectious organs from dead relatives were primarily eaten by women and children, the spread of this disease has been greatly limited (Huillard d’Aignaux et al., 2002).

1.4.2.1. Sporadic Creutzfeldt-Jakob disease

Sporadic Creutzfeldt-Jakob disease (sCJD) is characterized by wide clinical and pathological variability. The disease phenotype is mainly influenced by the methionine/valine (M/V) polymorphism at codon 129 in the PRNP gene and by the presence of two major types of protease-resistant form of the PrP (type 1 and type 2). These two PrPSc types are distinguishable based on the molecular weight of unglycosylated form of PrPSc after proteinase K (PK) digestion. In PrPSc type 1, the unglycosylated form migrates at 21 kDa, while in PrPSc type 2 it migrates at 19 kDa (Parchi et al., 1996). Different PK cleavage occurs probably due to existence of two major conformational states of PrPSc.
The major subtypes of sCJD are homozygous for methionine at codon 129 in \textit{PRNP} gene with PrP$^{Sc}$ type 1 (MM1-sCJD), homozygous for valine at codon 129 in \textit{PRNP} gene with PrP$^{Sc}$ type 2 (VV2-sCJD) and heterozygous with PrP$^{Sc}$ type 2 (MV2-sCJD), representing about 67%, 15% and 9% of all sCJD cases, respectively (Figure 4).

The most important differential diagnoses of sCJD include Alzheimer’s disease (AD), vascular dementia (VD), dementia with Lewy body, brain tumors and cerebellar degeneration (WHO, 1998).

![Figure 4. PrP$^{Sc}$ types in sCJD [adapted from (Gambetti et al., 2003)]](image)

The MM1-sCJD subtype was previously described as myoclonic or “classic” CJD. The typical clinical features of this subtype are short disease duration of 4 months with cognitive impairment, mental and visual signs. For MV1 subtype, which is usually grouped together with MM1-sCJD, ataxia rather than cognitive decline is present at onset. Within the first 3 months of disease course in about 80% of the MM1 and MV1-sCJD cases, periodic sharp wave complexes (PSWC) are detected in EEG (Parchi et al., 1999). MRI examination revealed the presence of basal ganglia hyperintensities in about 70% of MM1-sCJD cases (Meissner et al., 2009). Furthermore, the diagnostic test based on the presence of 14-3-3 protein in the cerebrospinal fluid (CSF) is positive in about 95% cases (Castellani et al., 2004; Sanchez-Juan et al., 2006). Similarly to other CJD forms, the histopathological features of these subtypes are spongiform degeneration, astrogliosis and neuronal loss. The spongiform degeneration is made of fine vacuoles and is fairly homogeneously distributed within the affected regions. The topography of the lesions shows that the cerebral neocortex, especially in the occipital lobe, is more severely affected than basal ganglia, thalamus and cerebellum while the brain stem is spared. PrP$^{Sc}$ immunodetection shows a characteristic punctate pattern of staining (synaptic) with a degree of intensity that is overall directly related to the severity of the affected brain regions. The immunostaining is often homogenous, but relatively large regions may remain either unstained or with variable staining intensities (Gambetti et al., 2003).
The VV2-sCJD subtype represents the cerebellar or ataxic variant, because ataxia is the most prominent presenting sign. With disease progression, dementia, myoclonus and pyramidal signs are developed. In contrast, cortical signs such as aphasia or apraxia are very rarely observed in this subtype. In the great majority of VV2-sCJD cases, EEG shows non-specific slowing while in less than 10% of cases PSWC are present (Gambetti et al., 2003). The basal ganglia hyperintensities occur in 77% of VV2-sCJD cases (Meissner et al., 2009). Moreover, the sensitivity of 14-3-3 is about 85% (Castellani et al., 2004; Sanchez-Juan et al., 2006). The lesion triad composed of fine spongiform degeneration, astrogliosis and neuronal loss is also present in VV2-sCJD subtype. Similarly to MM1-sCJD, the spongiform changes are made of fine vacuoles, but deeper cortical layers are preferentially affected. The topography of the lesions shows that the cerebral neocortex is usually more severely affected in the frontal than in the occipital lobe. The severity of the lesions depends on the disease duration so that the cerebral cortex is often spared in cases with rapid course. Overall, the cerebral neocortex is less affected than basal ganglia and thalamus, moreover, lesions are present in the brain stem, the dorsal regions and the substantia nigra. PrP$^{Sc}$ immunohistochemistry displays the presence of focal aggregates looking like plaques. Moreover, the intense immunostaining is found along cell processes and is especially prominent in basal ganglia and thalamus. The hallmark of VV2-sCJD subtype is the immunostaining pattern of the cerebellum showing intense immunostaining of the Purkinje cells and the upper granule cell layers (Gambetti et al., 2003). The MV2-sCJD is phenotypically and histopathologically similar to the VV2-sCJD subtype, but easily distinguishable due to significantly longer disease duration with a mean duration of 17 months and the presence of Kuru plaques in the cerebellum. However, cognitive, mental or pyramidal signs and myoclonus are often present in MV2-sCJD while they are absent in VV2-sCJD subtype. The sensitivity of 14-3-3 test is about 60%, thus significantly lower than for other sCJD subtypes (Castellani et al., 2004; Sanchez-Juan et al., 2006). The hyperintensity of basal ganglia has been found in 77% of cases (Meissner et al., 2009). In contrast to VV2-sCJD, MV2-sCJD subtype is characterized by the lack of any significant cerebellar cortical atrophy.

The presentation of MM2-sCJD subtype is dominated by cognitive decline observed in all cases. The disease duration for this subtype is 3-4 times longer when compared to disease duration of MM1-sCJD subtype. The EEG shows non-specific slowing and 14-3-3 test is
positive in about 75% of MM2-sCJD cases (Gambetti et al., 2003; Castellani et al., 2004; Sanchez-Juan et al., 2006). The typical feature of this subtypes is the spongiform degeneration with large vacuoles which was previously identified as status spongiosis and more recently as coarse spongiosis (Parchi et al., 1996). The vacuoles are several times larger than vacuoles found in MM1-sCJD and widespread in the cerebral cortex, basal ganglia and thalamus. They are often confluent resulting in the formation of tissue islands surrounded by vacuoles. The PrP<sub>Sc</sub> immunostaining shows two basic patterns: an intense staining of the large vacuoles rim and a spotted staining with loose plaque formations (Budka et al., 1995).

The VV1-sCJD subtype is the most uncommon one with characteristic features of early onset and dementia of frontotemporal type (Parchi et al., 1999). The EEG shows slowing, but not PSWC and 14-3-3 test are positive in all cases (Gambetti et al., 2003; Castellani et al., 2004; Sanchez-Juan et al., 2006). The basal ganglia hyperintensity was not detected in VV1-CJD subtype (Meissner et al., 2009). The hallmark of this subtype is dissociation between the histopathological lesions (severe fine spongiform degeneration, gliosis and occasionally neuronal loss) and synaptic pattern of PrP<sub>Sc</sub> staining. The hippocampal cortex is more affected while thalamus and cerebellum are less affected when compared to MM1-sCJD (Gambetti et al., 2003).

1.5. CSF proteomics and biomarker discovery in CJD

Proteomic approach has been already applied for the investigation of CSF proteome changes in CJD and the searching for novel biomarkers. Interestingly, First reported biomarkers in CJD were proteins 130 and 131, later identified as proteins belonging to 14-3-3 family (Harrington et al., 1986; Hsich et al., 1996).

Analyzing native CSF from MM1-sCJD, Piubelli and colleagues (Piubelli et al., 2006) detected 7 up-regulated and 6 down-regulated proteins. Besides sCJD-associated dysregulation in CSF, levels of ubiquitin, gelsolin and α-1-antichymotrypsin were also altered in CSF or/and blood of AD patients (Iqbal et al., 1997; Demeester et al., 2000; DeKosky et al., 2003; Puchades et al., 2003). This might suggest their role in general neurodegenerative processes caused by the presence of misfolded proteins in the brain. Similarly to the studies of Sanchez and colleagues (Sanchez et al., 2004) very high up-regulation of cystatin C, a inhibitor of cysteine proteinases, was found in CSF, thus this protein was proposed as a novel CJD
biomarker. Interestingly, gene encoding cystatin C was found be up-regulated in global expression profiling of sCJD affected brain (Xiang et al., 2005).

In other studies, comparison of CSF patterns between CJD, AD and controls revealed 5 protein spots present only in CJD. However, no further characterisation of these proteins was performed. Finally, Brechlin and colleagues (Brechlin et al., 2008) applied DIGE technology to investigate CSF in CJD, using fluorescent labelling, depleting albumin and IgG as well as including other dementia as controls significantly increased specificity of obtained results. Unfortunately, neither specific nor promising candidate for biomarker was detected in these studies.

Furthermore, native CSF from sCJD and vCJD to CSF from other dementia was also already compared and 7 proteins with different abundance were found in two CJD forms. Between them, apolipoprotein E showed significantly higher level in vCJD comparing to sCJD (Choe et al., 2002). Apolipoprotein A1, apolipoprotein E and prostaglandin-H2-D-isomerase showed different levels in both schizophrenia and CJD (Choe et al., 2002; Piubelli et al., 2006; Martins-De-Souza et al., 2010). These proteins are involved in cholesterol and phosholipid metabolism, thus the maintenance of cell membrane could be potentially altered in mental brain disorder as well as in fatal neurodegenerative brain disorder. In fact, one hypothesis postulates that schizophrenia is associated with disordered membrane lipid metabolism (Horrobin et al., 1994; Mahadik et al., 1994). Additionally, alterations in cholesterol metabolism in scrapie mice infected with ME7 strain have been also reported (Xiang et al., 2007).

Taken all together, emerging proteomics field give us tools to explore CSF proteome and reveal protein that may be involved in disease pathogenesis as well as give us a unique possibility to discover novel biomarkers of neurodegenerative disorders including human prion diseases.
2. FOCUS OF THE PRESENT WORK

2.1. Codon 129 polymorphism specific CSF proteome pattern in sporadic Creutzfeldt-Jakob disease and the implication of glycolytic enzymes in prion-induced pathology

2.1.1. Aims of the project

In this project, we determined CSF proteome alterations in living, symptomatic sCJD patients with two different codon 129 genotypes (MM and VV). This was acquired by applying a proteomic approach consisted of 2D Fluorescence Difference Gel Electrophoresis (2D-DIGE) and mass spectrometry.

At the molecular level, TSEs are caused by the conversion of a host cellular glycoprotein, the prion protein (PrP\textsuperscript{C}) into a pathological conformer called scrapie prion protein (PrP\textsuperscript{Sc}), which tends to aggregate and accumulate in the brain tissue. The presence of pathological form of PrP induces brain proteome changes, which might be different in particular disease subtypes. We hypothesize that these alterations may also be reflected in CSF from symptomatic sCJD patients.

A deep insight in prion-induced CSF proteome alterations in living symptomatic sCJD-patients will broaden our knowledge about pathological processes occurring in sCJD, as they are still not fully understood. Moreover, they could serve as protein source to identify novel biomarkers for a differential sCJD diagnosis.
2.2.2. Original publication

The results described below are published in:


Author contribution:
This study was designed, performed and interpreted by Joanna Gawinecka. Jana Dieks was involved in preparatory phase and sample collection. Julie Carimalo gave valuable advices for immunohistochemical analysis. Abdul R. Asif performed mass spectrometry analysis. Uta Heinemann helped with the patient selection. Jan-Hendrik Streich optimized conditions of the CSF fractionation. Hassan Dihazi provided fluorescent scanner used to obtain images of 2D gel. Walter Schulz-Schaeffer provided brain samples. The publication was written by Joanna Gawinecka and Inga Zerr.
Codon 129 polymorphism specific CSF proteome pattern in sporadic Creutzfeldt-Jakob disease and the implication of glycolytic enzymes in prion-induced pathology

Joanna Gawinecka, Jana Dieks, Abdul R. Asif, Julie Carimalo, Uta Heinemann, Jan-Hendrik Streich, Hassan Dihazi, Walter J. Schulz-Schaeffer, and Inga Zerr

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Abstract

Cerebrospinal fluid (CSF) contains a dynamic and complex mixture of proteins, which can reflect a physiological and pathological state of the central nervous system. In our present study we show CSF protein patterns from patients with the two most frequent subtypes of sporadic Creutzfeldt-Jakob disease (sCJD) defined by the codon 129 genotype (MM, MV and VV) and the protease-resistant form of prion protein (type1 and type 2).

The densitometric analysis of 2D gels showed up-regulation of 27 and down-regulation of 3 proteins in the MM-sCJD as well as the up-regulation of 24 proteins in the VV-sCJD as compared to non-demented control. Almost 40% of sCJD specific regulated proteins in CSF are involved in glucose metabolism, regardless of the codon 129 polymorphism. The increase in CSF levels of lactate dehydrogenase (LDH), glucose-6-phosphate isomerase (G6PI) and fructose-bisphosphate aldolase A (ALDOA) were validated on a larger group of sCJD patients including three possible codon 129 polymorphisms carriers and three control groups consisting of non-demented, neurological cases as well as patients suffering from Alzheimer’s disease or vascular dementia. Subsequently, the abundance of these glycolytic enzymes in the brain as well as their cellular localization were determined.

This study demonstrates for the first time the implication of G6PI in prion-induced pathology as well as its cellular translocalization in sCJD. The identification of sCJD-regulated proteins in CSF of living symptomatic patients in our study can broaden our knowledge about pathological processes occurring in sCJD, as they are still not fully understood.

Keywords:
CJD, CSF, proteome, 2D DIGE, Creutzfeldt-Jakob disease, cerebrospinal fluid, 2-D Fluorescence Difference Gel Electrophoresis
Sporadic Creutzfeldt-Jakob disease (sCJD), the most common form of human transmissible spongiform encephalopathies (TSEs), is characterized by wide clinical and pathological variability. Disease phenotype is mainly influenced by the methionine/valine (M/V) polymorphism at codon 129 in the human prion protein gene (PRNP gene) and by the presence of two major types of pathological, protease-resistant form of prion protein (PrPSc) leading to 2 different profiles in Western blot (type 1 and type 2)\(^1\). The major subtypes of sCJD are homozygous for methionine at codon 129 in PRNP gene with PrPSc type 1 (MM1-sCJD), homozygous for valine at codon 129 in PRNP gene with PrPSc type 2 (VV2-sCJD) and heterozygous for PrPSc type 2 (MV2-sCJD), representing about 67\%, 15\% and 9\% of all sCJD cases, respectively. The clinical and pathological characteristics of these 6 molecular disease subtypes differ markedly with respect to symptoms at onset, localisation and type of the pathological changes as well as PrPSc deposition pattern\(^1\), \(^2\). This might suggest the involvement of different molecular pathways in sCJD pathogenesis.

At the molecular level, TSEs are caused by the conversion of a host cellular glycoprotein, the prion protein (PrP\(^{C}\)) into an abnormal conformer called scrapie prion protein (PrP\(^{Sc}\)). PrP\(^{Sc}\) is characterized by increased β-sheet content, detergent insolubility, partial resistance to protease digestion and tendency to aggregate in the brain tissue. PrP\(^{Sc}\) accumulation and aggregation may induce proteome changes in the central nervous system. Furthermore, these prion-induced alterations of the brain proteome may also be reflected in the CSF of sCJD patients. However, due to high dynamic range of protein abundance in CSF spanning twelve orders of magnitude\(^3\) and the fact that around 10 very well known proteins (e.g. albumin, immunoglobulins, transferrin and haptoglobin) comprise up to 90-95\% of the total protein content, depletion of the high abundant proteins is indispensible for in-depth exploration of sCJD-associated alterations in the CSF proteome. The brain proteome alterations found in CSF can serve as a source of potential CJD biomarkers. The first identified diagnostic biomarker by applying proteomic approach was the 14-3-3 protein, which was introduced for CJD diagnosis\(^4\)–\(^7\) later on.

In this study, we show CSF proteome patterns specific for MM and VV codon 129 genotype in sCJD acquired by the application of 2D Fluorescence Difference Gel Electrophoresis (2D-
DIGE) approach. Moreover, the depletion of 12 high abundant proteins allowed a detailed view on proteome alterations induced by PrP$^{Sc}$ in CSF in living, symptomatic patients. The identification of sCJD-regulated proteins broadens our knowledge about pathological processes occurring in sCJD which are still not fully understood.

**Patients, Materials and Methods**

1. Patients

For 2D Fluorescence Difference Gel Electrophoresis (2D-DIGE) analysis 8 CSF samples from sCJD cases and 8 non-demented individuals were used. The sCJD group consisted of 4 patients homozygous for the methionine (MM) at codon 129 and 4 patients homozygous for the valine (VV) with a mean age of 69±3 years and 68±4 years, respectively. According to the WHO criteria, 5 patients were classified as having “probable” sCJD and 3 others as having “definite” sCJD (two MM1 and one VV2 type in Parchi’s classification)\(^1\). The CSF samples were taken for diagnostic purposes. However, due to very rapid progress of sCJD and unspecific symptoms at the onset, at the time of lumbar puncture the disease was progressed to either the middle or late stage. The control group consisted of 8 individuals with a mean age of 69±4 years and no signs of cognitive decline, which underwent lumbar puncture for various differential diagnostic purposes (Tab. 1). For both groups, CSF parameters did not show any evidence of blood-CSF barrier dysfunction, acute or chronic inflammation or intrathecal immuno response (Tab. 2). Furthermore, none of the sCJD and control patients was a heavy smoker, drug- or alcohol-abused.

To confirm up-regulation of selected proteins by Western blot, CSF samples from 8 non-demented controls (NDC), 8 patients suffering from vascular dementia (VD), 10 Alzheimer’s disease patients (AD) and 30 probable or definite sCJD patients (11 homozygous for MM, 9 homozygous for VV and 10 heterozygous at codon 129) were analyzed. The diagnosis of VD and AD was determined according to diagnostic criteria of the National Institute of Neurological Disorders and Stroke and the Association Internationale pour la Recherché et l'Enseignement en Neurosciences (NINDS-AIREN), and the National Institute of Neurological and Communicative Disorders and the Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), respectively.
Table 1. Detailed characteristics of sCJD and control patients in 2D-DIGE approach

All sCJD patients fulfilled WHO criteria for diagnosis of either probable or definite sCJD, neurological controls did not show any cognitive decline and were age-matched with the sCJD patients. The mean age of MM-sCJD, VV-sCJD and NDC group was 69±3, 68±4 and 69±4 years, respectively. The sCJD patients were characterized by the presence of 14-3-3 protein and elevated level of tau protein in CSF. ([\^] - disease stage when lumbar puncture for diagnostic purposes was performed; [\textsuperscript{\textdagger}] - presence of hyperintensities in basal ganglia; [*] - presence of periodic sharp wave complexes (PSWC); [#] - presence of 14-3-3 protein in CSF; [\$] - tau level above 1300 pg/ml; F – female; M – male; n.d. – not determined]

<table>
<thead>
<tr>
<th>Nr</th>
<th>Case</th>
<th>Age [years]</th>
<th>Sex</th>
<th>Disease duration [month]</th>
<th>Time point of lumbar puncture[^]</th>
<th>MRI[\textsuperscript{\textdagger}]</th>
<th>EEG[*]</th>
<th>14-3-3[^]</th>
<th>Tau[$] [pg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>definite MM1-sCJD</td>
<td>67</td>
<td>F</td>
<td>4.5</td>
<td>middle stage</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2282</td>
</tr>
<tr>
<td>2</td>
<td>definite MM1-sCJD</td>
<td>72</td>
<td>M</td>
<td>3.5</td>
<td>late stage</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>24000</td>
</tr>
<tr>
<td>3</td>
<td>probable MM-sCJD</td>
<td>65</td>
<td>M</td>
<td>3.5</td>
<td>middle stage</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>14550</td>
</tr>
<tr>
<td>4</td>
<td>probable MM-sCJD</td>
<td>70</td>
<td>M</td>
<td>8.5</td>
<td>late stage</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>8351</td>
</tr>
<tr>
<td>5</td>
<td>definite VV2-sCJD</td>
<td>66</td>
<td>F</td>
<td>3.5</td>
<td>middle stage</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>18014</td>
</tr>
<tr>
<td>6</td>
<td>probable VV-sCJD</td>
<td>67</td>
<td>F</td>
<td>7</td>
<td>middle stage</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>24020</td>
</tr>
<tr>
<td>7</td>
<td>probable VV-sCJD</td>
<td>64</td>
<td>M</td>
<td>11</td>
<td>late stage</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>20000</td>
</tr>
<tr>
<td>8</td>
<td>probable VV-sCJD</td>
<td>75</td>
<td>M</td>
<td>7</td>
<td>late stage</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>1997</td>
</tr>
</tbody>
</table>
To perform Western blot analyses, samples of frontal cortex from 6 pathologically confirmed sCJD cases as well as 6 non-neurological controls with only age-related changes in brain tissue were used. The post-mortem delay was around 24h for analyzed cases. For co-localization studies, samples of frontal cortex from 3 pathologically confirmed sCJD cases, 3 AD as well as 3 age-matched, non-neurological controls with only age-related changes in brain tissue were used.

<table>
<thead>
<tr>
<th>Cases</th>
<th>protein concentration [mg/l]</th>
<th>lactate [mmol/L]</th>
<th>QAlb x 10^3</th>
<th>cells/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM-sCJD</td>
<td>448±125</td>
<td>1.8±0.4</td>
<td>7.6±2</td>
<td>4±2</td>
</tr>
<tr>
<td>VV-sCJD</td>
<td>275±59</td>
<td>1.6±0.1</td>
<td>4.2±0.2</td>
<td>1±1</td>
</tr>
<tr>
<td>Controls</td>
<td>351±110</td>
<td>1.4±0.2</td>
<td>4.8±1.6</td>
<td>1±1</td>
</tr>
</tbody>
</table>

Table 2. CSF parameters of sCJD and control patients in 2D-DIGE approach
For both, sCJD and control group CSF parameters were within normal, physiological range.

2. 2D Fluorescence Difference Gel Electrophoresis (2D-DIGE)

2.1. Sample Preparation

Immediately after lumbar puncture, CSF samples were centrifuged at 1300xg for 10 min, snap frozen and stored at -80°C until analysis was performed. 2 ml of CSF were subjected to the depletion of 12 high abundant proteins (Albumin, IgG Total, IgM, IgA, Transferrin, Apo A-I, Apo A-II, α1-Acid Glycoprotein, α2-Microglobulin, α1-Antitrypsin, Haptoglobin and Fibrinogen) using ProteomeLab IgY-12 LC2 Proteome Partitioning Kit (Beckman Coulter). The procedure was performed according to manufacturer’s instruction.
2.2. 2D-DIGE and Image Analysis

25 μg of protein was precipitated overnight with acetone-methanol (8:1; vol:vol) at -20°C and centrifuged at 16 000xg for 15 min. The pellet was resuspended in lysis buffer containing 7 M Urea, 2.5 M Thiourea, 4% CHAPS, 30 mM TRIS and 5 mM magnesium acetate and subsequently labeled with 100 pmol of CyDye (GE Healthcare) as follows: pooled samples as internal standard with Cy2 as well as individual control and sCJD samples with Cy3 or Cy5. The dye-switch between control and sCJD samples was done in order to avoid dye-to-protein preferences.

Labeling reaction was performed on ice in the dark for 30 min and terminated by adding 10 mM Lysine before incubating for further 10 min. Equal volume of lysis buffer containing additionally 130 mM DTT and 0.4% 3-10 Bio-Lyte (Bio-rad) was added to the labeling mixture. After that samples were mixed together, diluted up to 350 μl with rehydration buffer composed of 7 M Urea, 2.5 M Thiourea, 4% CHAPS, 0.2% 3-10 Bio-Lyte and 65 mM DTT and loaded on ReadyStrip IPG nonlinear pH 3-10, 17 cm strip (Bio-rad). After 12 h of active rehydration at 50 V, isoelectric focusing was initiated at 500 V for 1 h, followed by ramping at 1000 V for 1 h and 5000 V for 2 h. The final focusing was carried out at 8000 V reaching the total of 60 000 Vh (PROTEAN IEF CELL, Bio-rad).

Then the strips were equilibrated 2 times for 20 min in buffer containing 6 M Urea, 2% SDS, 30% Glycerin and 150 mM Tris, pH 8.8, supplemented with 2% DTT in the first and with 2.5% Iodoacetamide in the second equilibration step. SDS-PAGE was performed overnight with homogenous 12% polyacrylamide gel using PROTEAN II XL Vertical Electrophoresis Cell (Bio-rad). CyDye-labeled protein gels were scanned by three different lasers with band pass filtered emission wavelengths of 510 nm (Cy2), 575 nm (Cy3) and 665 nm (Cy5) using FLA-5100 imaging system (Fujifilm).

Protein spot abundances within 20 CSF proteome patterns (4 MM-sCJD, 4 VV-sCJD, 8 NDC and 4 IS) were analyzed using the Delta2D software (v. 3.6) (DECODON). Differences in spot abundance detected by densitometric analysis were statistically evaluated using unpaired Student’s t test. Means and standard deviations were calculated from 4 sets of experiments. A protein spot was considered as differentially regulated when its densitometric analyses showed at least 2-fold change in abundance and when p-value was < 0.05 in unpaired Student’s t test.
2.3. Protein identification

Gel plugs containing proteins of interest were manually excised from silver- or Coomassie-stained gels and subjected to in-gel digestion. The detailed protocol of this procedure is given by Ramljak et al.\textsuperscript{8}. In-gel digested peptides were chromatographically separated peptides (C18 pepMap100 nano Series analytical column, LC Packings) and analyzed by ESI-Q-TOF Ultima Global mass spectrometer (Micromass). Data acquisition was performed using the MassLynx (v. 4.0) software and further processed on Protein- Lynx-Global-Server (v 2.1), (Micromass). The acquired data were searched against MSDB and SwissProt 2010_08 databases through the Mascot search engine using a peptide mass and MS/MS fragment mass tolerance of 0.5 Da. The searching criteria were set with one missed cleavage by trypsin allowed and protein modifications set to methionine oxidation and carbamidomethylcysteine when appropriate.

3. Western blotting

3.1. Brain homogenate

Samples of frontal cortex were homogenized in 5 volumes of buffer containing 20 mM HEPES (pH 7.4), 320 mM sucrose, 1 mM Sodium Orthovanadate, 1 mM EDTA and Complete Protease Inhibitor Cocktail (Roche). Brain homogenates were centrifuged at 15000 rpm for 10 min. Supernatants were collected and protein concentration was determined by BCA assay (Sigma).

3.2. Western blotting

30 μg of CSF protein or 20 μg of brain protein was separated on 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk in phosphate buffer saline with 0.2% Triton X-100 (PBST) for 1 h at RT. Subsequently, the membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-G6PI (1:2000, Abcam), mouse anti-ALDOA (1:1000, Abcam), mouse anti-β-actin (1:2000, Abcam) or goat anti-LDH (1:500, Abcam). Thereafter, membranes were washed with PBST and incubated for 1 h at RT with corresponding horseradish peroxide-conjugated secondary antibodies: donkey anti-goat (1:5000, Santa Cruz Biotechnology), goat anti-mouse
(1:7500, Abcam). The immunoreactivity was detected after immersing the membranes into enhanced chemiluminescence (ECL) solution and expositing to ECL-Hyperfilm (Amersham Biosciences). Films were scanned, densitometric and statistic analysis was performed with both ImageJ (Image Processing and Data Analysis free software) and Sigmaplot (Exact Graphs and Data Analysis software, Systat), respectively. The protein-regulation was considered as specific for sCJD when $p$-value was lower than 0.05 in Kruskal-Wallis ANOVA test.

4. Immunofluorescent staining

Formalin-fixed and paraffin-embedded brain tissue sections were deparaffinized and rehydrated by washing three times for 3 min in xylol and for 2 min in 100%, 95%, 70% and 50% isopropanol. Heat-induced antigen retrieval was performed by cooking in a microwave for 18 min in Tris/EDTA buffer at pH 9. Then brain sections were blocked with buffer containing 2% gelatin, 0.25% Triton-X and 1 mM Lysine for 1 h and lipofuscin-like autofluorescence was reduced with 0.5% Sudan black for 15 min. Subsequently, brain sections were incubated for 2 h with the following primary antibodies: goat anti-LDH (1:500, Abcam), mouse anti-G6PI (1:200, Abcam), mouse anti-ALDOA (1:150, Abcam), rabbit anti-GFAP (1:1000, DAKO), mouse anti-Neurofilament (1:100, DAKO) or rabbit anti-Neurofilament Light (1:200, Millipore). Brain sections were incubated for 1.5 h with the corresponding secondary antibodies: Cy3-labeled sheep anti-mouse (1:1000, Sigma), FITC-labeled goat anti-rabbit (1:500, Sigma) or Cy3-labeled donkey anti-goat (1:500, J. Research). Nuclei labeling was performed with 2 μg/ml Hoechst 33342 solution for 10 min. Finally, brain sections were mounted with Mowiol and stored at 4°C in a dark box. All steps were carried out in a humid dark chamber at RT and each incubation step was followed by rinsing four times with Tris buffer saline with 0.05% Tween 20 (TBST). Microscopic examination of brain sections was performed with the Olympus BX51 microscope using a fluorescence unit. Images were acquired using Olympus XM 10 camera and processed using the Cell F-software (Olympus).
Results

1. General observations

In order to achieve better coverage of low abundant proteins, the immunodepletion of 12 of the most abundant CSF proteins (albumin, IgG, IgM, IgA, transferrin, Apo A-I, Apo A-II, α1-Acid glycoprotein, α2-microglobulin, α1-antitrypsin, haptoglobin and fibrinogen) was performed. Then the fractions of low abundant CSF proteins were then subjected into labeling with CyDyes and separated by 2D-DIGE.

Using Delta2D’s 100% spot matching approach, 539 protein spots were detected on CSF 2D patterns. Densitometric and statistical analysis revealed that 51 protein spots showed significantly different expression level in sCJD comparing to non-demented, neurological controls, which comprises 9.5% of all detected protein spots. These protein spots corresponded to 33 different proteins (Fig. 1). In MM-sCJD, 27 up-regulated and 3 down-regulated proteins were found, whereas 24 up-regulated proteins were identified in the VV-sCJD cases (Tab. 3). Twenty one proteins were commonly up-regulated in both codon 129 polymorphisms. The group of MM-sCJD specific regulated proteins included: leucine-rich alpha-2-glycoprotein (LRG), actin, truncated form of complement C4-A, superoxide dismutase [Cu-Zn] (SOD-1), insulin-like growth factor-binding protein 6 (IGFBP-6), tetranectin, semaphorin-7A (Sema7A), protein FAM3C and transaldolase. The group of VV-sCJD specific regulated proteins included: DJ-1 protein, fibrinogen alpha chain (FGA) and angiotensinogen (ANGT).

When biological function was taken into consideration, the major alteration in CSF proteome was observed in proteins belonging to the glycolysis pathway or being involved in glucose metabolism. The remaining proteins fell into five functional groups: apoptosis / oxidative stress, signal transduction, amino acid / protein metabolism, immuno response / acute phase response signaling (APRS) and cell structure / transport (Fig. 2).
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Table 3. List of sCJD-regulated proteins in CSF

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<th>Nr</th>
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<th>MM genotype fold of change</th>
<th>VV genotype fold of change</th>
<th>UniProt Accession</th>
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<td>Gelsolin</td>
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<td>Angiotensinogen</td>
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<td>3.2, 8.1E-04</td>
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<td>Semaphorin-7A</td>
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Protein spot was considered as sCJD-regulated when its densitometric analyses showed at least -fold change in abundance and when $p$-value < 0.05 in unpaired Student’s $t$ test.
Figure 1. 2D maps of depleted CSF from codon 129 genotype in MM-sCJD (panel A), VV-sCJD (panel B), and NDC (panel C) patients acquired by the application of 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE). The densitometric and statistical analysis of CSF 2D maps revealed significantly different expression levels of 51 protein spots, which corresponded with to 33 different proteins.
The major alteration in CSF proteome was observed in proteins involved in the glycolysis pathway or involved in glucose metabolism. The remaining proteins fell into five functional groups: apoptosis / oxidative stress, signal transduction, amino acid / protein metabolism, immunoresponse / acute phase response signaling (APRS) and cell structure / transport.

2. CSF level of enzymes involved in glucose metabolism

Since we believe, that energy metabolism is strongly linked with cell death and survival, we focused particularly on three proteins related to glucose metabolism: glucose-6-phosphate isomerase (G6PI), lactate dehydrogenase (LDH) and fructose-bisphosphate aldolase A (ALDOA) in the following experiments.

In order to validate our findings from 2D-DIGE experiments, we performed Western blot analysis using a larger group of sCJD and control patients. Control patients included non-demented, neurological patients (NDC), patients suffering from vascular dementia (VD) and patients affected by Alzheimer’s disease (AD).

An increase of G6PI level in CSF was observed for all three codon 129 genotypes in sCJD (MM-sCJD, MV-sCJD and VV-sCJD) compared to all control groups The observed mean fold of change in protein abundance was 3 for VV-sCJD and 2 for other codon 129 genotypes in sCJD (Fig 3A).

The Western blot analysis revealed presence of two ALDOA isoforms slightly different in molecular weight. The isoform with lower molecular weight was predominant in NDC, VD and AD, whereas the dominance of the isoform with higher molecular weight was characteristic for sCJD. More than 3-fold of change in total ALDOA level were detected in MM-sCJD, MV-sCJD and VV-sCJD in comparison to NDC. Moreover, decreased ALDOA
concentration was found in VD, whereas its concentration in AD was found to be increased comparing to NDC (Fig 3B).

Finally, an enormous increase in LDH abundance was detected in the CSF of sCJD patients in comparison to all three control groups. The mean fold of change was 20 times higher for MM-sCJD and 40 times higher for VV-sCJD (Fig 3C).

Using Kruskal-Wallis ANOVA test all above-mentioned changes of protein abundance in CSF were found to be statistically significant.

![Figure 3](image)

**Figure 3.** Levels of G6PI (Panel A), ALDOA (Panel B) and LDH (Panel C) in CSF

3. **Brain level of enzymes involved in glucose metabolism**

Subsequently, levels of G6PI, ALDOA and LDH were determined in the frontal cortex of the MM1-sCJD and the VV2-sCJD patients. Both ALDOA and LDH showed no significant alterations in protein abundance in both sCJD subtypes comparing to the age-matched, non-demented controls (NDC) (Fig. 4A and 4B). The G6PI level was decreased by 2-fold in MM1-sCJD and it remained unchanged in VV2-sCJD comparing to NDC (Fig. 4C).

The above-mentioned change of protein abundance in brain was statistical significant using Kruskal-Wallis ANOVA test.
4. Cellular localization of enzymes involved in glucose metabolism

Pathological processes occurring in sCJD affect both neural populations of cells: glial cells (astrogliosis) and neurons (neuronal loss). Therefore, the next step of this study was the investigation of the cellular distribution of sCJD-regulated glycolytic enzymes. This was determined in paraffin-embedded sections of human frontal cortex by co-labeling of proteins of interest either with neuronal marker (Neurofilament – NF or NF-L) or astrocytic maker (Glial fibrillary acidic protein – GFAP).

The intensive LDH immunoreactivity was found in the cytoplasm of astrocytes in sCJD, AD, and in NDC (Fig. 5A). In NDC and AD, G6PI immunostaining was present in both cell bodies and processes of all cortical layers, whereas in sCJD, the G6PI intense immunoreactivity was predominantly observed in reactive astrocytes (Fig. 5B and Fig. 6). Diffuse ALDOA immunostaining was predominantly found in neurons in the gray matter and in astrocytes in the white matter of NDC and AD. This stands in contrast to the distribution in sCJD where ALDOA immunoreactivity was mostly detected in neuronal cell bodies (Fig. 5C).
Figure 5. Cellular localization of LDH (Panel A), G6PI (Panel B) and ALDOA (Panel C) in human frontal cortex

LDH intensive immunoreactivity was found in cytoplasm of astrocytes in sCJD, AD, and in NDC (Panel A). The G6PI staining was co-localized with GFAP-positive cells only in sCJD (Panel B). ALDOA diffuse immunostaining was predominantly found in neurons in the gray matter and in astrocytes in the white matter of NDC, sCJD and AD (Panel C). DAPI-labeling was used to visualize nuclei. Scale bar: 20 µm. [NDC – non-demented, neurological patients; sCJD – sporadic CJD; AD – Alzheimer’s disease]
Although G6PI-positive neurons were identified in sCJD brain, but the G6PI was mainly localized in reactive astrocytes. DAPI-labeling was used to visualize nuclei. Scale bar: 20 µm.

NDC – non-demented, neurological patients; sCJD – sporadic CJD; AD – Alzheimer’s disease

Discussion

Analysis of CSF is challenging in terms of the high dynamic range of protein concentration. The dominance of particular proteins like albumin or immunoglobulins leads to many low abundant proteins remaining undetected by conventional techniques such as 2D gel electrophoresis and mass spectrometry. Therefore, for a more effective coverage of low abundance proteins, the immunodepletion of 12 of the most abundant CSF proteins (albumin, IgG, IgM, IgA, transferrin, Apo A-I, Apo A-II, α1-Acid glycoprotein, α2-microglobulin, α1-antitrypsin, haptoglobin and fibrinogen) was performed. The fractions of low abundant CSF proteins were then subjected to labeling with CyDyes and separated by 2D-DIGE. The densitometric and statistical analysis of CSF 2D maps showed up-regulation of 27 and down-regulation of 3 proteins in MM-sCJD as well as up-regulation of 24 proteins in VV-sCJD in comparison to non-demented neurological controls. Twenty one proteins showed common up-regulation for both codon 129 polymorphisms in sCJD.
The leucine-rich alpha-2-glycoprotein (LRG), complement C4 and superoxide dismutase [Cu-Zn] (SOD-1), which belong to the group of MM-sCJD differentially regulated proteins, were previously shown to be implicated in the PrP pathobiology. The exact biological function of LRG still remains unclear. However, recent studies have shown that LRG can bind cytochrome C, which displays a neurotoxic effect when released from cells during apoptotic death processes. Thus LRG may have neuroprotective properties and promote cell survival. Furthermore, it has been shown that PrP106-126, a synthetic peptide corresponding to residues 106-126 of the human PrP sequence, which is used to mimic PrP Sc neurotoxicity, induces an endoplasmic reticulum (ER) stress in primary culture of rat cortical neurons leading to mitochondrial cytochrome C release, caspase 3 activation and subsequent apoptotic neuronal death. The increased expression level of the gene encoding LRG was found in mice intracerebrally infected with the Rocky Mountain Laboratory (RML) scrapie strain. However, no difference in expression level was found when mice were infected with the ME7 scrapie strain. Taking altogether, these data suggest that LRG may play a role in prion-induced pathological processes in a scrapie strain-dependent manner.

The C4 protein is essential for the activation of the complement system. Interestingly, active components of complement were detected in amyloid plaques in sCJD and GSS. Existing evidences suggest that prion-induced neurodegenerative processes may not be mediated by complement and that it is very unlikely that it plays a protective role in the CNS during prion infection. Nevertheless, expression profile studies performed in mice infected either with the ME7 or the RML scrapie strains revealed an up-regulation of genes encoding proteins involved in immuno response and participating in complement activation including C3 and C4. These findings might indicate that the enhancement of complement proteins and inflammatory factors may be important pathogenic events in prion disease.

The SOD-1 is a protein involved in protection against oxidative stress and cell death. The presence of oxidative stress events during prion infection was well proven by many studies. Moreover, the perturbation in SOD-1 level and activity as well as its involvement in PrP Sc-induced pathological processes have already been well described. VV-sCJD specific regulated proteins include DJ-1 protein, fibrinogen alpha chain (FGA) and angiotensinogen (ANGT).

The DJ-1 protein is abundantly expressed throughout the body and its biological implications are extremely diverse including fertility, oncogenesis and protection against oxidative stress. Moreover, mutations in the PARK7 gene encoding DJ-1 protein cause autosomal-
recessive early-onset Parkinson’s disease. It has also been reported that DJ-1 is required for the activity of the nuclear erythroid 2-related factor, a key player in the regulation of response to oxidative stress. This could suggest a potential protective role of DJ-1 in VV-sCJD pathophysiology.

The major role attributed to fibrinogen (FG) is the involvement in blood coagulation. However, it also displays a chaperone-like activity and is able to interact and suppress aggregation of a wide spectrum of stressed proteins. More interestingly, FG has been shown to inhibit fibril formation of yeast prion protein Sup35, which is known to share key features with the mammalian prion or amyloid proteins, suggesting a potential role of FG in protein misfolding diseases such as sCJD. Supporting this idea, it has been also shown that FG fractioned from murine blood can bind PrP Sc isolated from scrapie mice.

The angiotensinogen is a precursor molecule for angiotensins I, II and III belonging to renin-angiotensin system (RAS). Beside classical functions of RAS such as regulation of water and salt homeostasis or blood pressure, RAS has been implied in memory and learning processes. Recently, many evidences have shown that brain RAS may be involved in Alzheimer’s disease, stroke, depression or emotional stress. Moreover, an elevated level of ANGT in CSF has been found in multiple sclerosis indicating that it could be a potential biomarker of disease progression. However, no relationship between ANGT and prion disease is known so far.

The detection of regulated proteins specific for codon 129 genotype may confirm the hypothesis which assumes the involvement of different molecular pathways in sCJD pathogenesis depending on PrP Sc strain.

Some of the sCJD-altered proteins, which are found in these studies, are also previously reported as being associated either with prion protein or CJD. For instance, gelsolin, 14-3-3 protein, gamma enolase (NSE), and L-lactate dehydrogenase B chain (LDH-B) have been described to be differentially regulated in CSF of sCJD patients. Moreover, proteins related to glucose metabolism such as malate dehydrogenase, alpha and gamma enolase, glucose-6-phosphate isomerase (G6PI), pyruvate kinase isozymes M1/M2 as well as LDH-B were identified as interaction partners of fully functional myc-tagged PrP in supramolecular complex studies. In neuroblastoma cell-based disease model studies of PrP molecular network, 14-3-3 zeta protein and LDH-A were recognized as specific interactors of FLAG-tagged PrP. Further proteins such as actin, N(G),N(G)-dimethylarginine dimethylaminohydrolase 1, malate dehydrogenase, triosephosphate isomerase, fructose-bisphosphate aldolase A, alpha enolase, pyruvate kinase isozymes M1/M2 were also
indentified. However, they have been classified as unspecific interactors of PrP or its homologs, Shadoo and Doppel

Global studies of cerebral gene expression profile revealed that the expression of genes encoding 14-3-3 protein (beta, eta and zeta isoform), NSE, malate dehydrogenase, cytoplasmic aspartate aminotransferase and retinol-binding protein 4 were down-regulated whereas expression of encoding gene actin was up-regulated in brains of sCJD patients. In these studies, CSF level of all above mentioned proteins were increased. It may occur due to release or secretion mechanism of protein content from impaired neural cells into the extracellular space and then eventually into CSF. Moreover, this hypothesis could explain dominant up-regulation of proteins in CSF from sCJD affected patients. However, this far-reaching theory has to be proven by further investigations.

**Glycolytic enzymes in CJD**

Almost 40% of sCJD specific regulated proteins in CSF are involved in glucose metabolism, thus in energy metabolism. All these enzymes displayed up-regulation in sCJD regardless the codon 129 polymorphism. The increase in CSF level of glucose-6-phosphate isomerase (G6PI), lactate dehydrogenase (LDH) and fructose-bisphosphate aldolase A (ALDOA) was validated with further tests on a larger group of sCJD patients including three possible codon 129 polymorphisms as well as on three different types of controls consisting of non-demented, neurological controls (NDC), patients with vascular dementia (VD) and Alzheimer’s disease (AD). Subsequently, their abundance in the brain as well as their cellular localization was determined.

The G6PI catalyzes the conversion of glucose-6-phosphate into fructose 6-phosphate, whereas ALDOA catalyzes hydrolysis of fructose 1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Both enzymes belong to the second and fourth step of glycolysis, respectively. The LDH catalyzes the interconversion of lactate and pyruvate, which is formed in the final step of the glycolysis pathway.

The G6PI level showed 2-3-fold increase in CSF for all three codon 129 polymorphisms in the sCJD. However, its level in brain was 2-fold decreased specifically in the MM1-sCJD subtype and remained unchanged in the VV2-sCJD. This could suggest that G6PI expression and/or secretion may be modulated in a PrP Sc strain-dependent manner. Moreover, a shift in cellular distribution of G6PI was observed in sCJD. The intense immunoreactivity was predominantly localized in reactive astrocytes in sCJD, whereas in both control groups, NDC
and AD, it was mainly found in neurons. With this data we demonstrated for the first time the involvement of G6PI in prion-induced pathological process occurring in human brain.

Besides its glycolytic function, G6PI shows an activity of extracellular cytokine and acts as a growth factor promoting the survival and neurite outgrowth of motor and sensory neurons\(^{33}\), as an autocrine motility factor inducing cell motility\(^{34, 35}\), and as a maturation factor to mediate the differentiation of myeloid precursor cells to mature monocytes\(^{36}\). The inhibition of G6PI expression caused an increased susceptibility to caspase-dependent apoptosis in PC12 neuronal cells\(^{37}\). Furthermore, overexpression and subsequent secretion of G6PI protected NIH-3T3 fibroblasts against PI3K/Akt mediated apoptosis\(^ {38}\).

The positive G6PI immunostaining in astrocytes occurred two weeks after mechanical injury of murine cerebral cortex\(^ {39}\) and its expression was shown to be elevated in the later stage of brain infection with rabies virus in C3H mice model\(^ {40}\). This may suggest that detectable expression of G6PI in astrocytes is a response to signals coming from impaired neurons to increase their metabolism and prolong survival. The same motion of G6PI action might occur in sCJD-affected brain, but it is differentially modulated by different PrP\(^{Sc}\) strains.

The enormous increase in LDH level in CSF did not correlate with its unchanged level in the brain. This discrepancy could be explained by LDH release in extracellular space, which always accompanies cell death. However, LDH in CSF from sCJD patients exhibited significantly higher level than those determined in other dementias and proposed as a potential CSF marker for sCJD differential diagnosis\(^ {41}\). Moreover, as mentioned above, LDH is the interaction partner of cellular PrP\(^ {30, 31}\) and its expression was found to be up-regulated when PrP\(^ C\) was introduced into Prnp\(^ {0/0}\) cells\(^ 8\). Therefore, the role of LDH in prion-induced pathological processes may not be restricted only to being the marker of cellular damage.

According to an astrocyte-neuron lactate shuttle hypothesis (ANLSH), lactate is generated in an activity-dependent and glutamate-mediated manner by astrocytes and it is subsequently transported to active neurons, which are the target of the lactate use\(^ {42, 43}\). In more details, this hypothesis postulates that neuronal activation increases the extracellular concentration of glutamate, which is then taken up via Na\(^ +\)-dependent transporters by astrocytes. Subsequently, elevated levels of glutamate and Na\(^ +\) in astrocytes activate glutamine synthetase and Na\(^ +/K^+\) ATPase, respectively. This triggers astrocytic ATP consumption leading to activation of glycolysis and lactate production. As the next step, the lactate is released from astrocytes and taken up by neurons, where it serves as a fuel for activity-related neuronal energy needs\(^ {44-48}\). The cellular PrP regulates astrocytic \(\alpha_2/\beta_2\)-Na\(^ +/K^+\) ATPase activity and glutamate-triggered, basigin-associated lactate transport in primary culture of astrocytes. The Na\(^ +/K^+\) ATPase
activity was found to be reduced in PrP-deficient astrocytes or by binding of a monoclonal PrP antibody to its octarepeat, which further leads to regulation of MCT1-mediated lactate transport and the glutamate-independent elevation of lactate transport. Moreover, the CSF level of lactate is elevated in PrP-deficient mice compared to wild-type mice. The loss of PrP function in astrocytes of CJD-affected brain may result in elevated levels of extracellular lactate, thus neuronal damage followed by lactate-induced acidosis.

The ALDOA displayed 3-fold increased CSF concentration for all three codon 129 polymorphisms in sCJD. However, the elevated level of ALDOA was also found in AD. It suggests that ALDOA might also be implicated in pathological processes occurring in AD. The ALDOA abundance in the brain remained unchanged in MM1-sCJD and VV2-sCJD in comparison to the age-matched controls. Moreover, partial shift in its cellular distribution was observed in the sCJD. Diffuse ALDOA immunostaining was predominantly found in neurons in the gray matter and in astrocytes in the white matter of NDC and AD, whereas the ALDOA immunoreactivity in sCJD was only detected in neuronal cell bodies.

It has been shown, that glyceraldehyde-3-phosphate (G-3-P), an enzymatic product of ALDOA, is implied in the protection of cells against apoptosis via the inhibition of caspase-3 activity. Moreover, neurotoxicity of doppel (Dpl), the PrP C homolog, in Purkinje cells of the Ngsk Prnp 0/0 mouse model varies according to aldolase C expression. The higher sensitivity to Dpl-induced neurotoxicity, thus increased cell loss, is characteristic for Purkinje cells from aldolase C-negative compartments of the cerebellum. Summarizing, the presence of ALDOA in impaired neurons and its secretion may play an anti-apoptotic role in sCJD.

It is very likely that immuno-positive neural cells are origin of glycolytic enzymes which were found in elevated levels in CSF. Nevertheless, based on our experiments, a conclusive correlation between cellular localization of investigated glycolytic enzymes and their elevated level in CSF cannot be demonstrated. This hypothesis should be addressed in future studies.

Both, glycolysis and apoptosis are highly conserved and finely regulated multi-step processes maintaining cellular homeostasis. The activation of apoptotic pathway is dependent on energy status and, hence, apoptosis might be dependent on glucose metabolism. Based on numerous studies demonstrating that glucose metabolism is involved in cell death and survival, it is reasonable to speculate that these two crucial processes are linked. However, precise mechanisms underlying the regulation of glucose metabolism and the implication of glycolytic enzymes in apoptosis remain to be elucidated.
Concluding remarks

In summary, this study shows that 2D-DIGE approach can be successfully applied to explore in depth alterations in CSF proteome caused by CNS disorders. Furthermore, the identification of sCJD-regulated proteins in CSF provided a new insight into prion-induced pathological processes occurring in the brain of living, symptomatic patients. Moreover, revealed proteins could serve as a source of novel potential, diagnostic marker(s) for sCJD. Therefore, it would be of great interest to test utility of selected proteins in early diagnosis of sCJD. Another appealing aspect of our study, which would require further investigation concern the role of G6PI as well as its cellular translocalization in sCJD.
Bibliography


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2.2. Cellular prion protein overexpression disturbs cellular homeostasis in SH-SY5Y neuroblastoma cells but does not alter p53 expression: a proteomic study

2.2.1. Aims of the project

The aim of this study was to examine proteome and thereby biological processes affected by stable overexpression of human PrP<sup>C</sup> in human neuroblastoma SH-SY5Y cell line. Many evidences indicate that PrP<sup>C</sup>-overexpression in different cells line leads to increased sensitivity to apoptotic stimuli or triggers neurodegeneration and myopathy. The observed pro-apoptotic properties of PrP<sup>C</sup> seem to be related to the p53-dependent apoptotic pathway. Therefore, we applied a proteomic approach to reveal proteins which contribute to PrP<sup>C</sup> overexpression-mediated disturbances in cellular homeostasis as well as to investigate involvement of p53-dependent pathway in this pathological process.
2.2.2. Original publication

The results described below are published in:


Author contribution:

The study was designed, conducted and interpreted by Sanja Ramljak. Eva Weiss performed cell culture and partially Western blot analysis. Eva Weiss and Joanna Gawinecka performed 2D gel electrophoresis and densitometric analysis of gel images. Abdul R. Asif performed mass spectrometry analysis. Matthias Schmitz helped in review process by performing transient transfection of mouse neuroblastoma N2a cells and human neuroblastoma SK-N-LO cells. Christina Behrens generated stable PrP<sup>C</sup>-overexpressing SH-SY5Y human neuroblastoma cells. Walter Schulz-Schaeffer provided cell culture facilities and partially cell culture materials. Sanja Ramljak and Inga Zerr wrote manuscript.
CELLULAR PRION PROTEIN OVEREXPRESSION DISTURBS CELLULAR HOMEOSTASIS IN SH-SY5Y NEUROBLASTOMA CELLS BUT DOES NOT ALTER p53 EXPRESSION: A PROTEOMIC STUDY

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Abstract—The definite physiological role of the cellular prion protein (PrPc) remains elusive. There is ample in vitro and in vivo evidence suggesting a neuroprotective role for PrPc. On the other hand, several in vitro and in vivo studies demonstrated detrimental effects of PrPc overexpression through activation of a p53 pathway. Recently, we reported that transient overexpression of PrPc in human embryonic kidney 293 cells elicits proteome expression changes which point to deregulation of proteins involved in energy metabolism and cellular homeostasis. Here we report proteome expression changes following stable PrPc overexpression in human neuronal SH-SY5Y cells. In total 18 proteins that are involved in diverse biological processes were identified as differentially regulated. The majority of these proteins is involved in cell signaling, cytoskeletal organization and protein folding. Annexin V exhibited a several fold up-regulation following stable PrPc overexpression in SH-SY5Y cells. This finding has been reproduced in alternative, mouse N2a and human SK-N-D1 neuroblastoma cell lines transiently overexpressing PrPc. Annexin V plays an important role in maintenance of calcium homeostasis which when disturbed can activate a p53-dependent cell death. Although we did not detect changes in p53 expression between PrPc overexpressing SH-SY5Y and control cells, deregulation of several proteins including annexin V, polyglutamate tract-binding protein-1, spermine synthase and transgelin 2 indicates disrupted cellular equilibrium. We conclude that stable PrPc overexpression in SH-SY5Y cells is sufficient to perturb cellular balance but insufficient to affect p53 expression. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: prion protein, signal transduction, cytoskeleton, protein folding, annexin V, transgelin 2.

In recent years numerous studies have focused on revealing the physiological function(s) of PrPc. Recognizing cellular processes governed by PrPc might promote our understanding of molecular dysfunctions underlying the pathogenesis of transmissible spongiform encephalopathies mediated by the pathological isoform of PrPc. Strong evidence suggests PrPc entailment in activation of distinct signal transduction pathways (Mouillot-Richard et al., 2000; Chen et al., 2003; Vassallo et al., 2005), copper metabolism (Pauly and Harris, 1998; Kramer et al., 2001), anti-oxidant activities (Brown and Besinger, 1998; Wong et al., 2001), synaptic transmission (Collinge et al., 1994) and modulation of cell death (Kuwahara et al., 1999; Bourhar et al., 2001). Despite PrPc’s involvement in diverse cellular activities its exact physiological role is still not defined.

Various studies investigating the control of cell death by PrPc have led to somewhat opposite conclusions. Extensive data documented anti-apoptotic, neuroprotective properties of PrPc. Kuwahara and colleagues (1999) demonstrated that serum-deprivation induced cell death was more prominent in mouse prion protein knock-out (PrPc−/−) hippocampal neurons than in the control Prp c+/+ cells. Furthermore, PrPc protected human neurons from apoptotic triggers by an overexpression of pro-apoptotic Bax protein (Bourhar et al., 2001). In vivo studies confirmed PrPc-mediated neuroprotection. PrPc−/− deficient mice are more susceptible to acute seizures (Walz et al., 1999) and exhibit significantly increased infarction volumes following transient focal cerebral ischemia as compared to their wild-type counterparts (Weise et al., 2004). These data are apparently in contrast to other reports indicating that overexpression of PrPc in different cell lines results in increased sensitivity to apoptotic stimuli (Paitel et al., 2002, 2003). The observed PrPc−/− mediated hypersensitivity to apoptotic agents appears to be controlled via a p53-dependent pathway (Paitel et al., 2004; Sunyach et al., 2007). In vivo findings showed that PrPc overexpression triggers severe degeneration in the central and peripheral nervous system and causes primary myopathy in skeletal muscles (Westaway et al., 1994; Huang et al., 2007; Chiesa et al.,...
The primary myopathy observed in PrPc-overexpressing mice appears to be related to a p53-dependent pathway, too (Liang et al., 2008).

Using a proteome approach we demonstrated that transient overexpression of human PrPc in human embryonic kidney (HEK) 293 cells evokes perturbed expression of proteins involved in energy production and maintenance of cellular homeostasis (Ramljak et al., 2008). The present study employs proteomics techniques to examine proteome and thereby biological processes affected by stable overexpression of human PrPc in human neuroblastoma SH-SY5Y cell line. The SH-SY5Y cell line is well characterized and was previously used either as a model system for studying the physiological role of PrPc (Watt et al., 2007) or prion-induced neuronal death (Dupireux et al., 2006; Martinez and Pascual, 2007). We show that the majority of differentially regulated proteins can be either directly or indirectly associated with cell signaling, cytoskeletal organization or protein folding. Although the proteome patterns of HEK 293 cells transiently overexpressing PrPc (Ramljak et al., 2008) and SH-SY5Y cells stably overexpressing PrPc were largely non-intersecting with regard to affected protein groups, our results support the view that sole overexpression of PrPc is sufficient to subvert the cellular balance regardless of the cell type used (HEK 293 vs. SH-SY5Y) or transfection procedure (transient vs. stable).

EXPERIMENTAL PROCEDURES

Plasmid construction and generation of stable PrPc-overexpressing cells

SH-SY5Y human neuroblastoma cells were purchased from the American Type Culture Collection (ATCC, USA). To generate SH-SY5Y cells that constitutively and stably express full length human PrPc, wild-type human prion protein gene (PRNP) was cloned into the Xhol/XbaI site of the pcDNA vector (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. Forty-eight hours after transfection cells were grown without Geneticin in DMEM containing 10% fetal calf serum (FCS, Biochrom), 1% P/S (Biochrom) and 1% L-glutamine at 37 °C, 5% CO2 supply and 95% humidity. Six weeks after addition of 400 μg/mL Geneticin (Invitrogen, Karlsruhe, Germany) stably transfected SH-SY5Y cells were selected and further maintained in the same medium with a lower concentration of Geneticin (200 μg/mL). Medium was exchanged every 4 days. An overexpression of PRNP was checked by Western blot analysis. Parental SH-SY5Y cells were grown without Geneticin in DMEM containing 10% FCS, 1% P/S, 1% L-glutamine at 37 °C, 5% CO2 supply and 95% humidity. The morphological features of transfected and non-transfected SH-SY5Y cells were identical as shown by light microscopy (Fig. 1A, B).

Transient overexpression of PrPc in human neuroblastoma SK-N-LO and mouse neuroblastoma N2a cells

SK-N-LO human neuroblastoma (Cell lines service, Eppelheim, Germany) and N2a mouse neuroblastoma cells (ATCC, USA) were both transiently transfected with the pCMS-PRNP-EFGP vector, bearing human PRNP (Ramljak et al., 2008). In parallel, an empty pCMS-EGFP vector was used as a control. For the transfection of 1–1.5×106 cells in a six well plate 2 μg of plasmid DNA and 4–5 μL of Lipofectamine (Invitrogen) were dissolved in 250 μL OptiMEM (Gibco/Invitrogen, Karlsruhe, Germany) for 5 min. Stable DNA complexes were formed after 15–20 min and added to the transfection medium (Gibco OptiMEM containing 2% FCS). After 6–8 h the transfection medium was replaced by fresh culture medium. SK-N-LO cells were cultured in AlphaMEM (Stem Cell technologies, Grenoble, France) supplemented with 10% FCS and 1% P/S (Biochrom). N2a cells were maintained under same culture conditions as previously described for SH-SY5Y cell line. Cells were collected 48 h post-transfection.

Sample preparation for two-dimensional electrophoresis and Western blotting

Cell medium was removed from tissue culture flasks, cells were washed twice with cold phosphate-buffered saline (PBS), scraped and centrifuged at +4 °C, −4,000×g for 20 min. The supernatant was decanted and the pellet was resuspended in cold PBS and centrifuged once more at +4 °C, −4,000×g for 10 min. After discarding the supernatant, the pellet was lysed in 7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol (DTT) and a protease inhibitor mixture (0.1 mM phenylmethylsulfonylfluoride, 10 μM N-tosyl-L-lysinal-choromethylketone and 10 μM N-α-tosyl-L-lysinal-choromethylketone) was added. The lysate was centrifuged in a microcentrifuge at 14000 rpm for 10 min at +4 °C to remove cell debris. Proteins were quantified by the Bradford assay (Bio-Rad, Munich, Germany).

Western blotting

For immunoblotting analysis, equal amounts of protein were diluted in 4× sample buffer, boiled for 5 min and loaded onto 12% polyacrylamide gels. Following separation by gel electrophoresis, proteins were electrophoretically transferred to polyvinylidene fluoride membranes (AppliChem, Darmstadt, Germany) and subsequently blocked with 5% (v/v) non-fat dry milk in PBS and 0.1% Tween 20 (PBST) for 1 h at RT. Membranes were then incubated with different primary antibodies anti-PrP 12F10 monoclonal antibody (Krasemann et al., 1999; 1:1000), anti-annexin V polyclonal antibody (Abcam, Cambridge, UK; 1:500), anti-14-3-3 γ polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:4000), anti-transgelin monoclonal antibody (Abcam, 1:625) and anti-β actin monoclonal antibody (Abcam, 1:10 000) overnight at 4 °C, with gentle rocking. After extensive rinsing in PBST, membranes were incubated with a peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:2000; 1:5000) for 1 h at RT. Immunolabeled proteins were detected after immersion of the membranes into enhanced chemiluminescence (ECL) solution and exposition to ECL-Hyperfilm (Amersham Biosciences, Buckinghamshire, UK). Densitometric measurements were performed using the ArcSoft photo Studio 5 (ArcSoft Inc.) and LabImage V 2.52a software. For each condition analyzed, minimum three Western blots were prepared from three different protein extractions.

Two dimensional electrophoresis

Protein samples were diluted with rehydration buffer containing 7 M urea, 2 M thiourea, 15 mM DTT, 4% CHAPS and 2% ampholytes for first-dimension isoelectric focusing. One-hundred and thirty micrograms of protein were loaded on a 17 cm, pH 3–10 linear, immobilized pH gradient (IPG) strip (Bio-Rad). IPG strips were focused until 32000 Vh were reached and were first reduced for 25 min in the buffer containing 6 M urea, 2% sodium dodecyl
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**Identification of protein/peptide sequence analysis**

In-gel digestion was carried out according to a modified published protocol (Shevchenko et al., 1996). Spots of interest were excised from the silver stained gel into 1–2 mm² slices, destained with 15 mM potassium ferricyanide/50 mM sodium thiosulfate (Sigma-Aldrich, Steinheim, Germany) and then equilibrated with 50 mM ammonium bicarbonate/50% acetonitrile (ACN) (Sigma-Aldrich). Samples were dried for 15 min using the SpeedVac SVC100 (Savant Instruments, Farmingdale, NY, USA) vacuum concentrator. Dried spots were rehydrated on ice with 10–20 μl of trypsin digestion solution (Promega, Madison, WI, USA) for 45 min followed by an overnight incubation at 37 °C in digestion solution without trypsin. The peptides were first extracted with 0.1% trifluoracetic acid (TFA) for 30 min in the sonicating water bath Transonic 310 (Elma®, Pforzheim, Germany) followed by extraction with 30% ACN in 0.1% TFA and 60% ACN in 0.1% TFA. The eluate was collected in Eppendorf tubes and dried with the Speed-Vac. The extracted peptides were dissolved in 0.1% formic acid and one microliter of each sample was introduced using a CapLC auto sampler (Waters) onto a C18-precolumn cartridge C18 pepMap (300 μm × 5 mm; 5 μm particulate size) and further separated through a C18 pepMap100 nano Series™ (75 μm × 15 cm; 3 μm particulate size) analytical column (LC Packings). The single sample run time was set for 60 min. The chromatographically separated peptides were analyzed on a Q-TOF Ultima Global (Micromass, Manchester, UK) mass spectrometer equipped with a nanoflow ESI Z-spray source in positive ion mode. The data acquisition was performed using MassLynx (v 4.0) software on a Windows NT PC and data were further processed on Protein-Lynx-Global-Server (v 2.2), (Micromass, Manchester, UK). Processed data were searched against MSDB and Swiss-Prot databases through the Mascot search engine using a peptide mass tolerance and fragment mass tolerance of 0.5 Da. The search
Differential proteome analysis of human neuroblastoma SH-SY5Y cells stably overexpressing human PrP<sup>C</sup> and parental SH-SY5Y cells expressing only endogenous PrP<sup>C</sup> was done to study protein expression changes occurring due to permanent overexpression of PrP<sup>C</sup>. Western blot analysis confirmed markedly higher PrP<sup>C</sup> expression in SH-SY5Y cells stably overexpressing PrP<sup>C</sup> as compared to control cells (Fig. 1C). PrP<sup>C</sup> level was further quantified by ELISA in each cell lysate before proceeding to 2-DE. In average, PrP<sup>C</sup> expression level was nearly 16-fold higher (P<0.001) in PrP<sup>C</sup> overexpressing as compared to parental cells (Fig. 1D).

# Results

Densitometric analysis of silver stained 2-DE gels revealed a total of 18 differentially regulated protein spots between SH-SY5Y cells overexpressing PrP<sup>C</sup> and control cells (Table 1). Threshold for identification of up-/down-regulated proteins was set to at least 1.5 fold change. According to this criterion 13 proteins were up- and five proteins were down-regulated following PrP<sup>C</sup> overexpression in SH-SY5Y cells with respect to the control group. Nine out of 18 differentially regulated proteins showed a two-fold or higher regulation. The map of significantly regulated protein spots following 2-DE is shown in Fig. 2. In this study an overexpression of PrP<sup>C</sup> influenced various biological processes, especially, signal transduction, cytoskeleton organization and protein folding (Fig. 3). The proteins directly or indirectly involved in signal transduction displayed PrP<sup>C</sup>-induced up-regulation in SH-SY5Y cells: 14-3-3 gamma (1433G), growth factor receptor bound protein 2 (GRB2), Rho GTP-ase activating protein 1 (RHG01) and platelet-activating factor acetylhydrolase IB subunit gamma (PA1B3). Likewise, three proteins implied in cytoskeleton organization: transgelin 2 (TAGL2), translationally-controlled tumor protein (TCTP) and RHG01 were up-regulated. Only one protein, UPF0027 (CV028), which appears indirectly involved in cytoskeleton organization was down-regulated in PrP<sup>C</sup> overexpressing neuroblastoma cells. Among three proteins assisting in protein folding two were down-regulated: 40 kDa peptidyl-prolyl cis-trans isomerase (PPID) and FK506-binding protein 4 (FKBP4) whereas protein disulfide isomerase precursor (PDI1) was up-regulated by PrP<sup>C</sup>. Six out of eight remaining proteins that participate in various biological processes were up-regulated by PrP<sup>C</sup>: calcium and phospholipid binding protein, annexin V (ANXA5); polyglutamine binding protein-1 (PQBP1) involved in induction of neuronal cell death; oxidative stress protein, glutathione S-transferase-omega-1 (GSTO1); UV excision repair protein RAD23 homolog B (RD23B) participating in DNA repair; alcohol dehydrogenase class III (ADHX) with yet undefined physiological function in brain and mitochondrial protein, fumarate hydratase (FUMH), implied in energy metabolism. On the contrary, only two out of eight proteins categorized as having other biological functions were down-regulated:

### Table 1

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<th>Spot no.</th>
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<th>Score</th>
<th>State change</th>
<th>Fold change</th>
<th>P value</th>
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<td>Platelet-activating factor acetylhydrolase IB subunit gamma</td>
<td>PA1B3</td>
<td>Q15102</td>
</tr>
<tr>
<td>597</td>
<td>11</td>
<td>159</td>
<td>▲</td>
<td>1.70</td>
<td>0.043</td>
<td>UV excision repair protein RAD23 homolog B</td>
<td>RD23B</td>
<td>P54727</td>
</tr>
<tr>
<td>73</td>
<td>6</td>
<td>77</td>
<td>▼</td>
<td>0.52</td>
<td>0.035</td>
<td>Bifunctional methylene-tetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial precursor</td>
<td>MTDC</td>
<td>P13995</td>
</tr>
</tbody>
</table>

Abbr., Abbreviation; Acc., Accession.
spermine synthase (SPSY), an enzyme which catalyzes production of a free radical scavenger spermine and mitochondrial protein, bifunctional methylenetetrahydrofolate dehydratase/cyclohydrolase (MTDC) that is most probably involved in folate metabolism.

To reinforce the results obtained by densitometric analysis of 2-DE, the expression of ANXA5, TAGL2 and 1433G was additionally confirmed by Western blotting (Fig. 4A–C). PrPc overexpression induced nearly 4-fold \((P<0.01)\) up-regulation of ANXA5 in SH-SY5Y cells, as inferred from densitometric analysis of Western blot, although densitometric analysis of 2-DE showed 3-fold up-regulation. In order to further verify our data in an independent set of experiments we performed densitometric analyses of Western blots following transient overexpression of PrPc in mouse neuroblastoma N2a and alternative human neuroblastoma SK-N-LO cells which resulted in 2-fold \((P<0.001)\) and 1.8-fold \((P<0.01)\) higher expression of ANXA5 in PrPc-overexpressing as compared to control vector transfected cells (Fig. 5A–D).

Western blot analysis revealed more than 260-fold up-regulation of TAGL2 by PrPc \((P<0.001)\) in SH-SY5Y cells as compared to 2.5-fold up-regulation observed by 2-DE. We were not able to reproduce this result in two additional cell lines (N2a and SK-N-LO), following transient overexpression of PrPc, due to the complete lack of the signal with two different TAGL2 antibodies.

An up-regulation of 1433G by PrPc in SH-SY5Y cells was 1.6-fold following 2-DE analysis and was confirmed by Western blotting as being 1.4-fold \((P<0.01)\). The other two cell lines used in this study did not show

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**Fig. 2.** Silver stained 2-DE gel of SH-SY5Y cells stably overexpressing PrPc\(^{\text{c}}\). Linear 17 cm IPG strips (pH 3–10) were loaded with 130 µg of proteins. Labelling on the gel represents the location of the relevant spots. The protein identity of the spots is listed in Table 1.

**Fig. 3.** Schematic depiction of up-/down-regulated proteins following stable overexpression of human PrPc\(^{\text{c}}\) in human neuroblastoma SH-SY5Y cells. All 18 PrPc\(^{\text{c}}\)-regulated proteins are distributed into four groups based on their biological function. Only RHG01 could be simultaneously categorized into two groups. Light grey colour indicates up-regulated whereas dark grey colour indicates down-regulated proteins. Short abbreviations given for each protein are explained in Table 1.
1433G regulation following transient overexpression of PrPc.

Although tumor suppressor oncogene p53 was not detected as differentially regulated by PrPc overexpression in SH-SY5Y cells by 2-DE, we performed additional Western blot analysis showing that its expression remains unchanged between PrPc-overexpressing and control cells (Fig. 6). The reason to verify p53 expression by additional Western blot analysis was that an increase in p53 expression was already demonstrated in different cell lines overexpressing PrPc. However, we did not detect enhanced p53 expression following PrPc overexpression in our cell model.

**DISCUSSION**

Proteins involved in cell signaling

As proof of principle the 14-3-3 gamma protein (1433G), a well known cerebrospinal marker used for distinguishing sporadic Creutzfeldt–Jakob disease (sCJD) from other dementias (Van Everbroeck et al., 2005), exhibited PrPc-induced up-regulation in SH-SY5Y cells. Proteins belonging to the 14-3-3 family have significant influence on diverse signal transduction pathways, regulation of cell cycle progression and apoptosis (Fu et al., 2000). The finding that 1433G knock-out mice following intracerebral or intraperitoneal inoculation with the Rocky Mountain strain of scrapie do not show changes in survival rates as compared to wild-type mice (Steinacker et al., 2005), does not exclude the importance that 1433G might have for the physiological function of PrPc.

Besides 1433G, GRB2 was another signaling protein found to be up-regulated following overexpression of PrPc in the present study. A previous report demonstrated an interaction between GRB2 and PrPc in yeast two-hybrid system and co-purification in neuronal microsomal vesicles (Spielhaupter and Schätzl, 2001). This adaptor protein connects the signals from extracellular/transmembrane re-
Receptors to intracellular signaling molecules and is essential for the formation of signaling complexes.

RHG01 which also showed an enhanced expression by PrPc-overexpressing cells belongs to small GTPases of the Ras superfamily implied in the control of c-Jun N-terminal kinase and p38 mitogen activated protein kinase signaling cascades (Coso et al., 1995; Minden et al., 1995). A recent analysis of protein microarray data revealed that Rho GTPase activating protein 15 represents one of the PrPc interacting proteins (Satoh et al., 2009).

PA1B3, which was likewise up-regulated by PrPc, belongs to platelet-activating factor acetylhydrolases (PAF-AH). PAF-AH are not themselves signaling molecules but after the cleavage of the acyl group at the sn-2 position they inactivate platelet-activating factor (PAF) which is one of the most effective lipid messengers (Prescott et al., 2000). Identification of specific binding sites for PAF in subcellular fractions of gerbil brain and rat cerebral cortex suggests its physiological role in the brain (Domingo et al., 1988; Marcheselli et al., 1990). Interestingly, the intracellular PAF-AH can hydrolyze some oxidatively modified phospholipids and take part in their removal (Hattori et al., 1993). Thus, an up-regulation of PA1B3 by PrPc may indicate an increased level of oxidized phospholipids in PrPc overexpressing SH-SY5Y cells.

Proteins involved in cytoskeletal organization

The ability of PrPc to regulate microtubule dynamics and to reorganise actin cytoskeleton is well documented (Dong et al., 2008; Málaga-Trillo et al., 2009). TAGL2, a protein involved in the organization and stability of the actin cytoskeleton (Goodman et al., 2003) displayed striking, by Western blot validated, 260-fold up-regulation in PrPc-overexpressing cells. (A–D) Western blot analyses of PrPc and annexin V expression in mouse neuroblastoma N2a and human neuroblastoma SK-N-LO cells. PrPc and annexin V expression was determined in 20 μg of proteins from the total cell lysates of N2a (Panels A, B) and SK-N-LO (Panels C, D) cells. Note high PrPc expression in pCMS-PRNP-EGFP-transfected cells of both cell lines as compared to control (pCMS-EGFP) transfected cells (Panels A, C). Panel (B) displays markedly higher annexin (Anxa5) expression in PrPc-overexpressing N2a cells than in the control cells ($P<0.001$). Panel (D) shows likewise significant up-regulation of annexin (ANXA5) in PrPc-overexpressing SK-N-LO cells as compared to control cells ($P<0.01$). The displayed Western blots are representatives of four (N2a) and three (SK-N-LO) independent experiments and were reproduced three times. β-actin expression below each blot demonstrates an equal protein load.
overexpressing cells as compared to control cells. Deletion of the gene Scp1p encoding actin-bundling protein (vertebrate SM22/transgelin) in budding yeast is characterized by reduced production of reactive oxygen species and highly significant increase in longevity (Gourlay et al., 2004). Interestingly, transcriptome analysis following induction of amyloid precursor protein intracellular domain expression in human neuronal cell culture system revealed increased transgelin gene synthesis. Moreover, transgelin was significantly higher expressed in the frontal cortex of Alzheimer disease patients as compared to their age-matched controls (Müller et al., 2007). Transgelin can also suppress the expression of the metallo-matrix proteinase MMP-9 (Nair et al., 2006) which is involved in the extracellular matrix remodelling. Perhaps, recently reported PrPc-dependent down-regulation of MMP-9 transcript in neuronal cells (Pradines et al., 2008) may be additionally linked to an increase in transgelin expression. To our knowledge this is the first time evidence for PrPc-induced regulation of transgelin.

TCTP which exhibits properties of a tubulin binding protein that associates with microtubules in a cell cycle-dependent manner (Gachet et al., 1999), showed an up-regulation by PrPc overexpression. Elevated levels of TCTP lead to microtubule rearrangements (Gachet et al., 1999). Noticeably, recombinant prion protein induces tubulin oligomerization and thereby inhibits microtubule assembly (Nieznanski et al., 2006). Furthermore, TCTP interacts with the third cytoplasmic domain of Na+/K+-ATPase alpha subunit in yeast two-hybrid system and inhibits it in a dose-dependent manner (Jung et al., 2004). An overexpression of TCTP in vivo leads to inhibition of Na+/K+-ATPase activity and intracellular Ca2+ mobilization (Kim et al., 2008). Interestingly, third cytoplasmic domain of Na+/K+-ATPase alpha subunit was identified as a PrPc interacting protein (Petrakis and Sklaviadis, 2006).

RHG01 besides its role as a signaling protein (see Proteins involved in cell signaling) is also involved in the regulation and assembly of actin cytoskeleton (Hall, 1998). UPF0027 protein (CV028), more than a two-fold down-regulated by PrPc in the present study, has so far unknown function except that it interacts with vinculin (inferred from physical interaction from http://www.expasy.org/uniprot/Q9Y3I0), an actin filament binding protein that localizes in focal adhesions (Le Clainche and Carlier, 2008).

Proteins involved in protein folding

Influence of PrPc overexpression on proteins involved in protein folding is of special interest due to the fact that prion diseases are triggered by the accumulation of misfolded prion protein. Our study documented a PrPc-induced up-regulation of protein disulfide isomerase (PDI) precursor (PDIA1), an endoplasmic reticulum (ER)-resistant protein that functions as a chaperone catalyzing the isomerization of intra- and intermolecular disulfide bonds. An overexpression of PDIA1 could indicate a response to disrupted ER homeostasis. Importantly, an overexpression of PDI in brains of sCJD patients has also been reported (Yoo et al., 2002).

Conversely, another protein exhibiting chaperone activity, PPID, or cyclophilin 40 showed pronounced down-regulation upon PrPc overexpression. The results of Cohen and Taraboulos (2003) are indicative of the role of cyclophilins in the normal metabolism of PrPc. In detail, an addition of the immunosuppressant cyclosporine A inhibited the cyclophilin family of peptidyl-prolyl isomerases (PPIases) in cultured cells which led to the accumulation of proteasome-resistant “prion like” PrP species. The authors also suggested that the possible weakening of PPIase activity during aging may contribute to development of sporadic prion diseases.

In analogy, FK506-binding protein 4 (FKBP4) which exhibited more than a two-fold down-regulation by PrPc in this study, belongs to a highly conserved family of chaperone proteins that bind immunosuppressive drugs and have PPIase activity. FKBP4 is a part of the steroid receptor complexes to which it is linked via 90 kDa Hsp (Sanchez, 1990). Of interest, a microarray study revealed more than a two-fold down-regulation of another member of this family, FK506 binding protein 1B, in the frontal cortex of sCJD (Xiang et al., 2005).

Proteins involved in other biological processes

ANXA5 belongs to the annexin family of calcium and phospholipid-binding proteins with poorly understood physiological role. ANXA5 was the only protein that displayed a several fold up-regulation in human neuroblastoma SH-SY5Y cell line stably overexpressing PrPc, in mouse N2a and human SK-N-LO neuroblastoma cells, transiently overexpressing PrPc, and in the previous study where PrPc was likewise transiently overexpressed in HEK 293 cells (Ramljak et al., 2008). Hence, PrPc-mediated ANXA5 regulation appears neither cell type specific, vector specific nor dependent on transfection procedure (stable vs. transient). It was reported that ANXA5 knock-out DT-40 cells exhibit defect in cytosolic Ca2+-signaling and are therefore resistant to certain apoptotic agents which induce apoptosis via a Ca2+-dependent pathway, such as staurosporine (Hawkins et al., 2002). Conversely, an overexpression of ANXA5 stimulates apoptotic events (Wang and Kirsch, 2006). Interestingly, it was repeatedly demonstrated that
PrP<sup>c</sup> overexpression in different cell lines leads to an increased staurosporine-evoked cell death via a p53 pathway (Paitel et al., 2002, 2003, 2004). In the present study we did not observe p53 regulation upon PrP<sup>c</sup> overexpression on 2-DE. Moreover, Western blot analysis confirmed that the level of p53 expression indeed remains unchanged indicating that the sole PrP<sup>c</sup> overproduction in SH-SY5Y cells under basal conditions (no apoptotic agent added) may not be enough to activate p53 pathway. Nevertheless, it is tempting to speculate that there might be a functional link between earlier reported higher responsiveness of PrP<sup>PSC</sup>-overexpressing cells to staurosporine and an up-regulation of ANXA5. Moreover, gene expression profiling in the frontal cortex of sCJD patients detected an up-regulation of ANXA5 (Xiang et al., 2005) suggesting a possible role for this protein in the pathophysiology of CJD.

Expression of PQBP1 was prominently enhanced after PrP<sup>PSC</sup>-overexpression in SH-SY5Y cells as compared to parental cells. PQBP-1 is a nuclear, ubiquitously expressed protein found primarily in neurons of central nervous system with abundant levels registered in cerebellar cortex, hippocampus and olfactory bulb (Waragai et al., 1999). In vitro overexpression of PQBP-1 suppresses cell growth and enhances cell susceptibility to a variety of stress conditions (Waragai et al., 1999). Similarly, PQBP-1 overexpression in vivo results in late-onset neuronal reduction in CNS (Okuda et al., 2003). Induction of neuronal cell death by PQBP-1 seems to occur via mitochondrial stress, a key molecular event shared among distinct neurodegenerative disorders (Marubuchi et al., 2005). An up-regulation of PQBP-1 by PrP<sup>c</sup> let us assume that overexpression of PrP<sup>c</sup> in SH-SY5Y cells indeed disturbs cellular balance.

GSTO1 is a member of glutathione S-transferase family of enzymes that plays an important role in detoxification processes by conjugating xenobiotic compounds with reduced glutathione. An up-regulation of GSTO1 in PrP<sup>c</sup>-overexpressing SH-SY5Y cells indicates an increased oxidative stress. Likewise, we reported an up-regulation of glutathione S-transferase P by moderate level of PrP<sup>c</sup> overexpression in HEK 293 cells (Ramjak et al., 2008).

SPSY is an enzyme classified to a family of aminopropytransferases which catalyzes the addition of an aminopropyl group to polyamine spermidine in order to form spermine. The expression of SPSY was down-regulated in PrP<sup>PSC</sup>-overexpressing cells. Natural polyamines including spermene are ubiquitously distributed in mammalian tissues and are essential for cellular growth and differentiation (Pegg and McCann, 1982). Spermine can act directly as a free radical scavenger and can protect DNA from free radical-induced oxidative damage (Ha et al., 1998). Further, biological polyamines like spermine not only inhibit nucleic acid-induced polymerization of prion protein (Bera and Nandi, 2007) but their membrane targeting leads to inhibition of PrP<sup>PSC</sup> propagation and offers a possibility to degrade pre-existing PrP<sup>PSC</sup> aggregates in living cells (Winikhofer and Tatzei, 2000). Down-regulation of spermine synthase would obviously negatively interfere with the process.

Interestingly, UV excision repair protein RAD23B (RD23B) exhibited nearly two-fold up-regulation in SH-SY5Y cells overexpressing PrP<sup>c</sup>. RAD23B proteins play a role in both DNA repair and regulation of protein stability (Glockzin et al., 2003; Brignone et al., 2004). A role for RD23B in the nucleotide excision repair, one of the most important DNA repair pathways which eliminates wide variety of base lesions, was suggested (Sugasawa et al., 1996). Therefore, the finding that decrease in SPSY expression by PrP<sup>c</sup> was accompanied by an increase in RD23B expression might indicate enhanced susceptibility of PrP<sup>PSC</sup>-overexpressing SH-SY5Y cells to DNA damage.

ADHX, two-fold up-regulated in PrP<sup>PSC</sup>-overexpressing cells is the only class of these enzymes that is expressed in adult human brain and whose primary function is not ethanol oxidation. A possible explanation for an up-regulation of this enzyme by PrP<sup>c</sup> is still to be elucidated.

FUMH, an enzyme of tricarboxylic acid cycle which converts fumarate to malate, was up-regulated by PrP<sup>c</sup> overexpression. Another mitochondrial enzyme MTDC with unclear physiological function was down-regulated by PrP<sup>c</sup> overproduction. Nevertheless, MTDC knock-out mice die in utero suggesting its essential role during embryonic development possibly related to mitochondrial folate-metabolism (Di Pietro et al., 2002).

**CONCLUSION**

In summary, stable overexpression of PrP<sup>c</sup> in SH-SY5Y human neuroblastoma cells regulates expression of proteins involved in different cellular activities. The majority of PrP<sup>c</sup>-regulated proteins is associated with cell signaling, cytoskeletal organization and protein folding. Interplay between these protein groups governed by PrP<sup>c</sup> might hold the key to PrP<sup>c</sup> cellular function. An up-regulation of TAGL2, PQBP-1, PDIA1, GSTO1, ANXA5 as well as down-regulation of FPIID, FKBP4 and SPSY suggests disturbed cellular homeostasis in PrP<sup>PSC</sup>-overexpressing SH-SY5Y cells, but sole overexpression of PrP<sup>c</sup> is not enough to alter p53 expression and to possibly activate a p53-dependent apoptotic pathway in SH-SY5Y cells.

In the future experiments we intend to reveal the effects of staurosporine treatment on protein expression changes and phosphorylation patterns in SH-SY5Y cells overexpressing PrP<sup>c</sup>. The reason is repeatedly demonstrated higher susceptibility of PrP<sup>PSC</sup>-overexpressing cells to apoptotic agents such as staurosporine. Although it is known that a pro-apoptotic phenotype of PrP<sup>PSC</sup>-overexpressing cells following the treatment with staurosporine is controlled through a p53 pathway, other proteins involved in the process as well as their phosphorylation patterns are widely unknown.

An important issue of this and similar studies is the comparison of data obtained either by proteomics or transcriptome analysis in different experimental models. This is mandatory in order to filter candidate proteins that might play a decisive role in executing PrP<sup>c</sup> physiological function. In our opinion, ANXA5 could be one of the candidates considering its up-regulation by PrP<sup>c</sup> in this study, using
three different cell lines, two different vectors and two different transfection procedures, in our previous study (Ramljak et al., 2008) and after gene expression profiling in the frontal cortex of sCJD patients (Xiang et al., 2005).

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This study is dedicated to the memory of Prof. Dr. Victor W. Armstrong an outstanding scientist and a dear colleague.

REFERENCES


3. DISCUSSION

3.1. Codon 129 polymorphism specific CSF proteome pattern in sCJD

Disease phenotype of sCJD is mainly influenced by the methionine/valine (M/V) polymorphism at codon 129 in the human prion protein gene (PRNP gene) and by the presence of two major types of pathological, protease-resistant forms of the prion protein (PrP\textsuperscript{Sc}) leading to 2 different profiles in Western blot (type 1 and type 2). The clinical and pathological characteristics of these molecular subtypes markedly differ with respect to symptoms at onset, localisation and type of the pathological changes as well as PrP\textsuperscript{Sc} deposition pattern (Parchi et al., 1999; Gambetti et al., 2003). This might suggest the involvement of different molecular pathways in sCJD pathogenesis.

At the molecular level, TSEs are caused by the conversion of a host cellular glycoprotein, the prion protein (PrP\textsuperscript{C}) into a pathological conformer called scrapie prion protein (PrP\textsuperscript{Sc}), which tends to aggregate and accumulate in the brain tissue. The presence of the pathological form of PrP may induce brain proteome changes. In turn, these alterations may also be reflected in CSF.

However, due to a high dynamic range of protein abundance in CSF spanning twelve orders of magnitude (Anderson et al., 1998) and the fact that around 10 very well known proteins (e.g. albumin, immunoglobulins, transferrin and haptoglobin) comprise up to 90-95% of the total protein contents, depletion of the high abundant proteins is indispensible for in-depth exploration of sCJD-associated alterations in CSF proteome.

The densitometric and statistical analysis of CSF 2D maps showed up-regulation of 27 and down-regulation of 3 proteins in MM-sCJD as well as up-regulation of 24 proteins in VV-sCJD in comparison to non-demented neurological controls. Twenty one proteins showed common up-regulation for both codon 129 polymorphism in sCJD. For instance, the leucine-rich alpha-2-glycoprotein (LRG), complement C4 and superoxide dismutase [Cu-Zn] (SOD-1), which belong to the group of MM-sCJD dysregulated proteins, were already shown to be implicated in the pathobiology of PrP (Milhavet et al., 2002; Milhavet et al., 2000; Mabbott et al., 2004; Xiang et al., 2004). The group of VV-sCJD specifically regulated proteins includes DJ-1 protein, fibrinogen alpha chain (FGA) and angiotensinogen (ANGT). The detection of regulated proteins specific for codon 129 genotype may confirm the hypothesis which assumes the involvement of different molecular pathways in sCJD pathogenesis depending on PrP\textsuperscript{Sc} strain. Moreover, the dominant up-regulation of proteins in CSF from sCJD-affected
patients may occur due to release or secretion mechanisms of the protein content from impaired neural cells into the extracellular space and then eventually into CSF. However, this far-reaching hypothesis has to be proved by further investigations.

3.3. Implication of glycolytic enzymes in sCJD pathology

Almost 40% of sCJD specific regulated proteins in CSF are involved in glucose metabolism, thus in energy metabolism. All these enzymes displayed up-regulation in sCJD regardless of codon 129 polymorphism. The increase in CSF level of glucose-6-phosphate isomerase (G6PI), lactate dehydrogenase (LDH) and fructose-bisphosphate aldolase A (ALDOA) was confirmed on a larger group of sCJD patients including three possible codon 129 polymorphisms as well as on three different types of controls consisting of non-demented neurological controls (NDC), patients with vascular dementia (VD) and Alzheimer’s disease (AD). Subsequently, abundance of glycolytic enzymes in the brain as well as their cellular localization was determined.

Additionally to the increased level of G6PI in CSF, its decreased level was found in the brain of MM1-sCJD subtype. Moreover, in NDC and AD control groups, G6PI is predominantly localized in neurons, while in sCJD a shift in cellular distribution is occurring and G6PI is mostly present in reactive astrocytes. With these data we demonstrate for the first time the involvement of G6PI in prion-induced pathological process. Furthermore, different regulation of G6PI in the brain could suggest that its expression and/or secretion may be modulated in a PrPSc strain-dependent manner.

For ALDOA and LDH increase in CSF level did not correlate with unchanged level in the brain when compared to age-matched control group. Moreover, partial shift in ALDOA cellular distribution was observed in the sCJD. Diffuse ALDOA immunostaining was predominantly found in neurons in the grey matter and in astrocytes in the white matter of NDC and AD, whereas, the ALDOA immunoreactivity in the sCJD was mostly detected in neuronal cell bodies.

Beside their glycolytic function, all three investigated enzymes play other role in cellular physiology. For instance, G6PI possesses an activity of extracellular cytokine and acts as a growth factor promoting the survival and neurite outgrowth of motor and sensory neurons, as well as an autocrine motility factor inducing cell motility (Gurney et al., 1986; Niinaka et al., 1998; Watanabe et al., 1996). Furthermore, it has been shown that the inhibition of G6PI expression caused an increased susceptibility to caspase-dependent apoptosis in PC12 neuronal cells as well as its overexpression and subsequent secretion of G6PI protected NIH-
3T3 fibroblasts against PI3K/Akt mediated apoptosis (Romagnoli et al., 2003; Tsutsumi et al., 2003). This finding indicates anti-apoptotic properties of G6PI. Moreover, the positive G6PI immunostaining in astrocytes occurred two weeks after mechanical injury of murine cerebral cortex and its expression was shown to be elevated in the later stage of the brain infection with rabies virus in C3H mice model (Decourt et al., 2005; Prosniak et al., 2003). This may suggest that expression of G6PI in astrocytes can be a response to signals coming from impaired neurons to increase their metabolism and prolong survival. Taken together, the same motion of G6PI action might occur in sCJD-affected brain, but might be differentially modulated by different PrPSc strains.

LDH is an interaction partner of PrPC and its expression was found to be up-regulated when PrPC was introduced into Prnp0/0 cells (Ramljak et al., 2008; Rutishauser et al., 2009; Watts et al., 2009). Moreover, PrPC deficiency in astrocytes leads to glutamate-independent elevation of lactate transport (Kleene et al., 2007). Therefore, it should be considered that LDH is possibly not only a marker of cellular damage, but could play a more specific role in prion-induced pathological processes. We hypothesize that a loss or gain of PrP function in astrocytes of CJD-affected brain may result in elevated level of extracellular lactate leading to acidosis-induced damage in neurons.

It has been shown, that glyceraldehyde-3-phosphate, an enzymatic product of ALDOA, is implied in the protection of cells against apoptosis via the inhibition of caspase-3 activity (Jang et al., 2009). Moreover, neurotoxicity of doppel (Dpl), PrPC homolog, in Purkinje cells of the Ngsk Prnp0/0 mouse model varies according to aldolase C expression. The higher sensitivity to Dpl-induced neurotoxicity and subsequent increased cell loss, is characteristic for Purkinje cells from aldolase C-negative compartments of the cerebellum (Heitz et al., 2008). Therefore, the presence of ALDOA in impaired neurons and its secretion might play an anti-apoptotic role in sCJD.

Both, glycolysis and apoptosis are highly conserved and finely regulated multi-step processes maintaining cellular homeostasis. The activation of apoptotic pathway is dependent on energy status and, hence, apoptosis might be dependent on glucose metabolism. Based on numerous studies demonstrating that glucose metabolism is involved in cell death and survival, it is reasonable to speculate that these two crucial processes are linked. However, precise mechanisms underlying the regulation of glucose metabolism and the implication of glycolytic enzymes in apoptosis remain to be elucidated.
3.3. Disturbances in cellular homeostasis upon stably PrP\textsuperscript{C}-overexpression in human neuroblastoma SH-SY5Y cells

Differential proteome analysis of human neuroblastoma SH-SY5Y cells stably overexpressing human PrP\textsuperscript{C} was performed to study protein expression changes upon permanent overproduction of PrP\textsuperscript{C}.

Densitometric analysis of silver stained 2D gels showed 18 differentially regulated proteins in SH-SY5Y cells overexpressing human PrP\textsuperscript{C}. Between them, 13 proteins were up- and 5 down-regulated. Few biological processes, especially, signal transduction, cytoskeleton organization and protein folding are influenced by overproduction of PrP\textsuperscript{C} in SH-SY5Y cells.

To reinforce obtained results, the changes in expression were confirmed by Western blot for three selected proteins: annexin A5 (ANXA5), transgelin-2 (TAGL2) and 14-3-3 protein gamma (1433G). Additionally, in order to further verify our data, densitometric analysis of Western blots following transient overexpression of PrP\textsuperscript{C} in mouse neuroblastoma N2a and alternative human neuroblastoma SK-N-LO cells was performed. In both cell lines up-regulation of ANXA5 was found as compared to cells transfected with control vector. For two remaining proteins, TAGL2 and 1433G, changes of their expression were reproduced neither in N2a nor in SK-N-LO cells.

In different cell lines, PrP\textsuperscript{C}-overproduction was shown to increase expression of the tumor suppressor oncogene p53 (Paitel et al., 2002; Paitel et al., 2003). Therefore, supplemental Western blot analysis was performed in order to verify p53 expression in stably PrP\textsuperscript{C} overexpressing SH-SY5Y cells. However, no enhancement in p53 production following stable PrP\textsuperscript{C} overexpression was detected.

To the proteins involved in signal transduction which expression was modulated by stable PrP\textsuperscript{C} overexpression in SH-SY5Y cells belong: 14-3-3 protein gamma (1433G), growth factor receptor bound protein 2 (GRB2), Rho GTP-ase activating protein 1 (RHG01), and platelet-activating factor acetylhydrolase IB subunit gamma (PA1B3).

Proteins belonging to the 14-3-3 family modulate diverse signal transduction pathways regulating cell cycle progression and apoptosis (Fu et al., 2000). The 14-3-3 protein gamma is a well known as CSF diagnostic marker for differential diagnosis of CJD (Van Everbroeck et al., 2005; Zerr et al., 1996) and expression of other 14-3-3 proteins (beta, eta and zeta isoform) was found to be decreased in sCJD-affected brain (Xiang et al., 2005). Moreover, 14-3-3 proteins are interaction partners forming molecular complexes with PrP\textsuperscript{C} (Satoh et al., 2006; Watts et al., 2009). Therefore, 14-3-3 proteins may have an impact on the physiological function of PrP\textsuperscript{C}.
Growth factor receptor-bound protein 2 (GRB2) is another signaling protein which was found to be up-regulated in stable PrP<sup>C</sup> overexpressing SH-SY5Y cells. This protein connects signals from extracellular/transmembrane receptors with intracellular signaling molecules and is crucial for the formation of signaling complexes. Furthermore, yeast two hybrid system and co-precipitation in neuronal microsomal vesicles showed that GRB2 directly interact with PrP<sup>C</sup> (Spielhaupter and Schatzl, 2001).

To the proteins involved in cytoskeleton organization and dysregulated through PrP<sup>C</sup> overexpression, belong: transgelin 2 (TAGL2), translationally-controlled tumor protein (TCTP) and Rho GTP-ase activating protein 1 (RHG01).

The involvement of PrP<sup>C</sup> in the regulation of microtubule dynamics and reorganization of actin cytoskeleton has been already reported (Dong et al., 2008; Málaga-Trillo et al., 2009). TAGL2, which displayed enormous up-regulation in Western blot analysis, is involved in the organization and stability of the actin cytoskeleton (Goodman et al., 2003). Interestingly, transcriptome studies following induction of amyloid precursor protein intracellular domain expression in human neuronal cells revealed increased expression of the gene encoding TAGL. Moreover, TAGL was significantly higher expressed in AD brain as compared to age-matched controls (Müller et al., 2007). These findings suggest that TAGL2 might play some role in PrP pathophysiology.

TCTP, which exhibits properties of tubulin-binding protein and is associated with microtubules in cell cycle-dependent manner, showed up-regulation induced by PrP<sup>C</sup>-overexpression in SH-SY5Y cells. Its elevated level leads to microtubule rearrangements (Gachet et al., 1999). In contrast, recombinant PrP stimulates tubulin oligomerization and thereby inhibits microtubule assembly (Nieznanski et al., 2006). Moreover, TCTP was found to interact with the third cytoplasmic domain of Na<sup>+</sup>/K<sup>+</sup>-ATP-ase alpha subunit in yeast two-hybrid system and inhibits it in dose-dependent manner (Jung et al., 2004). Interestingly, the third cytoplasmic domain of Na<sup>+</sup>/K<sup>+</sup>-ATP-ase alpha subunit is also an interaction partner of PrP<sup>C</sup> (Petrakis and Sklaviadis, 2006).

To the proteins involved in the signal transduction which expression was modulated by stable PrP<sup>C</sup> overexpression in SH-SY5Y cells belong: 40 kDa peptidyl-prolyl cis-trans isomerase (PPID), FK506-binding protein 4 (FKBP4), and disulfide isomerase precursor (PDIA1).

Due to the fact that prion diseases are triggered by accumulation of misfolded PrP, the influence of PrP<sup>C</sup> overexpression on proteins involved in protein folding is of special interest.

The expression of PDIA1, a chaperone catalyzing the isomerisation of intra- and
intermolecular disulfide bonds, was up-regulated in the analyzed cell model. Interestingly, an overexpression of PDIA1 was also found in sCJD-affected brain (Yoo et al., 2002). PPID, also known as cyclophilin 40, showed up-regulation upon PrP\(^C\)-overexpression. It has been already shown that cyclophilins play a role in PrP\(^C\) metabolism. The inhibition of the cyclophilin family of peptidyl-propyl isomerases in cultured cells leads to the accumulation of proteasome-resistant scrapie-like PrP aggregates (Sunderland et al., 2003).

### 3.4. Annexin A5 as molecular partner for PrP pathobiology

The ANXA5 is the only protein which was up-regulated upon PrP\(^C\)-overexpression in all three different neuroblastoma cells used in our study as well as in a previous work where PrP\(^C\) was transiently overexpressed in HEK 293 cells (Ramljak et al., 2008). Therefore, PrP\(^C\)-mediated ANXA5 regulation appears neither cell type specific, vector specific nor dependent on transfection mode. Moreover, up-regulation of ANXA production was found in global gene expression profiling of sCJD-affected brain (Xiang et al., 2005). ANXA5 belongs to the family of calcium-dependent phospholipid-binding proteins, but its physiological role is still poorly understood. *In vitro* experiments showed that ANXA5 could be involved in apoptosis, blood coagulation as well as calcium singaling and transport (Yoshizaki et al., 1992; Gerke and Moss, 1997; Reutelingsperger et al., 1997). ANXA5 is also vulnerable to form voltage-dependent calcium channels in phospholipid bilayer (Huber et al., 1992; Liemann et al., 1996). Additionally to its cytoplasmic localization, ANXA5 can be secreted or can bind to phosphaditylserine on the outer cell surface. The presentation of phosphaditylserines on the cell surface occurs during apoptosis and it is a recognition signal for phagocytes. This process can provoke unnecessary coagulation and inflammatory response. ANXA5 can shield surface-exposed phosphaditylserines, thus it can inhibit their pro-coagulant and pro-inflammation activity. However, it does not block completely phagocytosis of apoptotic bodies which can still be efficiently utilized (van Engeland et al., 1996; Reutelingsperger et al., 1997). Taken all together, ANXA might play a role in the pathophysiology of PrP.

In conclusion, stable PrP\(^C\) overexpression in SH-SY5Y neuroblastoma cells disturbs cellular homeostasis, especially processes associated with cell signaling, cytoskeletal organization and protein folding. However, sole PrP\(^C\)-overexpression is not enough to alter p53 expression and possibly activates p53-dependent apoptotic pathway.
The comparison between data obtained from different proteomic and transcriptomic experimental models allows filtering candidate proteins that might play a decisive role in the physiology and the pathology of PrP\textsuperscript{C}. In our opinion, ANXA5 could be one of these candidates.
4. SUMMARY

This thesis includes 2 original publications that investigate the physiology and the pathology of the human prion protein.

In our first study, we analyzed CSF proteome alterations in living, symptomatic sCJD patients with two different codon 129 genotypes (MM and VV) using a proteomic approach consisted of 2D Fluorescence Difference Gel Electrophoresis (2D-DIGE) and mass spectrometry analysis.

Densitometric analysis of 2D gels showed the up-regulation of 27 and down-regulation of 3 proteins in the MM-sCJD as well as the up-regulation of 24 proteins in the VV-sCJD when compared to control. Beside proteins showing common regulation for both codon 129 genotypes in sCJD, some proteins seem to be specifically regulated in certain genotype. The detection of these proteins may confirm the hypothesis which assumes the involvement of different molecular pathways in sCJD pathogenesis depending on PrP<sup>Sc</sup> strain.

Almost 40% of sCJD specifically regulated proteins in CSF are involved in glucose metabolism, regardless of codon 129 polymorphism. The validation phase of selected glycolytic enzymes using Western blot technique confirmed up-regulation of ALDOA, LDH and G6PI when compared to three different control groups (NDC, AD and VD).

Furthermore, the immunolabeling showed that G6PI is present in reactive astrocytes in sCJD affected brain while it is predominantly localized in neurons in age-matched control brain. Additionally, decreased level of G6PI was found in the brain of MM1-sCJD subtype. With these data, we have demonstrated for the first time the implication of G6PI in prion-induced pathology.

The identification of sCJD-regulated proteins in CSF proteome alterations in living, symptomatic sCJD- patients will broaden our knowledge about pathological processes occurring in sCJD, as they are still not fully understood. Moreover, they could serve as protein source to identify novel biomarkers for differential sCJD diagnosis.

In our second study, we also applied a proteomic approach in order to reveal proteins, and thereby biological processes, affected by stable overexpression of human PrP<sup>C</sup> in human neuroblastoma SH-SY5Y cell line.

Densitometric analysis of silver stained 2D gels showed 18 differentially regulated proteins in SH-SY5Y cells overexpressing human PrP<sup>C</sup>. Between them, 13 proteins were up- and 5...
down-regulated. The PrP\textsuperscript{C} overexpression in SH-SY5Y cells affected mostly few biological processes such as signal transduction, cytoskeleton organization and protein folding. An increased expression of tumor suppressor oncogene p53 caused by PrP\textsuperscript{C} overproduction was already demonstrated in different cell lines (Paitel et al., 2002; Paitel et al., 2003). Therefore, supplemental Western blot analysis was performed to verify p53 expression in SH-SY5Y cells. However, no enhancement in its production following stable PrP\textsuperscript{C} overexpression was detected.

The ANXA5 is the only one protein which was up-regulated upon PrP\textsuperscript{C} overexpression in three different neuroblastoma cells, and in a previous study where PrP\textsuperscript{C} was transiently overexpressed in HEK293 cells (Ramljak et al., 2008). Therefore, PrP\textsuperscript{C}-mediated ANXA5 regulation appears neither cell type specific, vector specific nor dependent on transfection mode. ANXA5 belongs to the family of calcium and phospholipid-binding proteins with poorly understood physiological role. However, ANXA5 up-regulation was found in gene expression profiling of sCJD-affected brain (Xiang et al., 2005) suggesting its possible role in the pathophysiology of sCJD.

In conclusion, stable PrP\textsuperscript{C} overexpression in SH-SY5Y neuroblastoma cells is disturbing cellular homeostasis, especially processes associated with cell signaling, cytoskeletal organization and protein folding. However, sole PrP\textsuperscript{C} overexpression is not enough to alter p53 expression and possibly activate p53-dependent apoptotic pathway.

The comparison between data obtained from different proteomic and transcriptomic experimental models allows filtering candidate proteins that might play a decisive role in the physiology and the pathology of PrP\textsuperscript{C}. In our opinion, ANXA5 could be one of these candidates.

Proteomics gives a unique opportunity to analyze both physiological and pathological processes at the protein level on a global scale. Proteome analysis of SH-SY5Y human neuroblastoma cells stably overexpressing PrP\textsuperscript{C} revealed proteins whose expression is directly modulated through PrP\textsuperscript{C} and consequently physiological processes which are influenced by PrP\textsuperscript{C} level in cells. On the other hand side, identification of CSF proteome alterations in sCJD provides more information about pathological processes occurring in the brain and caused by presence of PrP\textsuperscript{Sc}. Both these studies broaden our knowledge about still not fully understood pathobiology of PrP.
4. LITERATURE


Mallucci GR, Ratte S, Asante EA, Linehan J, Gowland I, Jefferys JG, Collinge J, (2002). Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. EMBO J 21, 202-210


6. LIST OF PUBLICATIONS


6. CURRICULUM VITAE

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