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Altered gene transcription linked to astrocytes and oligodendrocytes in frontal cortex in Creutzfeldt-Jakob disease

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ABSTRACT

Targeted expression of genes coding for proteins specific to astrocytes, oligodendrocytes and myelin was performed in frontal cortex area 8 of Creutzfeldt-Jakob disease methionine/methionine and valine/valine (CJD MM1 and VV2, respectively) compared with controls. *GFAP* (glial fibrillary acidic protein) mRNA was up-regulated whereas *SLC1A2* (solute carrier family 1 member 2, coding for glutamate transporter 1: GLT1), *AQ4* (aquaporin 4), *MPC1* (mitochondrial pyruvate carrier 1) and *UCP5* (mitochondrial uncoupled protein 5) mRNAs were significantly down-regulated in CJD MM1 and CJD VV2, and *GJA1* (connexin 43) in CJD VV2. *OLIG1* and *OLIG2* (oligodendocyte transcription factor 1 and 2, respectively), *SOX10* (SRY-Box10) and oligodendroglial precursor cell (OPC) marker *NG2* (neuronal/glial antigen) 2 were preserved, but *GALC* (coding for galactosylceramidase), *SLC2A1* (solute carrier family 2 member 1: glucose transporter member 1: GLUT1) and *MCT1* (monocarboxylic acid transporter 1) mRNA expression levels were significantly reduced in CJD MM1 and CJD VV2. Expression levels of most genes linked to myelin were not altered in the cerebral cortex in CJD. Immunohistochemistry to selected proteins disclosed individual variations but GFAP, Olig-2, AQ4 and GLUT1 correlated with mRNA levels, whereas GLT1 was subjected to individual variations. However, MPC1, UCP5 and MCT1 decrease was more closely related to the respective reduced neuronal immunostaining. These observations support the idea that molecular deficits linked to energy metabolism and solute transport in astrocytes and oligodendrocytes, in addition to neurons, are relevant in the pathogenesis of cortical lesions in CJD.

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Introduction

Prion diseases are a group of transmissible encephalopathies linked to the prion protein (PrP^{C}) which is converted into an abnormally conformed proteinase K-resistant protein named prion (PrP^{SC}). The human prion diseases are sporadic, iatrogenic and genetic Creutzfeld-Jakob disease (sCJD, iCJD and gCJD, respectively), Gerstmann-Straüsler-Scheinker disease (GSS) disease, and fatal familial insomnia (FFI). gCJD, GSS and FFI are due to mutations in prion protein gene (*PRNP*) [1–7]. The main prionopathies in animals are scrapie (typical and atypical) in sheep and goats; chronic wasting disease in deer, elk, moose and reindeer; and bovine spongiform encephalopathy (BSE) in cattle [8].Variant CJD (vCJD) is considered a form of BSE transmitted to humans [9]. CJD is not homogeneous; different subtypes are distinguished in sCJD depending on the prion type (I and 2) and the composition of codon 129 in *PRNP* (methionine/methionine: MM, valine/valine: VV, and methionine/valine: MV), each one with singular clinical phenotypes [10,11]. The most frequent subtypes are sCJD MM1 and sCJD VV2.

Common neuropathological lesions in CJD are neuron loss, spongiform change and deposition of PrP^{SC}. Additional characteristics of sCJD MM1 are microvacuolar spongiform change in the frontal and occipital cortices, and molecular layer of the cerebellum, and synaptic-like PrP^{SC} deposition. Particular features of sCJD VV2 are microvacuolar or confluent spongiform change, major involvement of the cerebellum, and synaptic, perineuronal and plaque-like PrP^{SC} deposits [4–7]. Reactive astrocytosis and microgliosis, accompanied by a robust molecular inflammatory response, are important accompanying features [12–15].

Besides their contribution to astrocytic gliosis and inflammation, little is known about the defects of astrocytes in CJD. In addition to neuronal bodies, dendrites, synapses and axons, PrP^{SC} can be present in astrocytes

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and microglia [16]. Aquaporin 1 and 4 immunoreactivity is increased [17,18], thus suggesting adaptation to water transport. Moreover, recent studies have shown that human astrocytes have the capacity to take up and degrade normal and protease-resistant prion protein [19] and that they can transfer PrP^{SC} to neurons via nanotubules [20] thereby contributing to prion disease progression.

Regarding oligodendrocytes, less information is available in CJD. Oligodendrocytes are apparently resistant to PrP^{SC} infectivity [21]. However, axon and myelin damage occurs in prion diseases [22], abnormal interactions between oligodendroglia and astrocytes are found in experimental CJD and scrapie [23], and engulfment of oligodendrocytes by hypertrophic astrocytes has been reported in the white matter in CJD [24]. PRP^{SC} is also localized as arrays adjacent to myelin fibers in the cerebrum and cerebellum in CJD [25].

Based on these data, the present study was undertaken to gain understanding about transcription alteration of genes encoding proteins specifically expressed in astrocytes and oligodendrocytes in frontal cortex area 8 of CJD MM1 and CJD VV2. Selected genes include those encoding structural proteins of astrocytes and myelin, those involved in energy metabolism and axon maintenance, and genes of connexins of the gap junctions between oligodendrocytes and astrocytes. Protein expression of altered has been assessed by immunohistochemistry.

Results

Astrocytic markers

Covariance analysis (ANCOVA) of *GFAP* (coding for glial fibrillary acidic protein) mRNA expression levels revealed a significant group effect [F(2,19) = 4.383, $P = 0.027$] but did not show RIN effect [F(1,19) = 0.337, $P = 0.568$]. Subsequent one-way ANOVA analysis [F(2,20) = 7.918, $P = 0.0029$] revealed significant increase in CJD MM1 and CJD VV2 when compared with controls ($P = 0.012$ and $P = 0.005$, respectively). In contrast, ANCOVA of *ALDH1L1* (coding for *a*ldehyde dehydrogenase 1, family member L1) mRNA, used as marker of total astrocytes, had non-significant group [F(2,22) = 1.087, $P = 0.355$] and RIN [F(1,22) = 0.021, $P = 0.887$] effects; no differences of *ALDH1L1* mRNA were observed between CJD and controls. ANCOVA of *AQP4* (coding for aquaporin 4) mRNA showed significant group effect [F(2,21) = 4.307, $P = 0.027$] but not RIN effect [F(1,21) = 0.334, $P = 0.569$]. Subsequent one-way ANOVA analysis [F(2,22) = 8.596, $P = 0.0017$] showed significant decreased expression in CJD MM1 and CJD VV2 when compared with controls ($P = 0.26$ and $P = 0.002$, respectively). ANCOVA analysis of *SLC1A2* (coding for glutamate transporter 1) mRNA expression showed significant group effect [F(2,21) = 6.031, $P = 0.009$] but not RIN effect [F(3,25) = 0.754, $P = 0.395$]. One-way ANOVA analysis [F(1,21) = 8.865, $P = 0.0015$] revealed significant down-regulation reduction of *SLC1A2* in CJD VV2 when compared with controls ($P = 0.001$) and with CJD MM1 ($P = 0.028$).

ANCOVA of *MCT4* (coding for solute carrier family member 16: monocarboxylic acid transporter member 4) mRNA expression revealed no significant changes with respect to group [F(2,20) = 2.110, $P = 0.147$] and RIN [F (1,20) = 0.195, $P = 0.664$] effect. Conversely, ANCOVA of *MPC1* (coding for mitochondria pyruvate carrier 1) and *UCP5* (coding for mitochondrial anion carrier, mitochondrial uncoupling protein 5) showed significant group effect [F(2,21) = 6.282, $P = 0.007$] and [F(2,21) = 9.103, $P = 0.001$], respectively, as well as RIN effect [F(1,21) = 4.972, $P = 0.037$] and [F(1,21) = 8.734, $P = 0.008$], respectively. Next, ANCOVA revealed significantly decreased expression of *MPC1* and *UCP5* in CJD MM1 ($P = 0.019$ and $P = 0.008$ respectively) and CJD VV2 ($P = 0.002$ and $P = 0.001$, respectively) when compared with controls. ANCOVA of *UCP4* mRNA (coding for mitochondrial uncoupling protein 4) did not reveal significant alterations related to group [F(2,20) = 0.009, $P = 0.991$] but to RIN effects [F(1,20) = 11.258, $P = 0.003$].

Finally, the expression of *GJB6* (coding for connexin 30), which was preserved (although with a trend to decrease in CJD VV2), was not dependent on group [F(2,19) = 2.418, $P = 0.113$] or RIN [F(1,19) = 1.158, $P = 0.294$] effect. ANCOVA of *GJA1* (coding for connexin 43) mRNA expression indicated significant group effect [F(2,21) = 8.218, $P = 0.002$] but not RIN effect [F(1,21) = 4.105, $P = 0.056$]. Subsequent one-way ANOVA analysis [F(2,22) = 8.224, $P = 0.002$] revealed significantly reduced *GJA1* expression in CJD VV2 when compared with controls ($P = 0.007$) and with CJD MM1 ($P = 0.004$) (Figure 1(a)).

Oligodendrocytic markers

ANCOVA showed no group or RIN effect on mRNA expression levels of the oligodendroglial markers *OLIG1* [F(2,23) = 0.221, $P = 0.804$] and [F(1,23) = 0.336, $P = 0.568$], *OLIG2* [F(2,23) = 0.801, $P = 0.461$] and [F (1,23) = 0.276, $P = 0.604$], and *SOX10* [F(2,23) = 1.369, $P = 0.274$] and [F(1,23) = 3.930, $P = 0.059$]; and the OPC marker *NG2* [F(2,23) = 0.412, $P = 0.667$] and [F (1,23) = 0.001, $P = 0.978$]. Expression levels of *OLIG1*, *OLIG2* and *NG2* were similar in CJD and controls.

Likewise, ANCOVA showed no alterations in the expression levels of genes coding for different myelin

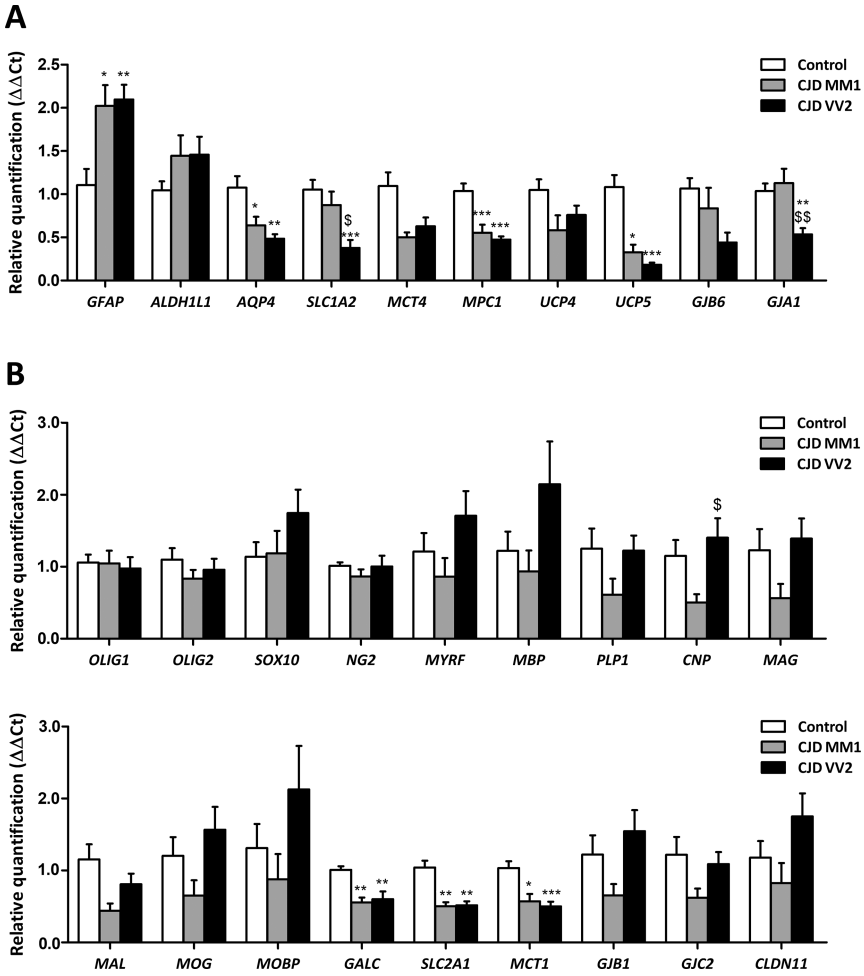

Figure 1. Expression levels, as revealed by RT-qPCR, of genes coding for specific proteins of astrocytes (a) and oligodendrocytes (b) in frontal cortex area 8 of CJD MM1 and CJD VV2 compared with expression levels in controls. Statistical analysis of the expression data between groups uses one-way analysis of variance (ANOVA) followed by Tukey post-test for *GFAP, AQP4, SLC1A2, MPC1, UCP4, GJB6, GJA1, OLIG1, OLIG2, NG2, CNP, MAL, GALC, MCT1, GJB1* and *GJC2*. Kruskal-Wallis test followed by Dunns post-hoc test was used for *ALDH1L1, MCT4, UCP5, SOX10, MYRF, MBP, PLP1, MAG, MOG, MOBP, SLC2A1* and *CLDN11* (SPSS software. IBM SPSS Statistics for Windows, Version 21.0). All data are expressed as mean ± SEM. Differences between groups are considered statistically significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when comparing CJD cases with controls; and set at $ $P < 0.05$ and $$ $P < 0.01$ when comparing CJD MM1 with CJD VV2.

proteins, such as *MYRF* (coding for myelin regulatory factor), *MBP* (coding for myelin basic protein), *PLP1* (coding for proteolipid protein 1), MAL (coding for Mal), *MOG* (myelin oligodendrocyte glycoprotein) and *MOBP* (myelin-associated oligodendrocyte basic protein) due to group effect [F (2,23) = 2.692, P = 0.0.89], [F(2,22) = 2.214, P = 0.133], [F(2,23) = 2.356, P = 0.117], [F (2,23) = 3.373, P = 0.052], [F(2,23) = 3.146, P = 0.062] and [F(2,23) = 2.409, P = 0.112], respectively, or due to RIN effect [F(1,23) = 3.664, P = 0.068], [F(1,22) = 0.918, P = 0.349], [F (1,23) = 0.991, P = 0.330], [F(1,23) = 0.311, P = 0.582], [F(1,23) = 1.968, P = 0.174] and [F (1,23) = 3.916, P = 0.060], respectively. However, ANCOVA analysis revealed significant group effect on *CNP* (coding for 2′,3′-cyclic nucleotide 3′ phosphodiesterase) and *MAG* (myelin associated glycoprotein) mRNA expression [F(2,21) = 3.970, P = 0.034] and [F(2,22) = 3.786, P = 0.039], respectively; but not RIN effect [F(1,21) = 1.697, P = 0.207], [F(1,22) = 3.307, P = 0.083], respectively. Subsequent analysis revealed no differences in *CNP* [F (2,22) = 2.328, P = 0.12] and *MAG* [KG (2,23) = 5.391, P = 0.0675] mRNA expression levels between CJD and controls. Regarding *GALC* (coding for galactosylceramidase), covariance analysis showed a significant effect of group [F(2,21) = 12.680, P = 0.000] and RIN [F(1,21) = 10.171, P = 0.004]. ANCOVA's contrast analysis revealed significant *GALC* mRNA down-regulation in CJD MM1 and CJD VV2 (P = 0.001 and P = 0.000, respectively).

ANCOVA applied to expression levels of *SLC2A1* (coding for solute carrier family 2: glucose transporter, member 1) and *MCT1* (coding for solute carrier family 16: monocarboxylic acid transporter, member 1) revealed significant group effect [F(2,22) = 9.304, $P = 0.001$] and [F(2,21) = 3.468, $P = 0.050$], respectively; but no RIN effect [F(1,22) = 0.034, $P = 0.855$] and [F(1,21) = 3.924, $P = 0.061$], respectively. Post-variance analysis of *SLC2A1* and *MCT1* ([KG (2,23) = 15.26, $P = 0.0005$] and [F(2,22) = 11.333, $P = 0.000$], respectively) showed significant *SLC2A1* and *MCT1* decrease in CJD MM1 ($P = 0.01$ and $P = 0.04$, respectively) and CJD VV2 ($P = 0.01$ and $P = 0.001$, respectively) when compared with control group.

Finally, ANCOVA of expression of genes coding for connexins 32 and 47 (*GJB1* and *GJC2*), and claudin 11 (*CLDN11*) did not reveal significant group [F (2,22) = 3.271, $P = 0.057$], [F(2,23) = 2.461, $P = 0.107$] and [F(2,23) = 2.608, $P = 0.095$], and RIN [F(1,22) = 1.326, $P = 0.262$], [F(1,23) = 0.730, $P = 0.402$] and [F(1,23) = 0.719, $P = 0.405$] effects, respectively. *GJB1, GJC2* and *CLDN11* expression was not modified in CJD, although there was a trend to decrease in CJD MM1, when compared with controls (Figure 1(b)).

Immunohistochemistry

Paraffin sections processed for immunohistochemistry showed marked increase in GFAP immunoreactivity in CJD when compared with controls, as expected. GFAP-immunoreacytive astrocytes were increased in number, and the amount of GFAP was increased per astrocyte with robust immunostaining of astrocyte processes (Figure 2). In contrast, OLig-2 immunostaining in the cerebral cortex did not reveal major differences between CJD and controls (Figure 2). Aquaporin 4 (AQP4) immunoreactive localized in astrocytes delineating fine and varicose network processes in control cerebral cortex. AQP4 immunoreactivity in CJD was variable from one case to another yet preserving the same morphological profile (Figure 2). In advanced cases, AQP4 immunoreactivity was increased and blurred as reported elsewhere. GLT1 immunoreactivity in control cases was seen as delicate meshwork of fine and varicose processes around the nuclei of astrocytes. This pattern was preserved in CJD, although with marked differences from one case to another; in some cases GLT1 immunoreactivity practically disappeared in areas with severe spongiform change while it was practically preserved in others; Moreover variations were also observed in different areas in the same tissue section (Figure 2). MPC1 was mainly localized in the

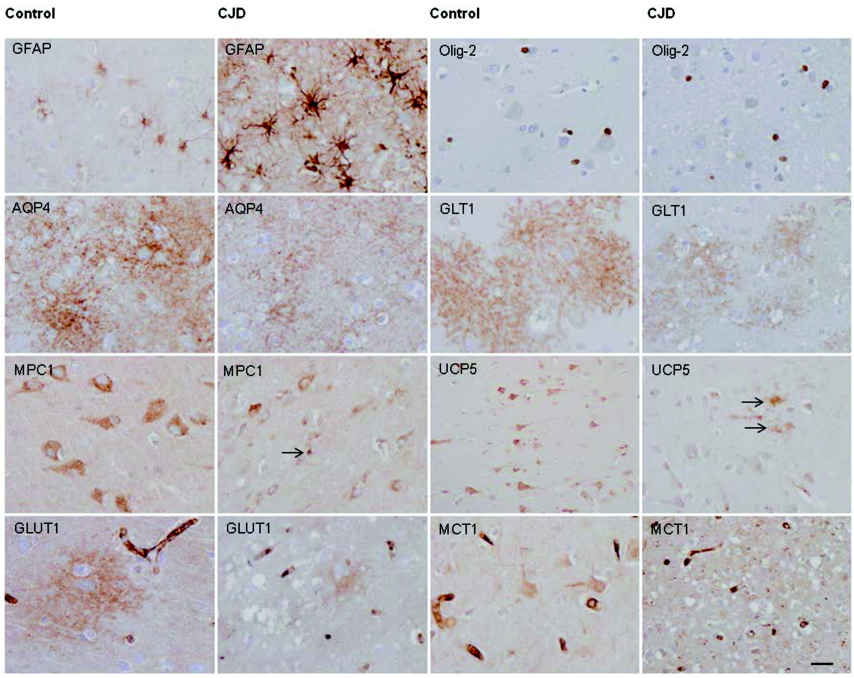

Figure 2. Representative images of glial fibrillary acidic protein (GFAP), Olig-2, aquaporin 4 (AQP4), solute carrier family 1, member 1 (SLC1A2: glial affinity glutamate transporter: GLT1), mitochondrial pyruvate carrier 1 (MPC1), mitochondrial uncoupling protein 5 (UCP5), solute carrier family 2 member 1 (glucose transporter 1: GLUT1) and solute carrier family 16 member 1 (monocarboxylic acid transporter 1: MCT1) immunoreactivity in the frontal cortex area 8 of controls and cases with CJD MM1. Paraffin sections processed for immunohistochemistry and slightly stained with hematoxylin, bar = 25 µm.

cytoplasm of neurons in the cerebral cortex of control cases as a punctuate immunostaining consistent with a mitochondrial localization. MPC1 immunoreactivity was dramatically reduced in neurons in CJD but increased in a few cortical glial cells (Figure 2). Similarly, UPC5 immunostaining mainly decorated neurons in controls. However, UPC5 immunoreactivity decreased in neurons but increased in glial cells, mainly in cells with astrocyte morphology, in CJD (Figure 2).

GLUT1 was mainly found in the walls of small blood vessels consistent with endothelial localization as expected. In addition, GLUT1 immunoractivity was found as blurred patches in the cortical neuropil in control cases; these patches were markedly reduced in size and intensity in CJD (Figure 2). Finally, MCT1 was mainly localized in endothelial cells and to a lesser degree in neurons and glial cells in controls. Marked reduction in MCT1 immunoractivity was found in neurons and glial cells, but not in endothelial cells, in CJD (Figure 2).

Discussion

The present study is focused on astrocytes and oligodendrocytes of the frontal cortex area 8 in CJD MM1 and VV2 compared to controls. In spite of the optically-monitored separation of the cerebral cortex from the underlying white matter, contamination by subcortical U fibers cannot be ruled out in our study. This is convenient remark as astrocytes and oligodendrocytes in grey matter differ from astrocytes and oligodendrocytes in white matter, and from one region to another.

Astrogliosis is one of the characteristic neuropathological lesions in cerebral cortex CJD which is here manifested by increased significant *GFAP* mRNA expression, as previously reported [15]. However, no significant differences are seen in the expression levels of the gene coding for aldehyde dehydrogenase 1 family member L1 (*ALDH1L1*) used as a marker of the total astrocyte population. Aquaporin 4 protein levels, as revealed by gel electrophoresis and western blotting, are markedly increased in CJD [17]. This is in contrast with the down-regulation of AQP4 mRNA in CJD MM1 and CJD VV2, here observed. Yet, AQP4 immunoreactivity is variable from one case to another:

AQP4 immunorecativity is apparently decreased in some cases, increased in others, and even increased and blurred in cases with severe spongiform change. We do not have an explanation for this point; enhanced translation and/or reduced degradation can account for discrepancies between mRNA and protein expression, and for case-to-case and area differences in protein immunoreactivity. Expression of solute carrier family 1A2 (*SLC1A2: GLT1*) is reduced in CJD VV2, pointing to the possibility of unbalanced glutamate concentrations between neurons and astrocytes in certain forms of CJD. However, immunohistochemistry reveals individual variations and areal variations in GLT1 expression in the same tissue section. Therefore, further studies in animal models are needed to evaluate GLT1 mRNA and protein expression in prion diseases.

In addition to these aspects, new information has been obtained in this study. Present quantitative data show altered expression of several genes coding for key specific proteins of astrocytes and oligodendrocytes in frontal cortex area 8 in CJD which may have functional implications in the pathogenesis of the disease. Reduced expression of mitochondrial pyruvate carrier 1 (*MPC1)* and mitochondrial uncoupling protein 5 (*UCP5, SLC25A14, BMCP1*: brain mitochondrial uncoupling protein 1) suggest altered mitochondrial function in CJD MM1 and CJD VV2. Reduced expression of solute carrier family 2A1 (*SLC2A1* or *GLUT1*) points to altered glucose transport, whereas reduced expression of solute carrier family 16 (*MCT1*) suggests impaired monocarboxylic acid transport. These are important aspects as blood-derived glucose through GLUT1 is metabolized via glycolysis to produce piruvate and lactate which are delivered to the axons through specific solute carriers, the monocarboxylase transporters (MCTs) located in cell membranes [26,27]. In glia, MCT1 is mainly expressed in developing oligodendrocytes and MCT4 in astrocytes [28,29]. Inhibition of MCT1 in organotypic cultures of the spinal cord in glucose-deprived media is toxic to neurons; the effects on neurons are reversed with the addition of lactate into the medium [29]; moreover, reduction of MCT1 activity *in vivo* results in axonal damage [29].

These changes are not accompanied by altered expression of oligodendroglial markers *OLIG1, OLIG2* and *SOX10* [30]; and adult oligodendroglial precursors (*NG2*) [31,32]. The expression of genes encoding main myelin proteins is also preserved in frontal cortex in CJD, including myelin basic protein (*MBP*), proteolipid protein (*PLP*), 2′,3′-cyclic nucleotide-3′phosphodiesterase (*CNP*), myelin-associated glycoprotein (*MAG*), myelin oligodendrocyte glycoprotein (*MOG*) and myelin/oligodendrocyte basic protein (*MOBP*) [33–35].The expression of *CLDN11*, the gene encoding for claudin 11, principal component of tight junctions of myelin connections at their edges [36,37], is not modified in CJD. The expression of *MYRF*, encoding myelin regulatory factor which triggers myelination following binding to several promoters of genes coding for myelin proteins [38], is maintained in CJD. In contrast, the expression of the gene coding for galactocerebrosidase (*GALC*) is reduced in

CJD MM1 and VV2, thus indicating possible impairment in glycosphingolipid metabolism. To our knowledge, most studies of brain lipids in CJD were carried out many years ago and the main focus was on ganglioside alterations [39–42]. Since the present studies are focused on the cerebral cortex, further investigation is needed to get information about brain lipid alterations in the white matter in prion diseases.

Oligodendrocytes are connected with each other and with astrocytes through gap junctions thus forming a glial syncytium in the white matter tracts [43,44]. Gap junctions are composed of connexins (Cx); Cx32 and Cx47 are synthesized by oligodendrocytes, and Cx30 and Cx43 by astrocytes [45]. The expression of genes coding for Cx32 (*GJB1*) and Cx47 (*GJC2*), and Cx30 (*GJB6*) is similar in CJD and in control cases, but *GJA1* (encoding Cx43) mRNA expression is reduced in CJD VV2 when compared with controls and CJD MM1. Whether these changes have implications in the normal function of the glial syncitium deserves further attention.

Interpretation of some of the present data must be taken with caution. MPC1 is expressed in brain [46] principally in neurons in controls. MPC1 immunoreactivity is reduced in neurons but increased in glial cells in CJD. Therefore, the origin of decreased *MPC1* mRNA expression in CJD here observed may be due to damaged neurons, whereas MPC1 protein is increased in glial cells. Similarly, UC5P in the brain is mainly expressed in neurons [47]; reduced *UCP5* mRNA expression here observed in CJD is accompanied by reduced UPC5 immunoreactivity in neurons, but this accompanied by increased immunoreactivity in glial cells with the morphology of astrocytes.

GLUT1 is largely expressed in red blood cells and endothelial cells, including brain microvessels [48,49]. No alterations in GLUT1 immunoreactivity is found in CJD microvessels; reduction of *GLUT1* mRNA correlates with decreased GLUT1 immunoreactivity in the neuropil in CJD. Finally, MCT1 is widely expressed in endothelial cells in brain [50–52], but also in glial cells especially during development [53,54]. MCT1 immunoreactivity in the adult brain is also observed in neurons. MCT1 immunoractivity is preserved in endothelial cells in CJD when compared with controls; reduced MCT1 mRNA and protein in CJD can be ascribed to reduced expression in neurons.

The concepts of astrocytopathy and oligodendrocytopathy, referring to molecular alterations in astrocytes and oligodendrocytes, are appropriate in neurodegenerative diseases with abnormal protein aggregates [55–60]. This study supports the idea that molecular flaws in astrocytes and oligodendrocytes, in addition to neurons, are relevant in the pathogenesis of cortical alterations in CJD.

Material and methods

Human cases

CJD cases were clinically diagnosed on the basis of rapid dementia together with variable accompanying symptoms, neuroimaging alterations and characteristic CSF biomarkers; all of them were tested for homozygosity of codon 129 in *PRNP*. Verification of clinical diagnosis was obtained after post-mortem neuropathological study of the brain. Controls did not show neurological symptoms; moreover, cases with metabolic syndrome, autoimmune diseases, fever and prolonged agonal state were not included in this series. Brain samples were obtained from the Brain Banks of the Institute of Neuropathology HUB-ICO-IDIBELL Biobank and the Hospital Clinic-IDIBAPS Biobank following the guidelines of the Spanish legislation on this matter and the approval of the local ethics committees. The post-mortem interval between death and tissue processing was between 2h 45min and 22h 40min. One hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks. The neuropathological study in control and CJD cases was carried out on, at a minimum, twenty selected 4μm-thick de-waxed paraffin sections of representative regions of the frontal, temporal, parietal, motor, primary visual, anterior cingulate and entorhinal cortices, hippocampus, amygdala, basal forebrain, caudate, putamen, globus pallidus, thalamus, midbrain, pons, medulla oblongata, cerebellar vermis, hilus and cerebral white matter. Sections were stained with haematoxylin and eosin, Klüver-Barrera, or processed for immunohistochemistry for microglia (Iba-1, Wako, Richmond, VA, USA), glial acidic protein (GFAP, Dako, Gostrup, Denmark), β-amyloid (Dako, clone 6F/3D), phospho-tau (Thermo Scientific, Rockford, USA, clone AT8), α-synuclein (Novocastra, Newcastle, UK, clone KM51), TDP-43 (Abnova, Taipei, Taiwan, clone 2E2-D3), PrP (clon 3F4, Millipore, Darmstadt, GE) following proteinase K incubation, ubiquitin (Dako, Polyclonal Rabbit) and p62 (BD Biosciences, San Jose, USA, Purified Mouse Anti-p62 LCK ligand) using EnVision+ System peroxidase (Dako), and diaminobenzidine and H_2O_2.

Samples of the frontal cortex and cerebellum of the other hemisphere were rapidly dissected, frozen on metal plates over dry ice and stored at −80ºC until use. Part of this material was used for gel electrophoresis and western blotting for identification of protease-resistant PrP type. CJD was diagnosed following well-established neuropathological criteria, codon 129 genotype and characteristics of PrP pattern on western blots [61]. None of the cases had suffered from panencephalopathic CJD [62]. Additional brain pathology consisting of neurofibrillary

tangles at stages I-II of Braak and Braak, argyrophilic grain disease stage 2, mild small blood vessel disease, and Lewy bodies in the brain stem in some individuals were expected with the age of the patients. Similar lesions were found in the control group and, therefore, they were not contemplated as discriminating variables.

The whole series included 7 CJD MM1 (4 men and 3 women; age: 70.5 ± 5.5), 10 CJD VV2 (4 men and 6 women; age 66.5 ± 6.85) and 10 controls (6 men and 4 women, age 67.1 ± 7.6). A summary of cases is shown in Table 1.

Biochemical studies were carried out in the frontal cortex area 8 after our best optically-monitored dissection of the cerebral cortex and white matter. However, inclusion of white matter subcortical U fibers cannot be ruled out in the present study.

RNA purification

RNA from frozen frontal cortex area 8 was extracted following the instructions of the supplier (RNeasy Mini Kit, Qiagen® GmbH, Hilden, Germany). RNA integrity and 28S/18S ratios were determined with the Agilent Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA). RIN values are shown in Table 1: control 6.76 ± 0.5; CJD MM1 5.68 ± 0.8 and CJD VV2 5.84 ± 0.6). Samples were treated with DNase digestion, and RNA concentration was evaluated using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. Summary of cases M: man; W: woman; Age in years; CJD: Creutzfeldt-Jakob disease, subtypes MM1 and VV2; PMD: post-mortem delay; RIN: RNA integrity number.

Case	Sex	Age	Diagnosis	PMD	RIN
1	M	66	Control	18 h 00 min	6.4
2	M	61	Control	03 h 40 min	7.0
3	M	74	Control	06 h 40 min	7.2
4	M	65	Control	05 h 15 min	6.8
5	M	63	Control	08 h 05 min	7,1
6	W	79	Control	03 h 35 min	6.8
7	W	67	Control	05 h 20 min	6,2
8	M	70	Control	03 h 45 min	7.2
9	M	52	Control	04 h 40 min	7.2
10	W	74	Control	02 h 45 min	5.7
11	M	69	CJD MM1	15 h 00 min	6.4
12	M	70	CJD MM1	N/A	4.7
13	W	64	CJD MM1	14 h 00 min	5.6
14	M	64	CJD MM1	14 h 00 min	4.7
15	M	77	CJD MM1	07 h 10 min	6,0
16	W	72	CJD MM1	08 h 00 min	7.0
17	M	78	CJD MM1	22 h 40 min	5.1
18	W	76	CJD VV2	05 h 00 min	5.5
19	M	65	CJD VV2	06 h 00 min	5.3
20	W	65	CJD VV2	07 h 15 min	5.4
21	W	72	CJD VV2	06 h 00 min	6.2
22	W	62	CJD VV2	08 h 45 min	4.7
23	M	71	CJD VV2	09 h 00 min	5.8
24	M	66	CJD VV2	05 h 00 min	6.8
25	M	52	CJD VV2	06 h 30 min	6.5
26	W	59	CJD VV2	09 h 00 min	6,7
27	W	67	CJD VV2	12 h 30 min	5.5

Table 2. TaqMan probes: gene identification, full name and reference.

Gene	Full name	Reference
ALDH1L1	Aldehyde Dehydrogenase 1 Family Member L1	Hs01003842_m1
AQP4	Aquaporin-4	Hs00242342_m1
CLDN11	Claudin 11	Hs00194440_m1
CNP	2′,3′-Cyclic Nucleotide 3′ Phosphodiesterase	Hs00263981_m1
GALC	Galactosylceramidase	Hs00164660_m1
GFAP	Glial fibrillary acidic protein	Hs00909233_m1
GJA1	Gap junction alpha-1 protein/connexin-43	Hs00748445_s1
GJB1	Gap junction beta-1 protein/connexin-32	Hs00939759_s1
GJC2	Gap junction beta-2 protein/connexin-47	Hs00252713_s1
GJB6	Gap junction beta-6 protein/connexin-30	Hs00922742_s1
GUS-β	β-glucuronidase	Hs00939627_m1
MAG	Myelin Associated Glycoprotein	Hs01114387_m1
MAL	Mal, T-Cell Differentiation Protein	Hs00360838_m1
MBP	Myelin Basic Protein	Hs00921945_m1
MCT1	Solute Carrier Family 16 (Monocarboxylic Acid Transporters), Member 1	Hs01560299_m1
MCT4	Solute Carrier Family 16 (Monocarboxylic Acid Transporters), Member 4	Hs01006127_m1
MOBP	Myelin-Associated Oligodendrocyte Basic Protein	Hs01094434_m1
MOG	Myelin Oligodendrocyte Glycoprotein	Hs01555268_m1
MPC1	Mitochondrial Pyruvate Carrier 1	Hs00211484_m1
MYRF	Myelin Regulatory Factor	Hs00973739_m1
NG2	Neural/glial antigen 2	Hs00426981_m1
OLIG1	Oligodendrocyte Transcription Factor 1	Hs00744293_s1
OLIG2	Oligodendrocyte Lineage Transcription Factor 2	Hs00377820_m1
PLP1	Proteolipid Protein 1	Hs00166914_m1
SLC1A2	Solute Carrier Family 1 (Glial High Affinity Glutamate Transporter), Member 2	Hs01102423_m1
SLC2A1	Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 1	Hs01102423_m1
SOX-10	SRY-Box 10	Hs00366918_m1
UCP4	Mitochondrial Uncoupling Protein 4	Hs00188687_m1
UCP5	Mitochondrial Uncoupling Protein 5	Hs00605850_m1

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Rt-qPCR

TaqMan RT-qPCR assays were performed in duplicate for each gene on cDNA samples in 384-well optical plates using an ABI Prism 7900 Sequence Detection system (Applied Biosystems, Life Technologies, Waltham, MA, USA). Probes are listed in Table II. For each 10µL TaqMan reaction, 4.5µL cDNA was mixed with 0.5µL 20x TaqMan Gene Expression Assays and 5µL of 2x TaqMan Universal PCR Master Mix (Applied Biosystems). Values of *GUS-β* were used as internal controls for normalization [63]. The parameters of the reactions were 50°C for 2min, 95°C for 10min, and 40 cycles of 95°C for 15sec and 60°C for 1min. Finally, capture of all TaqMan PCR data used the Sequence Detection Software (SDS version 2.2.2, Applied Biosystems). For the data analysis, threshold cycle (CT) values for each sample were processed to obtain the double delta CT (ΔΔCT) values. First, delta CT (ΔCT) values were calculated as the normalized CT values of each target gene in relation to the CT of endogenous controls *GUS-β*. Then, ΔΔCT values were obtained from the ΔCT of

each sample minus the mean ΔCT of the population of control samples.

Statistical analysis

The normality of distribution of fold change values was analyzed with the Kolmogorov–Smirnov test. Pearson's correlation coefficient was used to assess a possible linear association between two continuous quantitative variables. To determine the relationship between gene expression and RIN values according to pathologic variables, we used the analysis of covariance (ANCOVA). Statistical analysis of the expression data between groups was performed using one-way analysis of variance (ANOVA) followed by Tukey post-test or Kruskal-Wallis test followed by Dunns post-hoc test when required using the SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Outliers were detected using the GraphPad software QuickCalcs ($P < 0.05$). All data were expressed as mean values ± SEM. Differences between controls and CJD MM1 or CJD VV2 were considered statistically significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; and set at $ $P < 0.05$ and $$ $P < 0.01$when comparing CJD MM1 and CJD VV2.

Immunohistochemistry

De-waxed sections, 4 microns thick, were processed for immunohistochemistry (control n = 8; CJD MM1 n = 6; CJD VV2 n = 2). The sections were boiled in citrate buffer (20min) to retrieve tau antigenicity. Endogenous peroxidases were blocked by incubation in 10% methanol-1% H_2O_2 solution (15min) followed by 3% normal horse serum solution. Then the sections were incubated at 4ºC overnight with one of the primary antibodies against glial fibrillary acidic protein (GFAP) (rabbit polyclonal, used at 1:500, Dako, Glostrup, DK), Olig-2 (rabbit polyclonal, used at 1:500, Abcam, Cambridge, UK), aquaporine 4 (AQP4) (monoclonal, used at 1:400, Sigma, St Louis, Missouri, USA), solute carrier family 1 member 2 (GLT1) (guinea pig used at 1:100, Merck-Millipore, Billerica, MA, USA), mitochondrial pyruvate carrier 1 (MPC1) MPC1) (polyclonal, used at 1:100, Cell Signaling Technology, Danvers, MA, USA), mitochondrial uncoupled protein 5 (UCP5) (polyclonal, used at 1:10, Novus Biological, Littleton, CO, USA), solute carrier family 2 member 1 (GLUT1) (polyclonal, used at 1:100, Abcam, Cambridge, CB, UK) and solute carrier family 16 member 1 (MCT1) (monoclonal, used at 1:50, Sigma, St Louis, Missouri, USA). Following incubation with Following incubation with the primary antibody, the sections were incubated with EnVision + system peroxidase (Dako, DK) for 30min at room temperature. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Control of the immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody. Peptides for pre-absorption studies were not available.

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Disclosure statement

No potential conflict of interest was reported by the authors. AQ5

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