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PROTEOMICS REVEAL ENERGY METABOLISM AND MITOGEN-ACTIVATED PROTEIN KINASE SIGNAL TRANSDUCTION PERTURBATION IN HUMAN BORNA DISEASE VIRUS HU-H1-INFECTED OLIGODENDROGLIAL CELLS

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Abbreviations: 2-DE, two-dimensional electrophoresis; BDV C6BV, laboratory Borna disease virus strain C6BV; BDV He/80, laboratory Borna disease virus strain He/80; BDV Hu-H1, human Borna disease virus strain Hu-H1; BDV strain V, laboratory Borna disease virus strain V; BDV, Borna disease virus; CCK-8, Cell Counting Kit-8; CHAPS, 3-[(3-cholamido-propyl)-dimethylammonio]-1-propanesulfonate; CI%, protein score confidence index; CREB, cAMP-response element-binding protein; CrkL, Crk-like protein; DAVID, Database for Annotation, Visualization, and Integrated Discovery; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; EBV, Epstein-Barr virus; ECL, enhanced chemiluminescence; ERK, extracellular-regulated kinase; FBS, fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HBV, hepatitis B virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus; IEF, isoelectric focusing; IgG, immunoglobulin G; KEGG, Kyoto Encyclopedia of Genes and Genomes; MALDI-TOF-MS/MS, matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry; MSK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; MOI, multiplicity of infection; MS, mass spectrometry; pERK, a finding which warrants further research.

Abstract—Borna disease virus (BDV) is a neurotropic, non-cytopathic RNA virus which replicates in the cell nucleus targeting mainly hippocampal neurons, but also astroglial and oligodendroglial cells in the brain. BDV is associated with a large spectrum of neuropsychiatric pathologies in animals. Its relationship to human neuropsychiatric illness still remains controversial. We could recently demonstrate that human BDV strain Hu-H1 promoted apoptosis and inhibited cell proliferation in a human oligodendroglial cell line (OL cells) whereas laboratory BDV strain V acted contrariwise. Here, differential protein expression between BDV Hu-H1-infected OL cells and non-infected OL cells was assessed through a proteomics approach, using two-dimensional electrophoresis followed by matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry. A total of 63 differential host proteins were identified in BDV Hu-H1-infected OL cells compared to non-infected OL cells. We found that most changes referred to alterations related to the pentose phosphate pathway, glyoxylate and dicarboxylate metabolism, the tricarboxylic acid (TCA) cycle, and glycolysis/glucoseogenesis. By manual querying, two differential proteins were found to be associated with mitogen-activated protein kinase (MAPK) signal transduction. Five key signaling proteins of this pathway (i.e., p-Raf, p-MEK, p-ERK1/2, p-RSK, and p-MSK) were selected for Western blotting validation. p-ERK1/2 and p-RSK were found to be significantly up-regulated, and p-MSK was found to be significantly down-regulated in BDV Hu-H1-infected OL cells compared to non-infected OL cell. Although BDV Hu-H1 constitutively activated the ERK–RSK pathway, host cell proliferation and nuclear translocation of activated pERK in BDV Hu-H1-infected cells were impaired. These findings indicate that BDV Hu-H1 infection of human oligodendroglial cells significantly perturbs host energy metabolism, activates the downstream ERK–RSK complex of the Raf/MEK/ERK signaling cascade, and disturbs host cell proliferation possibly through impaired nuclear translocation of pERK, a finding which warrants further research.

Key words: Borna disease virus, BDV, oligodendroglial cell, proteomic, energy, ERK signaling.

INTRODUCTION

Borna disease virus (BDV), a member of the family Bornaviridae in the order Mononegavirales, is an...
enveloped virus with a non-segmented, negative-strand (NNS) ribonucleic acid (RNA) genome (Ludwig et al., 1988; de la Torre, 1994; Schneemann et al., 1995). BDV infects a wide variety of mammalian species (Ludwig and Bode, 2000; Hornig et al., 2003). Infected animal hosts develop a large spectrum of neuropathological and neuropsychiatric disorders ranging from immune-mediated neurological disease to non-inflammatory behavioral alterations (Ludwig and Bode, 2000), which are notably reminiscent of symptoms observed in certain human neuropsychiatric disorders (Hornig et al., 2001). Therefore, several studies have attempted to conclusively associate BDV with human psychiatric illness, but the findings remain controversial (Bode et al., 1995; Iwata et al., 1998; Kim et al., 1999; Fukuda et al., 2001; Ikuta et al., 2002; Bode and Ludwig, 2003; Hornig et al., 2012). Regardless of this debate, a few human BDV strains could have been finally recovered in Germany, through laborious, long-term co-cultivation of freshly isolated white blood cells from psychiatric inpatients with a human oligodendroglial cell line (OL cells) (Bode et al., 1996). Genetic analyses could validate both the identity of BDV RNA in the original samples and the corresponding isolates, and their authenticity as human viruses, as they differ genetically from the laboratory reference BDV strain V and another lab strain termed C6BV by few but distinct and meaningful mutations in each gene (de la Torre et al., 1996). Moreover, our previous metabonomic research has demonstrated that one of these human strains, BDV Hu-H1, which had been isolated from a severely depressed, hospitalized bipolar patient's PBMCs, perturbs energy metabolites in OL cells (Huang et al., 2012). Even more interesting, our group could have found last year that BDV Hu-H1 differed remarkably from the laboratory-adapted BDV strain V. In fact, BDV Hu-H1 inhibited proliferation and promoted apoptosis in OL cells, while strain V displayed the opposite effects (Li et al., 2013). Lab strain V was originally isolated from a diseased horse in Germany in the late 1920s, underwent numerous in vivo passages in rabbits and rats followed by multiple cell culture passaging. OL cells are a cell line derived from fetal human oligodendrocytes and passaged at least 100 times. Oligodendrocytes are a major cellular component of the brain white matter, which plays a pivotal role in maintaining neurological function via producing myelin proteins. The human oligodendrogial cell line termed OL cells, originally derived from fetal human oligodendrocytes, (112 passages), and the human BDV Hu-H1 strain (77 passages in OL cells) (Bode et al., 1996) were kindly supplied by Hanns Ludwig (Free University of Berlin, Berlin, Germany). Persistently-infected OL cells and non-infected OL cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, Utah, USA) with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin, and 100 µg/ml streptomycin (Hyclone) within a humidified incubator (5% CO₂, 37°C) and were passaged when they reached 90% confluence by trypsinization (Hyclone).

**Viral solution preparation, titration, and infection**

OL cells were sub-cultured in one 10-cm dish and infected with BDV Hu-H1 stock solution at a multiplicity of infection (MOI) of 1.0 as described previously (Huang et al., 2012). Specifically, cells were washed twice with serum-free DMEM before 800 µl of stock viral solution was added to the dish. Cells were then stored in a humidified incubator (5% CO₂, 37°C) for two hours with gentle shaking for 15 min. Excess virus was removed by washing with 5 ml of serum-free DMEM before bathing the cells in 10 ml of culture medium (10% FBS in DMEM). To achieve persistent infection and stable viral titration, the Hu-H1-infected OL cells (112 OL passages, 0 Hu-H1 passages) were cultured and passaged until all cells were infected with BDV Hu-H1 (142 OL passages, 30 Hu-H1 passages). An immunofluorescence assay was applied to stain BDV-specific nucleoprotein p40 in

**EXPERIMENTAL PROCEDURES**

**Cell lines and viral strain**

The human oligodendroglial cell line termed OL cells, originally derived from fetal human oligodendrocytes, (112 passages), and the human BDV Hu-H1 strain (77 passages in OL cells) (Bode et al., 1996) were kindly supplied by Hanns Ludwig (Free University of Berlin, Berlin, Germany). Persistently-infected OL cells and non-infected OL cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, Utah, USA) with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin, and 100 µg/ml streptomycin (Hyclone) within a humidified incubator (5% CO₂, 37°C) and were passaged when they reached 90% confluence by trypsinization (Hyclone).
order to monitor the state of the OL cells. The now persistently-infected (OL/Hu-H1 cells) and non-infected OL cells (control cells) were kept under identical conditions for the remainder of the study.

**Protein extract and 2-DE sample preparation**

After cell scraping and three washes with phosphate-buffered saline (PBS, pH 7.4, 0.01 M), cells were centrifuged at 500g for 5 min at 4°C. Separate pooled samples of OL/Hu-H1 cells and control cells were generated by combining equal volumes of the six 10-cm dishes from each group of cells. Proteins were dissolved in a dissociation solution (7 mM urea, 2 M thiourea, 4% 3-[3-cholamido-propyl]-dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 0.2% 3–10 Bio-Lyte; Bio-Rad Laboratories, Hercules, CA, USA), and the concentration of protein dilutions content was determined by the Bradford assay. Immediately prior to isoelectric focusing (IEF), each sample was further diluted to 100 μg/350 μl with dissociation solution.

**Two-dimensional electrophoresis (2-DE)**

Each sample was run in triplicate to control for gel variation; therefore, six analytical gels (three Hu-H1 gels and three control gels) with 100-μg loading were developed. In the first-dimension IEF phase, 17-cm IPG strips (pH 3–10 NL: Bio-Rad) were used. After passive rehydration for a minimum of 12 h, the strips were focused and stained as previously described (Yang et al., 2013). The six analytical gels were scanned using an Epson 10000XL scanner (Epson Co., Ltd., Beijing, China) at an optical resolution of 300 dpi. Image analysis and spot detection were accomplished with PDQuest software version 8.0.1 (Bio-Rad) using Gaussian spot modeling. For quantitative comparison of spots across gels, replicate images of the gels were created. To correct for the variability in silver staining, the individual spot volumes were normalized by dividing the sum total OD of all spots in the respective gel. This method controlled for differences in sample loading and color intensities among the gels. Automated and manual spot matching were also performed. Only integrated intensities demonstrating at least a 1.5-fold change were applied to determine the statistical differences in protein expression between the two groups (Yang et al., 2013).

**Protein identification by MALDI-TOF-MS/MS**

The protein spots of interest were excised from the preparative gels with 250-μg loading and destained. After reduction and alkylation, the gel slices were digested overnight with sequencing grade-modified trypsin (Promega, Madison, WI, USA). The digested peptides were extracted with 100 μl 60% acetonitrile (Merck, Darmstadt, Germany) containing 0.1% trifluoroacetic acid (TFA) (Merck) and concentrated in a Speed Vac (Savant Instruments, Inc., Hickenville, NY, USA). The peptides were redissolved using a matrix solution, spotted on a MALDI target plate, and analyzed using the 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) in the default mode. The MS spectra were recorded in reflector mode in a mass range from m/z 800 to 4000 with a focus mass of m/z 2000. MS used a CaliMix5 standard to calibrate the instrument (ABI 4800 Calibration Mixture). For each MS spectrum, 25 subspectra with 125 shots per subspectrum were accumulated using a random search pattern. For MS calibration, autolysis peaks of trypsin ([M + H] + 842.5100 and 2211.1046) were used as internal calibrates, and up to 10 of the most intense ion signals were selected as precursors for MS/MS acquisition (excluding the trypsin autolysis peaks and the matrix ion signals). In MS/MS-positive ion mode, 50 subspectra with 50 shots per subspectrum were accumulated using a random search pattern for each MS spectrum.

The data search was conducted on GPS Explorer (Version 3.6, AB SCIEX, Foster City, CA, USA) using the search engine Mascot (Version 2.2, Matrix Science, London, UK) against the following NCBI *Homo sapiens* database (248,112 sequences) and NCBI bornavirus database (372 sequences). Search parameters were set as follows: enzyme = trypsin, allowance = up to one missed cleavage, peptide mass tolerance = 100 ppm, fragment mass tolerance = 0.4 Da, fixed modification = carbamidomethylation (Cys), and variable modification = oxidation (Met). A protein score confidence index (CI%) of 95% was used for further manual validation (Yang et al., 2013).

**Bioinformatic analysis**

In order to identify the enriched pathways, the protein data identified from MALDI-TOF-MS/MS were entered into DAVID Bioinformatics Resources v6.7 (http://david.abcc.ncifcrf.gov/home.jsp) (Dennis et al., 2003) to obtain the Kyoto Encyclopedia of Genes and Genomes (KEGG) terms (www.genome.jp/kegg/). The KEGG pathways with a corrected P-value of less than 0.05 were deemed to be statistically significant.

**Western blotting**

To validate the effects of BDV Hu-H1 infection on phosphatidylethanolamine-binding protein 1 (PEBP-1), Crk-like protein (CrkL) and the Raf/MEK/ERK signaling cascade (i.e., p-Raf, p-MEK, p-ERK1/2, p-RSK, and p-MSK), the seven proteins were selected for Western blotting analysis. Beta-tubulin or Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control (both diluted 1:30,000). Monolayers of OL/Hu-H1 and control cells were lysed in the standard lysis buffer, sonicated on ice, and 10-μg lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated overnight at 4°C with anti-PEBP-1, and anti-CrkL rabbit antibodies (both diluted 1:1000; Abcam, Cambridge, MA, USA) in addition to anti-
p-Raf, anti-p-MEK, anti-p-ERK, anti-p-RSK, anti-p-MSK rabbit antibodies (all diluted 1:500; CST, Beverly, MA, USA), respectively. All membranes were washed and incubated with horseradish peroxidase-coupled anti-rabbit immunoglobulin G (IgG) (diluted 1:5000, Bio-Rad; diluted 1:2000, CST). After extensive washing, antibody-detected protein bands were visualized by enhanced chemiluminescence (ECL) and exposed to autoradiography film.

Assays for cell proliferation with and without ERK inhibitor U0126

OL/Hu-H1 cells and OL cells were plated into 96-well plates at 2.5 x 10^4 cells per well in DMEM/F12 medium containing 10% fetal calf serum (FCS), respectively. The cells were allowed to grow to 80% confluency and switched to serum-free medium in the absence or presence of ERK inhibitor U0126 (40 μM, CST). After 24 h, they were harvested and assayed using Cell Counting Kit-8 (CCK-8) (Beyotime, Jiangshu, China). The mean absorption of four independent assays was plotted with SD for each group.

Immunofluorescence and co-localization analysis

Both OL/Hu-H1 cells and control OL cells were grown on six-well dishes for 30 min at room temperature with 4% paraformaldehyde followed by permeabilization for 5 min in 0.4% Triton X-100. Thereafter, both lines were rinsed with PBS and blocked with 5% (w/v) skimmed milk solution for one hour at 37 °C. Overnight incubation with anti-pERK antigen primary monoclonal antibody (diluted 1:200; CST, Beverly, MA, USA) at 4 °C was followed by one hour of incubation with TRITC-labeled anti-rabbit IgG (diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, USA) at room temperature. After three PBS washes, immunofluorescence was detected by phase-contrast microscopy. We studied the co-localization of pERK in the nucleus using ImageJ software with the Intensity Correlation Analysis plugin (Li et al., 2004).

Statistical analysis

Statistical analysis was performed using the Statistical Package of Social Science (SPSS) for Windows version 19.0. The Wilcoxon test was used to analyze significant differences between the two cell groups. All tests were two-tailed, and the significance level was set at p < 0.05.

RESULTS

Experimental setup

To analyze the impact of BDV Hu-H1 persistence on the proteome of human OL cells, we applied a 2-DE-MALDI-TOF-MS/MS approach to cellular extracts prepared from OL cells infected or not infected with BDV Hu-H1 which were subsequently digested with trypsin. The whole experimental process is schematically shown in a work flow diagram (Fig. 1).

Fig. 1. Work flow diagram. Whole-cell extracts were prepared from BDV Hu-H1-infected and non-infected control OL cells. A 2-DE-MALDI-TOF-MS/MS approach was used to comparatively analyze the two groups. Further biochemistry was applied to validate the MS analysis results.

Differential protein spotting from 2-DE

Approximately 1900 protein spots on the 3–10 NL range gels were identified by silver staining (Fig. 2). Through differential analysis of the 3–10 NL pH range gels with PDQuest software, 86 differential spots were identified in OL/Hu-H1 cells compared to control cells of which 35 spots up-regulated and 51 spots down-regulated.

MALDI-TOF-MS/MS identification of differential proteins

Of the 86 spots originally detected using PDQuest analysis, several spots could not be obtained from the subsequent preparative gels after increasing the loading amount from 100 to 250 μg. As a result, only 76 protein spots (72 host protein spots and four BDV protein spots) were excised from the preparative gels for MALDI-TOF MS/MS analysis. Finally, 63 non-redundant differential host proteins originating from the 72 host protein spots, and three differential BDV proteins of the originally four BDV protein spots were successfully identified (Table 1), yielding a MS identification ratio of 90.7%.

Altered pathways by bioinformatic analysis

63 differential host proteins were analyzed for KEGG over-representation of pathways (“proteomic phenotyping”) to
obtain functional insights into the differences between Hu-H1 and control cells. Only 26 of the 63 differential host cell proteins were mapped onto the KEGG database. The resulting top ten-ranking canonical KEGG pathways are listed in Table 2. Energy metabolism was the most statistically over-represented set of pathways with pentose phosphate pathway ranking first \((p < 0.001)\), glyoxylate and dicarboxylate metabolism ranking second \((p < 0.01)\), tricarboxylic acid (TCA) cycle ranking fourth \((p < 0.05)\), and glycolysis/gluconeogenesis ranking eighth \((p < 0.05)\). Moreover and through manual querying, two proteins associated with the Raf/MEK/ERK signaling cascade, PEBP-1 and CrkL, were found to be dysregulated in OL/Hu-H1 cells compared to control cells.

**Detection of differential proteins and the Raf/MEK/ERK signaling cascade by Western blotting**

Based on the aforementioned analysis, two differential proteins – PEBP-1 and CrkL (Fig. 3), five key Raf/MEK/ERK signaling proteins – p-Raf, p-MEK, p-ERK1/2, p-RSK, and p-MSK, were selected for validation by Western blotting (Fig. 4). PEBP-1 \((p = 0.015)\), p-ERK1/2 \((p = 0.016)\) and p-RSK \((p = 0.0495)\) were found to be significantly up-regulated, CrkL \((p = 0.03)\) and p-MSK \((p = 0.024)\) was found to be significantly down-regulated, in OL/Hu-H1 cells compared to control cells. There was no significant dysregulation observed in p-Raf \((p = 0.744)\) or p-MEK \((p = 0.267)\).

**BDV Hu-H1 decreases OL cell proliferation**

Based on the finding of up-regulated pERK in response to BDV Hu-H1 infection, CCK-8 assay was performed on cells treated with or without ERK inhibitor U0126 in OL/Hu-H1 and control cells. The data, seen in Fig. 5, clearly demonstrated that BDV Hu-H1 infection decreased OL cell proliferation \((p < 0.05)\). ERK inhibitor U0126 decreased OL cell proliferation \((p < 0.05)\) but did not significantly decrease the impaired OL/Hu-H1 cell proliferation any further \((p > 0.05)\).

**Impaired nuclear translocation of pERKs**

Anti-pERK antibody labeling and subsequent immunofluorescence analysis were carried out in OL/Hu-H1 cells and control OL cells. Co-localization of pERK and nucleus was studied using ImageJ software with the Intensity Correlation Analysis plugin. As shown in Fig. 6a, BDV Hu-H1 induced lower presence of pERK in the nucleus and higher presence in the cytoplasm than control OL cells. As shown in Fig. 6b, analyzed by using ImageJ software with the Intensity Correlation Analysis plugin, the co-localization of pERK and nucleus in OL/Hu-H1 cells was significantly lower than in control cells \((p = 0.04)\), suggesting impaired nuclear translocation of pERK in response to BDV Hu-H1 infection.

**DISCUSSION**

In this study, a 2-DE-MALDI-TOF-MS/MS approach was used to comparatively analyze BDV Hu-H1-infected and non-infected control OL cells. Several previous studies have also applied 2-DE/MS-based proteomic approaches to identify and analyze differential host proteins across a range of viral infections (e.g., BDV, Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV-1), and severe acute respiratory syndrome coronavirus (SARS-CoV)) (Zhou et al., 2011). With respect to BDV, the study by Suberbielle et al. had focused on BDV-induced effects on the complex neuronal proteome. They found significant protein changes, among others referring to synaptic activity. They compared the proteomes of laboratory strain BDV He/80-infected and non-infected primary cultured embryonic Sprague–Dawley rat cortical neurons, using two-dimensional liquid chromatography fractionation, followed by protein identification through nanoliquid...
| Spot No. | GI No.    | Gene name            | Protein name                                      | Mascot score | Protein Score C. (%) | MW (Da) | PI | Biological function                                      | Fold-change (Hu-H1/CON) |
|---------|-----------|----------------------|--------------------------------------------------|--------------|----------------------|---------|----|----------------------------------------------------------|-------------------------|
| 1       | 116875831 | RMDN1                | Regulator of microtubule dynamics protein 1 [Homo sapiens] | 473          | 100                  | 36,013.4 | 8.64 | Microtubule-associated protein                           | -1.92                   |
| 2       | 119599451 | MRPS22               | Mitochondrial ribosomal protein S22, isoform CRA_e [Homo sapiens] | 645          | 100                  | 36,639.9 | 6.34 | Translation of mitochondrial mRNAs                      | -1.96                   |
| 3       | 12652799  | C22orf28             | Chromosome 22 open reading frame 28 [Homo sapiens] | 238          | 100                  | 55,722   | 6.77 | tRNA-splicing ligase complex                             | -3.23                   |
| 4       | 14249382  | ABHD14B              | Abhydrolase domain-containing protein 14B isoform 1 [Homo sapiens] | 281          | 100                  | 22,445.6 | 5.94 | Transcription activation                                | 1.54                    |
| 5       | 14495609  | CTPS1                | CTP synthase [Homo sapiens]                       | 81           | 99.807               | 67,358.3 | 6.02 | Pyrimidine metabolism                                   | 4.17                    |
| 6       | 81        | 99.807               | 67,358.3                                          | 6.02         | 4.17                 |          |     |                                                          |                         |
| 7       | 19438560  | cDNA FLJ58563, highly similar to CTP synthase 1 (EC 6.3.4.2) | 160          | 100                  | 50,233.4 | 7.23 |                                                         | -1.56                   |
| 8       | 15277503  | ACTB                 | ACTB protein, partial [Homo sapiens] (Beta-actin) | 525          | 100                  | 40,536.2 | 5.55 | Cell motility                                           | 3.45                    |
| 9       | 461       |                      | 40,536.2                                          | 5.55         |                      |          |     |                                                          |                         |
| 10      | 157426879 | NPLOC4               | Nuclear protein localization protein 4 homolog [Homo sapiens] | 241          | 100                  | 69,046.2 | 5.94 | NPLOC4-UFD1L-VCP complex                                 | -3.57                   |
| 11      | 158261431 | NSF                  | Vesicle-fusing ATPase                            | 452          | 100                  | 83,059.2 | 6.38 | Synaptic vesicle cycle                                   | -2.22                   |
| 12      | 1688076   | DNAJC7               | Tetratricopeptide repeat protein [Homo sapiens]  | 629          | 100                  | 56,185.6 | 7.08 | Steroid receptors folding                               | -5.26                   |
| 13      | 18645167  | ANXA2                | Annexin A2 [Homo sapiens]                        | 281          | 100                  | 38,779.9 | 7.57 | Heat-stress response, membrane-binding                   | -12.5                   |
| 14      | 189054178 | KRT1                 | Keratin, type II cytoskeletal 1                   | 1100         | 100                  | 40,730.9 | 8.41 | Nucleus, subunit of complex with a role in membrane         | 1.68                    |
| 15      | 193783553 | SOD2                 | Superoxide dismutase                             | 78           | 99.65                | 66,151   | 7.62 | Keratinization                                           | -1.96                   |
| 16      | 194380306 | AC02                 | cDNA FLJ51705, highly similar to aconitate hydratase, mitochondrial (EC 4.2.1.3) | 797          | 100                  | 84,102.3 | 7.62 | Glyoxylate and dicarboxylate metabolism, TCA cycle       | -3.03                   |
| 17      | 19438240  | FUBP1                | cDNA FLJ61021, highly similar to Far upstream element-binding protein 1 (FBP1) | 595          | 100                  | 66,361.9 | 7.12 | RNA binding                                             | 1.5                     |
| 18      | 28436809  | RAD51                | Radixin [Homo sapiens]                           | 493          | 100                  | 68,636.4 | 5.88 | Regulation of heat-shock-responsive genes induction       | 3.25                    |
| 19      | 30740877  | C12orf10             | Chromosome 12 open reading frame 10 [Homo sapiens] | 179          | 100                  | 42,823.6 | 6.35 | Regulation of actin cytoskeleton                         | -1.59                   |
| 20      | 32483337  | PRDX3                | Thioredoxin-dependent peroxide reductase, mitochondrial isoform b [Homo sapiens] (AOP-1) | 496          | 100                  | 26,107.4 | 7.04 | Redox regulation                                         | 2.86                    |
| 21      | 33469968  | MCM7                 | DNA replication licensing factor MCM7 isoform 1 [Homo sapiens] | 994          | 100                  | 81,883.8 | 6.08 | DNA replication                                          | 1.53                    |
| Spot No. | GI No. | Gene name | Protein name | Mascot score | Protein Score C. I.% | MW (Da) | PI | Biological function | Fold-change (Hu-H1/CON) |
|----------|--------|-----------|--------------|--------------|----------------------|---------|----|---------------------|------------------------|
| 32       | 33589854 | BLVRA    | Biliverdin reductase A precursor [Homo sapiens] | 293          | 100                  | 33,692.4 | 6.06 | Porphyrin and chlorophyll metabolism | 1.54                   |
| 33       | 350276247 | PPP1CC   | Serine/threonine-protein phosphatase PP1-gamma catalytic subunit isoform 2 [Homo sapiens] | 708          | 100                  | 39,234.7 | 5.8  | Regulation of actin cytoskeleton | -2.17                  |
| 34       | 386781221 | WDR4     | tRNA (guanine-N(7)-) - methyltransferase subunit WDR4 isoform 2 [Homo sapiens] | 350          | 100                  | 46,074.2 | 6.47 | tRNA modification, RNA (guanine-N7)-methylation | 3.66                   |
| 35       | 40788339 | MATR3    | KIAA0723 protein [Homo sapiens] (Matrin-3) | 317          | 100                  | 95,681.3 | 5.91 | Transcription regulation | 2.79                   |
| 36       | 40788883 | MLEC     | KIAA0152 protein [Homo sapiens] (Malecln) | 492          | 100                  | 34,385.8 | 5.6  | Carbohydrate metabolism | -2.27                  |
| 37       | 450201  | ANXA1    | Annexin A1 [Homo sapiens] | 635          | 100                  | 38,918.1 | 6.57 | Membrane fusion | 1.56                   |
| 38       | 4503729 | PPP1CB   | Protein phosphatase 1, catalytic subunit, beta isoform [Homo sapiens] | 182          | 100                  | 26,370   | 5.91 | RNA transport | 236.7                  |
| 39       | 4505621 | PEBP-1   | Phosphatidyethanolamine-binding protein 1 preproprotein [Homo sapiens] | 781          | 100                  | 52,057.2 | 5.35 | Estrogen signaling pathway | 2.33                   |
| 40       | 4507521 | TKT      | Transketolase isoform 1 [Homo sapiens] | 390          | 100                  | 21,157.7 | 7.01 | MAPK signaling pathway | 3.12                   |
| 41       | 460789  | HNRPK    | Transformation upregulated nuclear protein [Homo sapiens] | 554          | 100                  | 68,519   | 7.58 | Pentose phosphate pathway | 2.14                   |
| 42       | 4755358 | VDAC2    | VDAC2 [Homo sapiens] | 106          | 100                  | 51,325.5 | 5.13 | Spliceosome | 7.79                   |
| 43       | 4855585 | SAE1     | SUMO-activating enzyme subunit 1 isoform a [Homo sapiens] | 99           | 99.997               | 40,867.1 | 8.67 | Oxidative phosphorylation | 2.85                   |
| 44       | 48145673 | HNRPH1  | HNRPH1 [Homo sapiens] | 347          | 100                  | 49,384.4 | 5.79 | RNA Processing | -1.72                  |
| 45       | 48146045 | CRKL     | Crk-like protein [Homo sapiens] | 157          | 100                  | 30,849.2 | 6.81 | Calcium signaling pathway | 3.58                   |
| 46       | 4885153 | PPP1CB   | Protein phosphatase 1, catalytic subunit, beta isoform [Homo sapiens] | 365          | 100                  | 33,870   | 6.26 | Regulation of actin cytoskeleton, MAPK signaling pathway | -1.56                  |
| 47       | 49456481 | TWF1     | Ras GTPase-activating protein-binding protein 1 [Homo sapiens] | 521          | 100                  | 52,189.1 | 5.36 | Nucleic acid metabolic process | 1.67                   |
| 48       | 5031703 | G3BP1    | G3BP1 [Homo sapiens] | 321          | 100                  | 52,189.1 | 5.36 | Nucleic acid metabolic process | 1.67                   |
| 49       | 5031875 | LMNA     | Lamin isoform C [Homo sapiens] | 656          | 100                  | 65,152.6 | 6.4  | Cellular structural protein, cell senescence | -2.86                  |
| 50       | 54696354 | SNRPA1   | Small nuclear ribonucleoprotein polypeptide A' variant [Homo sapiens] | 559          | 100                  | 37,944.9 | 5.84 | Regulation of actin cytoskeleton | 13.71                  |
| 51       | 556514  | APEH     | Acylamino acid-releasing enzyme [Homo sapiens] (AARE) | 639          | 100                  | 82,210   | 5.29 | Serine-type endopeptidase activity | 1.94                   |
| 52       | 55956921 | HNRNPAB  | Heterogeneous nuclear ribonucleoprotein A/B isoform b [Homo sapiens] | 126          | 100                  | 30,683.1 | 7.68 | RNA Binding | -1.92                  |
| 53       | 5730023 | RUVBL2   | RuvB-like 2 [Homo sapiens] | 314          | 100                  | 51,295.6 | 5.49 | NuA4 histone acetyltransferase complex | 2.03                   |
| 54       | 6005942 | VCP      | VCP [Homo sapiens] | 602          | 100                  | 89,950   | 5.14 | DNA damage and repair | 2.2                     |
| 55       | 62087882 | HSPA4    | Heat shock 70 kDa protein 4 isoform a variant [Homo sapiens] | 866          | 100                  | 88,804.5 | 5.44 | Stress response | -2.5                   |
| 56       | 11962379 | CCT6A    | Chaperonin containing TCP1, subunit 6A (zeta 1), isoform CRA_b [Homo sapiens] | 70           | 97.216               | 39,433.9 | 6.74 | Protein folding | -2.08                  |
| 57       | 62089196 | TRAP1    | TNF receptor-associated protein 1 variant [Homo sapiens] | 605          | 100                  | 80,227.8 | 8.32 | Stress response | -1.54                  |
| 58       | 62896593 | ENO1     | Enolase 1 variant [Homo sapiens] | 112          | 100                  | 47,453.4 | 7.01 | Glycolysis/gluconeogenesis | -2                     |
| 59       | 62897701 | SNRPA1   | Small nuclear ribonucleoprotein polypeptide A' variant [Homo sapiens] | 150          | 100                  | 28,498.2 | 8.72 | Spliceosome | 12.44                  |

Table 1 (continued)
chromatography-tandem MS (Suberbielle et al., 2008). However, to our knowledge, this is the first proteomic-based investigation which uses a wild-type BDV virus of human origin (BDV Hu-H1) to analyze its impact on a white matter-derived human oligodendroglial cell line.

Applying the foregoing approach, 63 unique differential host proteins were identified (Table 1), and bioinformatic analysis revealed that energy metabolism and mitogen-activated protein kinase (Raf/MEK/ERK) signaling cascade, which are discussed in further detail below, were significantly altered in BDV Hu-H1-infected OL cells compared to the non-infected control cells.

**Energy metabolism**

Through KEGG analysis, energy metabolism (i.e., pentose phosphate pathway, glycolylate and dicarboxylate metabolism, the TCA cycle, and glycolysis/glucoseogenesis) was identified as the most significantly altered set of host biological pathways (Table 2). Viral replication requires energy and macromolecular precursors derived from the metabolic network of the host cell. Metabolic flux studies have revealed that large DNA viruses like herpes viruses are able to actively redirect energy metabolism in the host cell rather than passively relying on basal host cell metabolic activity (Vastag et al., 2011). Human cytomegalovirus (HCMV) and HSV-1 infection significantly perturb glycolysis, TCA cycle, and pentose phosphate pathway intermediates in host cells. Hepatitis C virus (HCV) has been shown to significantly upregulate host cell glycolysis (Diamond et al., 2010). Our own previous metabolic study has shown a downstream equilibrium shift away from glycolysis in conjunction with increased carbon flux through the TCA cycle in BDV-infected OL cells (Huang et al., 2012).

This study’s findings expand upon previous reports by showing energy metabolic dysfunction through significant alterations in six differential proteins in OL/Hu-H1 cells compared to control cells. As summarized in Table 1, three pentose phosphate pathway proteins (ribose-phosphate pyrophosphokinase, methylribosyl-5'-phosphate synthetase, and ribose-phosphate pyrophosphokinase), one glycolytic protein (enolase), and two TCA cycle and dicarboxylate metabolic proteins (cytoplasmic aconitate hydratase and cDNA FLJ51705 [highly similar to mitochondrial aconitate hydratase]) were found to be down- or up-regulated (see arrows in text).

Ribose-phosphate pyrophosphokinase, alternatively termed 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase, catalyzes the conversion of ribose 5-phosphate into PRPP and is essential to RNA synthesis. Previous studies on HCMV and a plant virus – potato virus Y (PVY) infecting tobacco have shown ribose-phosphate pyrophosphokinase upregulation in infected cells, suggesting the enzyme’s role in de novo viral nucleic acid biosynthesis via the pentose phosphate pathway (Sindelar and Sindelarova, 1987; Predmore, 2011). The here found down-regulation of ribose-phosphate pyrophosphokinase in OL/Hu-H1 cells is in contrast to these findings in DNA viruses,
suggesting that BDV Hu-H1 infection favors ribose 5-phosphate over RNA's biosynthetic precursor PRPP. However, this observation is consistent with BDV's characterization as a slow-replicating virus which is able to establish persistence with a lack of demonstrable viral particles (Ludwig et al., 1988), an infection type that would not require high levels of de novo viral RNA biosynthesis.

Mammalian transketolase 1 connects the pentose phosphate pathway to glycolysis, feeding/extracting sugar phosphates into/from the primary carbohydrate metabolic pathways by reversibly catalyzing the transfer of two-carbon glycoaldehyde units from ketose-donors to aldose-acceptor sugars (e.g., sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate → ribose 5-phosphate + xylulose-5-phosphate). Consistent with our

| KEGG pathway                      | Number of molecules | Fisher test p-value |
|-----------------------------------|---------------------|---------------------|
| Pentose phosphate pathway         | 3                   | 3e-4                |
| Glyoxylate and dicarboxylate metabolism | 2               | 2.5e-3              |
| Regulation of actin cytoskeleton  | 5                   | 4.1e-3              |
| Tricarboxylic acid (TCA) cycle    | 2                   | 1.07e-2             |
| Focal adhesion                    | 4                   | 1.79e-2             |
| Spliceosome                       | 3                   | 2.55e-2             |
| Insulin signaling pathway         | 3                   | 3.05e-2             |
| Glycolysis/gluconeogenesis        | 2                   | 3.71e-2             |
| Purine metabolism                 | 3                   | 4.19e-2             |
| Long-term potentiation            | 2                   | 4.66e-2             |

Fig. 3. Western blotting validation of PEBP-1 and Crkl. (a) Western blotting of PEBP-1 and Crkl with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a control. (b) PEBP-1 (p = 0.015) was found to be significantly up-regulated, and Crkl (p = 0.03) was found to be significantly down-regulated, in OL/Hu-H1 cells compared to control cells.

Fig. 4. Western blotting validation of Raf/MEK/ERK signaling proteins. (a) Western blots of five key Raf/MEK/ERK signaling proteins (i.e., p-Raf, p-MEK, p-ERK1/2, p-RSK, and p-MSK) with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a control. (b) P-ERK1/2 (P = 0.016) and p-RSK (P = 0.0495) were found to be significantly up-regulated, and p-MSK (P = 0.024) was found to be significantly down-regulated, in OL/Hu-H1 cells relative to control cells. There was no significant dysregulation observed in p-Raf (P = 0.744) or p-MEK (P = 0.267). (c) Activation of the Raf/MEK/ERK signaling cascade in human OL cells induces downstream phosphorylation of the transcription factor CREB through RSK and MSK.
current findings, transketolase (in addition to several other proteins functioning in nucleotide synthesis and homeostasis) has also been shown to be significantly up-regulated in HCV-infected human hepatocytes, supporting the metabolic rerouting into the pentose phosphate pathway that generates ribose 5-phosphate (Diamond et al., 2010).

Phosphoglucomutase 2 catalyzes the interconversion of ribose-1-phosphate and ribose-5-phosphate and also participates in glycolysis through interconverting glucose-1-phosphate and glucose-6-phosphate. Consistent with our findings in OL/Hu-H1 cells, previous studies on HCMV and H5N1 avian influenza virus have also shown phosphoglucomutase 2 upregulation in infected cells (Zou et al., 2010; Predmore, 2011). The phosphoglucomutase 2 upregulation observed in OL/Hu-H1 cells here may be associated with the aforementioned changes in ribose-5-phosphate pyrophosphokinase and transketolase 1, both of which affect ribose-5-phosphate levels in OL/Hu-H1 cells.

Enolase 1 is an isoenzyme of enolase, a key glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. Previous studies on enteroavivirus 71 and HCV have revealed significantly up-regulated enolase 1 in rhabdomyosarcoma and hepatocellular carcinoma cell lines (Takashima et al., 2005; Leong and Chow, 2006). The here observed opposite effect of down-regulated enolase 1 in OL/Hu-H1 cells suggests a different mechanism of affecting host cell glycolysis. Interestingly, a previous metabolic analysis of HSV-1-infected cells indicates a bottleneck in glycolytic efflux at the step catalyzed by pyruvate kinase, the enzyme that converts phosphoenolpyruvate to pyruvate. This 'glycolytic bottleneck' was accompanied by increased levels of pentose phosphate pathway intermediates, thereby increasing the availability of ribose-5-phosphate (Vastag et al., 2011). Therefore, the down-regulation of enolase-1 observed in OL/Hu-H1 cells here could be an alternative 'glycolytic bottleneck'-based mechanism to increase the availability of ribose-5-phosphate – the aforementioned upregulation of transketolase 1 and phosphoglucomutase 2 in OL/Hu-H1 cells is consistent with this conjecture.

The cytoplasmic aconitate hydratase (aconitase) and cDNA FLJ51705 (highly similar to mitochondrial aconitate hydratase) were significantly dysregulated in opposing directions. Aconitate hydratase catalyzes the isomerization of citrate to isocitrate in the TCA cycle and has a dual subcellular localization in the cytoplasm and mitochondria displaying differences in sensitivity to stimulation, inhibition, and stability (Hernanz and de la Fuente, 1988; Eprintsev et al., 2002). Therefore, one or more of these isoforms may be involved in regulatory activities independent of their traditional metabolic activities, producing the simultaneous up-regulation of one isoform and simultaneous down-regulation of another isoform observed here. Consistent with our findings, a previous proteomic study of BDV He/80-infected cortical neurons also found significant dysregulation of mitochondrial aconitate hydratase (Suberbielle et al., 2008). A comprehensive systems level study that includes transcriptomic, proteomic, and metabolic lines of analysis should clarify the precise

![Fig. 5. Cell proliferation assay with and without ERK inhibitor U0126. Cell proliferation was detected by CCK-8 assay. Hu-H1 infection decreased OL cell proliferation. ERK inhibitor U0126 decreased OL cell proliferation but did not decrease OL/Hu-H1 cell proliferation. Data were expressed as mean ± SD of four independent experiments with similar results. *p < 0.05 comparing to control.](image)

![Fig. 6. Immunofluorescence and co-localization analysis of pERK1/2 in nucleus. (a) OL/Hu-H1 cells and control OL cells were analyzed for the expression of activated ERK in nucleus by immunofluorescence. (b) BDV Hu-H1 induced lower pERK in nucleus than control OL cells (p = 0.04), suggesting impaired nuclear translocation of pERK.](image)
mechanism(s) by which BDV Hu-H1 impacts energy metabolism in human OL cells.

The Raf/MEK/ERK signaling cascade

Mitogen-activated protein kinase (MAPK) signal transduction cascades have been implicated in a variety of cellular functions including proliferation, differentiation, cell activation, immune responses and apoptosis (Pearson et al., 2001; Kurokawa and Kornbluth, 2009). In mammalian cells, three MAPK families have been thus far characterized: ERK, which is activated by growth factors, peptide hormones and neurotransmitters, Jun kinase (JNK) and p38 MAPK, which are both activated by cellular stress stimulus as well as growth factors (Frodin and Gammeltoft, 1999). The Raf/MEK/ERK signaling cascade is activated by many viruses, including BDV and several other human pathogenic RNA viruses (e.g., influenza, Ebola, HCV, and SARS-CoV) (Pleschka, 2008).

Here, by initial manual querying, two proteins associated with the Raf/MEK/ERK signaling cascade were found to be significantly altered in OL/Hu-H1 cells compared to control cells (arrows represent up/down-regulation in OL/Hu-H1 cells compared to control cells): PEBP-1 and CrkL. PEBP-1, alternatively termed Raf kinase inhibitor protein (RKim), regulates Raf/MEK/ERK signaling activity by competitively disrupting the interaction between Raf and MEK, thereby negatively interfering with the downstream activation of MEK and ERK (Yeung et al., 1999). CrkL has been shown to activate the Ras/Raf signaling pathway and transform fibroblasts in a Ras-dependent fashion (Senechal et al., 1996). CrkL is down-regulated in OL/Hu-H1 cells compared to control cells. Interestingly, in neurons, such down-regulation has been shown to block dendritogenesis during the development of the CA1 region in the hippocampus in vivo under specific conditions (Matsuki et al., 2008).

Based on these initial findings, we hypothesized that the Raf/MEK/ERK signaling cascade was perturbed in OL/Hu-H1 cells. Therefore, five key Raf/MEK/ERK signaling proteins (i.e., p-Raf, p-MEK, p-ERK1/2, p-RSK, and p-MSK) were selected for Western blotting validation (Fig. 4a). P-ERK1/2 and p-RSK were found to be significantly up-regulated, and p-MSK was found to be significantly down-regulated, in OL/Hu-H1 cells compared to control cells; however, there was no significant dysregulation observed in p-Raf or p-MEK (Fig. 4b). These combined findings indicate that BDV Hu-H1 activates the downstream ERK–RSK complex of the Raf/MEK/ERK signaling cascade in human OL cells (Fig. 4c). RSK, a substrate of ERK and a mediator of ERK signal transduction, is composed of two functional kinase domains that are activated in a sequential manner by a series of phosphorylations; MSK is a RSK-related kinase activated by ERK as well as p38 MAPK (Frodin and Gammeltoft, 1999). The activated ERK–RSK complex observed here has several proposed functions, including: (i) regulation of gene expression through phosphorylation of transcriptional regulators, such as NFκB/IkBα, cAMP-response element-binding protein (CREB), and CREB-binding protein; (ii) regulation of protein synthesis by phosphorylation of polyribosomal proteins and glycogen synthase kinase-3; and (iii) phosphorylation of the Ras GTP/GDP-exchange factor, Sos, leading to feedback inhibition of the Raf/MEK/ERK pathway (Frodin and Gammeltoft, 1999).

Many viruses manipulate the contributing kinases but differently in accord to their life cycles (Leong and Chow, 2006; Predmore, 2011; Vastag et al., 2011). Notably, BDV He/80 has also been shown to activate the Raf/MEK/ERK signaling cascade in several persistently-infected mammalian cell lines (Planz et al., 2001). In this study, BDV Hu-H1 was found to activate the ERK1/2 pathway, as well. As up-regulated pERK should increase the proliferation of cells (Seger and Krebs, 1995; Pearson et al., 2001), the CCK-8 experiment was conducted (Fig. 5) to detect proliferation and growth. In contrast to the higher ERK1/2 expression of BDV Hu-H1-infected OL cells, we found down-regulation of proliferation which was stronger than in uninfected control OL cells treated with ERK inhibitor U0126 which is apparently contradictory. However, a similar finding has been reported by Hans et al., namely that BDV activated the ERK1/2 pathway in a persistently-infected neural crest-derived cell line (PC12), but at the same time decreased the differentiation in PC cells, due to impaired translocation of ERK1/2 to the nucleus (Hans et al., 2001). We were able to show the same effects in pERK co-localization immunofluorescence assays, comparing OL/Hu-H1 cells and control OL cells (Fig. 6). Impaired translocation of key signal transduction kinases might be associated with the trafficking of viral proteins from the nucleus to the cytoplasm and vice versa, involved in nuclear replication, a unique feature of the family Bornaviridae among the order Mononegavirales (de la Torre, 1994).

Impaired translocation may at least partially explain our findings of up-regulated pERK and down-regulated proliferation in OL cells infected with a human strain of BDV. Inhibited cell proliferation was previously observed in human but not laboratory BDV (Li et al., 2013). Given the complex regulatory network between the ERK/RSK pathway, cell proliferation and programed cell death by apoptosis in multiple mammalian cell lines (for review Kurokawa and Kornbluth, 2009), the precise manner by which BDV Hu-H1’s activation of the ERK–RSK complex interferes with cell proliferation and the other biological processes warrant further research.

CONCLUSION

In summary, our findings using a 2-DE-MALDI-TOF-MS/MS-based proteomic approach indicate that human BDV strain Hu-H1 manipulates brain-derived human OL cells significantly. We found 63 differential host proteins on infected vs. non-infected cells. By bioinformatic analysis, energy metabolism was the most significantly altered set of host biological pathways in BDV Hu-H1-infected OL cells. In addition, Western blotting validation demonstrated significant perturbation of the host’s Raf/MEK/ERK signaling cascade: specifically, the downstream
ERK–RSK complex of the Raf/MEK/ERK signaling cascade was found to be activated by BDV Hu-H1 infection. Although BDV Hu-H1 produces constitutive activation of the ERK1/2 pathway, pERK’s nuclear translocation was impaired. Further investigation on cell proliferation and other biological processes in BDV-infected oligodendrocytes and other brain cell lines is key to better understanding the neuropathogenesis of BDV.

CONFLICT OF INTEREST

The authors (Xia Liu, Yongtao Yang, Liv Bode, Mingjun Zhao, Lujun Zhang, Junxi Pan, Lin Lv, Yuan Zhao, Siwen Liu, Liang Zhang, Xiao Wang, Rongzhong Huang, Jingjing Zhou, Peng Xie) declare no conflicts of interest. Liv Bode’s authorship in this study is independent of and has no relationship to her current affiliation at the Department of Epidemiology and Health Monitoring, Robert Koch Institute, Berlin, Germany.

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