**Proteomic profiling of epileptogenesis in a rat model:**

**focus on cell stress, extracellular matrix and angiogenesis**

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**Abstract**

Information about epileptogenesis-associated changes in protein expression patterns is of particular interest for future selection of target and biomarker candidates.

Bioinformatic analysis of proteomic data sets can increase our knowledge about molecular alterations characterizing the different phases of epilepsy development following an initial epileptogenic insult.

Here, we report findings from a focused analysis of proteomic data obtained for the hippocampus and parahippocampal cortex samples collected during the early post-insult phase, latency phase, and chronic phase of a rat model of epileptogenesis.

The study focused on proteins functionally associated with cell stress, cell death, extracellular matrix (ECM) remodeling, cell-ECM interaction, cell-cell interaction, angiogenesis, and blood-brain barrier function. The analysis revealed prominent pathway enrichment providing information about the complex expression alterations of the respective protein groups.

In the hippocampus, the number of differentially expressed proteins declined over time during the course of epileptogenesis. In contrast, a peak in the regulation of proteins linked with cell stress and death as well as ECM and cell-cell interaction became evident at later phases during epileptogenesis in the parahippocampal cortex. The data sets provide valuable information about the time course of protein expression patterns during epileptogenesis for a series of proteins. Moreover, the findings provide comprehensive novel information about expression alterations of proteins that have not been discussed yet in the context of epileptogenesis. These for instance include different members of the lamin protein family as well as the fermitin family member 2 (FERMT2). Induction of FERMT2 and other selected proteins, CD18 (ITGB2), CD44 and Nucleolin were confirmed by immunohistochemistry.

Taken together, focused bioinformatic analysis of the proteomic data sets completes our knowledge about molecular alterations linked with cell death and cellular plasticity during epileptogenesis. The analysis provided can guide future selection of target and biomarker candidates.

KEYWORDS: epilepsy, proteomics, status epilepticus, ECM, cell death, apoptosis

**Introduction**

Comprehensive cellular and network alterations characterize the process of epileptogenesis following an epileptogenic brain insult ([Dichter, 2009](#_ENREF_13); [Pitkanen and Lukasiuk, 2011](#_ENREF_59)). Thereby, cell stress along with associated neuronal cell loss affecting different susceptible brain regions serve as an important trigger for subsequent remodeling of brain tissue and its cellular composition ([Dingledine et al., 2014](#_ENREF_14)). Cellular plasticity is accompanied by intense molecular alterations in extracellular matrix (ECM) components, which in turn can play a crucial modulatory role following brain damage ([Dityatev and Fellin, 2008](#_ENREF_15); [McRae and Porter, 2012](#_ENREF_45); [Pitkanen et al., 2014](#_ENREF_60)). Remodeling does not only involve neuronal and glial cells but also cells of the cerebrovascular system ([Marchi and Lerner-Natoli, 2013](#_ENREF_42)). Enhanced angiogenesis and blood-brain barrier dysfunction are discussed as pro-epileptogenic factors contributing to the development of a hyperexcitable network ([Marchi and Lerner-Natoli, 2013](#_ENREF_42); [Morin-Brureau et al., 2012](#_ENREF_50)).

From an overarching perspective, neuronal cell loss, ECM alterations and angiogenesis can be considered a linked triad of events, which can significantly contribute to the development of structural epilepsy. ECM remodeling can be a consequence of insult-associated neuronal damage as well as blood-brain barrier alterations with subsequent activation of astrocytes, which are a major source of components and modulators of the ECM (Soleman et al., 2013; Kim et al., 2016). The other way round, various ECM molecules play an important role in the regulation of angiogenesis (Mongiat et al., 2016). In this context, we emphasize that targeting of both, ECM remodeling and angiogenesis, is discussed to reduce seizure susceptibility and epileptogenicity (Kim et al., 2016; Pitkänen et al., 2014; Morin-Brureau et al., 2012).

Whereas some molecular alterations contributing to the respective cellular alterations have been studied in chronic epilepsy models, there is only limited information about the complex changes in the total protein expression patterns. Transcriptomic analysis in models with development of structural epilepsy provided information about the regulation of mRNAs associated with neuronal death, ECM modulation and angiogenesis ([Hansen et al., 2014](#_ENREF_21); [Hunsberger et al., 2005](#_ENREF_29); [Okamoto et al., 2010](#_ENREF_51)). However, the findings from transcriptomic studies do not always translate into proteome alterations considering post-transcriptional and post-translational regulation mechanisms ([Vogel and Marcotte, 2012](#_ENREF_72)).

As we have demonstrated previously, large-scale proteomic profiling can render comprehensive data sets with information about the temporal profile of proteome alterations during the course of epilepsy development ([Keck et al., 2017](#_ENREF_31)), hypothesis-driven and focused analyses can in particular help to identify sets of proteins linked with a specific pathophysiological process ([Keck et al., 2017](#_ENREF_33); [Walker et al., 2016](#_ENREF_73)). We capitalize on a previous proteomics profiling approach based on liquid chromatography tandem mass spectrometry (LC-MS/MS) using individual hippocampal (HC) and parahippocampal cortex (PHC) tissue samples from a rat post-status epilepticus model. The proteome profile confirmed on one hand previously reported proteins which are dysregulated during epileptogenesis, but also identified a series of interesting novel proteins associated with immunity and inflammation that proved to be co-regulated in different phases of epilepsy development ([Walker et al., 2016](#_ENREF_73)).

Here, we now focused the bioinformatics analysis of the proteomics dataset on proteins and pathways linked with neuronal death, ECM changes and angiogenesis. Prominent and complex epileptogenesis-associated alterations were identified for all three protein groups. The analysis provides valuable information about the time course of expression patterns for various protein subgroups and individual proteins. The findings received further confirmation by immunohistochemical demonstration of the hippocampal induction of FERMT2 in the latency phase

**Materials and Methods**

*Animals*

The investigation has been approved by the responsible government (reference number 55.2-1-54-2532-94-11) and has been conducted in compliance with the German Animal Welfare act and the EU directive 2010/63/EU. Experiments were performed in female Sprague Dawley rats (n = 59; Harlan Laboratories, now Envigo, Udine, Italy) at 10–11 weeks age (200-224 g) after at least one week of acclimation. Animals were housed with a constant light/dark cycle of 12/12 h (lights on at 7:00 a.m. – lights off at 7:00 p.m.), under controlled temperature (20-24°C) and humidity (45-65%) conditions. Animals received nesting material and free access to standard food and tap water in their home cage. Every effort was made to avoid or reduce pain or discomfort and to minimize the number of animals used in the study.

*Post-status epilepticus model*

Rats underwent stereotactic implantation of the combined recording and stimulation electrode in the right anterior basolateral amygdala as previously described ([Walker et al., 2016](#_ENREF_73)). At least six weeks after electrode implantation, a status epilepticus (SE) was induced as described by [Ongerth et al. (2014)](#_ENREF_52). Only animals exhibiting a generalized SE (85%) were included in the subsequent experiments. Groups of five electrode-implanted control animals and five SE rats were euthanized by intraperitoneal pentobarbital injection (500 mg/kg; Narcoren, Sigma-Aldrich GmbH, Munich, Germany) two days, ten days, and eight weeks after SE induction. After removing the brain, hippocampus and parahippocampal cortex (containing the entorhinal, perirhinal und posterior-piriform cortex) were rapidly dissected on ice, for the PHC the ventrocaudal cortex was cut ventral to the rhinal fissure until approximately 5mm posterior to bregma. followed dissection the ipsilateral and contralateral tissue was pooled. Tissue samples were transferred to ice cold phosphate-buffered saline (PBS, pH 7.2). The subsequent mass spectrometry analysis was performed using tissue samples of the hippocampus and the parahippocampal cortex. Animals belonging to the group with tissue sampling eight weeks following SE induction underwent a continuous video- and EEG-monitoring (24 h per day / seven days a week over 19 days) starting 6 weeks following SE with a combined EEG- and video-detection system as previously described ([Pekcec et al., 2008](#_ENREF_56)). Animals that did not exhibit spontaneous generalized seizures were excluded from further analysis.

*Mass spectrometry, label-free quantification and protein identification*

The dataset analyzed here was previously published (Walker et al., 2016). It was generated by label-free LC-MSMS-based proteomics as described (Walker et al., 2016). Briefly, after tissue extraction, proteolysis and LC-MSMS, MS spectra were used for quantification by the Progenesis LC-MS software (Version 2.5, Nonlinear Dynamics) for label-free quantification and MS/MS spectra were used for protein identification with Mascot as described (([Hauck et al., 2010](#_ENREF_23); [Hauck et al., 2012](#_ENREF_24); [Walker et al., 2016](#_ENREF_73)). For quantification, only unique peptides of an identified protein were used and the total cumulative normalized abundance was measured by summarizing the abundances of all peptides assigned to the respective protein. Further analysis was performed with proteins quantified with a minimum of two peptides. The relation of protein abundances between control and SE animals (fold change) was determined to measure differential expression. All proteins exhibiting a fold change ≥ 1.5 (up- or downregulated) with a corresponding *p*-value (ANOVA) < 0.05 were considered differentially expressed.

*Pathway analysis*

Pathway analysis was performed as described previously by [Walker et al. (2016)](#_ENREF_73). Briefly, following LC-MS/MS, gene symbols for all quantified proteins were searched in the Ensemble database (http://www.ensembl.org/Rattus\_norvegicus/; version 69; 32971 sequences). If a rat genome annotation was not present, the human orthologue was used. Pathway enrichment analysis was performed using both a licensed (Genomatix Pathway System (GePS), Genomatix Software GmbH, Munich, Germany) and a publically available (ConsensusPathDB over-representation tool, [Kamburov et al. (2011)](#_ENREF_31); [Kamburov et al. (2009)](#_ENREF_32)) pathway tool for a combined pathway analysis.

The respective background lists for the three different time points consisted of the respective sets of total quantified hippocampal or parahippocampal proteins. Only enriched pathways linked with cell stress and cell death, ECM or angiogenesis (p-value < 0.05 and at least two protein members) were considered for further analysis.

*Expression analysis*

The regulation patterns of selected proteins were analyzed and visualized by heat maps. Individual fold changes were calculated for these proteins, proteins with missing values and zeros were excluded from further analysis. Fold changes were log2 transformed after which the heatmap.2 function included in the R package “gplots” ([Warnes et al., 2016](#_ENREF_74)) was used to create the heat maps.

*Tissue preparation and immunostaining*

To validate the results of the mass spectrometry analyses, immunohistochemistry was performed for four selected proteins, fermitin family member 2 (FERMT2), Integrin beta-2 (CD18), CD44 and Nucleolin. Animals were euthanized with an intraperitoneal injection of pentobarbital (500 mg/kg; Narcoren, Sigma-Aldrich GmbH, Munich, Germany). Subsequently, brains were dissected and post-fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) at 4°C for three days.

For FERMT2 and Nucleolin: An embedding workstation (Histomaster 2050/Di, Bavimed, Birkenau, Germany) was used to prepare paraffin embedded rat brain tissue blocks. Paraffin blocks were sectioned at 2 µm. Brain tissue 3,24 mm caudal bregma was used for immunohistochemical stainings. Tissue was deparaffinized using xylene and a xylene/ alcohol mixture (1:1; 100% EtOH). Afterwards, sections were rehydrated in alcohols (100, 95, 70, and 50% EtOH for 3 min each). Sections were then washed with tap water and distilled water. Heat-induced antigen retrieval was performed by placing the sections in 0.01 M sodium citrate (pH 6.0) in a microwave oven for 20 min. Following a washing step with Tris-buffered saline (TBS; 0.05% Tween 20), sections were incubated in 3% TBS-buffered H2O2 for 20 min and rinsed with TBS (0.05% Tween 20). Incubation with blocking solution (0.25% casein in TBS; 10 min) was followed by treatment with a primary antiserum overnight at 4 °C. Monoclonal mouse anti-rat FERMT2 (LSBio Life Span BioSciences Inc., Seattle, Washington, USA; 1:1000) or monoclonal rabbit anti-rat Nucleolin (Abcam, Cambridge, MA, USA; 1:1000) were used as the primary antibodies. Following another washing step (TBS; 0.05% Tween 20), sections were placed in respectively, biotin-labeled secondary antiserum (FERMT2: biotinylated donkey anti-mouse IgG (Dianova, Hamburg, Germany), 1:500 or Nucleolin: biotinylated goat anti-rabbit IgG (Dianova, Hamburg, Germany), 1.500) for 30 min. Following a washing step (TBS; 0.05% Tween 20), sections were incubated in, respectively horseradish peroxidase-labeled streptavidin (1:2000; Jackson Immunoresearch, West Grove, USA) or VECTASTAIN ABC-Peroxidase Kit (Vectastain Elite Kits, Vector Laboratories, Burlingame, CA) for 30 min. Finally, a nickel-intensified diaminobenzidine (0.025% 3,3’-diaminobenzidine, 0.6% ammonium nickel(II) sulfate and 0.025% H2O2) or diaminobenzidine (Sigmafast; Sigma-Aldrich GmbH, Munich, Germany) reaction was conducted. Sections were then air dried overnight and coverslipped with Entellan (Merck, Darmstadt, Germany).

For Integrin beta-2 (CD18) and CD44: Brain were placed into 30% sucrose in PBS for 24 hours after which 40µm sections were cut on a cryostat (HM560M, Microm International, Walldorf, Germany). Heat-induced antigen retrieval (at 80°C for 30 min) was performed using sodium citrate (pH 6.0). Following a washing step with Tris-buffered saline (TBS; 0.05% Tween 20), sections for CD18 were incubated in 3% TBS-buffered H2O2 for 20 min and rinsed with TBS (0.05% Tween 20). Incubation with blocking solution (TBS containing Triton X100 and bovine serum albumin (Millipore, Darmstadt, Germany); 90 min) was followed by treatment with a primary antiserum overnight at 4 °C. Polyclonal rabbit anti-rat CD18 (Abcam, Cambridge, MA, USA; 1:1000) or monoclonal mouse anti-human CD44 (R&D Systems, Minneapolis, MN, USA; 1:1000) were used as the primary antibodies. Following another washing step (TBS; 0.05% Tween 20), sections were placed in respectively, biotin-labeled secondary antiserum (CD18: biotinylated goat anti-rabbit IgG (Dianova GmbH, Hamburg, Germany), 1:1500 or CD44: biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA), 1.1000) for 90 min. Following a washing step (TBS; 0.05% Tween 20), sections were incubated in Vectastain ABC-Peroxidase Kit (Vectastain Elite Kits, Vector Laboratories, Burlingame, CA) for 30 min. Finally, a diaminobenzidine (Sigmafast; Sigma-Aldrich GmbH, Munich, Germany) reaction was conducted. Sections were then air dried overnight and coverslipped with Entellan (MerckMillipore, Darmstadt, Germany).

Immunohistochemical staining was analyzed using the StereoInvestigator software (11.03.1, MBF Bioscience, Burlington, Vermont) using a 16X and (Leica PL FLUOTAR 16X, Bensheim, Germany) and CCD colour camera (CX9000, MBF Europe, Magdeburg, Germany).

**Results**

*Enrichment of pathways linked with cell stress and cell death*

An overrepresentation of proteins associated with cell stress and cell death was confirmed during the course of epileptogenesis by pathway enrichment analysis using Genomatix and ConsensusPathDB (Tables 1-4). In the early post-insult phase, we identified seven (Genomatix) and 14 (ConsensusPathDB) overrepresented pathways in the HC and two (Genomatix) and three (ConsensusPathDB) overrepresented pathways in the PHC. Regulation of four (Genomatix) and six (ConsensusPathDB) pathways in the HC and of four (Genomatix) and ten (ConsensusPathDB) pathways in the PHC was evident during the latency phase. In the chronic phase following verification of the manifestation of epilepsy, three (Genomatix) pathways in the HC as well as three (Genomatix) and 14 (ConsensusPathDB) pathways in the PHC exhibited a significant regulation. Taken together, a different time course pattern became evident with an earlier regulation of respective protein pathways in the HC and a more delayed regulation in the PHC (Figure 1).

The list of pathways covered by regulated proteins included several pathways linked to apoptosis and its regulation, to heat-shock proteins and their regulation, and to DNA repair.

Please note that regulation of some of the pathways and proteins listed in Tables 1-4 has already been reported in our previous publication ([Walker et al., 2016](#_ENREF_73)) due to some overlap between groups of proteins linked with cell stress and cell death and linked with inflammation and immunity, which has been the focus of the previous analysis.

*Dynamic expression profile of proteins functionally linked with apoptosis*

The list of pathways, which are associated with cell stress and cell death and which proved to be overrepresented during the course of epileptogenesis, is dominated by pathways that are functionally involved in apoptosis and its regulation.

Considering the time course pattern, our data revealed tremendous differences between the HC and PHC (Figure 2). In the HC, an up-regulation of apoptosis-linked proteins proved to be most pronounced in the early post-insult phase and less pronounced during the latency phase. In the chronic phase, only two hippocampal proteins showed altered expression rates.

In the PHC, approximately half of the differentially expressed apoptosis-linked proteins proved to be up- or down-regulated, two days and ten days following SE. Following epilepsy manifestation, the majority of dysregulated apoptosis-linked proteins in the PHC exhibited overexpression.

Prohibitin (PHB) is a mitochondrial protein that can act as a chaperone or antichaperone depending on concentrations ([Zhou et al., 2012](#_ENREF_77)). This protein proved to be up-regulated in the HC in the early phase following the epileptogenic insult. For a more detailed description of the regulation of heat shock proteins, which also act as chaperones, we refer to our previous publication ([Walker et al., 2016](#_ENREF_73)).

Several proteins that have been proposed as modulators and regulators of apoptosis exhibited altered expression rates during the course of epileptogenesis. SE induced an early overexpression of P21 (Rac1) activated kinase 2 (PAK2) in the HC and a delayed overexpression in the PHC, which has been described as a putative regulator of apoptosis that is activated during caspase-mediated apoptosis ([Eron et al., 2017](#_ENREF_17)). Ankyrin repeat and KH domain containing 1 (ANKHD1) is thought to exert an antiapoptotic function based on the regulation of caspases ([Miles et al., 2005](#_ENREF_47)). Expression of this protein showed an early reduction two days following SE. B-cell receptor-associated protein 31 (BCAP31), which has been reported to be involved in caspase-8-mediated apoptosis ([Iwasawa et al., 2011](#_ENREF_30)), exhibited an up-regulation during the early post-insult phase and the latency phase in the PHC. Transglutaminase 2 (TGM2), another apoptosis-linked protein ([Kuo et al., 2011](#_ENREF_38)), showed increased expression rates in both brain regions in the first two phases of epileptogenesis.

Bassoon is a presynaptic protein, which has been suggested to play a regulatory role in apoptosis ([Heyden et al., 2011](#_ENREF_26)). The expression rates of Bassoon exhibited a reduction during the early post-insult phase and the latency phase in the PHC. A protein involved in a wide range of apoptotic processes is Nucleolin ([Srivastava and Pollard, 1999](#_ENREF_68)), SE induced an overexpression of Nucleolin in the early and latent phase in both the HC as well as the PHC. This upregulation was also confirmed using a immunohistochemical staining of Nucleolin in the HC where throughout all layers of the CA region of the hippocampus an increased expression of the protein was observed (Figure 5A).

Synaptic Ras GTPase activating protein 1 (SYNGAP1) can contribute to neuronal homeostasis ([Knuesel et al., 2005](#_ENREF_37)). Its expression was reduced in the PHC two days and in the HC ten days following the epileptogenic insult.

Lamin proteins are nuclear lamina proteins, which seem to play a crucial role in nuclear stability ([Dechat et al., 2008](#_ENREF_12)). Evidence exists that at least some members of the family control apoptosis ([Harborth et al., 2001](#_ENREF_22); [Yang et al., 2011](#_ENREF_76)). During the course of epileptogenesis an overexpression of different members of the protein was observed in both brain regions of interest, with lamin A (LMNA) being regulated in the HC two days and ten days following SE and in the PHC ten days and eight weeks following SE. Lamin B1 (LMNB1) proved to be induced during the latency phase in both brain regions with PHC expression remaining at elevated levels in the chronic phase. In contrast, Lamin B2 (LMNB2) was exclusively regulated in the PHC following manifestation of epilepsy.

Poly(ADP-ribose) polymerase 1 (PARP1) can contribute to recovery from DNA damage ([Kim et al., 2015](#_ENREF_35)). Its expression proved to be up-regulated during the latency phase in the HC and during the chronic phase in the PHC.

The histone family of nuclear proteins is relevant for the structure of the nucleosomes. Histone modifications contribute to the regulation of transcription and label sites of DNA damage. Histone proteins exhibited a regulation in both regions with an induction of histone cluster 1 H1 family member a (HIST1H1A) ten days in the PHC and an induction of HISTH1B ten days in the HC and the PHC and eight weeks following SE in the PHC. In addition, H1 histone family member 0 (H1F0) proved to be up-regulated in the chronic phase with spontaneous recurrent seizures.

Please see the Supplementary File for information about the complete list of regulated proteins functionally linked with apoptosis and its regulation.

*Enrichment of pathways linked with the ECM, cell-ECM and cell-cell interactions*

Pathway analysis indicated an overrepresentation of proteins associated with the ECM, with ECM-cell interactions and with cell-cell interactions (Tables 5-8). In the HC we identified four (Genomatix) and two (ConsensusPathDB) overrepresented pathways in the early post-insult phase, three (Genomatix) and four (ConsensusPathDB) overrepresented pathways in the latency phase, and two (Genomatix) and three (ConsensusPathDB) overrepresented pathways in the chronic phase. Respective analysis of PHC data sets revealed a much more pronounced regulation of respective protein groups. Four (Genomatix) and ten (ConsensusPathDB) pathways proved to be overrepresented two days following SE. Ten days following SE, we identified a total of eleven (Genomatix) and eight (ConsensusPathDB) overrepresented pathways. In the chronic phase an overrepresentation of respective protein groups was still evident with four (Genomatix) and eight (ConsenusPathDB) pathways exhibiting a significant regulation.

Among others, the list of overrepresented pathways comprised protein groups functionally associated with matrix metalloproteinases, tissue inhibitor of metalloproteinase, Beta catenin degradation signaling, integrin signaling, paxillin, syndecan-mediated signaling, fibronectin matrix formation, extracellular matrix organization, urokinase-type plasminogen activator, and focal adhesion kinase I.

Please note that regulation of some of the pathways and proteins listed in Tables 5-8 has already been reported in our previous publication ([Walker et al., 2016](#_ENREF_73)) due to some overlap between groups of proteins linked with ECM, ECM-cell interaction and cell-cell interaction and linked with inflammation and immunity, which has been the focus of the previous analysis.

*Dynamic expression profile of proteins functionally linked with the ECM, cell-ECM and cell-cell interactions*

A prominent regulation of these protein groups was evident in both brain regions. However, we observed the most extensive regulation of respective proteins in the PHC ten days following SE (Figure 1).

In our data sets, only single ECM proteins were identified with differential expression in the PHC during the course of epileptogenesis. Whereas Tenascin C (TNC) expression only proved to be induced following epilepsy manifestation, agrin (AGRN) expression showed an earlier up-regulation, which became evident ten days following SE and persisted in the chronic phase.

Various proteins mediating and regulating cell-ECM interactions exhibited altered expression patterns following the epileptogenic insult. CD44 serves as a receptor for hyaluronic acid and interacts with collagens and matrix metalloproteinases ([Roszkowska et al., 2016](#_ENREF_64)). We confirmed overexpression of this cell surface molecule two and ten days following SE in both brain regions of interest. In addition, the overexpression of CD44 in the PHC following SE was shown using an immunohistochemical staining (Figure 5B).

Dystroglycan proteins can act as receptors for ECM proteins comprising laminin-G domains ([Bozzi et al., 2009](#_ENREF_7)). In the PHC the expression rate of dystroglycan 1 (DAG1) proved to be increased following epilepsy manifestation. An early and persistent induction of annexin A2 (ANXA2), which represents a receptor for tenascin C splice variants ([Chung and Erickson, 1994](#_ENREF_11)), was evident in the PHC and the HC.

Moreover, TGM2, which contributes to the interaction between astrocytes and fibronectin ([van Strien et al., 2011](#_ENREF_71)), showed an induction in both brain regions two and ten days following SE.

In addition to ECM receptor molecules, proteins that regulate or contribute to ECM remodeling proved to be regulated during epileptogenesis. These proteins included low density lipoprotein receptor-related protein (LRP1) and NME/NM23 nucleoside diphosphate kinase 1 (NME1). We obtained evidence for an up-regulation of LRP1 ten days following SE in the HC and during the latency phase in the PHC. NME1 exhibited a delayed down-regulation ten days following SE in the PHC.

We also confirmed regulation of various proteins with a key role in cell-cell adhesion and its regulation. Among these proteins, junctional adhesion molecule 3 (JAM3) exhibited a hippocampal regulation at the two earlier time points and a regulation in the PHC eight weeks following SE. Hippocampal cadherin 4 (CDH4) was exclusively down-regulated in the chronic phase following onset of spontaneous seizures.

In addition, several integrin molecules and integrin interaction partners exhibited altered expression patterns along with the progression of epileptogenesis. For detailed information about the expression regulation of these proteins please see heat maps in Figure 3 for the HC and in Figure 4 for the PHC as well as the Supplementary File. Among these proteins, FERMT2 and CD18 (ITGB2) stood out with a prominent induction in the HC ten days following SE. This induction was further confirmed using an immunohistochemical staining of both proteins (Figure 5 C & D). Immunopositive cells were found throughout the different sub-regions of the HC ten days following SE, while low levels were observed in control animals. In animals with SE, the most pronounced expression for both proteins was observed in the stratum radiatum of the CA1 sub-region.

*Enrichment of pathways linked with angiogenesis and blood-brain barrier function*

In the HC a low number of pathways linked with angiogenesis and blood-brain barrier function exhibited a significant regulation. We identified only three (Genomatix) and two (ConsensusPathDB) overrepresented pathways ten days following SE and two (Genomatix) overrepresented pathways in the chronic phase following epilepsy manifestation.

In contrast, a more pronounced regulation of respective protein pathways became evident in the PHC with two (Genomatix) and six (ConsensusPathDB) pathways overrepresented in the early post-insult phase, seven (Genomatix) and six (ConsensusPathDB) pathways overrepresented in the latency phase and two (Genomatix) and three (ConsensusPathDB) pathways overrepresented in the chronic phase. The list of pathways with overrepresentation of the associated protein members included pathways linked with ATP binding cassette transporter function, caveolin 1, paxillin, vascular endothelial growth factor (VEGF) signaling, vasoactive intestinal peptide, endothelial growth factor signaling, and basigin interactions.

Please note that regulation of some of the pathways and proteins listed in Tables 9-12 has already been reported in our previous publication ([Walker et al., 2016](#_ENREF_73)) due to some overlap between groups of proteins linked with angiogenesis and blood-brain barrier function, and linked with inflammation and immunity, which has been the focus of the previous analysis.

*Dynamic expression profile of proteins functionally linked with angiogenesis and blood-brain barrier function*

VEGF is a key regulator of angiogenesis ([Santos et al., 2007](#_ENREF_65)). Several proteins, which modulate VEGF expression and signaling or serve as downstream signaling factors in VEGF receptor activated cascades, exhibited differential expression rates during the course of epileptogenesis.

Annexin A1 (ANXA1), which contributes to VEGF-triggered signaling events ([Pin et al., 2012](#_ENREF_58)), proved to be significantly induced in both brain regions of interest ten days following SE.

Vimentin (VIM) has recently been described as a downstream target of heme oxygenase-1 in VEGF-mediated angiogenesis ([Bauer et al., 2016](#_ENREF_3)). Its expression levels proved to be increased at all time points in the PHC as well as ten days following SE in the HC. Myristoylated alanine-rich C kinase substrate-like 1 (MARCKSL1) can exert anti-angiogenic effects based on suppression of VEGFR-2-dependent phosphorylation events ([Kim et al., 2016a](#_ENREF_34)). During epileptogenesis MARCKSL1 exhibited a significant reduction ten days following SE in the PHC and a significant induction eight weeks following SE in the HC. The growth factor receptor bound protein 2 (GRB2) is a phosphorylation target of VEGFR-2-signaling ([Anselmi et al., 2012](#_ENREF_1)). We observed a down-regulation of GRB2 during the latency phase in the PHC.

The small GTPase cell division cycle 42 (CDC42) seems to be involved in macropinocytic internalization of VEGFR2 ([Basagiannis et al., 2016](#_ENREF_2)). In the PHC expression levels of both isoforms of CDC42 were lowered as a consequence of SE at the two earlier time points. In the HC only one isoform CDC42 proved to be regulated with an induction in the early post-insult phase.

ANXA2 has pro-fibrinolytic activity, which can mediate pro-angiogenic effects ([Liu and Hajjar, 2016](#_ENREF_40)). As described above, ANXA2 overexpression was evident during all phases of epileptogenesis in both brain regions of interest.

Kininogen-1 (KNG1) can exert anti-angiogenic effects ([Ren et al., 2016](#_ENREF_62)). A strong hippocampal induction of KNG1 was evident ten days following SE.

Parahippocampal expression of melanoma cell adhesion molecule (MCAM or CD146), which can modulate angiogenesis ([Ouhtit et al., 2009](#_ENREF_53)), reached increased levels two and ten days following SE.

The serine/threonine protein kinase paralog ROCK2 serves as a modulator of angiogenesis ([Montalvo et al., 2013](#_ENREF_48)). During the latency phase we confirmed a transient reduction of ROCK2 in the PHC.

The role of migration inhibitory factor (MIF) in angiogenesis has been intensely studied ([Chesney and Mitchell, 2015](#_ENREF_10)). A delayed down-regulation of MIF persisting into the chronic phase became evident in the PHC.

Several proteins, which play a role in cytoskeleton regulation and re-organization exhibited altered expression profiles during epileptogenesis. In the PHC ezrin (EZR) overexpression was evident throughout the different phases of epileptogenesis. In contrast, a transient reduction of stathmin 1 (STMN1) occurred during the latency phase. Whereas talin 1 (TLN1) expression showed an early induction in the HC, a delayed up-regulation of this protein was observed in the PHC.

Filamin A (FLNA) exhibited increased expression rates at all time points in both brain regions except for the chronic phase in the HC.

Proteins playing a crucial role in caveola formation have been described as indirect regulators of angiogenesis. Here, we obtained evidence for regulation of polymerase I and transcript release factor (PTRF). Whereas PTRF overexpression was evident in both brain regions of interest ten days following SE, the protein proved to be only up-regulated in the PHC eight weeks following SE.

Nitric oxide can stimulate angiogenesis directly as well as indirectly by regulation of angiogenic factor expression ([Morbidelli et al., 2004](#_ENREF_49)). Analysis of the PHC data set of differentially expressed proteins revealed an early down-regulationof guanylate cyclase 1 soluble subunit beta (GUCY1B3), which constitutes a receptor for nitric oxide ([Chang et al., 2011](#_ENREF_9)).

For detailed information about the expression regulation of these proteins please see heat maps in Figure 6 as well as the Supplementary File.

*Comparison with published proteomics data sets*

In addition to the described analyses of the proteomics data set the list of differently expressed proteins for the different focus areas were compared to a number of published proteomics studies. A numeric overview of this comparison can be found in table 13 (the table with the full protein names can be found in the supplementary file). Due to methodological differences little overlap in proteins was found between studies using two-dimensional gel electrophoresis and the presented data. The only study showing a significant overlap was by Bitsika et al. (2016), where expression of proteins involved in ECM remodeling, cell stress and cell death were significantly increased in the presented electrically stimulated rat model as well as in the kainic acid mouse model used in the Bitsika et al. study.

**Discussion**

Focused bioinformatics analysis of protein expression patterns during different phases of epileptogenesis revealed a prominent regulation of numerous proteins functionally associated with neuronal cell loss, ECM regulation, cellular interaction, and angiogenesis. The findings confirm that dysregulation of proteins linked with these events characterize epileptogenesis-associated molecular alterations in a significant manner. Our data sets provide comprehensive and valuable information about the complex time course patterns of protein groups throughout the course of epilepsy development. Respective information can render a basis for future selection of novel target and biomarker candidates.

Experimental and clinical evidence exists that neuronal cell loss triggered by epileptogenic brain insults can contribute to the long-term consequences including cognitive deficiencies ([Pearson et al., 2015](#_ENREF_55); [Shetty, 2014](#_ENREF_66)). In the HC, the dysregulation of proteins linked with cell stress and death reached an early peak with a subsequent gradual decline in the number of regulated proteins during the latency period towards the chronic phase. This finding is in line with neuronal cell damage and cell loss evident in different hippocampal sub-regions at very early phases following a SE ([Borges et al., 2003](#_ENREF_6); [Holopainen, 2008](#_ENREF_28); [Uemori et al., 2017](#_ENREF_70)) In contrast, the dysregulation of respective proteins was rather consistent in parahippocampal brain regions during the different phases of epileptogenesis. However, the overlap among the proteins regulated at different time points is rather limited. The regulation might imply pro-apoptotic molecular alterations in the post-insult phase and more protective molecular alterations during later phases.

Proteomic data that we obtained for the different phases of epileptogenesis demonstrated a prominent regulation of proteins known to be induced during apoptosis. On the other hand, various regulators of apoptosis and proteins contributing to neuronal homeostasis exhibited reduced expression rates. ANKHD1 can regulate caspases resulting in an anti-apoptotic function ([Miles et al., 2005](#_ENREF_47)). Its early reduction might pave the way for hippocampal neuronal death in the early post-insult phase. Bassoon has also been discussed to play a regulatory role in apoptosis ([Heyden et al., 2011](#_ENREF_26)). Thus, reduced expression rates of this protein might contribute to early and delayed neuronal cell loss in the PHC.

Other protective proteins contributing to nuclear stability and controlling apoptosis like Lamin proteins or contributing to recovery from DNA damage like PARP1 proved to be induced with peaks at later time points. Lamin proteins are key regulators of nuclear stability ([Dechat et al., 2008](#_ENREF_12)). Strong evidence exists that several members of the protein family limit apoptotic events ([Harborth et al., 2001](#_ENREF_22); [Yang et al., 2011](#_ENREF_76)). To our knowledge, regulation of lamin proteins has not been studied yet in detail in epilepsy models. Our findings reveal a prominent regulation of the lamin protein family involving three different members including LMNA, LMNB1, and LMNB2. Whereas LMNA proved to be regulated in both brain regions, LMNB1 and LMNB2 were exclusively regulated in the PHC. In this brain area, the induction of these proteins occurred in a delayed manner with persistence in the chronic phase following epilepsy manifestation. Considering the function of the proteins, overexpression of lamin proteins might serve an important role in neuronal protection during epileptogenesis and following epilepsy manifestation. In the same manner, PARP1 overexpression during the latency phase in the HC and during the chronic phase in the PHC may mediate neuroprotection.

The nuclear histone protein family is part of the nucleosomal structure. Their modification contributes to epigenetic regulation of transcription and to labeling of DNA damage ([Henshall and Kobow, 2015](#_ENREF_25)). A delayed induction of different family members including HIST1H1A, HISTH1B, and H1F0 became evident in our proteomic analysis. This up-regulation of histone proteins might contribute to epigenetic alterations, which are discussed to play a key role during epileptogenesis ([Henshall and Kobow, 2015](#_ENREF_25)).

Pathway analysis revealed an overrepresentation of protein groups comprising ECM components as well as their interaction partners and their regulators. Integrity of the ECM and homeostasis of its components is an important mediator of structural and synaptic stability ([Dityatev et al., 2010](#_ENREF_16); [McRae and Porter, 2012](#_ENREF_45)). Alterations in ECM components and their organization can pave the way for any type of cellular plasticity including cell proliferation, migration, differentiation, synaptic rearrangement, and circuit rewiring ([Frischknecht et al., 2014](#_ENREF_19); [Kim et al., 2016b](#_ENREF_36); [McRae and Porter, 2012](#_ENREF_45)). Over the past years, ECM remodeling and its role in CNS diseases has gained significant interest ([Soleman et al., 2013](#_ENREF_67)). In epileptology, ECM alterations are discussed to play a key role during the development of a hyperexcitable network following an epileptogenic brain insult ([Kim et al., 2016b](#_ENREF_36); [McRae and Porter, 2012](#_ENREF_45); [Pitkanen et al., 2014](#_ENREF_60)). Moreover, an impact on ictogenesis is considered according to various studies ([Pitkanen et al., 2014](#_ENREF_60)).

In the HC, a limited but persistent dysregulation of ECM, ECM-cell interaction and cell-cell interaction proteins was evident through all phases of epileptogenesis with the number of regulated proteins declining towards the chronic phase. [McRae et al. (2012)](#_ENREF_44) have previously described a decrease in different components of perineuronal nets as an ECM structure, which surrounds inhibitory interneurons. This reduction became evident one week following SE and still persisted two months later. Our data suggest that hippocampal ECM remodeling can also be evident earlier during the first days following an epileptogenic insult. Differences between the studies might for instance be related to the use of different modes of SE induction as well as the specific focus on perineuronal net components in the study by [McRae et al. (2012)](#_ENREF_44).

In PHC regions, the number of regulated proteins in these functional groups indicated a dysregulation that proved to be in the same range as in the HC in the early post-insult phase. However, at later time points respective molecular alterations in the PHC exceeded those in the HC with a substantially higher number of regulated proteins. To our knowledge, none of the earlier expression analyses studying selected ECM or ECM-cell interacting proteins have focused on sub-regions of the PHC, so that there are no expression data available for direct comparison at the protein level. Our findings indicate that ECM remodeling might play an even larger role for epileptogenesis-associated structural and synaptic plasticity in the PHC as compared to the HC. This finding is of particular interest in view of the fact that these brain regions are considered key areas, which can play a critical role for ictogenesis and epileptogenesis. The interconnections among these brain regions as well as their connections with the hippocampus, including the rhinal-hippocampal loop, can significantly contribute to excessive propagation and amplification, which characterizes temporal lobe epilepsy (Cataldi et al., 2013; Vismer et al., 2015). Thus, ECM remodeling in the entorhinal, perirhinal, and posterior piriform cortices, and its consequences may critically affect cellular plasticity and network alterations, which might increase seizure susceptibility and contribute to development of post-insult epilepsies.

Among ECM proteins, TNC and AGRN were the only proteins exhibiting a regulation during epileptogenesis. An induction of these proteins occurred in a delayed manner becoming evident ten days following SE (AGRN) or eight weeks following SE (TNC and AGRN). The fact that only two proteins proved to be regulated might reflect the limited number of proteins in the ECM along with technical sensitivity limitations for extracellular proteins. Previous studies reported a TNC up-regulation after chemical SE induction in hippocampal brain regions. They discussed a role for TNC in synaptic remodeling ([Ferhat et al., 1996](#_ENREF_18)) and a link between TGF-β signaling pathway and TNC up-regulation ([Mercado-Gomez et al., 2014](#_ENREF_46)). Therefore, the induction of TNC in the PHC following epilepsy manifestation may reflect ongoing ECM remodeling in this brain region. Agrin induction might be functionally relevant as it can regulate responses to excitatory neurotransmitter release ([Hilgenberg et al., 2002](#_ENREF_27)).

Several proteins mediating cell-ECM interactions exhibited an up-regulation during different phases of epileptogenesis. These proteins comprised the CD44 receptor for hyaluronic acid, which can interact with collagens and matrix metalloproteinases ([Roszkowska et al., 2016](#_ENREF_64)) as well as DAG1, ANXA2 and TGM2. The induction of these proteins indicates that compensatory changes are triggered that might serve a stabilizing function. In view of earlier studies ([Bausch, 2006](#_ENREF_4)), CD44 induction in both brain regions during the early phase of epileptogenesis may restrict neuronal plasticity. Along this line, up-regulation of DAG1 observed in the chronic phase might serve a protective function taking into account that the protein can contribute to homeostatic scaling up of inhibitory GABAergic synaptic transmission ([Pribiag et al., 2014](#_ENREF_61)).

Proteins that are considered modulators of ECM remodeling also proved to be regulated as a short term consequence of SE. These proteins, comprising LRP1 and NME1, might trigger and shape the alterations of the ECM during the early post-insult and latency phases. Furthermore, we demonstrated alterations in the expression of several cell-cell interaction proteins and proteins linking extracellular with intracellular events. Among these proteins FERMT2 stood out with a prominent regulation in the HC. To our knowledge, the protein has not been described in the context of epileptogenesis yet. Thus, we have selected this protein for further validation by immunohistochemistry. The immunohistochemical detection confirmed the strong overexpression of FERMT2 in the HC ten days following SE. The immunohistochemical analysis of FERMT2 further validated our proteomic data sets along with previous analyses of other selected proteins ([Walker et al., 2016](#_ENREF_73)). The regulation of FERMT2 might be of functional interest in the context of epilepsy development considering that the protein can bind to the intracellular part of the β-integrin subunit thereby activating integrin receptors ([Park and Goda, 2016](#_ENREF_54); [Rognoni et al., 2016](#_ENREF_63)). Thus, the protein plays a crucial role in the crosstalk between extra- and intracellular molecular components and signaling events. A putative functional role of FERMT2 during epileptogenesis needs to be further addressed by genetic targeting approaches.

Taken together, we have identified complex alterations in ECM proteins, cell-ECM and cell-cell interacting proteins that characterize the different phases of epileptogenesis. The comprehensive data sets provide an excellent basis for the development of ECM targeting approaches, which are intensely discussed for different neurological disorders including epilepsy ([Soleman et al., 2013](#_ENREF_67)).

Pathologic angiogenesis has been reported in epilepsy models and in specimen from patients with temporal lobe epilepsy ([Morin-Brureau et al., 2012](#_ENREF_50)). It has been attributed to an excessive activation of VEGF signaling ([Morin-Brureau et al., 2012](#_ENREF_50)).

In our data sets, we observed a far more pronounced regulation of protein pathways linked with angiogenesis in the PHC. The dysregulation of this protein group peaked during the latency period, but became already evident in the early post-insult phase and persisted into the chronic phase. Interestingly, the proteins of the respective pathways exhibited a substantial overlap with proteins that we identified by a bioinformatics analysis of the proteomic data set focused on inflammation and immune response during epileptogenesis ([Walker et al., 2016](#_ENREF_73)).

VEGF acts as a key regulator of angiogenesis ([Santos et al., 2007](#_ENREF_65)). We demonstrated regulation of several proteins modulating VEGF expression or signaling, or acting as VEGF downstream signaling factors and effectors during different phases of epilepsy development.

The proteins regulating angiogenesis included MCAM and ROCK2, which have been described as modulators of angiogenesis in earlier studies ([Montalvo et al., 2013](#_ENREF_48); [Ouhtit et al., 2009](#_ENREF_53)).

Among the pro-angiogenic proteins ANXA2 stood out with a continuous overexpression during all phases of epileptogenesis. According to [Liu and Hajjar (2016)](#_ENREF_40), ANXA2 can exert indirect pro-angiogenic effects. Thus, ANXA2 might trigger excessive angiogenesis throughout epileptogenesis. On the other hand, down-regulation of anti-angiogenic factors such as MARCKSL1 may promote epileptogenesis-associated increases in angiogenesis. MARCKSL1, which can suppress VEGFR-2-dependent phosphorylation ([Kim et al., 2016a](#_ENREF_34)), proved to be reduced during the latency phase in the PHC, whereas its expression rates were increased in the chronic phase in the HC. Thus, regulation of MARCKSL1 might also restrict angiogenesis following epilepsy manifestation.

Caveolae are membrane invaginations structures that are frequently identified in endothelial cells ([Massimino et al., 2002](#_ENREF_43); [Stan, 2005](#_ENREF_69)). PTRF constitutes a functionally relevant component of the caveolar coat ([Chadda and Mayor, 2008](#_ENREF_8)). Deletion of PTRF results in flattening of caveolae and internalization and degradation of caveolin-1 ([Chadda and Mayor, 2008](#_ENREF_8)). Taking into account that alterations in caveolae can affect angiogenesis ([Massimino et al., 2002](#_ENREF_43)), delayed differential expression of PTRF during epileptogenesis may also indirectly influence the induction of angiogenesis.

Finally, we have compared our findings with available proteomic data sets from rodent models and human tissue. The comparison faces major limitations considering the profound differences in the experimental study designs, and the fact that human tissue is from patients following manifestation of epilepsy. The only study indicating a relevant overlap is the study by Bitsika et al. (2016), which we have previously applied to a comparative weighted gene-coexpression network analysis (WGCNA) (Keck et al., 2017). The present data further confirm parallels in the data sets, which several differentially expressed proteins exhibiting a regulation in both studies. However, the outcome of the comparison also underlines the differences in the time course patterns when comparing the mouse intrahippocampal kainic acid models with the rat electrical post-status epilepticus model.

In conclusion, our proteomic analysis revealed complex alterations in expression patterns of proteins associated with cell stress and cell death, ECM remodeling, cell-ECM interactions, cell-cell interactions, angiogenesis, and blood-brain barrier function. The time course information about the development of expression patterns during epileptogenesis provides valuable information for future selection of target and biomarker candidates. Immunohistochemical analysis of FERMT2, nucleolin, CD18, and CD44 further confirmed the validity of the data.

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The authors declare that they do not have any competing interest.

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**Tables**

Table 1: Overrepresented pathways linked with cell stress and cell death in the HC – Genomatix

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.002 | p38 signaling mediated by MAPKAP kinases | NCI-nature | YWHAE, HSPB1, YWHAZ, YWHAG |
| 0.008 | Antiapoptotic | Genomatix | HSPA5, CLU, HSPB1, ANKHD1, PLSCR3, HNRNPL, ANXA5, CD44 |
| 0.010 | Apoptosis | Genomatix | TGM2, HSPA5, CLU, HSPB1, PHB, LMNA, CALR, PLSCR3, HSPA1A, HNRNPC, HSP90B1, SDHC, CTSB, EEF1A1, ANXA5, ANP32A |
| 0.029 | Apoptosis ( Fas signaling pathway) | INOH | YWHAE, YWHAZ, YWHAG |
| 0.029 | Aurora B signaling | NCI-nature | RASA1, VIM, NCL |
| 0.037 | Dna repair | Genomatix | COPS8, LMNA, UBE2V2, COPS5, PPP5C, DDB1 |
| 0.044 | hypoxia-inducible factor in the cardivascular system | BioCarta | COPS5, LDHA |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.019 | Caspase cascade in apoptosis | BioCarta | LMNA, LMNB1, PARP1 |
| 0.026 | Stress | Genomatix | OGT, HSPB1, PPIB, CALR, APOE, ACADVL, HSPA1A, FLNB, LMNB1, CP, ACTN1, G6PD, FAM120A, HSP90B1, CLIC4, RDX, HYOU1, SRM, P4HB, PFN1, TGM2, CLU, USP7, RPL11, VCL, PARP1, TXNRD1, EIF5B, HAGH, EIF2S1, KIF1B |
| 0.043 | FAS signaling pathway (CD95) | NCI-nature | LMNA, GSN, PARP1 |
| 0.048 | Caspase cascade in apoptosis | NCI-nature | LMNA, LMNB1, GSN, PARP1, VIM |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.002 | Glial cell line derived neurotrophic factor | Genomatix | GFAP, ITGB1 |
| 0.014 | Mixed lineage kinase | Genomatix | ABI2, ANXA2 |
| 0.034 | 14 3 3 protein | Genomatix | GFAP, ABI2 |

Genomatix Pathway System was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 2: Overrepresented pathways linked with cell stress and cell death in the HC – ConsensusPathDB

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.004 | Apoptosis-related network due to altered Notch3 in ovarian cancer | Wikipathways | VIM, ANXA5, PAK2, HSPA5, HSPB1 |
| 0.010 | Cellular response to heat stress | Reactome | EEF1A1, HSPA1A, HSPB1, SERPINH1, YWHAE |
| 0.013 | Apoptosis | Reactome | YWHAZ, HMGB1, VIM, LMNA, YWHAG, PAK2, YWHAE |
| 0.013 | HSF1 activation | Reactome | EEF1A1, HSPA1A, SERPINH1, HSPB1 |
| 0.015 | Activation of BAD and translocation to mitochondria | Reactome | YWHAZ, YWHAG, YWHAE |
| 0.018 | Programmed cell death | Reactome | YWHAZ, HMGB1, VIM, LMNA, YWHAG, PAK2, YWHAE |
| 0.023 | Cell cycle | Wikipathways | YWHAZ, YWHAG, YWHAE |
| 0.032 | Aurora B signaling | PID | NCL, RASA1, VIM |
| 0.032 | Cell cycle - Homo sapiens (human) | KEGG | YWHAZ, YWHAG, YWHAE |
| 0.032 | Activation of BH3-only proteins | Reactome | YWHAZ, YWHAG, YWHAE |
| 0.032 | Intrinsic pathway for apoptosis | Reactome | YWHAZ, YWHAG, YWHAE |
| 0.036 | Hypoxia-inducible factor in the cardivascular system | BioCarta | LDHA, COPS5 |
| 0.036 | Bile acid biosynthesis, neutral pathway | HumanCyc | ACAA2, POR |
| 0.036 | Metabolism of polyamines | Reactome | SMS, SRM |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.000 | L13a-mediated translational silencing of Ceruloplasmin expression | Reactome | RPL8, RPL6, RPL7, RPL4, RPL5, RPL18A, RPL35A, RPS15A, RPS6, RPS5, EIF2S1, RPS9, EIF2S2, RPS18, RPL7A, EIF3C, RPS19, RPS26, RPS25, RPL3, RPL14, RPL15, RPL10, RPL11, RPSA, RPL36, RPL10A |
| 0.018 | Caspase cascade in apoptosis | BioCarta | LMNA, LMNB1, PARP1 |
| 0.022 | Apoptosis-related network due to altered Notch3 in ovarian cancer | Wikipathways | APOE, ANXA5, HSPB1, VIM, PTK2B, CTNNA1 |
| 0.028 | Caspase cascade in apoptosis | PID | LMNA, LMNB1, PARP1, VIM, GSN |
| 0.034 | FAS pathway and stress induction of HSP regulation | Wikipathways | LMNA, LMNB1, PARP1, HSPB1 |
| 0.034 | NRAGE signals death through JNK | Reactome | KALRN, TRIO, ARHGEF2, RASGRF2 |

ConsensusPathDB overrepresentation tool was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 3: Overrepresented pathways linked with cell stress and cell death in the PHC – Genomatix

|  |  |  |  |
| --- | --- | --- | --- |
| *P*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.023 | Aurora B signaling | NCI-nature | PSMA3, RASA1, VIM, NCL L |
| 0.037 | 14 3 3 protein | Genomatix | NF1, FLNA, BSN, ITGB2, GFAP, MVP, YWHAZ, VIM, KPNA3, NEFL, PPP1R9B, YWHAB |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.006 | Mixed lineage kinase | Genomatix | PSME1, PARK7, HSPB1, SLK, PSMC3, NEFH, ANXA2, SH3KBP1, CDC42, FLNA, MAPK8IP3, WNK2, PRDX2, RPS5, YWHAQ, COPS2 |
| 0.013 | Glial cell line derived neurotrophic factor | Genomatix | GFAP, ITGB1, HSPB1, GAP43, SNCA, ENO2, CALB1, ITGA5 |
| 0.017 | Aurora B signaling | NCI-nature | PSMA3, RASA1, PEBP1, STMN1, VIM, NCL |
| 0.022 | il 3 signaling pathway | BioCarta | PTPN6, GRB2, HRAS |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.001 | Caspase cascade in apoptosis | BioCarta | LMNA, LMNB1, PARP1, LMNB2 |
| 0.003 | Caspase cascade in apoptosis | NCI-nature | LMNA, APP, LMNB1, GSN, PARP1, VIM, LMNB2 |
| 0.018 | Glial cell line derived neurotrophic factor | Genomatix | MAOB, GFAP, ITGB1, HSPB1, SLC1A3 |

Genomatix Pathway System was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 4: Overrepresented pathways linked with cell stress and cell death in the PHC – ConsensusPathDB

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.010 | Regulation of Ras family activation | PID | PRKCA, SYNGAP1, PRKCE, RASA1, NF1 |
| 0.025 | Aurora B signaling | PID | PSMA3, NCL, RASA1, VIM |
| 0.034 | L13a-mediated translational silencing of Ceruloplasmin expression | Reactome | RPS27A, RPL29, RPL23A, RPL27, EIF3C, RPL30, RPL31, RPL11, RPL12, RPL22, RPS19 |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.001 | Apoptosis | Reactome | YWHAZ, BCAP31, LMNB1, DBNL, PPP3R1, HIST1H1A, YWHAQ, VIM, SFN, HIST1H1B, MAPT, YWHAB, LMNA, YWHAG, PAK2, GSN |
| 0.001 | Programmed cell death | Reactome | YWHAZ, BCAP31, LMNB1, DBNL, PPP3R1, HIST1H1A, YWHAQ, VIM, SFN, HIST1H1B, MAPT, ADAM17, YWHAB, LMNA, YWHAG, PAK2, GSN |
| 0.004 | Aurora B signaling | PID | PEBP1, STMN1, VIM, PSMA3, NCL, H3F3A, RASA1 |
| 0.005 | Activation of BAD and translocation to mitochondria | Reactome | YWHAZ, PPP3R1, YWHAQ, SFN, YWHAB, YWHAG |
| 0.010 | LKB1 signaling events | PID | YWHAZ, SFN, YWHAQ, CTSD, EZR, CDC37, MAP2, MAPT, YWHAB, YWHAG |
| 0.015 | Apoptotic execution phase | Reactome | BCAP31, LMNB1, DBNL, VIM, PAK2, MAPT, LMNA, HIST1H1A, HIST1H1B, GSN |
| 0.016 | Cellular responses to stress | Reactome | EHMT1, MINK1, HIST1H4A, LMNB1, DNAJB6, HIST1H1A, HSPB1, PTGES3, PRDX6, PRDX5, RPS27A, ERO1L, PRDX2, HSPA1A, H3F3A, SERPINH1, P4HB, HIST1H1B, TXNRD1 |
| 0.021 | Intrinsic pathway for apoptosis | Reactome | YWHAZ, PPP3R1, YWHAQ, SFN, YWHAB, YWHAG |
| 0.041 | Apoptotic cleavage of cellular proteins | Reactome | BCAP31, LMNB1, DBNL, VIM, MAPT, LMNA, GSN |
| 0.041 | RhoA signaling pathway | PID | ITGB1, EZR, TLN1, MSN, ROCK2, CDC42, SH3GL2 |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.000 | Dna damage/telomere stress induced senescence | Reactome | HIST1H1D, H1F0, LMNB1, HIST1H1B, HMGA1 |
| 0.001 | Caspase cascade in apoptosis | BioCarta | LMNB2, LMNA, LMNB1, PARP1 |
| 0.001 | Caspase cascade in apoptosis | PID | LMNB2, LMNB1, APP, PARP1, VIM, LMNA, GSN |
| 0.002 | Apoptotic execution phase | Reactome | TJP2, LMNB1, DSP, VIM, H1F0, HIST1H1D, LMNA, TJP1, HIST1H1B, GSN |
| 0.002 | Apoptosis-related network due to altered Notch3 in ovarian cancer | Wikipathways | APOE, ANXA5, HSPA5, HSPB1, APP, GCLC, VIM |
| 0.008 | FAS pathway and Stress induction of HSP regulation | Wikipathways | LMNB2, LMNA, LMNB1, PARP1, HSPB1 |
| 0.009 | Apoptotic cleavage of cellular proteins | Reactome | TJP2, LMNB1, DSP, VIM, TJP1, LMNA, GSN |
| 0.015 | Apoptotic cleavage of cell adhesion proteins | Reactome | TJP2, TJP1, DSP |
| 0.027 | Breakdown of the nuclear lamina | Reactome | LMNA, LMNB1 |
| 0.034 | Activation of DNA fragmentation factor | Reactome | HIST1H1D, H1F0, HIST1H1B |
| 0.034 | Apoptosis induced DNA fragmentation | Reactome | HIST1H1D, H1F0, HIST1H1B |
| 0.040 | Apoptosis | Reactome | TJP2, LMNB1, DSP, VIM, H1F0, HIST1H1D, LMNA, TJP1, HIST1H1B, GSN |
| 0.046 | RhoA signaling pathway | PID | ITGB1, VCL, RDX, EZR, TLN1, MSN |
| 0.048 | Programmed cell death | Reactome | TJP2, LMNB1, DSP, VIM, TJP1, HIST1H1D, LMNA, H1F0, HIST1H1B, GSN |

ConsensusPathDB overrepresentation tool was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 5: Overrepresented pathways linked with ECM, cell-ECM and cell-cell interactions in the HC – Genomatix

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.027 | Focal adhesion kinase 1 | Genomatix | CDC42, TGM2, TLN1, TRIO, RASA1, CD151, ACTN1, CD44, LDHB |
| 0.040 | Beta catenin degradation signaling (Canonical) ( Canonical Wnt signaling pathway Diagram ) | INOH | YWHAE, YWHAZ, YWHAG, CD44 |
| 0.042 | Tissue inhibitor of metalloproteinase | Genomatix | SERPINH1, CD63, CTSB, CD44 |
| 0.049 | Alpha6Beta4Integrin | CellMap | YWHAE, YWHAZ, CD151, VIM |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.015 | Integrin | Genomatix | LCP1, ITGAM, CD9, HNRNPU, ITGAL, FLNA, ITGB2, PTK2B, TLN1, VCL, JAM3, CD63, FERMT3, CD44, FERMT2 |
| 0.018 | amb2 Integrin signaling | NCI-nature | KNG1, LRP1, ITGAM, ITGB2, TLN1, JAM3 |
| 0.048 | Paxillin | Genomatix | KNG1, PLCG1, GNB2L1, FLNA, ITGB2, PTK2B, TLN1, VCL |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.006 | Integrin linked kinase | Genomatix | ITGB1, ANXA2 |
| 0.008 | Paxillin | Genomatix | ITGB1, MARCKSL1 |

Genomatix Pathway System was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 6: Overrepresented pathways linked with ECM, cell-ECM and cell-cell interactions in the HC – ConsensusPathDB

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.015 | MAPK signaling pathway | Wikipathways | HSPA5, HSPB1, PPP5C, HSPA1A, RASA1, PAK2, CDC42 |
| 0.043 | amb2 integrin signaling | PID | HMGB1, JAM3, TLN1 |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.010 | Rho cell motility signaling pathway | BioCarta | PFN1, TRIO, GSN, VCL, TLN1 |
| 0.013 | Amoebiasis - Homo sapiens (human) | KEGG | RAB7A, ITGB2, HSPB1, PRKCB, VCL, ACTN1, PRKCG, ITGAM |
| 0.017 | amb2 integrin signaling | PID | JAM3, LRP1, ITGAM, ITGB2, TLN1 |
| 0.018 | Cell-extracellular matrix interactions | Reactome | FERMT2, ACTN1, RSU1 |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.006 | Cell junction organization | Reactome | ITGB1, CDH4 |
| 0.016 | Cell adhesion molecules (CAMs) - Homo sapiens (human) | KEGG | ITGB1, CDH4 |
| 0.019 | Cell-cell communication | Reactome | ITGB1, CDH4 |

ConsensusPathDB overrepresentation tool was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 7: Overrepresented pathways linked with ECM, cell-ECM and cell-cell interactions in the PHC – Genomatix

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.000 | Syndecan-2-mediated signaling events | NCI-nature | NF1, PRRT2, CDC42, EZR, ITGB1, RASA1, ITGA5, GNB2L1 |
| 0.004 | Syndecan-4-mediated signaling events | NCI-nature | PRKCA, ITGB1, ACTN1, ITGA5 |
| 0.010 | Paxillin | Genomatix | CDC42, CTTN, FLNA, ITGB2, ITGB1, PTK2B, MCAM, GNB2L1 |
| 0.024 | Focal adhesion kinase 1 | Genomatix | CDC42, CTTN, TGM2, EZR, ITGB1, PTK2B, MCAM, RASA1, ACTN1, CD44, ITGA5, GNB2L1 |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.000 | a6b1 and a6b4 integrin signaling | NCI-nature | PRKCA, ITGB1, ITGA6, YWHAG, YWHAZ, SFN, YWHAQ, GRB2, HRAS, YWHAB |
| 0.001 | Integrin family cell surface interactions | NCI-nature | ITGB1, ITGAM, ITGA6, ITGB8, ITGA1, ITGB2, ITGA5 |
| 0.007 | Syndecan-4-mediated signaling events | NCI-nature | PRKCA, DNM2, ITGB1, ACTN1, ITGA5 |
| 0.012 | Alpha6Beta4Integrin | CellMap | PRKCA, ITGA6, YWHAZ, SFN, CD151, MBP, YWHAQ, VIM, GRB2, YWHAB |
| 0.012 | Tyrosine protein kinase src | Genomatix | DNM2, CRK, ITGB1, PRKCE, ASAP1, RASA1, GPRIN1, ACTN1, ACP1, ANXA2, PTPRC, ALB, PRRT2, SH3KBP1, CDC42, EZR, GSN, TOM1L2, PTK2B, MAPK8IP3, KIFAP3, FHIT, PTPN6, ANPEP, MIF, ADRBK1, ADAM17, ITGA5, GRB2, ARRB1 |
| 0.016 | Focal adhesion kinase 1 | Genomatix | CRK, ITGB1, ITGA6, SLK, RASA1, ACTN1, NME1, CDC42, TGM2, EZR, PTK2B, TLN1, MAPK8IP3, MCAM, PPAP2B, SFN, CD151, CD44, ITGA5, GRB2 |
| 0.020 | Beta catenin degradation signaling (Canonical) ( Canonical Wnt signaling pathway Diagram ) | INOH | YWHAG, SKP1, AGRN, YWHAZ, SFN, YWHAQ, CD44, YWHAB |
| 0.027 | Integrin | Genomatix | ITGB1, ITGAM, ITGA6, EDIL3, NME1, ITGB8, ITGA1, AGRN, MFGE8, CDC42, HNRNPU, FLNA, ITGB2, PTK2B, TLN1, CD151, ANXA7, CD44, ITGA5 |
| 0.028 | Paxillin | Genomatix | CRK, ITGB1, SLK, CDC42, MARCKSL1, FLNA, ITGB2, PTK2B, TLN1, MCAM, PPAP2B |
| 0.033 | Integrin signaling pathway | BioCarta | ITGB1, ACTN1, ITGA1, TLN1, GRB2, HRAS |
| 0.048 | Filopodium formation ( Integrin signaling pathway ) | INOH | CRK, ITGB1, ITGAM, ITGA6, ITGB8, ITGA1, AGRN, CDC42, ITGB2, TLN1, CD44, ITGA5, GRB2, HRAS |
| 0.049 | mcalpain and friends in cell motility | BioCarta | ITGB1, ITGA1, EZR, TLN1, PRKAR2A, PRKAR1B, GRB2, HRAS |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.000 | Integrin | Genomatix | LCP1, ITGB1, ITGA6, CD9, ITGAV, AGRN, RAP1A, HNRNPU, SLC3A2, FLNA, TLN1, SLC12A2, TNC, VCL, DAG1, JAM3 |
| 0.000 | Beta1 integrin cell surface interactions | NCI-nature | ITGB1, ITGA6, ITGAV, CD81, TNC |
| 0.001 | Matrix metalloproteinase | Genomatix | LRP1, STOML2, ITGB1, CD9, ITGAV, HMGA1, NDRG2, CACNA2D3, SLC3A2, FLNA, CLU, JAM3, MIF |
| 0.004 | Integrin family cell surface interactions | NCI-nature | ITGB1, ITGA6, ITGAV |

Genomatix Pathway System was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 8: Overrepresented pathways linked with ECM, cell-ECM and cell-cell interactions in the PHC – ConsensusPathDB

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.003 | Syndecan-4-mediated signaling events | PID | PRKCA, ITGB1, ACTN1, ITGA5 |
| 0.010 | Syndecan-2-mediated signaling events | PID | GNB2L1, ITGB1, RASA1, EZR, NF1 |
| 0.015 | Fibronectin matrix formation | Reactome | ITGB1, ITGA5 |
| 0.015 | Phospholipase c delta in phospholipid associated cell signaling | BioCarta | TGM2, PLCD1 |
| 0.015 | Syndecan interactions | Reactome | PRKCA, ITGB1, ACTN1 |
| 0.021 | Extracellular matrix organization | Reactome | ITGB1, ACTN1, ITGB2, PRKCA, CD44, P4HB, ITGA5, SERPINH1, PPIB |
| 0.025 | Urokinase-type plasminogen activator (uPA) and uPAR-mediated signaling | PID | ITGB1, ITGB2, NCL, ITGA5 |
| 0.036 | a6b1 and a6b4 Integrin signaling | PID | YWHAZ, PRKCA, YWHAB, ITGB1 |
| 0.041 | Cell-extracellular matrix interactions | Reactome | ITGB1, ACTN1 |
| 0.049 | Integrin-linked kinase signaling | PID | CDC42, ACTN1, NACA, CDC37 |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.001 | a6b1 and a6b4 integrin signaling | PID | YWHAZ, ITGB1, PRKCA, HRAS, YWHAQ, SFN, YWHAB, YWHAG |
| 0.005 | Syndecan-4-mediated signaling events | PID | PRKCA, ITGB1, ACTN1, ITGA5, DNM2 |
| 0.007 | Extracellular matrix organization | Reactome | ITGB1, ACTN1, ITGB8, ITGB2, PRKCA, ITGA1, P4HB, ADAM17, ITGA5, PRSS1, CD151, AGRN, ITGAM, CD44, SERPINH1, PPIB |
| 0.011 | Integrin cell surface interactions | Reactome | ITGB1, ITGB8, ITGB2, ITGA1, ITGA5, AGRN, ITGAM, CD44 |
| 0.012 | Integrin-mediated cell adhesion | Wikipathways | ITGB1, ITGB2, ITGA1, HRAS, ITGA5, ROCK2, TLN1, ITGAM, ITGB8, CRK, PAK2, CDC42 |
| 0.021 | Urokinase-type plasminogen activator (uPA) and uPAR-mediated signaling | PID | ITGB1, ITGB2, ITGA5, NCL, ITGAM, CRK |
| 0.040 | Interleukin-3, 5 and GM-CSF signaling | Reactome | YWHAZ, CRK, HRAS, PTPN6 |
| 0.042 | MAPK Signaling Pathway | Wikipathways | MINK1, STMN1, PPP3R1, PPM1A, HSPB1, HSPA1A, MAPT, ARRB1, CRK, MAPK8IP3, RASA1, PAK2, CDC42 |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.000 | Integrin cell surface interactions | Reactome | ITGB1, JAM3, ITGAV, AGRN, DAG1, TNC |
| 0.003 | Beta1 integrin cell surface interactions | PID | CD81, ITGB1, ITGAV, TNC |
| 0.004 | ECM-receptor interaction - Homo sapiens (human) | KEGG | ITGB1, AGRN, TNC, DAG1, ITGAV |
| 0.008 | Non-integrin membrane-ECM interactions | Reactome | AGRN, ITGB1, TNC, DAG1, ITGAV |
| 0.027 | Laminin interactions | Reactome | ITGB1, ITGAV |
| 0.029 | amb2 integrin signaling | PID | JAM3, LRP1, RAP1A, TLN1 |
| 0.033 | Cell surface interactions at the vascular wall | Reactome | ITGB1, JAM3, SLC3A2, ATP1B3, ITGAV, SLC16A1, MAG |
| 0.034 | ECM proteoglycans | Reactome | AGRN, ITGB1, TNC, DAG1, ITGAV |

ConsensusPathDB overrepresentation tool was used for pathway enrichment analysis. All pathways with a p-value < 0.05 and at least two overlap members were considered for further analysis.

Table 9: Regulated pathways linked with angiogenesis and blood-brain barrier function in the HC – Genomatix

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *10 days after SE* | | | |
| 0.016 | Atp binding cassette, sub family G (white) | Genomatix | ATIC, APOE, ABCA1 |
| 0.016 | Very low density lipoprotein receptor | Genomatix | LRP1, APOE, ABCA1 |
| 0.048 | Paxillin | Genomatix | KNG1, PLCG1, GNB2L1, FLNA, ITGB2, PTK2B, TLN1, VCL |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.008 | Paxillin | Reactome | ITGB1, MARCKSL1 |
| 0.019 | Caveolin 1 | BioCarta | ITGB1, ANXA2 |

Genomatix Pathway System was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 10: Overrepresented pathways linked with angiogenesis and blood-brain barrier function in the HC – ConsensusPathDB

|  |  |  |  |
| --- | --- | --- | --- |
| *p*-Value | Pathway | Source | Overlap members |
| *10 days after SE* | | | |
| 0.010 | Lipoprotein metabolism | Reactome | P4HB, APOE, PLTP, ABCA1 |
| 0.018 | HDL-mediated lipid transport | Reactome | APOE, PLTP, ABCA1 |

ConsensusPathDB overrepresentation tool was used for pathway enrichment analysis. All pathways with a p-value < 0.05 and at least two overlap members were considered for further analysis.

Table 11: Overrepresented pathways linked with angiogenesis and blood-brain barrier function in the PHC – Genomatix

|  |  |  |  |
| --- | --- | --- | --- |
| *P*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.001 | ATP binding cassette, sub family A (abc1) | Genomatix | PRKCA, CDC42, PLTP, CAMK4, APOE, SCP2 |
| 0.017 | Caveolin 1 | Genomatix | PRKCA, FLNA, ITGB1, PRKCE, SCP2, TOLLIP, ANXA2, TXNRD1, ALB |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.007 | VEGFR3 signaling in lymphatic endothelium | NCI-nature | CRK, ITGB1, ITGA1, PRRT2, ITGA5, GRB2 |
| 0.010 | ATP binding cassette, sub family A (ABC1) | Genomatix | PRKCA, PLTP, APOE, CDC42, CAMK4, SCP2, MIF |
| 0.028 | Paxillin | Genomatix | CRK, ITGB1, SLK, CDC42, MARCKSL1, FLNA, ITGB2, PTK2B, TLN1, MCAM, PPAP2B |
| 0.030 | EGFR1 | CellMap | PRKCA, CRK, ASAP1, DNM1, RASA1, SH3GL2, RAB5A, SNCA, SH3GL3, SH3KBP1, CDC42, PTK2B, ABI1, PTPN6, GRB2, HRAS, YWHAB |
| 0.032 | Internalization of erbb1 | NCL-nature | SYNJ1, DNM1, SH3GL2, RAB5A, SH3KBP1, CDC42, GRB2, HRAS |
| 0.033 | Vasoactive intestinal peptide | Genomatix | ISYNA1, STMN1, KCNMA1, ENO2, CALB1 |
| 0.039 | Caveolin 1 | Genomatix | PRKCA, DNM2, ITGB1, PRKCE, ROCK2, ANXA2, PTRF, ITGA1, ALB, FLNA, SCP2, TOLLIP, TXNRD1 |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.001 | ATP binding cassette, sub family C (CFTR/MRP) | Genomatix | RDX, EZR, GSTA1, SLC16A1, GCLC |
| 0.013 | ATP binding cassette, sub family G (WHITE) | Genomatix | ATIC, APOE, GSTP1 |

Genomatix Pathway System was used for pathway enrichment analysis. All pathways with a *p*-value < 0.05 and at least two overlap members were considered for further analysis.

Table 12: Overrepresented pathways linked with angiogenesis and blood-brain barrier function in the PHC – ConsensusPathDB

|  |  |  |  |
| --- | --- | --- | --- |
| *P*-Value | Pathway | Source | Overlap members |
| *2 days after SE* | | | |
| 0.007 | HDL-mediated lipid transport | Reactome | PLTP, APOE, ALB |
| 0.015 | Nitric oxide stimulates guanylate cyclase | Reactome | ITPR1, PRKG2, GUCY1B3 |
| 0.021 | Signaling by ERBB2 | Reactome | PRKCA, RPS27A, PRKCE, CAMK4, CDC37, STUB1, PRKAR1B, YWHAB, ITPR1 |
| 0.047 | EGFR interacts with phospholipase C-gamma | Reactome | PRKCA, ITPR1, PRKCE, CAMK4, PRKAR1B |
| 0.047 | PLCG1 events in ERBB2 signaling | Reactome | PRKCA, ITPR1, PRKCE, CAMK4, PRKAR1B |
| 0.050 | EGFR1 | NetPath | MVP, ITGB1, ACTN1, ANXA2, ANXA4, TOLLIP, SLC38A2, PRKCA, ALB, RPL30, RASA1, MINK1, PTK2B, CTTN, TAGLN2, VIM, YWHAZ, PTPN6, SH3GL3, CDC42 |
|  |  |  |  |
| *10 days after SE* | | | |
| 0.001 | EGFR1 | NetPath | ANXA1, ACP1, HRAS, RPLP0, PTK2B, DNM1, ADAM17, CRK, STIP1, TLN1, ADRBK1, SH3GL3, SH3GL2, YWHAZ, PEBP1, STAM, CALM1, HNRNPR, MYH9, RASA1, GSN, SH3KBP1, MVP, ANXA2, RAB5A, ANXA4, MINK1, PRKCA, SFPQ, ITGB1, PTPN6, ACTN1, TOLLIP, ABI1, ALB, MYO6, RPL30, VIM, DDX6, TOM1L2, PTRF, WBP2, ASAP1, CDC42 |
| 0.017 | EGF-EGFR signaling pathway | Wikipathways | PEBP1, STMN1, PRKCA, HRAS, ABI1, SH3KBP1, RAB5A, PTK2B, CDC42, SYNJ1, RASA1, CRK, DNM1, STAM, ASAP1, SH3GL3, SH3GL2 |
| 0.040 | VEGFR3 signaling in lymphatic endothelium | PID | ITGB1, ITGA1, ITGA5, CRK |
| 0.044 | EGFR interacts with phospholipase C-gamma | Reactome | CALM1, PRKCA, PRKCE, CAMK4, PRKAR2A, PRKAR1B, ITPR1, ADRBK1 |
| 0.044 | PLCG1 events in ERBB2 signaling | Reactome | CALM1, PRKCA, PRKCE, CAMK4, PRKAR2A, PRKAR1B, ITPR1, ADRBK1 |
| 0.044 | Signaling by Ligand-Responsive EGFR Variants in Cancer  Signaling by EGFRvIII in Cancer  Signaling by EGFR in Cancer | Reactome | ITPR1, CALM1, PRKCA, SH3KBP1, HRAS, RPS27A, PRKCE, CAMK4, CDC37, PRKAR2A, CDC42, PRKAR1B, ADAM17, YWHAB, STAM, ADRBK1, SH3GL2 |
|  |  |  |  |
| *8 weeks after SE* | | | |
| 0.004 | Lipoprotein metabolism | Reactome | P4HB, APOE, ALB |
| 0.023 | Basigin interactions | Reactome | SLC3A2, SLC16A1, ITGB1, ATP1B3, MAG |
| 0.027 | HDL-mediated lipid transport | Reactome | APOE, ALB |

ConsensusPathDB overrepresentation tool was used for pathway enrichment analysis. All pathways with a p-value < 0.05 and at least two overlap members were considered for further analysis.

Table 13: Comparison of results with published proteomics studies.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | *Bitsika et al. 2016* | *Bitsika et al. 2016* | *Bitsika et al. 2016* | *Greene et al. 2007* | *Li et al. 2010* | *Liu et al. 2008* | *Persike et al. 2012* | *Wu et al. 2015* | *Wu et al. 2015* | *Wu et al. 2015* |
| Species | Mouse | Mouse | Mouse | Rat | Mouse | Mouse | Human | Rat | Rat | Rat |
| Model | kainic acid | kainic acid | kainic acid | pilocarpine | pilocarpine | pilocarpine | - | pilocarpine | pilocarpine | pilocarpine |
| Time after SE | 1 day | 3 days | 30 days | 2 days | 4 weeks | 12 & 72 hours | - | 2 days | 3 weeks | 8 weeks |
| 2 days | 0 / 22 | 2 / 53 | 8 / 175 | 1 / 6 | 2 / 23 | 1 / 42 | 0 / 9 | 0 / 26 | 0 / 28 | 0 / 45 |
| 10 days | 1 / 22 | 5 / 53 | 15 / 175 | 1 / 6 | 2 / 23 | 1 / 42 | 0 / 9 | 1 / 26 | 1 / 28 | 1 / 45 |
| 8 weeks | 0 / 22 | 2 / 53 | 1 / 175 | 0 / 6 | 0 / 23 | 0 / 42 | 0 / 9 | 1 / 26 | 1 / 28 | 1 / 45 |
|  |  |  |  |  |  |  |  |  |  |  |
|  | *\*number of overlapping differentially expressed proteins / total number of differentially expressed proteins in study* | | | | | | | | |  |

**Legends to figures**

Figure 1: Differentially expressed proteins assigned to the analyzed foci

(A) shows the numbers of differentially expressed proteins assigned to the analyzed foci for the HC, (B) for the PHC. Please note that proteins could be allocated to more than one focus.

Figure 2: Expression analysis of proteins assigned to pathways linked with apoptosis

(A), (B), and (C) show the individual protein abundances assigned to selected apoptosis associated pathways during the time course of epileptogenesis for the HC and (D), (E), and (F) for the PHC. For hierarchical row clustering protein data was log2 transformed. Red cell color indicates an up-regulation, blue cell color a down-regulation. The respective color code is given above each heat map. (A) and (D) = 2 days after SE. (B) and (E) = 10 days after SE. (C) and (F) = 8 weeks after SE.

Figure 3: Expression analysis of proteins assigned to pathways linked with ECM, cell-ECM and cell-cell interactions

(A), (B), and (C) show the individual protein abundances assigned to ECM, cell-ECM and cell-cell interactions associated pathways during the time course of epileptogenesis for the HC. For hierarchical row clustering protein data was log2 transformed. R package “gplots” was used for generating heat maps. Red cell color indicates an up-regulation, blue cell color a down-regulation. The respective color code is given above each heat map. (A) = 2 days after SE. (B) = 10 days after SE. (C) = 8 weeks after SE.

Figure 4: Expression analysis of proteins assigned to pathways linked with ECM, cell-ECM and cell-cell interactions

(A), (B), and (C) show the individual protein abundances assigned to ECM, cell-ECM and cell-cell interactions associated pathways during the time course of epileptogenesis for the PHC. For hierarchical clustering protein data was log2 transformed. R package “gplots” was used for generating heat maps. Red cell color indicates an up-regulation, blue cell color a down-regulation. The respective color code is given above each heat map. (A) = 2 days after SE. (B) = 10 days after SE. (C) = 8 weeks after SE.

Figure 5: Immunohistochemical staining of selected proteins in the hippocampus and parahippocampal cortex

Representative images showing increased expression of Nucleolin, CD44, FERMT2 and CD18 (ITGB2) ten days following SE compared to control animals throughout the hippocampus (A, C and D) and parahippocampal cortex (B). In animals with SE, the most pronounced difference in FERMT2, CD18 and CD44 expression was observed in the stratum radiatum of the CA1 region (A, C and D). Scale bars are 100 µm. Hippocampal layers abbreviations: so = Stratum oriens, sps = Stratum pyramidale and sr = Stratum radiatum. Inserts show high magnification images of immunopositive cells in animals with SE.

Figure 6: Expression analysis of proteins assigned to pathways linked with angiogenesis and blood-brain barrier

(A) and (B) show the individual protein abundances assigned to angiogenesis and blood-brain barrier associated pathways during the time course of epileptogenesis for the HC and (C), (D), and (E) for the PHC. For hierarchical row clustering protein data was log2 transformed. R package “gplots” was used for generating heat maps. Red cell color indicates an up-regulation, blue cell color a down-regulation. The respective color code is given above each heat map. (C) = 2 days after SE. (A) and (D) = 10 days after SE. (B) and (E) = 8 weeks after SE.