Altered Functional Protein Networks in the Prefrontal Cortex and Amygdala of Victims of Suicide

Katalin Adrienna Kékesi1,2*, Gábor Juhász1, Attila Simor1, Péter Gulyássy1, Éva Mónika Szegő1†, Éva Hunyadi-Gulyás3, Zsuzsanna Darula3, Katalin F. Medzihradszky3,4, Miklós Palkovits5, Botond Penke6, András Czurkó1,6

1 Laboratory of Proteomics, Institute of Biology, Eötvös Loránd University, Budapest, Hungary, 2 Department of Physiology and Neurobiology, Eötvös Loránd University, Budapest, Hungary, 3 Proteomics Research Group, Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary, 4 Department of Pharmaceutical Chemistry, School of Pharmacy, University of California San Francisco, San Francisco, California, United States of America, 5 Neuroradiology Research Group, Hungarian Academy of Sciences, Semmelweis University, Budapest, Hungary, 6 Medical Chemistry Department, University of Szeged, Szeged, Hungary

Abstract
Probing molecular brain mechanisms related to increased suicide risk is an important issue in biological psychiatry research. Gene expression studies on post mortem brains indicate extensive changes prior to a successful suicide attempt; however, proteomic studies are scarce. Thus, we performed a DIGE proteomic analysis of post mortem tissue samples from the prefrontal cortex and amygdala of suicide victims to identify protein changes and biomarker candidates of suicide. Among our matched spots we found 46 and 16 significant differences in the prefrontal cortex and amygdala, respectively; by using the industry standard t test and 1.3 fold change as cut off for significance. Because of the risk of false discoveries (FDR) in these data, we also made FDR adjustment by calculating the q-values for all the t tests performed and by using 0.06 and 0.4 as alpha thresholds we reduced the number of significant spots to 27 and 9 respectively. From these we identified 59 proteins in the cortex and 11 proteins in the amygdala. These proteins are related to biological functions and structures such as metabolism, the redox system, the cytoskeleton, synaptic function, and proteolysis. Thirteen of these proteins (CBR1, DPYSL2, EFHD2, FKBP4, GFAP, GLUL, HSPA8, NEFL, NEFM, PGAM1, PRDX6, SELENBP1 and VIM) have already been suggested to be biomarkers of psychiatric disorders at protein or genome level. We also pointed out 9 proteins that changed in both the amygdala and the cortex, and from these, GFAP, INA, NEFL, NEFM and TUBA1 are interacting cytoskeletal proteins that have a functional connection to glutamate, GABA, and serotonin receptors. Moreover, ACTB, CTSD and GFAP displayed opposite changes in the two examined brain structures that might be a suitable characteristic for brain imaging studies. The opposite changes of ACTB, CTSD and GFAP in the two brain structures were validated by western blot analysis.

Introduction
Suicide is a human attribute without a proper equivalent in animals; however, some behavioural traits, such as aggression, hopelessness, and impulsivity, are correlated with suicide and can be reproduced in animals [1]. Suicidal behaviour often occurs in conjunction with different psychiatric diseases, such as major depression or schizophrenia [2]. Major depression and bipolar disorder generally increase the incidence of suicide [3]. Although suicide is a complex behaviour that is often preceded by suicidal thoughts, it can occur as the outcome of an impulsive action [4]. The altered serotonergic mechanism theory is the most widely emphasised cellular mechanism of suicide [4,5]. Suicide is linked with the downregulation of serotonin (5HT) release and/or uptake [6] together with 5-HT1A receptor dysfunction. These dysfunctions are thought to be major factors in several mental disorders, including major depression [7]; however, the current gene expression data suggest that suicide is possibly correlated with extensive changes in the brain and is not restricted to only one neurotransmitter system [8,9,10]. In addition to changes that have been observed in the serotonergic system, studies on brain samples of people who have committed suicide suggest that GABAergic and glutamatergic transmissions are also involved [11,12]. Furthermore, changes in the expression of glia-derived genes and glial fibrillary acidic protein (GFAP) in depression and other psychiatric illnesses indicate that suicide-related molecular alterations may not be restricted to neurons [13]. Most likely, molecular mechanisms in the brain that lead to suicide coexist with pathological changes along several functional protein networks. Suicide-brain studies that show that hyper-
methylation of the ribosomal-RNA gene promoter could cause aberrant changes in protein synthesis [14] support this idea. Psychoactive drugs can change the risk of suicide, and there are ongoing efforts to find potential biomarkers to predict suicidal behaviours [15,16,17,18,19,20]. Thus, understanding the molecular brain mechanisms involved in suicide is important for the development of both psychoactive drugs and predictive diagnostic tools.

Screening technology progress in the past two decades (e.g., the gene chip and the 2D gel-based and liquid-based proteomic techniques) have provided new insights into the molecular processes of the brain [21]. Because suicide cannot be observed in animals, investigating post mortem human brains with a relatively short post mortem delay is a good alternative. Particularly, the post mortem human brain proteome reflects the complex pathological changes of protein expression in the human brain while alive [21]. A homogeneous sample is usually unlikely in such studies because suicide and its associated psychiatric disorders and medications differentially influence various underlying molecular mechanisms. Therefore, in the present study we used brain samples from people who had hanged themselves and from individuals who died due to acute cardiac arrest to decrease the heterogeneity of data. We examined prefrontal cortex and amygdala samples because mood disorders involve several neuronal mechanisms in these brain areas and are correlated with suicide [1,7].

Our aim was to find changes in the proteome of the prefrontal cortex and amygdala that correlated with suicide. Changes in protein expression patterns may reflect molecular changes of psychopathological states and could provide biomarkers for suicide risk.

### Table 1. Description of participants in the present study.

| Brain No. | Gender | Age | Post mortem interval (PMI) | Cause of death | Neuropathological diagnosis |
|-----------|--------|-----|---------------------------|-----------------|-----------------------------|
| #138 S    | male   | 52  | 3 h                       | suicide (hanging)| lack of specific neuropathological alteration |
| #139 S    | male   | 79  | 4 h                       | suicide (hanging)| lack of specific neuropathological alteration |
| #143 S    | male   | 43  | 3 h                       | suicide (hanging)| lack of specific neuropathological alteration |
| #144 S    | male   | 42  | 4 h                       | suicide (hanging)| lack of specific neuropathological alteration |
| #1735 S   | male   | 43  | 6 h                       | suicide (hanging)| NA                          |
| #1745 S   | male   | 57  | 6 h                       | suicide (hanging)| NA                          |
| #11 C     | male   | 47  | 2 h                       | acute cardiac insufficiency, chronic myocardial infarction, chronic heart failure, coronary sclerosis | NA                          |
| #12 C     | male   | 80  | 2 h                       | acute cardiac insufficiency, acute heart failure, coronary sclerosis, senile, hypertensive arteriosclerosis | NA                          |
| #111 C    | male   | 55  | 3 h                       | cardiac insufficiency, coronary stenosis | NA                          |
| #151 C    | male   | 47  | 2 h                       | acute myocardial infarction | encephalopathy alcoholic |
| #164 C    | male   | 85  | 3 h                       | cardiorespiratory insufficiency | lacunar encephalopathy |
| #213 C    | male   | 75  | 5 h                       | cardiac insufficiency | vascular leuocencephalopathy small vessels disease lacunar stroke |

NA: not available; S: suicide; C: control.

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### Methods

#### Ethics Statement

The human brains were obtained from the Lenhossek Human Brain Program, Human Brain Tissue Bank, Budapest. Brains were taken from persons who had died without any known neurodegenerative diseases. The collection of brains and the microdissection of the brain samples for research have been performed by the approval of the Regional Committee of Science and Research Ethics of the Semmelweis University, Budapest (TUKEB: 32/92) and the Ethics Committee of the Ministry of Health, Hungary, 2002 according to the principles expressed in the Declaration of Helsinki. Tissues were collected only after a family member gave informed (written) consent.

#### Sample Collection and Preparation for Proteomics

We used brain samples from male subjects. The age distributions of suicide (6 brains; age range: 41–79 years; mean age: 52.7; SD: 14.2) and control (6 brains; age range: 47–85 years; mean age average: 64.8; SD: 17.2) groups did not differ significantly (p = 0.1491, Wilcoxon test; Table 1). Suicide group brain samples came from subjects who had hanged themselves, control group brain samples came from victims of cardiac arrest. No information was available whether the cardiac arrest in control subjects happened during sleep or not. The post mortem interval (PMI) did not differ significantly between groups (p = 0.0683, Wilcoxon test; Table 1). We used two brain areas - the prefrontal cortex and the amygdala – to conduct proteomic analyses. We treated and handled brain samples as described in a previous publication [22]; briefly, brains were removed from the skull 2–6 hours after death, frozen, and sliced into 1– to 1.5 cm-thick coronal sections. We used the punch technique to micro-dissect the brain areas. Tissue samples were stored at –80˚C until used. In this study, we...
processed one cortex and one amygdala samples from 6 suicide and 6 control subjects, meaning a total of 24 human post mortem brain tissue samples.

The brain sample preparation protocol was similar to previous studies [23,24]; briefly, we mechanically homogenised tissue samples in a cooled lysis buffer (7 M urea; 2 M thiourea; 20 mM Tris; 5 mM magnesium acetate, 4% CHAPS; Protease Inhibitor Mix (1:1000), GE Healthcare, Uppsala, Sweden). Samples were then sonicated and centrifuged (1 h, 14,000 g, 4°C). The pH of the supernatant was adjusted to 8.0 and protein concentrations of the samples were measured by PlusOne Quant Kit (GE Healthcare). We labelled 5 µg of each protein sample with CyDyeTM DIGE Fluor Labelling kit for Scarce Samples (GE Healthcare) at a concentration of 4 nmol/5 µg proteins according to instructions.

We labelled the experimental samples (control and suicide samples) as Cy5 and the pooled internal standard samples (reference or standard sample, is a pool comprising equal amounts (2.5 µg) of each of the experimental samples being compared) as Cy3. The pooled standard represents the average of all the samples being analyzed and ensures all proteins present in the experimental samples are represented. The pooled standard is used to normalize protein abundance measurements across multiple gels in an experiment. As a consequence each gel will contain an image with a highly similar spot pattern, simplifying and improving the confidence of inter-gel spot matching and quantification [25].

We multiplexed the differently labelled samples in the same gel. Sample multiplexing in DIGE greatly refines the detection of changes at the protein level between samples [26], as variation in spot intensities due to experimental factors, for example protein loss during sample entry into the strip, will be the same for both samples within a single DIGE gel [25].

The multiplexed, differently labelled samples (5 µg protein of Cy5-labelled and 5 µg protein of Cy3-labelled reference) were dissolved in isoelectric focusing (IEF) buffer containing ampholytes (0.5 v/v %), DTT (0.5 m/v %), 8 M urea, 30% glycerine, 2% CHAPS, and rehydrated passively onto 24 cm nonlinear IPG strips (pH 3–10 NL, GE Healthcare) overnight at room temperature. After rehydration, the strips were placed to first dimension isoelectric focusing (IPGPhore, GE Healthcare) for 24 h to attain a total of 80 kVh. The applied currents were: 30 V for 3.5 h step, 500 V for 5 h gradient, 1000 V for 6 h gradient, 8000 V for 3 h gradient, and 8000 V for 6.5 h step mode. Focused proteins were reduced by equilibrating with buffer containing 1% (w/v) mercaptoethanol for 20 min. After reduction the IPG strips were loaded onto 10% polyacrylamide gels (24 x 20 cm), and SDS-PAGE was conducted at 2 W/gel for 1 h and at 10 W/gel in the second dimension.

We prepared 12 gels from both areas because one experimental sample and one pooled standard reference sample can be loaded into one gel with the Labelling kit for Scarce Samples (GE Healthcare). Following electrophoresis, gels were scanned by a Typhoon TRIO+ Variable Mode Imager (GE Healthcare) using appropriate lasers and filters with the photomultiplier tube (PMT) biased at 600 V. Cy3 images were scanned using a 532 nm laser and an emission filter of 380 nm BP (band pass) 30. Cy5 images were scanned using a 633 nm laser and a 670 nm BP30 emission filter. All gels were scanned at 100 µm resolution. Images in different channels were overlaid using selected colours, and differences were visualised using Image Quant software (GE Healthcare). We used the DeCyder 6.3 2D gel evaluation software (GE Healthcare); the Differential In-gel Analysis (DIA) module to perform differential protein analyses and the Biological Variance Analysis (BVA) module to gel-to-gel matching and statistical analysis of protein-abundance change between samples.

In the DIA module the scanned images of the sample and the internal standard were overlaid and the algorithms within the software co-detected the spots in the gel. The estimated number of spots for each co-detection procedure was set to 2500. When calculating the abundance ratios for spot pairs in co-detected sample images, the spot volumes of the component spot maps needed to be normalized and the log standardized abundances were calculated.

The statistical analysis of protein-abundance change between samples was made by the BVA module. The BVA matched the quantified spots of all gels to a chosen master gel. According to the standard proteomic protocol [25], the threshold for the differential expression was set at a minimum fold change of 1.3 as we used human samples and the quality of the gels were adequate. We determined the p-values (Student's t-test) for each protein spot (p<0.05).

To identify proteins in the spots of interest, we performed preparative 2D electrophoresis using 800 µg of proteins per gel. We made four preparative gels and picked the relevant spots for protein identification.

**Protein Identification**

We extracted peptides from gel spots after in-gel digestion by Trypsin Gold (for a detailed protocol, see http://ms-facility.ucsf.edu/ingel.html). Peptide separation before MS analysis was done by HPLC started by inline trapping on to a nanoACQUITY UPLC trapping column (Symmetry, C18 5 µm, 180 µm × 20 mm; 15 µl/min with 3% solvent B) followed by a linear gradient elution (solvent B: 10% to 50% in 40 min, flow rate: 250 nl/min; nanoACQUITY UPLC BEH C18 Column, 1.7 µm, 75 µm × 200 mm). Solvent A was composed of 0.1% formic acid in water; solvent B was composed of 0.1% formic acid in acetonitrile. MS measurements started by using information-dependent acquisition mode, using a Waters nanoACuity nanoUPLC system coupled to a Micromass qTOF tandem mass spectrometer (Waters, USA). Next, 3 s collision-induced dissociation (CID) analyses on multiple computer-selected ions were performed for amino acid sequence determination.

**Database Search**

We converted raw MS data into a Mascot generic file using the Mascot Distiller software (version 2.1.1.0). We used the Mascot search engine (version 2.2.2) to search the resulting peak lists against the NCBI non-redundant database without species restriction (6,833,826 sequences), to eliminate false positive hits. We submitted monoisotopic masses with a peptide mass tolerance of at least 50 ppm and a fragment mass tolerance of at least 0.1 Da. We set the carbamidomethylation of Cys as a fixed modification, and we permitted acetylation of the protein N-termini, methionine oxidation and pyroglutamic acid formation from N-terminal Gln residues as variable modifications. The acceptance criterion was the identification of at least two significant peptides per protein (i.e., peptide score >52, p<0.05).

**Correction for False Discovery Rate (FDR)**

When applying statistical tests to 2-D gel data, one is faced with the so-called multiple hypothesis testing problem: for each matched and quantified spot series, a separate test is done. Each test has a certain probability of giving a false positive result, and the large number of tests can produce a high number of false positives [27]. This has led to the application of methodologies to control the false discovery rate (FDR) where FDR is the rate of
false positive results among all profiles that were tested positive (type I errors).

The original FDR methodology was considered to be too conservative for discovery experiments consequently, an extension to the FDR was developed by Storey that calculates a q-value [28].

The q-values were calculated from the p-values obtained for all features within the study with the statistics software, R [R Development Core Team (2011)]. R: A language and environment for statistical computing [R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org/] [29] by using an easy to use tool (qVALUE software version 1.0) developed by Storey and Tibshirani [28]. The frequency distributions of P-values were used to estimate the proportion of features that are unchanging; this is then used to estimate the false discovery rate (Fig. S1).

Careful observation of the P-values histograms suggested that the shape of the histograms were not the most desirable shape, although they were acceptable. Note, that the Student’s t test we used is a simple test that assumes the data are randomly sampled from normal distributions and shows homogeneity of variance. In DIGE with the traditional three-dye approach, Karp et al. demonstrated that the final standardized abundance data for the spots are not truly independent [30]. However, we used the two-dye design in this study where the Student’s t test was adequate [30,31].

The histograms of P-values of the prefrontal cortex and amygdala were dense near zero and became less dense as the P-values increased. The amygdala P-histogram contained a wider peak indicating that less spots were detected as significantly changing. By observing their q-value cut-off histograms (Fig. S1) we used 0.06 and 0.4 as q-values alpha thresholds for FDR adjustment of significant spots of the prefrontal cortex and amygdala, respectively.

Functional Clustering of Identified Proteins

Following an extensive literature search, we formed the functional protein clusters using PDB (http://www.pdb.org, La Jolla, CA, USA), ExPASy and UniProt databases (http://www.expasy.org and http://www.uniprot.org, respectively; Swiss Institute of Bioinformatics, Switzerland). From our data pool we selected 11 proteins that changed in both the amygdala and the cortex for detailed protein interaction modelling analyses using PathwayStudio® 6.2 software (Ariadne Genomics, Inc., Rockville, MD, USA). The protein network model created was manually verified using the PubMed database (http://www.ncbi.nlm.nih.gov, MD, USA).

Western Blot

Frozen brain samples were homogenized as described earlier [24]. Protein lysates (20 μg) were resolved on a 10% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad, USA). Membranes were blocked in 5% BSA in TRIS-Tween buffer (500 mM TRIS, 150 mM sodium chloride, pH 7.4, and 0.05% Tween 20 (Sigma)) for 1 h, incubated with polyclonal anti-cathepsin 1B (1:1000, Santa Cruz Biotechnology, CA, USA), anti-GFAP (1:1000, DAKO, Denmark) or anti-actin (1:5000, Sigma, Hungary) antibodies in TRIS-Tween buffer for 24 h at 4°C. After incubation with ECL-HRP-conjugated secondary antibody (1:5000, GE Healthcare, Germany), bands were visualized using a Chemiluminescence kit (BioRad, CA, USA). Ponceau staining was used as control for equal protein load and transfer.

Results

We used DIGE proteomics technology to investigate the differences in the protein expression pattern of suicide compared to control brain samples. We detected a total of 2,465 spots (after exclusion of false spots) from the prefrontal cortex and 2,115 from the amygdala on the master gels, defined to be the gel containing the most spots. Representative gel is shown in Figure 1. We performed the gel-to-gel matching with the DeCyder 6.5 software (GE Healthcare) BVA module and after careful and rigorous manual validation we matched 681 spots in the prefrontal cortex samples and 696 in the amygdala samples. From these matched spots with the t test and 1.3 fold change as cut off we found 46 significant differences between the control and suicide prefrontal cortex samples from which we could identify 84 proteins (see Table S1). Regarding the amygdala, 16 matched spots showed significant differences, and 20 proteins were identified from them (see Table S2). After FDR adjustment we had 27 significant spots in the prefrontal cortex and 9 significant spots in the amygdala. This way the number of protein “hits” in the proposed profile reduced to 59 proteins in the prefrontal cortex and 11 proteins in the amygdala (see bold-italic gene names in Tables 2 and 3).

Clustering of proteins in the prefrontal cortex revealed the following categories: cytoskeleton, signalling, metabolism, protein processing, development, synapse and neuron, proteolysis, RNA/DNA metabolism, redox system, and glia cell marker (see Table 2). Changes in the protein expression pattern of the amygdala were smaller, but they formed almost the same clusters as the cortical protein changes (see Table 3). The direction of change in the two brain structures was the opposite for several proteins. We identified several proteins in more than one spot of the 2D gel, most likely due to posttranslational or post mortem processing. Thus, whenever more than one arrow is included, they represent the number of spots in which the protein was identified; the direction of each arrow shows the direction of change in a certain spot (see Tables 2 and 3). The numerical values of changes and p-values of significance are shown in the Supplementary material (see Table S1 and S2).

Functional protein clusters of the amygdala and prefrontal cortex demonstrated both similarities and differences in the brains of suicide victims compared to controls. Of the nine proteins whose levels were altered in both the brain structures (Table 4), three (actin (ACTB), glial fibrillary acidic protein (GFAP) and cathepsin D (CTSD)) showed altered levels in opposing directions; elevated in the amygdala and lower in the cortex.

In an attempt to validate our proteomic results, western blot analysis was carried out on the proteins that showed opposing directions of change in the two brain structures as these proteins are the most promising biomarker protein candidates, e.g. for brain imaging PET probe targets. Expressions of cathepsin (p = 0.0321) and GFAP (p = 0.0192) were significantly decreased in the suicide prefrontal cortex samples compared to the control samples, while in the amygdala, the expression of cathepsin (p = 0.0164) and GFAP (p = 0.0383) significantly increased in suicide samples (Figure 2). In case of the actin we also observed decreased level in the cortex and increased level in the amygdala of suicide samples although these changes were not significant because of high SD and low n (Figure 2).

Another set of proteins displayed parallel changes in both brain structures: creatin kinase B-type (CKB), alpha-internexin (INA), neurofilament light polypeptide (NEFL), neurofilament medium polypeptide (NEFM), tubulin alpha-1B chain (TUBA1A) and heat shock cognate 71 kDa protein (HSPA8). We did not find proteins
change simultaneously in the prefrontal cortex and amygdala in functional categories: signalling, redox system and development.

Interestingly, nearly half of the altered proteins in the wider data pool had already been identified as indicative factors of suicide risk (see Table 2 and 3). In our study, we identified 35 proteins from the cortex and 16 proteins from the amygdala that had been previously linked to schizophrenia. We also identified 21 protein changes from the cortex and 9 from the amygdala that are related to depression as well as 5 proteins from the cortex and 2 proteins from the amygdala that have never been connected to schizophrenia or depression.

Discussion

In this study, we found changes in the expression of several proteins in the amygdala and the prefrontal cortex of suicide victims using proteomics technology. Our data reflect the widely accepted idea that suicide is the result of complex interactions of psychopathology-related molecular events [32,33,34,35,36] because several of the altered proteins have already been linked to psychiatric disorders such as schizophrenia or depression (see Table 2 and 3). Thus, our results are in agreement with the clinical observations that report coexisting psychopathological symptoms that can lead to suicide [37,38,39]. The proteomic changes detected in our study and the results of gene chip studies [9,11,40] show little overlap, which is in agreement with the fact that only a fraction of transcribed genes result in protein expression. In addition, differences in sample preparation, differences in sensitivity of protein or DNA/RNA detection and differences in the brain structures sampled may explain these differences. Similarly, the hyper-methylation of ribosomal-RNA gene promoter observed in suicide victims [14] might explain the widespread protein changes observed. Therefore, our data complement gene-chip and target-oriented mRNA studies [11,12].

Methodological Considerations

The applied proteomics methodology provides information on only a fraction of the proteome at one time; thus, although our results indicate certain functional processes, they do not reveal the complete functioning protein network [41]. The number of different proteins in a cell is estimated to be around 30,000, and the DIGE technology can detect only 2,000-4,000 (detecting 2,000 proteins is routine) [42,43]. Nevertheless, the number of detected proteins is large enough to treat as a multi-spot index of change in the cellular protein network and suggests possible biomarker proteins of suicide. Additional information can be
### Table 2. Functionally clustered protein changes in the prefrontal cortex.

#### CYTOSKELETON

| Gene     | Protein name                      | Up/down regulation | Accession number | Cellular localization                        | Molecular function                                                                 |
|----------|-----------------------------------|--------------------|------------------|---------------------------------------------|-------------------------------------------------------------------------------------|
| *ACTB    | Actin, cytoplasmic 1              | ↓                  | P60709           | Cytoplasm, cytoskeleton                     | Structural constituent of cytoskeleton, cell motion                                   |
| *INA     | Alpha-internexin                   | ↑                  | Q16352           | Neurofilament                               | Cell differentiation, nervous system development, structural constituent of cytoskeleton |
| *NEFL    | Neurofilament, light polypeptide 68kDa | ↑ ↑ ↓             | P07196           | Axon, neurofilament                         | Maintenance of neuronal caliber, axon cargo transport                                |
| *NEFM    | Neurofilament, medium polypeptide  | ↑ ↑ ↑              | Q4QRK6           | Axon, intermediate filament, neurofilament, neuromuscular junction | Axon cargo transport, microtubule/neurofilament cytoskeleton organization, regulation of axon diameter |
| SERPINC3 | Serpin B3                          | ↓                  | P29508           | Cytoplasm                                   | Protein binding, serine-type endopeptidase inhibitor activity                         |
| *TUBA1A  | Tubulin alpha-1B chain            | ↑ ↑ ↓              | P68363           | Microtubule                                 | Microtubule-based movement, protein polymerization                                   |
| *TUBA1B  | Tubulin alpha-1C chain            | ↑                  | Q9BQE3           | Microtubule                                 | Major constituent of microtubules, microtubule-based movement, protein polymerization |
| *TUBA1C  | Tubulin alpha-4A chain            | ↑ ↑ ↓              | P68366           | Cytoplasm, microtubule                      | Microtubule-based movement, protein polymerization, major constituent of microtubules |
| TUBB4    | Tubulin beta-4 chain              | ↓                  | P04350           | Cytoplasm, microtubule                      | Major constituent of microtubules, microtubule-based movement, protein polymerization |

#### SIGNALING

| Gene     | Protein name                      | Up/down regulation | Accession number | Cellular localization                        | Molecular function                                                                 |
|----------|-----------------------------------|--------------------|------------------|---------------------------------------------|-------------------------------------------------------------------------------------|
| CALB2    | Calbindin 2                       | ↓                  | P22676           | N.D.                                        | Calcium ion binding                                                                |
| CAP2     | Adenylyl cyclase-associated protein 2 | ↑                | P40123           | Cell membrane                               | Signal transduction, establishment/maintenance of cell polarity, cytoskeleton organization, activation of adenylate cyclase activity |
| CRKL     | Crk-like protein                   | ↑                  | P46109           | Cytoplasm                                   | JNK cascade, Ras protein signal transduction, protein tyrosine kinase activity       |
| *EFHD2   | EF-hand domain family, member D2   | ↓ ↓               | Q96C19           | Membrane raft                               | Calcium ion binding, regulator of the NF-kappa-B-activating branch, apoptosis        |
| *GRB2    | Growth factor receptor-bound protein 2 | ↓                | P62993           | Golgi apparatus, cytosol                    | Ras protein signal transduction, cell-cell signaling, interspecies interaction between organisms, insulin receptor signaling pathway, EGF signaling pathway   |
| MAPK1    | Mitogen-activated protein kinase 3 (ERK1) | ↑              | P27361           | Cytoplasm, cytoskeleton, nucleoplasm        | Cell cycle, Ras protein signal transduction, protein amino acid phosphorylation, interspecies interaction between organisms |
| PARK7    | Protein DJ-1                      | ↓                  | Q99497           | Cytoplasm, nucleus                          | Chaperone, Ras protein signal transduction                                           |
| PGK1     | Phosphoglycerate kinase 1 Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform | ↑ ↑ ↑            | Q08209           | Cytosol, nucleus                            | Calcium ion binding, calmodulin binding, iron ion binding, zinc ion binding           |
| YWHAB    | Tyrosine 3-monooxygenase/trypthphan 5-monooxygenase activation protein, beta polypeptide 14-3-3 protein beta/alpha | ↑              | P31946           | Cytoplasm, melanosome                       | Signal transduction, regulation of amino acid dephosphorilation, apoptosis            |
| YWHAG    | 14-3-3 protein gamma             | ↓                  | P61981           | Cytoplasm                                   | Signal transduction, synaptic plasticity, neuron differentiation, regulation of protein kinase activity |
| *YWHAH   | 14-3-3 protein epsilon           | ↑                  | P62258           | Cytosol, melanosome                         | Apoptosis, intracellular signaling cascade, interspecies interaction betweenorganisms |
| *YWHAH   | 14-3-3 protein eta               | ↑                  | Q04917           | Cytoplasm                                   | Glucocorticoid catabolism/signaling, synaptic plasticity, dendrite morphogenesis, regulation of transcription, protein transport |
### SIGNALING

| Gene          | Protein name               | Up/down regulation | Accession number | Cellular localization         | Molecular function                                      |
|--------------|----------------------------|--------------------|------------------|-------------------------------|----------------------------------------------------------|
| YWHAZ        | 14-3-3 protein zeta/delta  | ↑                  | P63104           | Cytoplasm, melanosome         | Anti-apoptosis, signal transduction                       |

### METABOLISM

| Gene          | Protein name               | Up/down regulation | Accession number | Cellular localization         | Molecular function                                      |
|--------------|----------------------------|--------------------|------------------|-------------------------------|----------------------------------------------------------|
| ABHD14B      | Abhydrolase domain-containing protein 14B | ↓                  | Q96U4            | Cytoplasm, nucleus            | Hydrolase activity                                       |
| ALDOC        | Fructose-bisphosphat aldolase C | ↑                  | P09972           | Cytoskeleton                  | Glycolysis, fructose 1,6-bisphosphate catabolism          |
| APOA1        | Apolipoprotein A-I          | ↑                  | P02647           | Endocytic vesicle, ER lumen, plasma membrane, secretory granule | Cholesterol metabolism, lipid metabolism/transport, sterol metabolism, transport |
| ATP5B        | ATP synthase subunit beta, mitochondrial | ↓                  | Q13510           | Membrane, mitochondria        | ATP synthesis, hydrogen ion transport, ion transport, transport, angiogenesis, regulation of intracellular pH |
| ATP5C1       | ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide d | ↓                  | P06576           | Mitochondria                  | ATP synthesis, hydrogen ion transport, ion transport, transport |
| ATP5H        | ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d | ↓                  | O75947           | Mitochondria                  | Mitochondrial ATP synthesis coupled proton transport, hydrogen ion transport, |
| ATP6V1D      | ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D | ↑                  | P21281           | N.D.                          | ATP synthesis, hydrogen ion transport, ion transport, transport |
| C5orf33      | Chromosome 5 open reading frame 33 | ↑                  | Q4G0N4           | N.D.                          | Metabolic process, NAD+ kinase activity                  |
| CB1          | Carbonyl reductase          | ↑ ↑ ↓             | P16152           | Cytoplasm                     | Drug metabolism, vitamin K metabolism                    |
| CBR1         | [69]                        |                    |                  |                               |                                                          |
| +CKB         | [85], [88], [90], [105], [100], [106] | ↑ ↑ ↓             | P12277           | Cytoplasm                     | Creatine metabolism                                      |
| +CS          | [104], [107], [108]        | ↑ ↑ ↑             | O75390           | Mitochondria                  | Cellular carbohydrate metabolism, tricarboxylic acid cycle |
| ECHS1        | Enoyl-CoA hydratase, mitochondrial | ↓ ↓              | P30084           | Mitochondria                  | Fatty acid metabolism, lipid metabolism                  |
| FH           | Fumarate hydratase, mitochondria | ↑              | P07954           | Mitochondria                  | Fumarate metabolism, tricarboxylic acid cycle           |
| GLD4         | Glyoxalase domain-containing protein 4 | ↑              | Q9HC38           | Mitochondria                  | N.D.                                                      |
| +GLUL        | Glutamate-ammonia ligase, glutamine synthetase | ↑ ↑             | P15104           | Cytoplasm, Golgi apparatus    | Cell proliferation, glutamine biosynthesis               |
| GOT1         | Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1) | ↑ ↑             | P17174           | Cytoplasm                     | Aspartate catabolism, cellular response to insulin stimulus, response to glucocorticoid stimulus |
| GUK1         | Guanylate kinase            | ↓                  | Q16774           | Cytosol                       | Purine nucleotid metabolism                              |
| HADH         | Hydroxyacyl-CoA dehydrogenase, mitochondrial | ↑             | Q16836           | Mitochondria                  | Fatty acid metabolism, lipid metabolism                  |
| IDH2         | Isocitrate dehydrogenase 2 [NADP+], mitochondrial | ↑ ↑             | P48735           | Mitochondria                  | Isocitrate metabolism, tricarboxylic acid metabolism, glyoxylate cycle |

Proteome of Victims of Suicide
### METABOLISM

| Gene      | Protein name                                      | Up/down regulation | Accession number | Cellular localization           | Molecular function                                                        |
|-----------|---------------------------------------------------|--------------------|------------------|--------------------------------|----------------------------------------------------------------------------|
| IDH3A     | Isocitrate dehydrogenase 3 [NAD+] subunit alpha, mitochondrial | ↑                  | P50213           | Mitochondria                   | Carbohydrate metabolism, tricarboxylic acid cycle                         |
| *IMP1     | Inositol(myo)-1(4)-monophosphatase 1              | ↓                  | P29218           | Cytoplasm                      | Phosphate metabolism, phosphatidylinositol biosynthesis, signal transduction |
| *NDUFS1   | NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase) | ↓                  | P28331           | Mitochondrial inner membrane space, respiratory chain complex 1            | ATP metabolism, transport, electron transport, ROS metabolism, apoptosis  |
| NDUFV2    | NADH dehydrogenase Fe protein                    | ↓                  | Q6IPW4           | Mitochondria                   | NAD binding                                                               |
| PDHA1     | Pyruvate dehydrogenase (lipoyamide) alpha 1      | ↑↑↑               | O00330           | Mitochondria                   | Pyruvate metabolism                                                       |
| PGLS      | 6-Phosphogluconolactonase                        | ↓↓                | O95336           | Cytoplasm                      | Pentose-phosphate shunt                                                   |
| PKM2      | Pyruvate kinase isozymes M1/M2                    | ↑↑                | P14618           | Cytoplasm, nucleus             | Glycolysis, programmed cell death                                         |
| TALDO1    | Transaldolase 1                                  | ↑                 | P37837           | Mitochondria                   | Pentose shunt                                                             |
| UCHL1     | Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) | ↑↑                | P08559           | Mitochondria                   | Glycolysis, pyruvate metabolism                                           |
| UQRC2     | Ubiquinol-cytochrome c reductase core protein II Cytochrome b-c1 complex subunit 2, mitochondrial | ↑↑↑               | P22695           | Mitochondria                   | Electron transport, respiratory chain, proteolysis, transport, oxidative phosphorylation |

### PROTEIN PROCESSING

| Gene      | Protein name                                      | Up/down regulation | Accession number | Cellular localization           | Molecular function                                                        |
|-----------|---------------------------------------------------|--------------------|------------------|--------------------------------|----------------------------------------------------------------------------|
| BRCC3     | BRCA1/BRCA2-containing complex, subunit 3 Lys-63-specific deubiquitinase BRCC36 | ↑                  | P46736           | Nucleus                        | DNA repair, modification-dependent protein catabolism, Ubl conjugation pathway |
| CAPZA2    | Capping protein (actin filament) muscle Z-line, alpha 2 | ↑                  |                   | Cytoplasm                      | Chaperon protein folding                                                  |
| FKBP4     | FKS06-binding protein 4, 59kDa                    | ↑                  | Q02790           | Cytoplasm, nucleus             | Protein binding, HSP binding, FKS06 binding, peptidyl-prolyl cis-trans isomerase activity |
| HSPA8     | Heat shock cognate 71 kDa protein                 | ↑↓                | P11142           | Cytoplasm                      | Chaperone, response to unfolded proteins, membrane organization, interspecies interaction between organisms, post-Golgi vesicle-mediated transport |
| HSPB1     | Heat shock 27kDa protein 1 (beta-1)               | ↓                 | P04792           | Cytoplasm, nucleus, cytoskeleton | Anti-apoptosis, cell death, cell motion, response to heat, response to unfolded proteins, regulation of translational initiation |
| VTA1      | Vps20-associated 1 homolog                        | ↑                  | Q9NP79           | Cytoplasm, endosome, cell membrane | Protein transport                                                         |

### DEVELOPMENT

| Gene      | Protein name                                      | Up/down regulation | Accession number | Cellular localization           | Molecular function                                                        |
|-----------|---------------------------------------------------|--------------------|------------------|--------------------------------|----------------------------------------------------------------------------|
| DPYSL2    | Dihydropyrimidinase-like 2                        | ↑↑↑               | Q16555           | Cytoplasm                      | Cell differentiation, nervous system development, nucleotide and nucleic acid metabolism, intracellular trafficking of heterooligomeric forms of steroid hormone receptors |
**DEVELOPMENT**

| Gene       | Protein name                      | Up/down regulation | Accession number | Cellular localization                      | Molecular function                                                                 |
|------------|-----------------------------------|--------------------|------------------|--------------------------------------------|-------------------------------------------------------------------------------------|
| *SELENBP1  | Selenium binding protein 1        | ↑ ↑                | Q13228           | Cytoplasm, membrane, nucleus               | Protein transport, transport, selenium binding                                        |
| SEPT2      | Septin-2                          | ↑                  | Q15019           | Cytoplasm, cytoskeleton, nucleus           | Cell division, mitosis, cell cycle                                                  |
| SERT2      | NAD-dependent deacetylase sirtuin-2| ↑ ↑                | Q8IXJ6           | Cytoplasm, microtubule                      | Regulation of mitosis, regulation of phosphorylation, chromatin silencing, cell division |

**SYNAPSE, NEURON**

| Gene       | Protein name                      | Up/down regulation | Accession number | Cellular localization                      | Molecular function                                                                 |
|------------|-----------------------------------|--------------------|------------------|--------------------------------------------|-------------------------------------------------------------------------------------|
| APOL1BP    | Apolipoprotein A-I-binding protein | ↑                  | Q6PGN4           | N.D.                                        | Lipid transport, lipoprotein metabolism, acut-phase response, multicellular orgamisal development |
| *APOL2     | Apolipoprotein L2                 | ↑                  | Q9QQE5           | Cytoplasm                                   | Hydrogen ion transport, ion transport, transport                                      |
| *ATP6V1B2  | V-type proton ATPase subunit B, brain isoform | ↑ ↑ ↑ | P36542           | Endoplasm reticulum, cytosol                | Apoptosis, host-virus interaction, ion transport, transport                           |
| SYN1       | Synapsin-1                        | ↑                  | P17600           | Synaptic vesicles                           | Neuronal phosphoprotein that coats synaptic vesicles, neurotransmitter release regulation |

**PROTEOLYSIS**

| Gene       | Protein name                      | Up/down regulation | Accession number | Cellular localization                      | Molecular function                                                                 |
|------------|-----------------------------------|--------------------|------------------|--------------------------------------------|-------------------------------------------------------------------------------------|
| S CAPNS1   | Calpain small subunit 1           | ↓                  | P04632           | Cytoplasm, cell membrane, nucleus          | Regulation of cell proliferation                                                      |
| S *CTSD    | Cathepsin D                       | ↓ ↓                | P07339           | Lysosome, melanosome, extracellular region  | Cell death, proteolysis                                                              |
| +PSMB4     | Proteasome subunit beta type-4     | ↓                  | P28070           | Centrosome, nucleus, proteasome core complex | Regulation of ubiquitin-protein ligase activity during mitotic cell cycle, ubiquitin-dependent protein catabolism |

**RNA/DNA METABOLISM**

| Gene       | Protein name                      | Up/down regulation | Accession number | Cellular localization                      | Molecular function                                                                 |
|------------|-----------------------------------|--------------------|------------------|--------------------------------------------|-------------------------------------------------------------------------------------|
| HNRPD     | Heterogeneous nuclear ribonucleoprotein D-like | ↑                  | O14979           | Cytoplasm, heterogeneous nuclear ribonucleic complex | Regulation of transcription, RNA processing, transcription                           |
| PHB        | Prohibitin                        | ↓                  | P35232           | Mitochondria, nucleoplasm                   | DNA replication, cell proliferation, transcription, apoptosis, signal transduction    |
| +PURA      | Transcriptional activator protein Pur-alpha | ↑                  | Q00577           | Nucleus                                    | DNA replication initiation, transcription                                            |

**REDOX SYSTEM**

| Gene       | Protein name                      | Up/down regulation | Accession number | Cellular localization                      | Molecular function                                                                 |
|------------|-----------------------------------|--------------------|------------------|--------------------------------------------|-------------------------------------------------------------------------------------|
and the agonal state, metabolic state, the use of drugs, infections, hypoxia and the PMI duration [44] and these proteins include e.g. GFAP and INA that also changed in this study. However, the PMI duration was short and overlapping in our study, and the spot positions in the gel and the peptide coverage of the identified protein (see Fig. S2 and Table S3, S4), as well as the opposite change of some proteins in the two brain structures, do not suggest simple protein degradation. We think, that at least some of these cytoskeleton related protein abundance changes observed in our study could be in vivo existing protein isoforms reflecting the pathophysiological processes of psychiatric illnesses rather than protein degradation.

However, one question is open, whether the changes in protein expression present before the suicide or the result of the trauma from the suicide. Post mortem brain tissue studies on suicide brains can not elucidate this question. Protein expression changes presented here can be the result of pre-suicide psychotic state, or a longer major depressive agitated state because of the long turnover time of proteins. The hypoxia caused by hanging might not have changed the brain proteome directly because hypoxia activated proteins were not found in great number. Since we are searching for biomarkers of suicide, it would be very important to know which biomarker protein candidates are correlating with the pre-suicide psychosis however we must leave the question open.

### Extensive Protein Changes in the Brains of Suicide Victims Reflect an Altered State of Cellular Functions

Different psychiatric diseases, such as major depression [48,49] and schizophrenia [50], may increase the risk of suicide; in turn, protein expression changes in the brains of suicide victims reflect several overlapping molecular mechanisms of different psychiatric illnesses. They may also reflect preceding psychiatric abnormal-
Table 3. Functionally clustered changes in proteins of the amygdala.

| CYTOSKELETON | | | | | |
|--------------|--------------|----------------|----------------|----------------|----------------|
| Gene        | Protein name | Up/down regulation | Accession number | Cellular localization | Molecular function |
| *+ACTB       | Actin, cytoplasmic 1 | ↑ | P60709 | Cytoplasm, cytoskeleton | Structural constituent of cytoskeleton, cell motion |
| *INA        | Alpha-internexin (68 kDa neurofilament protein) | ↑ | Q16352 | Neurofilament | Cell differentiation, nervous system development, structural constituent of cytoskeleton |
| *+NEFL      | Neurofilament, light polypeptide 68 kDa | ↑↑↑↑ | P07196 | Axon, neurofilament | Maintenance of neuronal caliber, axon cargo transport |
| *NEFM       | Neurofilament, medium polypeptide | ↑ | Q4QRK6 | Axon, intermediated filament, neuromuscular junction | Cytoskeleton organization, axon cargo transport |
| *+TUBA1A    | Tubulin alpha-1A chain | ↑↑ | Q71U36 | Cytoplasm, melanosome | Microtubule-based movement, protein polymerization |
| *+TUBB3     | Tubulin beta-3 chain | ↑ | Q13509 | Microtubule | Microtubule-based movement, protein polymerization |
| *+VIM       | Vimentin | ↑ | P08670 | Cytoplasm, intermediated filament | Cell motion, structural constituent of cytoskeleton |

| SIGNALLING | | | | | |
| Gene        | Protein name | Up/down regulation | Accession number | Cellular localization | Molecular function |
| *ANXA5      | Annexin A5 | ↑ | P08758 | Cytoplasm | Anti-apoptosis, coagulation, signal transduction |
| *EFHD2      | EF-hand domain family, member D2 | ↑ | Q96C19 | Membrane raft | Calcium ion binding, regulator of the NF-kappa-B-activating branch, apoptosis |
| *+PHB       | Prohibitin | ↑ | P35232 | Membrane, mitochondria | Proliferation, transcription, apoptosis, replication, signal transduction |

| METABOLISM | | | | | |
| Gene        | Protein name | Up/down regulation | Accession number | Cellular localization | Molecular function |
| ATP5A1      | ATP synthase subunit alpha, mitochondrial (precursor) | ↓ | P25705 | Mitochondria inner membrane | ATP synthesis, ion transport, transport, embryonic development, lipid metabolism |
| *+CA2       | Carbonic anhydrase II | ↓ | P00918 | Cytoplasm, nucleus | One-carbon metabolic process, bone resorption, osteoclast differentiation |
| *CKB        | Creatine kinase B-type | ↑ | P12277 | Cytoplasm | Creatine metabolism |
| Table 3. Cont. |
|----------------|
| **METABOLISM** |
| Gene | Protein name | Up/down regulation | Accession number | Cellular localization | Molecular function |
| *MDH1* [87], [141], [142] | Malate dehydrogenase, cytoplasmic | † | P40925 | Cytoplasm | Glycolysis, malate metabolism, tricarboxylic acid cycle |
| **PROTEIN PROCESSING** |
| Gene | Protein name | Up/down regulation | Accession number | Cellular localization | Molecular function |
| *HSPA8* [69], [116] | Heat shock 70 kDa protein 8 | ↓ | P11142 | Cytoplasm | Chaperone, protein folding, membrane organization, post-Golgi vesicle-mediated transport |
| *HSPA9* | Heat shock 70 kDa protein 9 (mortalin) Stress-70 protein, mitochondrial | † | P38646 | Mitochondria | Control of cell proliferation and cellular aging; probably a chaperone |
| *HSPD1* [69] | Heat shock 60 kDa protein 1 (chaperonin) 60 kDa heat shock protein, mitochondrial | ↑ † | P10809 | Mitochondria matrix | Chaperone |
| **DEVELOPMENT** |
| Gene | Protein name | Up/down regulation | Accession number | Cellular localization | Molecular function |
| *GAP43* [143] | Neuromodulin | ↑ | P17677 | Cell junction, synapse, plasma membrane | Nerve growth regulation, neurogenesis, differentiation, signal transduction |
| **PROTEOLYSIS** |
| Gene | Protein name | Up/down regulation | Accession number | Cellular localization | Molecular function |
| S*CTSD* [126], [125] | Cathepsin D | ↑ | P07339 | Lysosome, melanosome | Cell death, proteolysis |
| **GLIA CELL MARKER** |
| Gene | Protein name | Up/down regulation | Accession number | Cellular localization | Molecular function |
| S*GFAP* [96], [88], [69], [13], [132], [133], [134], [119], [101] | Gial fibrillary acidic protein | ↑ † † † † † | P14136 | Cytosol, intermediate filament | Central nervous system development, structural constituent of cytoskeleton |

*proteins involved in schizophrenia; ++ proteins involved in depression; S: proteins involved in suicide.
*proteins involved in schizophrenia; +: proteins involved in depression; S: proteins involved in suicide. Bold-italic gene names highlighting those proteins that were found in those differently expressed protein spots that proved significant with both statistical tests. ↑ or †: the direction of the spot intensity change of a given spot compared to control.

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re-suicide stress and/or psychopathology. Thus, we did not expect to find a pathway or protein network directly responsible for suicide; rather, we expected that molecular markers for predicting the risk for committing suicide can be uncovered. As expected, we identified several proteins already reported in the suicide and psychiatric disorder literature (see Tables 2 and 3). Some of our results may probably indicate an altered monoaminergic neurotransmission [51] while mitochondrial enzymes, such as different ATP synthase subunits (ATP5B,

Table 4. Altered proteins in the prefrontal cortex and amygdala.

| Gene name         | Protein name                           | Up/down regulation in the cortex | Up/down regulation in the amygdala |
|-------------------|----------------------------------------|----------------------------------|-----------------------------------|
| Cytoskeleton      |                                        |                                  |                                   |
| ACTB              | Actin, cytoplasmic 1                   | ↓*                               | ↑*                                |
| INA               | Alpha-internexin                       | ↑↑                               | ↑                                 |
| NEFL              | Neurofilament, light polypeptide 68 kDa| ↑↑↓                              | ↑↑↓                               |
| NEFM              | Neurofilament, medium polypeptide,     | ↑↑↑↓↑                           | ↑↑↑↓↑                             |
| TUBA1A            | Tubulin alpha-1B chain                 | ↑↑↓                              | ↑↑↓                               |
| Glia cell marker  |                                        |                                  |                                   |
| GFAP              | Glial fibrillary acidic protein        | ↑↓#↓↓↓↓↓↓↓↓↓↓*                    | ↑↑↑↑↑↑↑↑*                         |
| Metabolism        |                                        |                                  |                                   |
| CKB               | Creatine kinase B-type                 | ↑↓↓                             | ↑                                  |
| Protein processing|                                        |                                  |                                   |
| HSPA8             | Heat shock cognate 71 kDa protein      | ↑↓                               | ↑↑↓                               |
| Proteolysis       |                                        |                                  |                                   |
| CTSD              | Cathepsin D                           | ↓↓↓↓*                           | ↑*                                |

Proteins labelled by * were changed in both the cortex and the amygdala, but the directions of the changes were in reverse directions. Bold-italic gene name as in previous tables. ↑ or ↓: the direction of the spot intensity change of a given spot compared to control, for details see the Suppl. Materials Table 3 and 4, Suppl.

Figure 2. Western blot validation of GFAP, cathepsin and actin expressions in the cortex and amygdala of suicide and control subjects. The expressions of GFAP and cathepsin were significantly decreased in the suicide prefrontal cortex compared to the control samples while in the amygdala their expressions were significantly increased. In case of the actin similar but non-significant changes were found. The loading control was Ponceau, mean ± SEM.

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ATP5C1, etc.), citrate synthase (CS), enoyl-CoA hydratase (ECHS1), and fumarate hydratase (FH) may reflect the glucose metabolism down-regulation theory of suicide [52]. On the other hand, lower amounts of peroxiredoxin 6 (PRDX6) and glutathione peroxidase (GPX1), in the brains of suicide victims support the relevance of the redox imbalance hypothesis in psychiatric patients [53]. We found changes in the expression of cytoskeleton proteins (see Tables 2 and 3), which probably reflects altered receptor trafficking and signalling [54]. Unbalanced glutamatergic and GABAergic neurotransmission are also important risk factors in developing suicide behaviour [11,55]. Furthermore, changes in GABAA receptor subunits accompanied by alterations in NMDA and AMPA receptor signalling have been found in psychopathological states related to suicide [8,56]. Contrary to our finding in the cortices of suicide victims, decreased glutamine synthetase (GLUL) levels have been detected in schizophrenia and depression models [57], and down-regulated GLUL gene has been found among depressed suicide victims [9,11]. This discrepancy might indicate that a suicide by hanging and its associated stress elevates excitatory events, whereas depression decreases excitatory events. Increased GLUL levels may not only indicate increased glutamate-to-glutamine conversion, but also increased glutamatergic transmission [58]. We found other proteins that indicate that elevated excitatory events may play a role in suicide; e.g., decreased cortical levels of calbindin (CALB2) suggest - as a consequence of decreased Ca²⁺ binding capacity - an elevated concentration of free Ca²⁺ that can be excitatory above a certain level [59].

Glutamine synthetase is mainly located in astrocytes, and its changes in relative level were investigated after deprivation of paradoxical sleep in rats [60]. A significant increase in GLUL level

![Figure 3. The protein network of altered cytoskeleton proteins in the brains of suicide victims (green) is connected to the receptor-interaction network of glutamate and serotonin (red) via NEFL and GFAP.](image-url)

Abbreviations: GRIA1 – Glutamate receptor, ionotropic, AMPA1, GRIA3 - glutamate receptor, ionotrophic, AMPA 3, GRIK1– Glutamate receptor, ionotropic, kainate 1, GRIN1 – Glutamate receptor, ionotropic, N-methyl-D-aspartate, HTR1A – 5-Hydroxytryptamin (serotonin) receptor 1A, HTR2A (5-hydroxytryptamine (serotonin) receptor 2A, HTR1B (5-hydroxytryptamine (serotonin) receptor 1B, CKB - Creatine kinase B-type, ACTB - Actin, cytoplasmic 1, TUBA1A – Tubulin alpha-1B chain, NEFL – Neurofilament, light polypeptide 68 kDa, NEFM – Neurofilament, medium polypeptide, INA – Alpha-internexin, GFAP – Glial fibrillary acidic protein, CTSD - Cathepsin D, HSPA8 - Heat shock 70 kDa protein 8.

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was observed e.g. in the frontoparietal cortex after paradoxical sleep deprivation that rises the issue that stress and prolonged waking could affect the physiological regulation of GLUL. In our study, it can not be excluded that the cardiac arrest in control subjects would had happened during sleep (see methods section) and the difference between those who died asleep opposed to those who died awake could influence our result. The heterogeneity of data from this regard also could increase the data dispersion. Nevertheless, we think that the stress and the prolonged waking in case of the suicide victims could be an important issue.

In accordance with previously published changes in the GFAP of suicide victims and patients with psychiatric disorders [61], we also found an increased level of GFAP in the amygda but decreased expression in the cortex. GFAP concentration is generally believed to be an index of the number of glia cells [13]; however, astrocyte dysfunction, without a reduction in cell number, may be a factor in suicide [52]. We identified GFAP from several different gel areas (see Table 2 and 3 and Table S1, S2, S3, S4 Fig. S2), which indicates that GFAP is probably highly processed. Furthermore, a lower level of PRDX6 is known to be present in astrocytes [62]. Therefore, our data suggest that focused studies on changes in glial morphology and glial protein functions in the brains of suicide victims could be beneficial in understanding the role of glia cells in suicide.

The extensive changes detected in the proteome of suicide brain are not surprising because the ribosomal RNA level is likely decreased in the brains of suicide victims due to hyper-methylation in the RNA-promoter region [14]. Epigenetic factors, such as DNA methylation, are known to exist in different psychiatric disorders related to high suicide risk [63,64,65].

Can Some Proteins be Used as Biomarker Molecules of Suicide?

Our proteomic study revealed that protein changes might be considered as a potential starting point for identifying biomarker candidates of suicide. Fifteen of the proteins we detected (carbonyl reductase [CBR1], dihydroorotidase-like 2 [DPYS1.2], EF-hand domain gel family, member D2 [EFHD2], FK506-binding protein 4 [FBKP4], GFAP, GLUL, HSPA8, NEFL, NEFM, phosphoglycerate mutase 1 [PGAM1], PRDX6, SELENBP1, VIM, 14-3-3 protein eta [YWHAH] and 14-3-3 protein zeta/delta [YWHAZ]) have already been suggested as potential biomarker candidates for depression or schizophrenia [66,67,68,69,70,71,72,73]. Additionally, 14-3-3 protein epsilon [YWHAE] was found to be a potential suicide susceptibility gene [74]. There were 9 protein expression changes in both the cortex and the amygdala in the brains of suicide victims compared to controls (Table 4), and four of these (GFAP, HSPA8, NEFL and NEFM) were overlapped with the previous fifteen. These 9 proteins indicate that at least some of the protein changes are global in the brains of suicide victims. Three of these proteins (ACTB, CTSD and GFAP) had opposite changes in the cortex compared to the amygdala and these opposite changes were validated by western blot analysis.

These proteins with opposite changes in the amygdala and prefrontal cortex could be particularly interesting in the scope of the functional neuroimaging studies of suicide. Greater fMRI activity of the amygdala were demonstrated on threatening stimuli in association with serotonin transporter gene promoter polymorphism [75,76] that is known to be associated with suicidal behaviors in psychiatric patients, especially with violent suicides [77,78]. In the prefrontal cortical regions however, lower metabolism (measured by PET) was found in association with greater suicidal ideation and greater lethality in suicide attempts in depressive patients [79,79].

The protein interaction networks of the 9 proteins that changed both in the cortex and the amygdala (see Figure 3) contained a direct interaction sub-network of cytoskeletal proteins (INA, NEFL, NEFM and GFAP) which interact with binding or expression regulation. This direct interaction network of the cytoskeletal proteins is connected to the network of glutamate and serotonin receptors involved in psychotic illnesses through GRIN1 (Glutamate receptor, ionotropic, N-methyl-D-aspartate; NMDA receptor, e.g. [80]). CTSD connected to HSPA8, ACTB, CKB and TUBA1A were not directly linked to the other selected proteins. ACTB, CKB, NEFL, INA and GFAP had link to both schizophrenia and depression, while CTSD, HSPA8, NEFM and TUBA1A had link to schizophrrenia.

We regard these 9 proteins as biomarker candidates of suicide risk. Furthermore, the development of quantitative brain imaging probes based on selected proteins shows promise. Prior to developing these, however, several additional studies must be performed to confirm the identity of candidate biomarkers (e.g., in other forms of suicide and in suicide trait behaviour in animals).

Conclusion

In this study, the proteome of the prefrontal cortex changed more extensively than the amygdala of suicide victims. This result is in accordance with the fact that the prefrontal cortex is highly involved in mental disorders and suicide [81]. Because the direct interaction network of cytoskeletal proteins is changed in the brains of suicide victims, new perspectives for studying suicide-related mechanisms in receptor anchoring and ultra-structural plasticity including glia cell function have been introduced.

Supporting Information

Figure S1 The q-values were calculated from the p-values with the statistics software R (www.r-project.org; see text). The frequency distributions of P-values were used to estimate the proportion of features that are unchanging; this is then used to estimate the false discovery rate. The q-values were graphed twice for both p-value range 0.0–1.0 and 0.0–0.15. (DOC)

Figure S2 Gel image from the prefrontal cortex, GFAP containing spots are highlighted with grey colour, the spot marked with orange is GFAP isoform containing spot. See. Table S3. (TIF)

Table S1 The full list of the identified triptic peptides of GFAP by MS analysis according to spot numbers from the prefrontal cortex. Bold gene names highlighting those proteins that were found in those differently expressed protein spots that proved significant with both statistical tests. (DOC)

Table S2 The full list of the identified triptic peptides of GFAP by MS analysis detected in different spots from the prefrontal cortex. (DOC)

Table S3 The list of the identified triptic peptides of GFAP by MS analysis detected in different spots from the prefrontal cortex. (DOC)
Table S4 The list of the identified triptic peptides of GFAP by MS analysis detected in different spots from the amygdala.

Author Contributions
Conceived and designed the experiments: KAK GJ EMSZ MP BP AC. Performed the experiments: KAK GJ AS PG EMSZ EHG MP ZSD KFM. Analyzed the data: KAK GJ AS PG EMSZ EHG MP ZSD KFM. Contributed reagents/materials/analysis tools: KAK GJ AS PG EMSZ EHG MP ZSD KFM. MP BP AC. Wrote the paper: KAK GJ AC. Drafting the article: KAK GJ KFM AC. Critically revising the Manuscript: AS PG EMSZ EHG MP ZSD KFM.

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