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DATASET BRIEF

Proteomic analysis of human hippocampus shows differential protein expression in the different hippocampal subfields

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Abbreviations: LAM: Laser assisted microdissection; LC-ESI-MS/MS: Liquid chromatography electrospray ionisation tandem mass spectrometry

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In the current investigation we aimed to characterize the differential protein expression in each of the hippocampal subregions in healthy control samples (n=20). We used laser-assisted microdissection and Difference-in-Gel-Electrophoresis to enrich for these tissues and to compare protein profiles. Image analysis was carried out using Progenesis-SameSpots. Samples with a False Dicovery Rate smaller than 5%, a p-value of <0.01 and an expression of at least +/- 1.2 were considered significant.

Proteins were identified using Mass Spectrometry (Liquid chromatography electrospray ionisation tandem mass spectrometry). The raw mass spectral data was analysed using DataAnalysis software. Data was searched against the Swissprot database using MASCOT.

Samples were grouped according to the different subregions and we found 182 spots to be differentially expressed between the different hippocampal subregions. These have been made available as part of the UCD-2DPAGE database at http://proteomics-portal.ucd.ie:8082. The associated mass spectrometry data have been submitted to PRIDE (Accession numbers 21593-21745).

This baseline data will be helpful in helping us to understand the central role of the hippocampus in health and the evidence that particular hippocampal subregions are differentially affected in disease.

The hippocampus is an important brain region that has critical roles in learning and memory and is centrally implicated in e.g. ageing, stress, epilepsy, and schizophrenia. It is divided into subregions, and these differ with regards to morphology, connectivity, electrophysiology and susceptibility to insults. These different subregions have distinct roles in the anatomy of hippocampal circuitry and alterations within them are believed to contribute in a primary way to the clinical presentation [1].

The distinct subfields of the hippocampus, which comprise the corona ammonis (CA) regions 1-4 and the dentate gyrus (DG), differ anatomically, functionally [2-3], and in their vulnerability to neurological disorders [4]. The hippocampus is typically described in the context of the trisynaptic circuit, a pathway that relays information from the perforant path to the dentate gyrus, dentate to area CA3, and CA3 to area CA1. However, the trisynaptic circuit may not be the only way information is processed in hippocampus [5].

Some work has been done to create whole hippocampal protein maps, see for instance Yang and Chez et al. [6] and studies by Edgar and co-workers [7-8] for human hippocampus as well as rat and mouse maps [9-10]. Information on protein expression in the human hippocampus is limited and we therefore decided to generate a protein data set of this pivotal brain structure adding information on the different subregions of the hippocampus.

However, a limitation of all current proteomic methods, whether 2-DE or MS based, is their relatively limited dynamic range [9], which leads to an inability to resolve the complex proteome of whole tissue samples [10]. There is consequently a need to focus on subproteomes such as those containing proteins from distinguishable subregions in order to identify and quantify low abundance proteins. In this study we have extended our 2-DE-based proteomic analysis to the sub-proteome within the human hippocampus, thus complementing previous work in hippocampus [6]. No 2-DE

map is currently available which demonstrates the proteome in hippocampal subregions, whereas in rat brain a series of proteins were identified in hippocampal subareas CA1 and CA3 [11-12].

In the current study we assessed 4 hippocampal subregions separately (CA1, CA2/3, CA4, and DG see Supplementary Figure 1), using laser assisted microdissection to achieve anatomical separation (see Supplementary Table 1 and Supplementary Methods for details on samples and procedures).

The pH 4-7 proteome data has been incorporated into the UCD-2DPAGE online database available at http://proteomicsportal.ucd.ie:8082 , which contains several 2-DE maps for human brain, heart tissue and neutrophils.

Samples were processed and separated by 2D-Difference-in-Gel-Electrophoresis (2D-DIGE) as described previously by our group [13-17]. Briefly, samples were lysed in standard lysis buffer (9.5 M urea, 2% CHAPS, 0.8% Pharmalyte pH 3–10, 1% DTT, Protease Inhibitors) and the total protein content was determined using a Bio-Rad protein assay kit with BSA as standard. All solubilized protein samples were stored at −80°C until analysis. An internal standard for the study was prepared with equal fractions of human hippocampus and bulk labeled with 200 pmol Cy2 per 25 μg of protein. 25 μg of the protein from each sample was fluorescently labeled with 200 pmol of either Cy3 or Cy5. The Cy3 and a Cy5 labeled sample (25 μg each) were run alongside a Cy2 labeled internal standard (25 μg), making a total protein loading of 75 μg per gel. IPG strips, 24 cm in length and with a separation range of pH 4–7 (GE Healthcare) were passively rehydrated overnight at room temperature in 350 μL rehydration buffer (8 M urea, 0.5% w/v CHAPS, 0.2% w/v DTT, 0.2% w/v Pharmalyte pH 3–10) as described by Rabilloud et al. [18] and Sanchez et al. [18]. Subsequent to this the proteins were then focused at 0.05 mA/IPG strip for 75 kVh at 20° [19].

Strips were equilibrated in 6 M urea containing 30% v/v glycerol, 2% w/v SDS, and 0.01% w/v bromophenol blue with the addition of 1% w/v DTT for 15 min. The strips were then equilibrated in the same buffer, without DTT but with the addition of 2.5% w/v iodoacetamide for 15 min [20]. The

second dimension was carried out overnight at 1 W/gel at 15°C using a Bio-Rad Protean Plus Dodeca Cell system (Amersham Biosciences, GE Healthcare, UK) and was terminated when the dye front had migrated off the lower end of the gels.

After electrophoresis, scanning of the gels with CyDye-labeled proteins was performed on a Typhoon^{TM}9410 Image scanner (Amersham Biosciences/GE Healthcare UK). Pre-scans were performed to adjust the photomultiplier tube voltage to obtain images with a maximum intensity of 60,000 to 80,000 units. Images were cropped using ImageQuant^{TM} software (Amersham Biosciences/GE Healthcare UK). For analysis the gels were converted to digital images and we used Progenesis SameSpots® (Nonlinear Dynamics, UK; (http://www.nonlinear.com/products/progenesis/samespots/overview/) for background subtraction, spot matching across gels, data transformation and normalization. Normalized spot volume data was extracted from the Progenesis® software and log-base-10 transformed prior to analysis in order to eliminate distributional skew and to give approximate normality. Analysis of covariance (ANCOVA) was performed on the normalized spot volumes for each spot with brain region as a factor for comparison and age, PMI, RI and brain pH as covariates. Estimated differences between brain regions were obtained using linear contrasts and exponentiated to obtain fold-changes. Statistical significance was assessed using a False Discovery Rate (FDR) of 5% [21]. In addition, only protein spots with an expression level greater than +/- 1.2-fold change were considered for further investigation with Mass Spectrometry. The management of data and statistical analyses were performed using STATA version 11 and R version 9.1.

For protein identification, preparative 2-D gels (300 µg protein loading) were run as described above and fixed for a minimum of 1 h in a methanol: acetic acid:water solution (4:1:5 v/v/v). The resulting 2-D protein profiles were visualised using the PlusOne silver staining kit (Amersham

Biosciences) with slight modifications to ensure compatibility with subsequent MS analysis [22]. Spots of interest were manually excised and in-gel tryptic digestion was performed as described in the on-line Supporting Information material.

Protein extracts were separated by 2-DE and the constructed 2-DE reference map for the human hippocampus pH 4-7 subregions is shown in Figure 1. After image analysis, 182 spots with fold change > +/- 1.2 and FDR < 0.05 were clearly separated and identified on preparative 2-D gels were excised and analyzed, of which, 153 were successfully identified by MS. Details of the MS identification, including spot numbers, accession numbers, the significant subregion, theoretical Mw and pI as well as information on functional ontology have been compiled into the data set (Supplementary Table 2). The 2-DE proteome map of the human hippocampal subregions is available via the UCD 2-DE Proteome Database (http://proteomics-portal.ucd.ie:8082) using the public login. The database displays all relevant information on all identified spots including protein identification data.

Figure 1: A representative 2-D gel separation of 300 µg proteins, pH 4–7, indicating the protein spots found to be significantly altered between the different hippocampal subregions and subsequently identified with mass spectrometry. Protein identification numbers relate to the proteins listed in Supplementary Table 2.

All protein identifications were exported to Excel and analysed using the Gene Ontology database (amigo.geneontology.org/cgi_bin/amigo/slimmer). The gene ontology terms cellular components, molecular function, and biological processes were explored for all identified proteins. Identified proteins were found to be distributed across various cellular compartments including cytoplasm,

membrane, mitochondria and nucleus with the majority of identified proteins originating from the cytoplasm (70 proteins). Furthermore, proteins were also grouped according to their molecular function and biological process and are shown in Figure 2. Proteins grouped in the molecular function category were mostly involved in protein binding as well as catalytic and structural molecule activity. Other functions included transporter and enzyme regulator activity. In biological process, the proteins identified were predominantly involved in cellular and metabolic processes but also have a role in stimulus response and transport.

Figure 2: Diagram showing the assignment of Gene Ontology terms to the proteins identified in this study for each of the three categories: (A) cellular component, (B) molecular function, and (C) biological process in the human hippocampal subregions.

When the individual subregions were compared 47 of the identified proteins were regulated when comparing CA4 versus dentate gyrus, 74 were significantly changed between CA4 versus CA2/3, 66 changed between CA2/3 versus DG and finally 55 when comparing CA2/3 with CA1. Sixty-nine of these proteins were significantly differentially expressed in at least two subregions. Glial fibrillary Acidic Protein (GFAP) protein expression was markedly up-regulated in the CA2/3 region compared to both, dentate gyrus and CA4, indicating an increase in the number or size of astrocytes in these regions. This finding may have relevance to our understanding of differential cellular activity levels within the hippocampal circuitry as astrocytic function is closely coupled with oxidative phosphorylation and metabolic activity [23].

In summary, we detail here a pH 4-7 2-D reference map for the hippocampus subregions of the human brain. The data set has been deposited in PRIDE (accession numbers 21593-21745 and is available to view at the UCD 2-DE Proteome Database (http://proteomics-portal.ucd.ie:8082). It is envisaged that the data set will contribute to the further characterization of human brain. The results of our study show spatial regulation of proteins within the main subregions of the hippocampus for the first time, using proteomic methods. They are relevant for the design and interpretation of further studies at the protein level and may provide valuable information about the molecular mechanisms involved in health and disease.

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