SWATH DATA ANALYSIS OF THE MARMOSET AND HUMAN HIPPOCAMPAL SYNAPTOSOME FRACTION: EVALUATION OF TECHNICAL VARIATION IN PROTEOMIC ANALYSIS

Sigrid K. Franke, Nikhil J. Pandya, Frank Koopmans, Iryna Paliukhovich, Pim van Nierop, Ingrid H.C.H.M. Philippens, Ronald E. van Kesteren, Ka Wan Li, August B. Smit
ABSTRACT

Technical measurement variation can have a significant impact on proteomics results. Limiting this source of variation is necessary to identify subtle differences between experimental groups and conditions. Filter-aided sample preparation (FASP) combined with SWATH (Sequential Window Acquisition of all THeoretical fragment-ion spectra) is expected to reduce technical variation. Here we determined variability in the measurement of synaptic proteins from marmoset and human hippocampal synaptosomes using FASP/SWATH. The SWATH-derived data obtained from the marmoset hippocampus are discussed in particular in the context of technical variation compared with other experimental approaches. We conclude that FASP/SWATH is a method that leads to less variation measured between samples. Moreover, this method is faster and less labour intensive compared to in-gel digestion methods and subsequent mass spectrometry analysis. FAPS-SWATH is thus the preferred method for proteome analysis of samples obtained from species with inherently high biological variation, such as marmosets and humans. Comparison of the marmoset and human data further shows that hippocampal synaptosomes are very similar in terms of protein expression, supporting the idea that the common marmoset is a relevant model to study human diseases of the brain.
INTRODUCTION

The use of non-human primates as models in research on neurodegenerative diseases has many advantages ([3, 47], see also Chapters 2 and 3). Primates are more similar to humans than non-primate models, which is particularly relevant when brain anatomy, behaviour, molecular makeup are critical determinants of a successful testing of new treatment and medication. Primates at the same time offer a particular challenge in biomedical research, as test results will always show a larger inter-individual variability compared to most used laboratory animals: rodents. Inter-individual or biological variation occurs intrinsically when genetic diversity is present between individuals possibly accompanied by, or combined with, environmental factors [15, 117]. Biological variation is a given fact in the human population and likewise in outbred animal strains of non-human primates. Genetic diversity among non-human primates to some extent mimics diversity among humans. This could be considered a benefit of primate research, but at the same time it limits the power to obtain statistically significant results and precludes fast and easy translation of research findings to the clinic.

Reducing the impact of variation from biology can be achieved to a certain extent by extending group size, however, this is limited by costs involved and by ethical considerations.

In the previous Chapters we used MPTP treatment of marmosets as a primate model for Parkinson's disease (PD) (Chapters 2 and 3). Using this paradigm, proteomics analyses of the substantia nigra (SN) and putamen were performed to assess the changes in protein levels at various time points resulting from exposure to and recovery from MPTP treatment (Chapter 4). These proteomics analyses were largely inconclusive due to a lack of statistical significance, which could be contributed partly to high inter-individual variation in measured protein levels. Biological variation may be particularly high in these types of experiments because both the trigger, i.e., sensitivity to MPTP, and the response, i.e., protein expression, may be different already among individuals even under basal conditions (see Chapters 3 and 4, respectively).

Whereas biological variation is difficult to control experimentally, reproducibility in proteomics experiments can benefit significantly from minimizing technical variation, and new measurement and analysis methods are continuously being developed to this end. Technical variation arises during the procedures of measuring proteins, starting from sample preparation all the way up to mass spectrometry (MS). Typical label-free quantitative proteomic experiments are carried out using in-gel digestion (IGD) en liquid chromatography (LC) separation of peptides and MS/MS measurement in a data-dependent acquisition (DDA) mode (Figure 1, left panel) [25, 101, 179]. When isobaric labeling techniques, such as iTRAQ, are used this allows the relative quantification of protein levels between different treatment groups. More recently, several innovations for reproducible and sensitive label-free quantification of proteins have been made. The first involves simplified sample preparation, i.e., filter-aided sample preparation (FASP), allowing protein digestion into peptides using a single filter step, which reduces sample handling and is less labor intensive compared to IGD [187]. Another innovation is the use of SWATH (Sequential Window Acquisition of all Theoretical fragment-ion spectra), which in contrast to DDA allows, in a single sample injection, time-resolved fragment ion spectra for all analytes detectable within the
400–1200 m/z precursor range and the user-defined retention time window [65]. The use of a predefined spectral library is essential in order to subsequently successfully identify peptides using SWATH. The combination of FASP and SWATH is thought to increase peptide identification and reduce variation (Figure 1, right panel).

In this study we tested whether FASP/SWATH reduces technical variation in protein samples derived from marmoset brain tissue, in which biological variation is inherently high, and increases reproducibility compared with IGD/DDA-based methodologies (Chapter 4). To further validate the FASP/SWATH approach we also systematically compared variability in hippocampal synaptic protein measurements in two primate species, i.e. marmoset and human.

Figure 1: Comparison of DDA and SWATH proteomics workflows as used in this thesis. The total lysates of marmoset SN and putamen were prepared using in-gel digestion and subsequently subjected to DDA-proteomics (left side of the panel). The hippocampal synaptosome samples from marmoset and human were prepared using FASP followed by a SWATH approach (right side of the panel).
MATERIAL AND METHODS

Study design
Synaptosomes were isolated from hippocampal tissue of marmosets and humans and analysed using FASP/SWATH. Results obtained within this experiment were used for species comparison in synaptic protein expression. In addition, variation in hippocampal synaptic protein expression in the marmoset obtained here with FASP/SWATH was compared with protein expression measurements obtained by IGD/DDA proteomics of total lysate from SN and putamen of the marmoset (Chapter 4).

Origin and dissection of hippocampal tissue across species
Hippocampal tissue of 7 common marmosets (Callithrix jacchus; Biomedical Primate Research Centre, Rijswijk, The Netherlands) was isolated from brain slices taken between Bregma -2.00 and +1.50. The use of marmoset brain material was approved by the Biomedical Primate Research Centre (BPRC) ethics committee before the start of experiments, according to Dutch law. Whole human hippocampus of 6 individuals was sectioned coronally and equal parts of the hippocampus were taken using laser dissection. Sections of 20 µm were prepared from fresh frozen brain tissue and a total of approximately 50 mg of hippocampal tissue was isolated. Human post-mortem brain tissue was obtained from the Netherlands Brain Bank (NBB, Netherlands Institute for Neuroscience, Amsterdam, Netherlands). All human brain tissue was collected from donors with previous written informed consent for brain autopsy, and approval to use this tissue for research purposes has been obtained by the NBB. Tissue from the two species was collected in cooled pre-weighed Eppendorf tubes and stored at -80 °C until use.

Synaptosome isolation
Synaptosomes were isolated from hippocampal tissue as described previously [178]. In short, tissue was homogenized in 50 µl 0.85 M sucrose buffer per mg tissue. After homogenization samples were spun at 1,000 x g for 10 min and the supernatant was loaded on a sucrose gradient of 1.2-0.85 M followed by ultracentrifugation at 100,000 x g for 2 h. Synaptosomes were collected at the interface of 1.2-0.85 M sucrose, mixed with 5 ml homogenization buffer and centrifuged at 20,000 x g for 30 min to obtain the synaptosomal pellets, which were stored at -80 °C prior to the FASP procedure.

FASP in-solution digestion of proteins
Samples were digested using the FASP in-solution digestion protocol [195] with minor modifications. 10 µg of synaptosomes from each sample was incubated with 75 µl reducing agent (2% SDS, 100 mM TRIS, 1.33 mM TCEP) at 55 °C for 1 h at 900 rpm. Next, the samples were incubated with MMTS for 15 min at RT while shaking. Samples were then transferred to YM-30 filters (Microcon, Millipore, Amsterdam, the Netherlands) followed by addition of 200 µl 8 M urea in 100 mM Tris (pH 8.8). Samples were washed with this solution 5 times by spinning at 14,000 x g for 10 min each time, followed by 4 washing steps with 50 mM
NH4HCO3. Finally, the samples were incubated with 100 µl of trypsin overnight in a humidified chamber at 37 °C for 12 h. Digested peptides were eluted from the filter with 0.1% acetic acid. The samples were dried using a speedvac and stored at -20 °C until LC-MS analysis.

**SCX fractionation**

Peptides obtained from 100 µg of synaptosomes following the FASP procedure were fractionated using strong cation exchange chromatography. Peptide samples were loaded onto a 4.6 x 100 mm polysulfoethyl A column (PolyLC) and separated using a non-linear gradient of 60 min at 200 µl/min solvent A (10 mM KH2PO4, 20% acetonitrile, pH 2.9) and solvent B (solvent A containing 500 mM KCl). The first 10 min a flow of 100% solvent A was applied, from 10-35 min solvent B was increased to 65%, from 35-40 min solvent B was increased to 100%, followed by 8 min of washing with 100% solvent A. In total 40 fractions of 200 µl each were collected. Fractions were pooled as follows: 16-20 min (fraction 1), 21-22 min (fraction 2), 23-24 min (fraction 3), 25-26 min (fraction 4), 27-28 min (fraction 5), 28-38 min (Fraction 6). Fraction 6 was desalted using Oasis column (Waters Corporation, Milford, MA, USA), prior to LC-MS/MS DDA analysis.

**Data-dependent acquisition**

Peptides were analysed by micro LC MS/MS using an Ultimate 3000 LC system (Dionex, Sunnyvale, CA, USA) coupled to a TripleTOF 5600 mass spectrometer (Sciex, Framingham, MA). Peptides were trapped on a 5 mm Pepmap 100 C18 column (300 µm i.d., 5µm particle size, Dionex) and fractionated on a 200 mm Alltima C18 column (100 µm i.d., 3 µm particle size). The acetonitrile concentration in the mobile phase was increased from 5 to 18% in 88 min, to 25% at 98 min and to 40% at 108 min and to 90% at 110 min, at a flow rate of 5 µl/min. Eluted peptides were electro-sprayed into the TripleTOF MS with the micro-spray needle voltage set at 5500 V. The mass spectrometer was operated in a data-dependent mode with a single MS full scan (m/z 350−1250, 150 msec) followed by a top 25 MS/MS (m/z 200- 1800, 150 msec) in high sensitivity mode in UNIT resolution (precursor ion >150 counts/s, charge state from +2 to +5) with an exclusion time of 16 sec once the peptide was fragmented. Ions were fragmented in the collision cell using rolling collision energy, and a spread energy of 5 eV. LC-MS data measured in DDA mode was analysed using MaxQuant 1.5.2.8 [28]. An initial search using a 0.07 Da peptide mass tolerance was followed by a correction of systematic mass errors. The calibrated data was then subjected to the main search with a 0.006 Da peptide mass tolerance. The minimum peptide length was set to 7, with a maximum of two miss-cleavages allowed. Methionine oxidation and N-terminal acetylation were set as variable modifications and cysteine beta-methylthiolation as fixed modification.

MS/MS spectra were matched against the proteome using the UniProt (release April 2015) human FASTA database which includes both reviewed (Swiss-Prot) and unreviewed (TrEMBL) records, and both canonical and isoform sequences. The Biognosys iRT FASTA database was also searched in order to ensure that iRT peptides were included in the search results, as these were used to normalize retention times in downstream analysis. For both peptide and protein identification a false discovery rate of 0.01 was set. Match between runs was enabled within a 30 sec retention time window. MaxQuant computes iBAQ [7] protein abundances, an
approximation of absolute protein abundances, by dividing the total intensity of a protein by its number of digestible peptides. To accurately compare such pseudo-absolute protein abundances between experiments, the same approach was used to convert the MaxLFQ normalized protein intensities to normalized iBAQ abundances using the MaxQuant output tables.

**SWATH**

A spectral library was generated from the MaxQuant analysis of DDA for each species using Spectronaut 6.0 [14]. The Q-value threshold for peptides imported from the MaxQuant output table was set to 0.01, all other settings were default. Next, Spectronaut was used to extract peptide abundances from the raw SWATH data. The retention time prediction type was set to dynamic iRT and profiling peak refinement was enabled. Finally, cross-run normalization was performed based on total peak areas. Peptide abundances were exported as a Spectronaut report and further processed using the R language for statistical computation, in which each unique precursor was considered as a peptide (e.g., identical peptide sequences observed with multiple charges were considered distinct peptides). Based on Spectronaut’s FragmentGroup Q-values, only confidently quantified peptides and present in both species were considered.

The spectral library contains the mapping of all peptides to their respective protein groups. The protein abundance matrix was built by summation of the normalized peak area of all peptides that belong to protein groups with the same gene name. Analogous to the DDA analysis, ambiguous protein groups that map to multiple indistinguishable gene names are disregarded. Finally, protein abundances were Loess normalized using the ‘normalizeCyclicLoess’ function from the limma R package, method was set to ‘fast’ and iterations was set to 10.

**Statistical analysis**

Data was analysed using the R language for statistical computation (version 3.0.2). The reproducibility of proteomics studies was evaluated using the distribution of Coefficients of Variation (CoV) within biological replicates. Similarities between samples were quantified using hierarchical clustering. Hierarchical clustering by complete linkage was applied to the Euclidean distance matrix of the log10 transformed protein abundances. The R package pvclust was used to compute edge confidences for the cluster dendrogram using multiscale bootstrap resampling in 1,000 iterations. We used the Approximately Unbiased p-values reported by pvclust (as percentages) as the likelihood that a sample is in a separate cluster.

Outliers in the SWATH data within species were determined by cluster distances between all replicates. Differential abundance analysis between species was preceded by Loess normalization on the respective subset of the data matrix. Empirical Bayes moderated t-statistics were used with multiple testing correction by False Discovery Rate (FDR), as implemented by the eBayes and topTable functions from the ‘limma’ R package. An FDR-adjusted p-value threshold of 0.005 was used to discriminate proteins of interest after differential abundance analysis.
RESULTS

Species comparison

Hippocampal tissue was dissected from marmoset and human. Species samples were age-matched and all individuals were in the adult phase of their respectively lives (Table 1). Synaptosomal fractions were analysed using FASP/SWATH. The total number of individual identified proteins found was 2465 and 2726 for marmoset and human, respectively. From these, 1716 proteins were confidently detected in both species and selected for further analysis.

There were 6 biological replicates for human and 7 for marmoset. The ‘human 1’ sample, yielded only hundreds of peptides after SWATH analysis whereas we found thousands of peptides in all other samples; this persisted after re-measuring the same sample. This sample was discarded from further analysis. Sample ‘marmoset 13’ was an outlier when compared to the other biological replicates within their respective species with 99% and 100% confidence respectively according to the Approximately Unbiased p-values from sample clustering. Removal of the outliers yielded 6 marmoset and 5 human biological replicate samples in the final dataset.

When clustering samples on protein abundance, a clear separation became apparent of species, whereas within species samples were very similar, as indicated by the approximately unbiased (AU) probability values and bootstrap probability (BP) values (Figure 2). Species are separated with BP and AU values of 100%, indicating that based on protein expression profiles samples are assigned to species with absolute certainty.

We then analysed the variation among protein samples of the same species. The CoV of the abundance values of each of the 1716 selected proteins was calculated and plotted as boxplots. Human CoV values were smaller (0.09), than of marmoset tissue, which showed significantly more variation (0.12) (Figure 3).

Subsequently, the correlation in protein expression was calculated between individual marmoset and human sample (Figure 4, page 80). Samples of same species showed a higher correlation within species than between species, in agreement with hierarchical cluster analysis (cf. Figure 2). All samples showed a significant correlation to each other ($r^2$ for marmoset-marmoset = 0.959, $r^2$ for human-human = 0.969 and $r^2$ marmoset-human = 0.782, p<0.001 for all), which is in agreement with the cluster analysis and correlation plots.

We next examined the degree of differential protein expression between species. We were able to detect different levels in roughly half of the proteins when comparing these two species (Figure 5, page 81). 415 proteins were higher expressed in marmosets and 422 proteins were found lower expressed in marmosets compared to humans.

Table 1: Data on animal numbers used, ages, and proteins detected by mass spectrometry.

<table>
<thead>
<tr>
<th>Species</th>
<th>Age (in years)</th>
<th>Unique proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marmoset (n=6)</td>
<td>4.2 ± 0.7</td>
<td>2465</td>
</tr>
<tr>
<td>Human (n=5)</td>
<td>53.1 ± 3.7</td>
<td>2726</td>
</tr>
</tbody>
</table>
Figure 2: Hierarchical clustering of synaptosome samples across species based on protein expression values. Samples were hierarchically clustered using euclidian distance matrix analysis based on the protein expression values. Indicated are the Approximately Unbiased (AU) probability values (red), and Bootstrap Probability (BP) values (green). Samples cluster according to the species from which they were derived.

Figure 3: Samples were hierarchically clustered using euclidian distance matrix analysis based on the protein expression values. CoVs of protein abundance within species of all 1716 selected proteins are plotted as boxplots. Data on marmoset show a larger CoV than human data.
Figure 4: Correlation in protein expression between individual marmoset and human samples. All samples were pairwise compared and correlation coefficients ($R^2$) are indicated. Protein expression is represented by log10 transformed LFQ values on the x- and y-axis.
Protein expression measured using FASP/SWATH of hippocampal synaptosomes was compared with data obtained in Chapter 4 on total cell homogenate of SN and putamen tissue using IGD/DDA. It is important to note that we made this comparison with the notion that both sets of sample preparation are quite different from each other. This comparison is discussed below. The total number of individually measured proteins was in the same order of magnitude between the brain areas (SN: 3491, hippocampus: 2465, putamen: 2222).

Protein expression measured in the synaptosomal hippocampal fraction measured with FASP/SWATH showed significantly less variation compared to measurements in total lysate of the SN and putamen measured after in-gel digestion and LC-MS/MS in a DDA-mode (Figure 6) (p<0.001, for both comparisons). Additionally, the variation measured in the putamen was also found to be larger than the SN (p<0.001).

**Figure 5: Volcano plot showing differentially expressed proteins between two primates in a synaptosome preparation.** Indicated were individual proteins that are significantly higher expressed in humans (red upward triangles), and proteins that were significantly higher expressed in marmosets (blue downward triangles).
The current study was aimed at assessing variability in protein expression measurements when using different proteomics analysis methods (i.e., technical variation) and between different individuals or species (i.e., biological variation). SWATH analysis of the synaptosomal fraction showed that human and marmoset (both primates) are closely linked. We were able to confidently detect proteins due to the fact that protein sequences overlap greatly and homology between human and marmoset is approximately 96 % in their amino acid sequences (as shown in Chapter 4 and by others [88, 192]).

Interestingly, we detected many proteins that have a statistical differential expression between marmoset and human. It will be of importance to confirm differential expression of some of these proteins that are differentially expressed between species in an independent experiment. This would validate the approach.

**Figure 6: Variation in protein levels in three different brain areas.** Synaptosomal fraction of marmoset hippocampus was measured using FASP/SWATH, and total lysate of putamen and SN were measured using IGD/DDA (see text for explanation).
Importantly, we found that variation in protein expression measured using FASP/SWATH within different species is lower compared to IGD/DDA analysis and to data obtained by others [110, 115]. However, marmoset samples did show more variation than human samples. This suggests that some of the observed variation in the marmoset samples still is of technical origin. Possibly, differences in tissue collection methods may account for this variation, as marmoset tissue was manual macro dissected whereas laser dissection was used to isolate human brain tissue. The total number of synaptic proteins identified was similar between species.

Comparing FASP/SWATH to IGD/DDA derived proteomics, significantly more variation for the IGD/DDA derived data set was observed, whereas similar numbers of proteins were measured. A complicating factor in this comparison is that besides the different methods of proteomics used, different cellular fractions were used for the comparative analysis. However, it seems unlikely that a cell fractions that involves significantly more experimental steps to prepare, i.e., the synaptosome sucrose gradient preparation, would yield less variation in terms of detection technology, if not the FASP/SWATH analysis itself would be superior. In addition, a sample measured with FASP/SWATH is performed in a single MS-run, as opposed to a sample that is sliced in 6 or 12 slices, and that needs 6 or 12 MS-runs per sample using IGD/DDA. Nevertheless, the IGD/DDA analysis as described in Chapter 4, also seems to suffer from protein expression variability that is not related to the method but might be due to in-between sample dissection differences.

It is important to mention that a direct analysis of a total lysate fraction using FASP/SWATH is not yet possible, because the high protein diversity will meet the limitations of the capacity of the mass spectrometer. This is not only the case when measuring proteins with SWATH, also DDA-analysis can only identify a limited number of spectra depending on the type of mass spectrometer used. Steps to reduce complexity should therefore be included. Alternatively, to complete insight in other cellular compartments such as mitochondria, membranes and myelin fractions, it would be a possibility to isolate these separately using a sucrose gradient and subject the fractions to MS analysis separately. It is important to realize though that more separation steps will lead to more variation. Combining in-gel digestion or more extended column fractionation with SWATH analysis might reduce sample complexity without increasing the variation to the same extent as observed with IGD/DDA.

Methods for proteome analysis are continuously being optimized and further developed to reduce variation and thereby increase statistical power. FASP/SWATH reduces the variation compared to IDG-DDA and is most certainly a suitable method for identification of subtle differences in protein expression compared to IGD/DDA, when small effect sizes are to be expected. Current data encourages measuring subcellular fractions of tissue using FASP/SWATH at minimal variation. We show here that the basal levels of marmoset protein expression in the hippocampus can be measured accurately between samples. Moreover, FASP/SWATH methods are also less labor-intensive, need less tissue presented to the MS, and use less total time for measurement of the mass spectrometer. These improvements may be of use in future studies in marmoset as there is still much insight to gain in the molecular pathogenesis of PD.