Advances in neuromembrane proteomics: efforts towards a comprehensive analysis of membrane proteins in the brain

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Abstract
Proteomic investigation of normal and diseased brain states has the potential to reveal novel molecular therapeutic and diagnostic targets for a multitude of pathological central nervous system conditions. Due to their unique properties, integral membrane proteins are likely to play a central role in the aetiology of these disorders. These properties, however, have prevented comprehensive analysis of this important class of proteins. Recent advances in sample preparation and proteomic quantification platforms, specifically focused on recovery and enrichment of integral membrane proteins, are discussed.

Keywords: membrane proteins; mass spectrometry; neuroproteomics; proteomics; brain; quantification; sample preparation

The brain is an extraordinary multifaceted organ, controlling nearly every function of the body and consisting of hundreds or even thousands of different cell types [1]. With such amazing complexity, it is no surprise that there are over 1000 different disorders that produce dysfunction in the nervous system. Recent applications of proteomics to investigate the normal and diseased states of the brain could provide indispensable insights into the aetiology of these conditions and aid in the development of new therapeutics and diagnostics. In fact, the Human Proteome Organization (HUPO) has recently launched an initiative to characterize the brain proteome under normal and diseased conditions [2, 3].

Due to their unique properties and cellular functions, it is likely that proteins with functional domains exposed on the extra-cellular surface play central roles in the cause and treatment of these various disorders. Even though technological advancements have propelled discoveries in neuroproteomics, comprehensive analysis of membrane embedded proteins remains an elusive challenge. Integral membrane proteins (IMPs) represent a class of proteins which are hydrophobic in nature and comprise up to 30% of all proteins in any given genome [4]. Present at the cell surface, they play essential roles in signal transduction, transport of solutes, cell motility and anchoring, as well as comprise more than two-thirds of all pharmaceutical drug targets [5].

Systematic and quantitative analysis of these biologically important proteins has remained intangible due to great difficulty in the sample preparation process, owing primarily to limited solubility in water, but also to their low abundance, making enrichment strategies a required part of any means of sample preparation. The impact of such analyses would have far reaching consequences, advancing areas such as immunology, oncology and pharmacology, as well as opening doors for deeper investigation into diseases and disorders such as anxiety, depression, Parkinson’s disease, Alzheimer’s disease and cancer.
This review will focus on recent advancements in sample preparation and quantification methods which are directed at membrane proteins in the brain, and discuss current platforms for analysis.

CURRENT PLATFORMS FOR PROTEOMIC ID OF MEMBRANE PROTEINS

Gel-based methods

Two-dimensional gel electrophoresis (2-DE)

Traditional proteomic methods have relied on the use of 2-DE, in combination with mass spectrometry (MS), for resolving and identifying proteins in a complex mixture, which has been the method of choice for gel-based techniques [6, 7]. Using this method, proteins are separated in the first dimension according to their isoelectric point (pI) through isoelectric focusing (IEF), which can be accomplished using carrier-ampholytes or immobilized pH-gradients (IPGs) [7]. Following IEF, SDS-PAGE is conducted and proteins are separated according to molecular weight in the second dimension. Protein spots are then visualized by applying stains (Coomassie or silver stains are common) or fluorescent dyes. When comparing two conditions, differences in stain intensity can be evaluated across gels for the same spot and quantified using densitometry. Further analysis can be conducted by excising spots of interest, proteolytically digesting (in-gel, usually with trypsin), extracting peptides from the gel, and subjecting to MS for identification of the protein(s) (and/or post-translational modifications) contained within the spot [7, 8].

The resolving power of 2-DE makes the technique a desirable one to use; several hundred to 1000 proteins are routinely identified per gel with the upper limit approaching 10,000 individual spots per gel [1]. However, there are several decidedly negative drawbacks with the use of this platform that must be considered, especially in neuroproteomic studies. First, resolution of membrane proteins is limited using this approach because most brain integral membrane proteins (bIMPs) of interest are considerably less abundant than average neuronal housekeeping proteins, and tend to be obscured by these more abundant proteins when run on a gel. Additionally, since resolution with IEF fractionation combined with PAGE is ideally suited for proteins with acidic to weakly basic pIs, most bIMPs would not fall among the ideal candidates for this type of analysis because most membrane proteins have pI which are decidedly alkaline, typically >8.0 [9]. Second, most of these hydrophobic membrane proteins are very difficult to solubilize in the traditional buffers used for first dimension IEF, and even many that can be solubilized have a tendency to precipitate out of solution at their pI – making entry into the gel during the second dimension impossible [10].

Even with the aforementioned caveats, this platform shows future promise for neuroproteomic profiling and quantitation as many improvements continue to be made. New surfactants, buffers, and narrow-range IPGs have been made to more appropriately address hydrophobic proteins and their resolution and solubility issues [11, 12]. Investigators such as Myung and Lubec, have taken advantage of these new tools to make advances in the neuroproteomics field. Using whole mouse brain homogenates, these researchers were able to resolve 2673 spots corresponding to 581 proteins, of which 48 were predicted to have transmembrane domains. This was achieved by first pre-fractionating homogenates using microscale solution IEF (in the form of the ZOOM-IEF Fractionator, Invitrogen), prior to standard 2-DE, followed by digestion with trypsin, and protein identification via MALDI-MS [13]. Although the results reported in this article were quite an achievement in extending the amount of brain proteins usually found through this type of analysis, it is certainly not all-inclusive. Because of the low percentage of bIMPs that are typically identified using 2-DE, even with technological improvements and biochemical enrichments, many investigators are choosing to use gel-free methods, which, when coupled with appropriate enrichment strategies, greatly improve the proportion of bIMPs that can be identified through MS analysis.

Gel-free methods

Shotgun proteomics/multidimensional protein identification technology (MudPIT)

In recent years, there has been a surge in the number of laboratories using shotgun proteomics, or MudPIT, as the primary MS-based method for global characterization of proteomes (Figure 1). Using this platform, complex protein samples are first digested, using protease(s), to generate an even more complex peptide mixture. Following digestion, the peptide mixture is loaded onto a pulled microcapillary liquid chromatography (LC) column,
which is typically loaded with two chromatography materials that separate based on orthogonal physical properties such as charge and hydrophobicity [14]. This biphasic column is then placed in-line with the mass spectrometer, and peptides are displaced from the chromatography material using varying buffer conditions (increasing salt concentrations and organic mobile phases).

Because a peptide mixture is generated prior to chromatographic separation, the MudPIT platform is largely unbiased, meaning that proteins from all subcellular fractions, including those with high or low pI, molecular weight, abundance and hydrophobicity are identified. This indicates that, unlike the 2-DE method, IMPs can be identified with the same sensitivity as any other protein [15]. Although this strategy offers significant advantages over the 2-DE method, there are shortcomings, including limited dynamic range, and problematic informatics issues due to extreme complexity of digested samples [16]. One of the major disadvantages with the use of this platform for identification of IMPs results from low recovery of peptides representing the transmembrane domains [17]. Because of the low number of Arg and Lys residues located within membrane spanning domains, trypsin (commonly used for enzymatic digestion of proteins) cannot cleave them into smaller peptides, leaving

Figure 1: Schematic representation of MudPIT.
large, hydrophobic peptides which are very difficult to elute from reverse phase materials using standard gradients, and, due to their size, are also difficult to identify using ion trap mass spectrometers commonly used for shotgun proteomics; thus, many protein IDs of membrane proteins are represented by single peptide recoveries [17, 18].

Still, using the current shotgun platform, in conjunction with up-front sample fractionation (such as differential centrifugation and sucrose density gradients) in order to reduce complexity, has the ability to generate thousands of unambiguous protein IDs—with hundreds containing at least one transmembrane domain. In fact, Wang et al. [19] recently reported the most comprehensive analysis of mouse whole brain to date. Using an enrichment strategy for cysteinyl containing peptides in addition to a standard global enzymatic digestion method up front, whole mouse brain homogenates were subjected to LC-MS/MS. The resulting tandem mass spectra were analysed using the Mouse International Protein Index Database, and these efforts resulted in an astounding number of non-redundant protein IDs. In all, 7792 brain proteins were identified with 1447 (~18%) predicted to have at least one transmembrane domain by the TMHMM algorithm [19]. The results of this analysis represent over a 13-fold increase in the number of proteins identified and a 30-fold increase in the number of bIMPs versus the numbers reported using the aforementioned 2-DE platform.

From the results of this analysis, it is clear that large steps toward a comprehensive analysis of the brain proteome are being made. However, in order to identify and account for more potential drug targets, mainly the bIMPs, enrichment strategies will need to be employed in order to overcome the dynamic range problem encountered when attempting to analyse such complex samples with such low-abundance membrane proteins. Additionally, in order to determine diagnostic predictors of pathology, it is necessary to provide relative or absolute quantitative comparisons of proteins. The following sections will cover recent advances in enrichment strategies of bIMPs, as well as recent ingenuity in quantitative platforms which can be coupled to these strategies, providing more useful and powerful data.

Sample preparation methods
Incumbent upon either of the above mentioned platforms is the necessity for sample preparation techniques which effectively solubilize the brain membrane proteins. Although shotgun methods are largely unbiased during LC separation and MS detection, the challenge of membrane protein solubility remains. In 2003, Wu and Yates, [6] reviewed four methods for addressing solubility, which included the use of organic solvents, organic acids, detergents, and membrane fractionation at high pH. While this review did not specifically address the membrane proteins found in brain tissue, any of these solubilization strategies can be adapted for sample preparation in neuroproteomics studies; however, there are few if any publications which have taken advantage of solubilization with organic solvents and organic acids applied to brain. In fact, most have relied on tried and true detergent methods with only a few labs utilizing the high pH method [20]. For example, Peng et al. [21] routinely used detergents such as SDS and Triton X-100 during preparation of synaptosomal and post-synaptic density (PSD) proteins obtained from rat forebrain, while Nielsen et al. [22] utilize high pH Na₂CO₃ buffer as part of their mouse cortex and hippocampus sample preparation.

In addition to solubilization strategies, sample fractionation can dramatically increase the percentage of bIMPs identified via either platform (Figure 1). Taking into account the average number of splice variants (10 on average), coding genes, and PTMs, there is the possibility for over 250 000 different protein forms within the CNS [23], which also means that approximately 30% or 75 000 of these protein forms will be bIMPs. Currently, however, neither of the aforementioned proteomic platforms can identify this extensive number of proteins nor can they deal with the extraordinary complexity and dynamic range of such samples. As a way to overcome this hurdle, sample fractionation techniques have been shown by many groups to enrich for specific subcellular components within the brain, thereby decreasing the dynamic range problem that is often encountered when attempting to analyse and identify low-abundance membrane proteins. For example, in the previously mentioned Peng et al. [21] study, sucrose density gradients were used to first enrich for synaptosomes which were then collected and further separated and purified to yield a PSD-rich fraction for MS analysis. Several protocols have been optimized for enrichment of specific brain components, such as for the enrichment of membranous synaptosome structures.
or for the PSD-enriched fractions [21, 24]. In the global analysis of mouse brain conducted by Wang et al. [19] (see earlier text), no sample fractionation was conducted and this resulted in identification of only 18% bIMPs. Additionally, by combining solubilization strategies and meticulous subcellular fractionation, some laboratories have been able to achieve remarkable enrichment of bIMPs. Nielsen et al. [22] employed frozen mouse cortex and developed a method which not only utilized high pH Na2CO3 buffer but also included high salt, 4M urea, digitonin and subcellular fractionation via Percoll density gradient. This new method proved quite useful for this group, and resulted in the identification of over 60% bIMPs [23] and, although the number of proteins containing at least one transmembrane domain was not reported, this enrichment and identification of bIMP proteins is a notable achievement. It should be noted however that reproducibility remains an important part of any method and the more extensive the sample preparation process, the harder it will be for other groups to replicate. In a recent publication, Bihan and colleagues [25] employed the Nielsen et al. [22] method for use in differential analysis of membrane proteins in mouse fore- and hind brain, but only reported obtaining coverage of 38% bIMPs containing at least one transmembrane domain. The same instrumentation was used during analysis of protein fractions, but, because of the substantial steps involved during the sample preparation process, a significant number of protein identifications may have been lost due to mere differences in the personnel performing the initial steps of the experiment.

As research in neuroproteomics continues it will be important to explore the methods that have been used to investigate other tissues and apply them to brain, as well as to develop new, robust techniques for enrichment and subsequent analysis of bIMPs. The reproducibility of the sample preparation, enrichment methods, and all of the tissue manipulation that is conducted prior to analysis by MS becomes even more important when considering quantitation of the bIMPs identified in the various brain fractions that are generated. While identification alone of different proteins and PTMs is useful in many circumstances, the ability to quantify thousands of proteins between different samples and conditions takes proteomic data from useful to powerful by allowing insight into functionality. In fact, RNA levels, while informative, do not provide a comprehensive readout of phenotypic state because they do not take into account post-translational processing or protein turnover rates, and new evidence suggests that many essential proteins, as well as transcription factors, are present in levels other than what their mRNA suggest [26]. In light of this discrepancy, it will become increasingly important in the future to complement global quantitative microarray studies with corresponding proteomic analysis. The last section of this review will cover established and emerging quantitation platforms which can be applied to the field of neuroproteomics, especially those that are particularly suited for quantitation of bIMPs.

**Current quantitation platforms in neuroproteomics**

**DIGE: 2D-Difference gel electrophoresis**

DIGE is a technique which involves differential labelling of complex protein samples with amine-reactive cyanine fluorescent dyes prior to electrophoresis. Up to three labelled samples (one usually a control) can be loaded onto the same gel in equal protein amounts, after which 2D-PAGE is conducted. Determination of changes in relative abundance of proteins between samples is determined by a software which calculates changes in intensity of the different dyes [27]. Spots which indicate differences in intensity can then be cut from the gel and protein(s) identified using the methods described in earlier text under 2-DE. Because three samples can be loaded and compared on one gel, there is an increase in accuracy compared to quantitation using traditional 2-DE techniques, which can be confusing due to spot matching across different gels. Additionally, the new fluorescent dyes used in this technique provide an increase in dynamic range over stains traditionally used for 2-DE, such as silver stain. However, the same caveats apply to the use of this method for quantification that applied for the use of 2-DE in regard to the identification of bIMPs. Other non-gel MS-based quantification techniques, such as those which utilize labelling with stable isotopes, are much less biased against hydrophobic, alkaline membrane proteins. This is not to say that this technique should not be used for quantification in neuroproteomics; instead, it should prove very beneficial when used in conjunction with stable isotope platforms,
as a complementary approach for global neuroproteome analysis.

**Use of stable isotopes in quantification**

Several non-gel-based quantitative proteomic platforms have been developed over the past few years which centre around the use of stable isotopes, and which are gradually becoming routine methods for quantification in the neuroproteomics field (Figure 2) [28]. These stable isotope techniques are most often used in conjunction with shotgun proteomics, which results in increased proteomic coverage compared to the DIGE technique, improved identification, and therefore quantification, of bIMPs.

The first of these platforms, which utilizes isotope coded affinity tags (ICAT), was developed by Gygi and Aebersold in 1999 [29]. These reagents typically consist of three distinct modules, with the first consisting of a thiol reactive group, which selectively couples to the side chain of reduced cysteinyl residues, a second module which contains either an isotopically ‘heavy’ (usually either deuterium or $^{13}$C) or ‘light’ (isotopically normal) ethylene glycol linker, and the third module which is a biotin moiety that allows the user to enrich for labelled peptides by avidin affinity chromatography [30]. In a typical protocol, two protein samples of different biological condition or state are first denatured and cysteines reduced. Isotopically heavy ICAT reagents are then added to one sample and isotopically light ICAT reagents are added to the other sample. Following covalent modification, the heavy and light labelled samples are mixed together, digested with protease, and run over a cation exchange column to remove excess ICAT reagent. The purified mixed sample is then run over an avidin column to isolate ICAT containing peptides, after which MS analysis is conducted. The relative abundance of peptides can be determined by the ratio of the signal intensity of the heavy and light forms of the individual peptides. The sequence of the peptide is determined from its tandem mass spectrum and correlated to the protein from which it originated via software tools; the

![Stable isotopes in quantification](image)

**Figure 2:** Overview of two primary quantification platforms utilizing stable isotopes.
relative abundance of the parent protein is assumed to be reflective of the relative abundance of the identified labelled peptide [31]. This approach offers a distinctive advantage over other quantitative methods in that it reduces sample complexity by affinity isolation of peptides containing the biotin moiety, which can enhance identification of low-abundance peptides. Additionally, by using ICAT reagents coupled to MudPIT, a bias should not exist against membrane embedded proteins, so more bIMPs should be able to be identified and quantified. However, ICAT reagents selectively label only cysteine-containing peptides, so those proteins which do not contain a cysteine residue (or even multiple cysteine residues) in their sequence will be underrepresented using this type of analysis. Moreover, samples are denatured, reduced and labelled separately, so differential efficiency in any of these three events could lead to the introduction of systematic errors in quantification.

When conducting quantitative comparisons using MS, it is most optimal to include an internal standard for every protein that is to be quantified. However, when conducting global proteomic comparisons, it is impossible to know all of the internal standards that must be included. One approach is to utilize the method described earlier in this article, which employs the use of ‘heavy’ and ‘light’ chemical derivatization tags, which can be of great use to complete a comprehensive proteomic analysis of the brain without bias against hydrophobic membrane proteins. However, not all protein residues are labelled, and thus proteins which do not contain the residue to be labelled cannot be quantified. As an alternative, an in vivo metabolic labelling approach can be employed which minimizes the drawbacks of in vitro tagging. In the first of the two metabolic labelling techniques that will be discussed, cells in culture are grown in media that contains an essential amino acid which is labelled with a stable isotope (based on SILAC [32]); this allows all newly synthesized proteins to contain a heavy isotopic label, and within 5 or 6 cell doublings all proteins within a culture should contain the heavy isotope labelled residue. These labelled cells can then be combined with an unlabelled sample (e.g. tissue or cells), and protein fractionation, proteolytic digestion, MS analysis and quantification conducted as described above for the ICAT reagents. Ishihama et al. [33] recently used this technique to identify and quantify 1000 proteins from mouse whole brain. In their approach, Neuro2A cells were grown in 13C-labelled leucine-rich medium to create an internal standard for subsequent quantitative analysis of whole mouse brain. Equal protein amounts of extract from labelled Neuro2A cells and homogenized whole mouse brain were mixed, digested with trypsin and analysed by LC-MS/MS. Using this metabolic labelling method, these researchers found that there was 98% protein overlap between the Neuro2A cells and the 1000 proteins identified in whole mouse brain. This high percentage of overlap between their labelled cell and unlabelled whole brain samples is quite surprising given the high diversity of cell types located within the brain [33]. In addition, this group conducted a comparison of their metabolic labelling strategy with the ICAT method by using the same amount of brain extract, and found that their original approach was more efficient at identifying and quantifying proteins. Results of this comparison showed that they were able to quantify 602 brain proteins using the metabolic labelling approach while only 339 proteins were quantified using ICAT. In addition to not being able to quantify peptides which did not contain cysteines, these researchers also speculated that inefficiency of the ICAT approach could be due to steps which are required to attach the chemical tags, remove excess reagents, as well as in difficulty identifying complicated MS/MS spectra resulting from the added ICAT tag [33]. Although they did not list the percentage of bIMPs identified, these researchers clearly showed that, by using a metabolic labelling platform, they were able to conduct a more comprehensive analysis in which they were able to identify and quantify more brain proteins, with less bias.

As can be seen from the aforementioned analysis, metabolic labelling offers a noticeable advantage over other quantification techniques, such that all proteins in an internal standard can be labelled and used to quantify corresponding unlabelled proteins in a biological sample. One of the major drawbacks to using cells in culture as an internal standard for quantification of proteins in tissue is that, even if the correct cell line is chosen for an internal standard, not all proteins will be represented in both samples, and thus quantification will not be possible. In fact, with brain being the most complex organ in the body, containing thousands of different cell types, it is unlikely that one or even several cell lines will contain all of the proteins located within such a
remarkably intricate tissue. An alternative quantitative analysis would be conducted by comparing an internal standard and tissue that are not only similar, but identical. This was the logic behind the study conducted by Wu et al. [34], in which rats were metabolically labelled using $^{15}$N enriched diet. Rats were metabolically labelled for 44 days by feeding them a diet which consisted of 2 parts protein-free rodent diet and 1 part algae ($Spindula platensis$) enriched with >99% atomic percent excess $^{15}$N. No visible abnormalities were shown as a result of feeding a diet enriched in the stable nitrogen isotope. Amino acid enrichment in each of the selected tissues was determined by hydrolysing proteins extracted from the tissues and measuring $^{15}$N amino acid enrichment by GC/MS; results varied from 92.2 mole percent excess (mpe) in plasma to 74.3 mpe in brain. This difference in enrichment, as described in the publication, is likely due to slower protein turnover in some tissues. Ultimately, relative quantification of proteins in liver tissue was conducted by combining equal amounts of labelled and unlabelled rat liver post-nuclear supernatant (PNS), digesting with proteinase K in high pH buffer (to avoid bias against membrane proteins), and MudPIT conducted; relative abundance ratios were determined using the program RelEx [35]. Quantification and determination of relative abundance ratios in brain tissue was not conducted and would have been difficult for several reasons involving the low enrichment of the tissue. As enrichment of individual isotopically labelled residues decreases, the isotope distribution of a given peptide widens and shifts to lower m/z, which might cause a decrease in precision. In addition, peptides with broader isotope distributions tend to have lower signal to noise ratios, again complicating identification and quantification. However, it is useful to note at this point that MacCoss et al. [35] have shown that, by summing an entire isotope distribution window, quantitative peptide ion current ratios will be unaffected by the enrichment of the material used to label the internal standard. Thus, (i) as long as the atomic enrichment of the labelled atoms is known, (ii) the protein has completely equilibrated with the enriched atoms from which it was synthesized and (iii) the labeled and unlabelled isotope distributions do not overlap, incompletely labelled internal standard can be used to make quantitative comparisons. Finally, metabolic labelling of mammalian brain tissue shows incredible promise for use in the field of neuroproteomics and while it is not possible to isotopically label human brain, it is possible that longer periods of labelling applied to rodent brain would generate higher enrichment of stable isotope, allowing for easier identification and quantification of low-abundant brain proteins, such as bIMPs, when used in conjunction with a non-biased MS method.

**Label-free quantification techniques**

Although isotopic labelling platforms have proved to be sensitive enough to measure changes in relative abundance ratios significant to ~1.5-fold, it would be advantageous to have an alternative quantitative technique which would allow the user to be able to quantify samples when metabolic labelling or chemical derivatization is impossible or cost prohibitive [36]. Recently, there has been a resurgence of activity in the development of ‘label-free’ and differential display platforms which are capable of quantification of proteomic MS data without the use of isotopes or tags of any kind.

The first of these methods that will be discussed is conceptually simpler than the other and has been termed ‘spectrum counting’. The inception of this technique came about due to the seemingly simple observation that in a MudPIT analysis of a complex protein mixture, proteins having higher abundance will be identified by more peptides than will proteins with low abundance [37]. Since then, several groups have shown that the total number of peptide identifications in a MudPIT analysis of a complex mixture is linearly correlated with the abundance of the proteins [37, 38].

Spectrum counting is usually conducted by summing all MS/MS observations of any peptide for a given protein, including redundant spectra resulting from different charge states. The advantages of this method for quantification of redundant spectra over isotopic labelling are fairly straightforward; no labelling or tagging of any kind is necessary, thereby reducing the cost of the analysis, and multiple conditions can be compared to each other. Additionally, because the number of samples is essentially infinite, good statistical analyses can be attained. This is in contrast to techniques like isotopic labelling, which are restricted to pair-wise comparisons. Zybailov et al. [37] recently conducted a study comparing quantification using spectrum counting and quantification using relative abundance ratios generated from peptide ion chromatograms following stable isotope labelling. Complex samples,
which included membrane proteins, were generated from *S. cerevisiae* followed by MudPIT analysis [37]. This study showed that spectrum counting has a broader dynamic range and may be more reproducible than stable isotope labelling and quantification of extracted ion chromatograms [37]. However, spectrum counting may not be as accurate or precise as measuring peak area intensities, and is complicated by identification of peptides which are shared between different database entries containing overlapping sequences, such as for protein isoforms [36]. Additionally, the performance of spectrum counting depends strongly on the depth of MS/MS sampling, because ratios are most significant for proteins with large numbers of spectra, meaning that the ratios estimated can be significantly suppressed when the minimum spectral count is zero and the maximum count is limited by sampling due to low protein abundance [36]. This would clearly need to be a consideration when attempting to quantify bIMPs.

Another label-free method, differential MS, recently described by Weiner *et al.* [39], may offer greater sensitivity than spectral counting, and also does not require the use of isotopic labels or chemical tags. The differential MS quantification platform is fully automated and utilizes LC-MS chromatography to compare and quantify proteins in different samples. The approach described by Weiner’s group utilizes software to directly compare intensity at each *m/z* ratio and return a ranked list of differences between samples. Multiple LC-MS runs are conducted, and chromatography profiles averaged in order to account for variability of intensity measurements; this allows the detection of small but statistically significant differences in low-abundance peptides [39]. A key requirement of accurate quantification using this platform is reproducible chromatography, however, it is virtually impossible to eliminate small shifts in retention time, no matter how reproducible the chromatography. In order to account for these differences in retention time, an alignment algorithm is applied which can correct for peak shifts of up to 1.5 min (Figure 3). Meng *et al.* [40] recently applied this method to quantify peptides that were spiked into two rat plasma samples at different concentrations. Results of this investigation showed that this group was able to detect minimum fold changes of ~1.5 with an error of <20% [40]. The benefits of performing this type of quantitative analysis are similar to that of spectral counting in that multiple samples can be compared—not just two states or conditions, no costly isotopes or chemical tags need to be used, and good statistical analysis can easily be conducted. However, the vital requirement for both techniques is the reproducibility of not only the LC, but of the sample preparation as well. Both of the label-free platforms show tremendous promise toward the application of quantification of bIMPs, but taking into account the caveats of these methods is important when considering their practical use.

**CONCLUSIONS**

The field of neuroproteomics is rapidly growing and techniques used in other proteomic endeavours
should and can be adapted to identify more brain membrane proteins. None of the methods and platforms discussed in this article has the ability to provide a comprehensive global proteomic analysis of bIMPs; however, each of them can provide a complementary analysis that will lead the field in the right direction. Accurate reproducibility remains a key component for all of the platforms and quantitative methods discussed, and the ability to quantify low-abundance proteins, such bIMPs, within a complex sample will be necessary in order for a comprehensive analysis to be conducted. Concentrated efforts should be made to advance sample preparation methods, which have not seemed to keep pace with technological advancements, to increase solubility and separation of bIMPs, so that bias against identification and quantification of this very important class of proteins can be diminished.

Key Points
- Integral membrane proteins (IMPs) comprise up to 30% of all proteins in any given genome and represent more than two-thirds of all pharmaceutical drug targets.
- Sample complexity and solubility must be addressed during sample preparation for optimal identification of IMPs during proteomic analysis.
- Accurate reproducibility of sample preparation and tissue manipulation is crucial for identification and quantification of bIMPs via mass spectrometry.
- No one method or platform reviewed here is capable of comprehensive identification or quantification of IMPs; however, when used together, each of them can provide a complementary analysis, leading to a more thorough global analysis of these low-abundance membrane proteins.
- Further advances in sample preparation methods are needed to optimize solubility and separation of IMPs to keep pace with instrumentation improvements.

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