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Quantitative multiple reaction monitoring analysis of synaptic proteins from human brain

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HIGHLIGHTS

- Peptide verification through manual sequencing is essential.
- Target peptide signals can be swamped by co-eluting peptides.
- Normalized peak area showed less than 4% inter-day coefficient of variation.
- Synaptic proteins from human autopsy tissue were quantitatively analysed.

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ABSTRACT

Background: The recent introduction of multiple reaction monitoring to proteomics research has allowed many researchers to apply this technique to study human diseases.
New methods: Here we combine subcellular fractionation of human autopsy brain with label-free multiple reaction monitoring to quantitatively analyse proteins in synapses. The protein enolase, from *Streptococcus pyogenes* serotype M6, which is sufficiently different from human proteins, was spiked into the sample mixture prior to trypsin digestion and used as an internal standard across samples.
Results: Three synaptic proteins and an internal standard analysed with four injections over four consecutive days gave consistent differences with a coefficient of variation of <4%. Consistent retention time was recorded across the replicates. Comparison with existing methods: Previously, multiple reaction monitoring analysis has been utilized to study human autopsy and animal tissues. Utilizing the synaptosomal fraction prior to analysis reduced sample complexity and allowed the enriched synaptic proteins to be quantitatively assessed in a highly reproducible manner, without the need for expensive fluorescent labels and synthetic peptides.
Conclusion: Protein expression can be measured with accuracy using label-free multiple reaction monitoring mass spectrometry in relatively complex human brain samples. Synaptic functions are critical for neuronal communication and function, and synapse dysfunction underlies many neurodegenerative diseases, including Alzheimer's disease. This method can be applied to study a range of brain disorders.

1. Introduction

For the past decade, great efforts have been made to identify novel pathways and unravel the complex molecular mechanisms involved in the pathogenesis of various neurological disorders. Despite such effort, the molecular pathways that underlie many neurological disorders are poorly understood. Animal models are valuable preclinical tools to explore neurological disorders, but neuropsychiatric and cognitive symptoms are difficult to model and convincingly reproduce in animals. In addition, differences in structure and functionality between human and animal brains make the extrapolation of results from animal model studies to the human brain questionable. Ethical and practical issues make ante mortem sampling of human brain tissue difficult, and sample availability for research is very limited. Hence, autopsy tissue is a valuable source for exploring and understanding the pathogenesis of neurological disease. The introduction of sensitive proteomic technologies has facilitated the use of autopsy tissues in neurobiology.

Abbreviations: MRM, multiple reaction monitoring; MS, mass spectrometry; HPLC, high performance liquid chromatography; IDA, information dependent acquisition; CE, collision energy.

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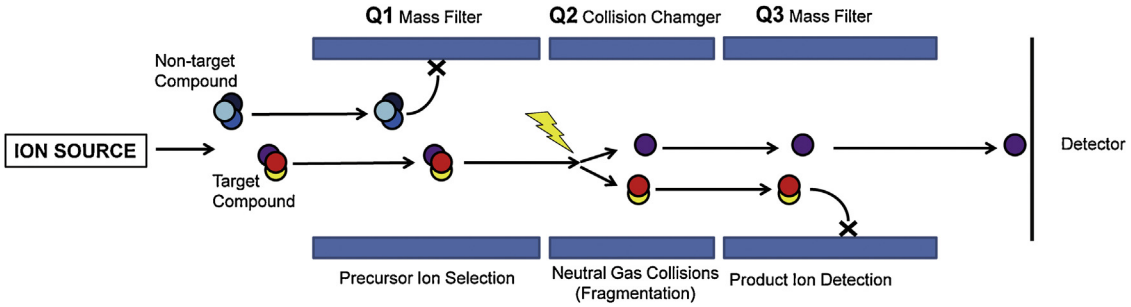

Fig. 1. Schematic diagram of MRM experiment. The first quadrupole only permits a predefined precursor ion to pass through, which then fragments into product ions in the second quadrupole. The third quadruple only transmits a predefined fragment ion which is recorded by the detector.

The human brain is complex. Whole brain tissues are composed of glial cells and neurons: the former account for 90–95% of all cells (Williams and Herrup, 1988). Since glial cells are predominantly astrocytes (Hansson and Ronnback, 2003), their overabundance could mask expression differences in neuronal proteins. Detection and characterization of low-abundant proteins remains a major challenge in the field of neuroproteomics, including within the synaptic proteome. Analysis of the synaptic proteome has gained much interest because synapses are responsible for cell-to-cell communication essential for neuronal function. By isolating synaptic proteins, we can focus on the business-end of the central nervous system and reduce sample complexity by enriching less-abundant proteins. Several studies have demonstrated the feasibility of using subcellular fractionation for protein enrichment through the analysis of rat brain cytosolic, microsomal, and mitochondrial fractions (Krapfenbauer et al., 2003). In addition, synaptic protein isolation has been applied in rat (Gredilla et al., 2012; Yang et al., 2011), mouse (Choi et al., 2012; Valencia et al., 2013) and human (Chang et al., 2012; Etheridge et al., 2009; Shi et al., 2009) brains to study various neurological disorders.

Multiple reaction monitoring (MRM) has become a popular tool for quantitative protein analysis in the past few years (Ackermann and Berna, 2007; Wolf-Yadlin et al., 2007). MRM exploits the capability of the triple–quadrupole mass spectrometer (MS) to selectively isolate predetermined precursor ions of peptides from targeted proteins in the first quadrupole, which then undergo collision-induced dissociation in a second quadrupole to generate fragment ions, of which one predefined fragment is allowed to pass into the third quadrupole for monitoring (Kondrat et al., 1978; Fig. 1). The combination of precursor and fragment ion mass-to-charge ratios is called a transition. For each protein, several of these transitions are collected over time, which gives a chromatographic trace represented with retention time and signal intensity on x and y axes respectively. A suitable set of transitions with accurate precursor ion retention times constitutes a definitive assay for that protein (Lange et al., 2008). Once a set of transitions is established, the assay can be multiplexed with great reproducibility, even across laboratories (Addona et al., 2009).

MRM coupled with subcellular fractionation can provide a comprehensive analysis of protein quantitation with wide dynamic range in human autopsy brain. A recent study used similar combinations (subcellular fraction with LC–MS/MS) to analyse the postsynaptic density in human Alzheimer's disease brain (Zhou et al., 2013). The aim of this paper is to illustrate the quantitative analyses of three synaptosomal proteins – synaptotagmin 1, cathepsin D and tubulin α-1A chain – from human autopsy tissue using label-free MRM with high-performance liquid chromatography (HPLC)-triple quadrupole-linear ion trap MS.

2. Materials and methods

2.1. Reagents

Sucrose, deoxycholate, trichloroacetic acid, 2D-Quant kit (GE Healthcare Life Sciences, Rydalmere, NSW, Australia), acetone, urea, thiourea, ammonium bicarbonate, dithiothreitol, iodoacetamide, trypsin, acetonitrile, formic acid, and enolase from *Streptococcus pyogenes* serotype M6. All the reagents used were of MS purity.

2.2. Tissue collection and storage

Brain tissue was obtained from the Queensland Brain Bank, School of Chemistry and Molecular Biosciences, University of Queensland, in collaboration with the Australian Brain Bank Network, with the informed written consent from the next of kin. At autopsy, brain tissues were immersed in an ice-cold 0.32 M sucrose for cryoprotection and stored at −80 °C. This project was approved by the Medical Research Ethics Committee of The University of Queensland (#2010000105).

2.3. Sample preparation

All tissue sectioning was performed on dry ice. Synaptosomes were prepared as per Etheridge et al. (2009). In brief, a total of 0.5 g frontal tissue was fractionated using density-gradient centrifugation. Tissue was homogenized with ice-cold 0.32 M sucrose (10×, v/w) in a Teflon homogenizer using 8–10 pestle strokes. The homogenate was transferred to a 15 mL polypropylene centrifuge tube and centrifuged at 750 × g for 10 min in a Beckman JA 20 centrifuge (Beckman Coulter P/L, Lane Cove, NSW, Australia) at 4 °C. The pellet, the crude nuclear fraction, was resuspended in 10× (v/w) of 0.32 M sucrose for storage at −80 °C. Following centrifugation of the supernatant at 19,000 × g for 20 min at 4 °C, the resultant supernatant was the crude microsomal fraction, while the pellet, the crude synaptosomal fraction was resuspended in 10× (v/w) of 0.32 M sucrose. A sucrose gradient was prepared by layering 0.8 M sucrose solution over a layer of 1.2 M sucrose. The resuspended fraction was layered onto this gradient and the whole centrifuged at 82,500 × g in a swinging bucket rotor (SW41 Ti, Beckman L8-60M ultracentrifuge) for 2 h at 4 °C. Myelin remained at the junction between the 0.32 M and 0.8 M sucrose layers, synaptosomes accumulated at the 0.8–1.2 M junction, and mitochondria deposited as a pellet that was resuspended in 0.32 M sucrose. The myelin and synaptosome fractions were collected by aspiration with a Pasteur pipet in minimal volumes; myelin was frozen for storage while synaptosomes were processed further (*v.i.*).