Metabolic phenotyping and systems biology approaches to understanding neurological disorders
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Abstract
The development of high-throughput metabolic profiling and the study of the metabolome are particularly important in brain research where small molecules or metabolites play fundamental signalling roles: neurotransmitters, signalling lipids, osmolytes and even ions. Metabolic profiling has shown that metabolic perturbations in the brain go beyond alterations of neurotransmission and that variations in brain metabolic homeostasis are associated with neurological disorders. In this report, we will focus on recent developments in the field of metabolic phenotyping that have contributed to unravelling the pathophysiology of neurological diseases. Also, we will highlight the necessity of implementing systems biology approaches to integrate metabolic data and tackle the structural and functional complexity of the brain in normal and pathological conditions.

Introduction
The study of the metabolome is particularly suited for brain research as small molecules play fundamental roles. Among other metabolites, neurotransmitters are the chemical messengers between neurons via synapses, signalling lipids act as secondary messengers and relay information from the synapse to the neuronal soma, osmolytes are essential for maintaining osmotic balance in the brain, and ions play a crucial role in neurotransmission and as enzymes cofactors. Given the variety of neuronal pathways regulated by small molecules, it comes as no surprise that perturbations in the metabolic balance of the brain can provoke important neurological disorders. However, several studies have demonstrated that the importance of brain metabolism extends beyond neurotransmission and secondary messengers, and that neurological disease may be associated with variations in intermediary and energy metabolism [1-4].

In this report, we will review the recent developments in metabolic phenotyping that have contributed to unravelling the pathophysiology of neurological diseases. Also, we will introduce systems biology approaches to integrate metabolic data and tackle the structural and functional complexity of the brain in normal and pathological conditions. Importantly, we will show that metabolomics and systems biology might not only lead to a better understanding of diseases of complex aetiology but could also be key methodologies to predict neurological diseases and individual drug responses.

Metabonomics, metabolomics and neurodegenerative disorders, a recent affair
Metabonomics has been defined as understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological nuclear magnetic resonance (NMR) spectroscopic data [5,6]. This is essentially a differential profiling approach, whereas metabolomics was originally defined as the large-scale analysis of the entire complement of small molecules (<1KDa), or metabolome, produced by a biological system: cell, tissue, organ or organism [7], just like the term genome refers to the entire gene content of an individual. The distinction
between these two terms has now faded and the use of the alternative terms ‘metabolic profiling’ or ‘metabolic phentypotyping’ is increasing. Quantifying the metabolome is of great biological interest since metabolites are the endpoints of many biological processes in the body and their identity and abundance directly reflect biological perturbations originating from collective modifications of the genome, transcriptome and proteome [5]. While transcriptomic or proteomic data do not grant access to the physiological status of the analysed matrix, metabonomics provide a characteristic fingerprint of the metabolic state of a given sample. As such, metabonomics was originally applied to the study of a metabolic response to toxic stimuli [5,6], such as metabolic dysfunctions in systemic metabolic disorders, i.e. insulin resistance-associated non-alcoholic fatty liver disease [8], diabetes [9,10], hypertension [11] or coronary artery diseases [12]. Over the last decade, the study of the metabolome has driven considerable attention in the field of neurological disorders since it enables the quantification of neurochemicals with signalling properties, such as neurotransmitters and signalling lipids, osmolytes, oxidative stress markers and even ions. Furthermore, metabonomics can be applied to a variety of biological matrices: intact biopsies or post-mortem tissue, tissue or cell extracts and biofluids such as cerebrospinal fluid (CSF), plasma and urine (Figure 1A).

**Analytical and chemometric techniques used for metabonomics**

Metabolic profiling is typically performed using two main analytical techniques: High Resolution proton NMR ($^1$H NMR) and mass spectrometry, which undergo continual refinement (Figure 1B). $^1$H NMR spectroscopy has been widely used for structural identification purposes in organic chemistry for decades, and is now used extensively in metabolic applications, providing low-cost per sample, rapid, highly reproducible and high-throughput analyses with minimal preparation for liquid samples (biofluids, brain extracts). Developments in the NMR field now allow metabolic profiling directly on intact tissues using high-resolution magic-angle spinning NMR [13]. This preserves sample integrity that can be further analysed using other “omics”. The main advantage of mass spectrometry is its high sensitivity and therefore its ability to detect low concentration metabolites. Sample extraction is a prerequisite prior to mass spectrometry, which is usually coupled to a separation step using either gas or liquid chromatography depending on the type of matrix analysed. Metal ions such as zinc, copper or iron, or elements such as calcium, potassium or sodium with essential roles in neuronal functions can be identified and quantified using inductively coupled plasma mass spectrometry data acquisition (B). Multivariate statistical analysis of spectral data enables classification and/or prediction of case and control samples based on statistical scores. Model coefficients or loadings (black triangles) are derived to highlight the single or group of peaks assigned to metabolites (here, to simplify representation, only 5 metabolites are represented: m1 to m5) whose variations maximize the discrimination between case and control samples (C). Pattern recognition statistical methods define disease-specific metabolic signatures (D), which correspond to the pattern of significantly affected metabolites (purple dots) representing candidate biomarkers of the disease.

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**Figure 1. Typical metabonomics workflow**

Standardized samples collection (A) is followed by NMR or mass spectrometry data acquisition (B). Multivariate statistical analysis of spectral data enables classification and/or prediction of case and control samples based on statistical scores. Model coefficients or loadings (black triangles) are derived to highlight the single or group of peaks assigned to metabolites (here, to simplify representation, only 5 metabolites are represented: m1 to m5) whose variations maximize the discrimination between case and control samples (C). Pattern recognition statistical methods define disease-specific metabolic signatures (D), which correspond to the pattern of significantly affected metabolites (purple dots) representing candidate biomarkers of the disease.
of the neurochemical and metabolic profile of murine models of neurological disorders [20], including neurodegenerative diseases such as spino-cerebellar ataxia [21], Huntington’s disease [22] or Alzheimer’s disease [18]. Recently, a neuroprotective treatment in a mouse model of epilepsy was proven to be effective in normalizing the brain metabolic signature of epilepsy using a $^1$H NMR-based metabolic approach [23]. Using HR magic-angle spinning $^1$H NMR-based metabolic profiling, we have identified a metabolic signature and biomarkers associated with fragile X syndrome, the leading genetic cause of intellectual disability and autism-spectrum disorders, in various brain regions of its mouse model, the Fmr1-deficient mouse [3]. Our study has highlighted that Fmr1 gene inactivation has profound, albeit coordinated, consequences on 25 brain metabolites, notably leading to alterations in levels of neurotransmitters (e.g. $\gamma$-aminobutyric acid (GABA), glutamate, acetylcholine and aspartate) and their precursors (e.g. glutamate, acetate, choline), secondary messenger precursors (e.g. inositol), energy metabolism products (e.g. lactate, acetate and acetoacetate) and oxidative stress markers (e.g. lipid oxidised species). Metabonomic studies in a mouse model of Huntington’s disease have identified characteristic metabolite profiles across several biological matrices (urine, plasma and intact brain tissue), suggesting that brain dysfunctions can also be detected in biofluids [24]; this is particularly timely as metabolic profiling holds promise for minimally invasive detection of brain disorders.

**Metabonomics as a predictive and analytical tool to study human neurological diseases**

Strikingly, the application of metabonomics to the analysis of biopsies and biofluids (e.g. plasma, urine, CSF) of patients affected by neurological diseases has seen tremendous developments in the last 10 years, highlighting the fact that metabonomics have a unique and specific potential for applications in the fields of physiological evaluation, diagnosis and drug therapy monitoring of neurological diseases.

CSF is a matrix of choice to study brain dysfunctions [15]. Given this, a seminal study used a metabonomics approach to detect a unique combination of subtle metabolic changes in the CSF of drug-naïve patients with first-onset schizophrenia, resulting in alterations in glucoregulatory processes [1]. Strikingly, the CSF metabolic profile was able to distinguish patients from healthy controls with a confidence of 85%. Further analysis revealed that early antipsychotic treatment normalizes the metabolic profile of treated subjects towards a control profile in half the patients well before a clinical improvement was
observed [1]. A $^{1}H$ NMR-based metabonomics study on ex vivo brain tissue indicated the molecular changes associated with the pathophysiology and drug treatment of bipolar disorder patients leading to the identification of biomarkers of this disorder in post-mortem brain samples, but also of secondary side effects associated with drug treatment [19]. A similar study has provided evidence for disease and antipsychotic medication effects in post-mortem brain from schizophrenia patients [25]. More recently, mass spectrometry-based metabolomics were used to predict the disease status of post-mortem Alzheimer’s disease biopsies [17]. NMR-based metabonomics have also been used to define biomarkers and to grade malignancy of CNS cancers using oligodendroma [26] or meningioma [27] biopsies. One study has determined a metabolic signature of malignant glioma in human CSF, enabling accurate discrimination between affected patients and controls [28]. Quite strikingly, it appears that brain dysfunctions can be mirrored in other biofluids, such as plasma and urine. In fact, individuals with neurological disorders have distinct metabolic profiles that can contribute to accurate diagnosis using metabolic profiling of these easily accessible samples. Interestingly, an ultra-performance liquid chromatography-mass spectrometry and NMR-based metabonomic study has even identified a metabolic signature in plasma samples from schizophrenia patients upon treatment with the anti-psychotic risperidone [4]. Also, plasma metabolic profiling of Parkinson’s Disease patients differentiated between idiopathic and genetic forms of the disease [29]. Importantly, one study has reported that metabolic profiling of urine samples can discriminate between samples from children affected by autism spectrum disorders, their unaffected siblings and age-matched controls [30]. Lately, an NMR-based metabonomic analysis of the serum of first-trimester pregnant women reported novel metabolic markers for the prediction of foetal Down Syndrome [31].

These studies highlight the huge potential and versatility of metabolic phenotyping to identify biomarkers of neurological diseases across various biological matrices, including the easily collectable biological matrices such as plasma and urine. Also, metabolomics can be used to assess treatment efficacy and determine secondary side effects associated with the metabolic breakdown products of drugs with potential toxicity. This is particularly pertinent in the context of psychiatric conditions of complex aetiology, where patients are often subjected to heavy medication with metabolic secondary side effects, e.g. typical and atypical antipsychotics with associated metabolic syndromes.

The importance of systems biology for the interpretation and integration of metabolomic data in the context of neurological diseases

Once metabolic biomarkers of a disease of interest have been assigned and adequately quantified, systems biology can then be applied to enhance the biological interpretation of metabolic signatures. For example, mapping metabolic biomarkers onto biological networks enhances the understanding of complex metabolic signatures at the pathway level. The combination of metabonomics with systems biology in the field of neurological diseases is only beginning but has a very promising future. First, metabolite-set enrichment analysis (MSEA, [32]), an extension of the gene-set enrichment analysis (GSEA) [33], can be used to identify which metabolic pathways are enriched in the metabolic signature and highlight biologically relevant pathways. We used MSEA to highlight alterations of glutamate metabolism and oxidative stress response in the cortex of a fragile X syndrome mouse model [3]. However, to enhance our understanding of the mechanisms linking the disease-causing gene (Figure 2A) to the metabolic signature of fragile X syndrome, we developed a novel alternative strategy, named “integrated metabolome and interactome mapping” (iMIM). iMIM is based on mapping metabolic phenotypes (Figure 2B) directly onto protein-protein interactions, metabolite-enzyme interactions and ligand-receptor interactions networks extracted from literature and databases (Figure 2C) [3]. As the estimated number of protein-protein interactions approaches 650,000 [34], it has become necessary to analyse the topology of the resulting network to identify key proteins regulating the metabolic phenotype associated with the disease-causing genes (Figure 2D). In this type of network, the shortest paths between causal genes and the downstream metabolic consequences are computed and compiled to identify the central proteins (i.e. the busiest proteins) in the interaction network [3]. This is achieved using network statistics such as the “betweenness” [35], which can be used to highlight key proteins that relay the signal between the causal genes and the metabolites. Further developments of the iMIM methodology could introduce weighting factors integrating other “omics” data, for example, taking into account variations in transcriptomic or proteomic profiles could help in refining the bioinformatic predictions of key proteins and key pathways and increase biological relevancy towards the studied disease.

When it comes to the integration of “omics” data, one important development is the statistical integration of multiple data levels (i.e. different “omics”), which is commonly used in the field of metabolic diseases. A recent study used hierarchical clustering for the integration of
metabolic and transcriptomic data to assess brain chemistry [36]; however, such an integrative approach has yet to be applied to neurological diseases. Such combined multi-omics platforms analysis should be developed using mouse models or clinical samples of neurological diseases to increase the confidence in disease prediction.

**Future directions**

In the future, one important development of metabolomics and systems biology and their application to the study of neurological diseases would be to search for genetic determinants of the observed metabolic signature. Brain metabolic profiles could be correlated to DNA polymorphisms and haplotypes to identify quantitative trait loci for metabolic traits (mQTL), following the mQTL approach originally applied to the study of metabolic diseases [9]. The affordability of next-generation sequencing methods holds promise for high-throughput acquisition of large-scale single nucleotide polymorphism (SNP) data which, in combination with corresponding metabolomic data, would enable mQTL applications to highlight susceptibility loci and genes for neurological diseases of complex aetiology, such as schizophrenia or autism-spectrum disorders in the near future.

Finally, another critical application of metabolic phenotyping is pharmacometabonomics, which predicts the response of an individual to a stimulus (e.g. drug, toxin, surgery, disease) prior to the treatment [37]. For example, pharmacometabonomics typically uses pre-dose measurements for prediction of toxicity or efficacy of specific drugs [38]. It is an integral part of predictive biology, aimed at understanding pre-existing phenotypic differences and their medium- to long-term beneficial or pathological impact on interventions. This is particularly suited for psychiatric conditions such as schizophrenia, in which treatment optimization and subsequent stabilization of patients can often be a complex and long process. The approach has major implications for improved efficiency in drug discovery efforts, for example, identifying non-responders and characterizing the molecular basis of their non-responsiveness in preclinical studies, or for better stratification of patients in drug clinical trials and for individualized therapies [37].

In conclusion, the use of state-of-the-art spectrometry, statistics and systems biology has initiated a shift in neurological sciences. There is now a body of evidence showing that brain metabolism goes beyond neurotransmitter synthesis and that intermediate metabolism contributes to complex brain disorders. It is now anticipated that from the availability of comprehensive profiling methods, coupled with innovative network biology approaches, will eventually emerge a high-precision mapping of metabolic phenotypes onto the
genome and the interactome, enhancing our understanding of brain metabolism in health and disease.

**Abbreviations**

CSF, cerebral spinal fluid; GABA, γ-aminobutyric acid; HR $^1$H NMR, high resolution proton nuclear magnetic resonance; iMIM, integrated metabolome and interactome mapping; mQTL, metabolic quantitative trait loci; MSEA, metabolite set enrichment analysis; NMR, nuclear magnetic resonance; PCA, principal component analysis; PLS, partial least squares.

**Disclosures**

The authors declare that they have no disclosures.

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