The Application of NMR-Based Metabonomics in Neurological Disorders

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Summary: Advances in postgenomic technologies have radically changed the information output from complex biological systems, generating vast amounts of high complexity data that can be interpreted by means of chemometric and bioinformatic methods to achieve disease diagnosis and prognosis. High-resolution nuclear magnetic resonance (NMR) spectroscopy of biofluids such as plasma, cerebrospinal fluid (CSF), and urine can generate robust, interpretable metabolic fingerprints that contain latent information relating to physiological or pathological status. This technology has been successfully applied to both preclinical and clinical studies of neurodegenerative diseases such as Huntington's disease, muscular dystrophy, and cerebellar ataxia. An extension of this technology, ^{1}H magic-angle-spinning (HRMAS) NMR spectroscopy, can be used to generate metabolic information on small intact tissue samples, providing a metabolic link between metabolic profiling of biofluids and histology. In this review we provide a summary of high-resolution NMR studies in neurodegenerative disease and explore the potential of metabonomics in evaluating disease progression with respect to therapeutic intervention. **Key Words:** Metabonomics, NMR spectroscopy, neurodegeneration, chemometric, biomarker.

INTRODUCTION

As we progress toward a systems approach to understanding disease etiology and evolution via the use of postgenomic disciplines such as transcriptomics, proteomics, and metabonomics, the conceptual framework for analysis and data interpretation has shifted. Traditionally, strategies have been employed whereby a few response vectors have been measured in sequence on the basis of a predetermined hypothesis. The framework adopted favored a single stimulus—single response structure, such as protein phosphorylation and therefore was designed to model simple linear pathways. However, there is increasing recognition of biological complexity and the need to integrate technologies and their outputs into a systems-based approach. Therefore, the conceptual paradigm has been shifted to accommodate the complexity of biological networks[2,3] (as described by Miller and Federoff[4] and Olson[5] in the current issue).

As with all "omics" methodologies, *metabonomic* technology is approaching a deeper level of maturity and is beginning to fulfill its potential in the diagnostic characterization of disease with promise of prognostic capabilities in some disease areas. Moreover, it has a key role in the discovery and development of new treatments for human disease. Metabonomics, which is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification,"[6] involves the determination of comprehensive metabolite profiles of biological matrices (biofluids, cells, tissues). Metabonomic technology largely relies on advanced spectroscopic platforms such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) that generate high-density data from biological samples providing a characteristic "fingerprint pattern" for a range of biologically important endogenous metabolites[6,7] that reflect the physiological or pathological status of an organism. Latent information pertaining to the "global" metabolic status of an individual and encoding metabolic responses to genetic and environmental factors, such as nutrition, aging, gender, stress, and disease, can be extracted from the spectral data using multivariate mathe-

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matical modeling tools[6,8] (see Olson[5] in the current issue for further explanation). A wide range of bioinformatic and chemometric modeling strategies can be applied to define the metabolic boundaries of disease and to monitor progression of disease with and without therapeutic intervention. Several such examples exist in the literature based on NMR spectroscopy, HPLC–MS, GC–MS, and also MS-based methods with electrochemical detection, spanning a range of diseases including cancer,[9–11] diabetes,[12] coronary heart disease,[13,14] neurological disease,[15–17] metabolic disorders[18] and infectious diseases.[19–21]

In the current review we focus on the development and application of NMR-based metabonomic technology in the field of neurological disease and explores its potential in evaluating therapeutic strategies.

HISTORY OF NMR TECHNOLOGY IN STUDYING NEUROLOGICAL DISORDERS

The history of magnetism in medicine dates back to the legendary discovery by the shepherd Magnes around 1000 BC of a magnetic substance later known as lodestones ("lead" stones) or "stones that point the way."[22] In the 15th century, a Swiss physician named Paracelsus used lodestones to filter diseases such as epilepsy, diarrhea, and hemorrhage from the body. He believed that the ability of magnets to attract iron could be replicated by attracting disease away from the body. In the late 18th century, the Austrian physician Franz Anton Mesmer, who popularized the use of hypnotism in medicine in France, described the healing properties of passing magnets over the open veins of patients. A Board of Inquiry containing prominent scientists was established by King Louis XVI to validate the effects of "animal magnetism" or "mesmerism." In what is considered to be perhaps the first placebo-controlled trial of a therapy ever conducted, Benjamin Franklin constructed an experiment in which a blindfolded patient was shown to respond as much to a nonprepared tree as to one that had been "magnetized." In the mid-19th century, magnetic ointments produced in New York were introduced as remedies for a whole spectrum of illnesses such as headaches, inflammation of the bowels, burns, fever sores, rheumatism, gout, and toothache.

In 1952 the Nobel Prize in physics was awarded to Edward Purcell and Felix Bloch for their discovery of nuclear magnetic resonance in 1945, and since then a further seven Nobel Prizes have been issued to pioneers in applications of NMR in chemistry, biochemistry, and medicine. Magnetic resonance works on the principle that the electrons and the nucleus of many isotopes possess a property called spin, which causes these charged particles to behave like small bar magnets when placed in a magnetic field, aligning themselves with or against the magnetic field. When energy is introduced into the system in the form of radiofrequency, the particles absorb energy and are said to be on resonance. Since the different atoms of a given nuclei within a molecule, such as ^1H, resonate at slightly different frequencies, dependent upon their electronic environment, information about the chemical structure of a molecule can be obtained.

Nuclear magnetic resonance technology has been used extensively in the field of neurodegeneration, both as an imaging tool to generate anatomical information via the measurement of water molecules in different physical environments and as magnetic resonance spectroscopy (MRS), which provides chemical structural information from *in vivo* tissues. Magnetic resonance imaging (MRI) has been established as one of the most important clinical diagnostic tools for diseases such as multiple sclerosis,[23–25] and Alzheimer's disease (AD).[26] Magnetic resonance imaging has also been used to characterize neural changes in diseases such as Creutzfeldt–Jakob syndrome,[27] Huntington's disease (HD),[28] Hallervorden–Spatz disease,[29] Alexander's disease,[30] Cushing's syndrome,[31,32] and bipolar disorder.[33] Although MRI is an extremely important clinical tool, MRS and high-resolution NMR spectroscopy of biofluids and tissues are more useful in the context of systems biology since they generate multivariate information on a wide range of molecules.

^1H MRS has sufficient sensitivity to detect a range of neurochemicals including amino acids (alanine, aspartate, glutamate, glutamine, taurine), organic acids (γ-aminobutyric acid, lactate), phosphocreatine, phosphoethanolamine, phosphocholine, glycerophosphocholine, inositols (*myo*-inositol, *scyllo*-inositol) and its use in neurological studies has been reported in a number of reviews.[22,34] Other nuclei that are of use include ^{13}C and ^{31}P, although the sensitivity of these nuclei preclude them from being used routinely in MRS studies. Since lesions in the brain are generally localized, MRS is performed on small tissue volumes or voxels, typically 1–2 cm^3. Absolute quantitation of metabolites from *in vivo* tissues is problematic since it is not possible to use an internal reference standard, and therefore metabolite concentrations are usually expressed as a ratio to a metabolite that does not change with disease or intervention. Many neurological conditions that have been successfully characterized by MRS include epilepsy,[35] amyotrophic lateral sclerosis (ALS),[36,37] Parkinson's disease (PD),[38,39] HD,[28,39] and AD,[40,41] to name but a few. MRS has also been used to increase the specificity of noninvasive diagnosis of brain tumors.[42] Although alanine may be present in several types of tumor, it is only clearly distinguishable from lactate in meningiomas. An overview of the use of MRS in the study of transgenic models of PD, AD, HD, and ALS is given by Choi et al.[34]

High-resolution NMR spectroscopy can be used to analyze *ex vivo* tissues and biofluids such as CSF, blood plasma, and urine and provides a uniquely powerful means of investigating the metabolic composition of such samples, since the rich atom-centered information generated often allows direct structural elucidation of metabolites. Unlike MRS, high-resolution NMR spectroscopy is not compromised by poor spectral resolution and can routinely access metabolites present at micromolar concentrations. For example, whereas alterations in relative concentrations of lipoprotein fractions may contribute to characterization and diagnosis of disease tissues, MRS commonly "edits out" the contributions of these signals. The use of more specialized NMR probes, such as cryoprobes and microprobes, offers further enhancement in sensitivity, lowering the detection limit to the nanogram range. Historically, high-resolution NMR spectroscopy originated in the elucidation of chemical structures. In 1977, Brown and coworkers[43] applied ^{1}H NMR spectroscopy to the study of red cell metabolism and in 1979 the technology was applied to blood serum and identified elevated lactate in patients with malignant tumors.[44] In the 1980s, Nicholson and coworkers[45–47] adapted the technology for routine measurement of biofluids and over the next decade high-resolution NMR spectroscopy was used progressively, mainly for drug metabolism and toxicology studies in experimental animals. A number of human clinical studies were also undertaken in the 1980s and early 1990s,[45] but such studies were restricted to overt disease, since the capacity for sample acquisition was limited and spectral interpretation was performed manually. Nuclear magnetic resonance spectroscopic analysis has now been used to investigate the mammalian brain and has been particularly useful as a diagnostic tool.[48–51] For example, Cheng and coworkers[49] were able to distinguish between normal tissues and a number of brain tumor types, reporting correlations in metabolite ratios with tumor grade. In recent years, NMR technology has undergone revolutionary improvements in terms of capacity for sample throughput, increased sensitivity, physical hyphenation with other analytical spectroscopic tools, and improved processing and modeling software. The analytical features that have maximized the performance of NMR spectroscopy in clinical studies are summarized below.

KEY ANALYTICAL DEVELOPMENTS IN NMR SPECTROSCOPY

Recent advances in both NMR spectrometer hardware and the mathematical tools available for spectral interpretation have revolutionized the field, conferring a huge increase in analytical sensitivity. A sample throughput of 200–300 samples/day/spectrometer can be achieved via the use of flow-injection NMR under optimized condi-

tions. Typically, high-throughput screening and evaluation of drug toxicity and metabolism analyses are conducted on 600 MHz NMR spectrometers since this gives an adequate performance with a good cost:benefit ratio and much of the research to date has been conducted at 600 MHz. However, the increase in sensitivity and dispersion achieved at higher fields such as 800–900 MHz is sometimes necessary for metabolite identification.[52,53] One of the major limiting technical factors in NMR spectroscopy, in general, is its rather low inherent sensitivity compared with other types of analytical detection methods. A critical development in overcoming this limitation has been the recent commercialization of NMR detectors (probes) cooled to near cryogenic temperatures, usually around 20 K, which improve the signal-to-noise ratio (SNR) via reduction of the thermal (Johnson) noise level by a factor proportional to the square root of the temperature ratio.[54] In practice, this results in an improvement of around 400% in sensitivity (for a single scan) and reduces the data acquisition time by ~16 fold. With the gain in SNR, the use of other less sensitive nuclei in biofluid analysis such as ^{13}C, ^{15}N, or ^{31}P becomes possible.[55,56] In addition, the improved SNR of cryoprobes allows the acquisition of two-dimensional (2D) NMR data; i.e., the use of a second frequency domain to improve the dispersion of the spectra and to generate structural information on correlation between neighboring nuclei in a time frame suitable for metabonomic screening.

Since biological samples are chemically complex, often containing hundreds or thousands of metabolites, one-dimensional (1D) spectroscopy produces crowded spectra in which low concentration metabolites may be obscured. For example, biofluids such as serum, bile, or intact tissues, which contain a mixture of macromolecular (e.g., lipoproteins, cholesterol) and low molecular weight components (e.g., amino acids, carboxylic acids), can be difficult to interpret using standard 1D sequences, since the sharper resonances arising from low molecular weight species are superimposed with the broader macromolecular resonances. In this case, it is often appropriate to use more than one pulse sequence, for instance a spin-echo sequence, to enhance the contribution of the low molecular weight metabolites or diffusion edited sequences to edit out the low molecular weight components and focus on the lipoprotein and higher molecular profile.[57,58] Methods for reducing overlapped metabolite resonances and increasing the molecular structural information include dispersion of the spectral data using a second dimension, for which a range of 2D pulse sequences exist.[59,60] Once a potential biomarker candidate of disease class or response has been identified, then the NMR spectroscopic detection can be physically integrated with HPLC–MS to effect simultaneous molecular characterization of separated biomolecules from biofluid

samples.[61] The recent development of ultra high-pressure liquid chromatography (UPLC) has further enhanced the capabilities for metabolite isolation and detection. The advances offered by UPLC include higher metabolite concentration per band, more rapid separation, and improved resolution. UPLC–MS can be used in its own right to generate information-rich metabolite profiles for disease diagnosis.[62] Coupled with the increase in sensitivity offered by the cryo-flow probes, UPLC offers the possibility of a dramatic improvement in the ability to perform high-throughput metabolic analysis and substantially improves the limits of biomarker detection.

In the past decade it has become possible to analyze intact tissues using a technique called magic-angle-spinning (MAS) NMR spectroscopy based on technology adapted from solid-state studies. This technique has been applied to the study of organ-specific toxicity,[63] characterization of tissue composition and structure,[64] the analysis of needle biopsies of human samples,[65] cell systems,[55] tumors,[51,66] and to study dynamic information in tissue compartments.[19] Since only ~10 mg of tissue is needed, this approach is directly applicable to clinical biopsy samples. Like MRS, the exact quantitation of metabolite changes is difficult, as it is not possible to add an external quantitation standard. Therefore, metabolite changes are usually expressed as ratios to a selected metabolite or relative to the sum of the spectrum, adjusted for tissue weight. However, MAS provides a direct metabolic link between biofluid profiles and histopathology and can also be used to observe compartmentalization of metabolites and dynamic tissue changes in real time.[19]

DEVELOPMENTS IN MATHEMATICAL PROCESSING AND MODELING TOOLS

Since analytical spectra are highly complex objects and the dynamic range of signal intensities results in a visual bias toward high-intensity signals, computer-based Pattern Recognition (PR) algorithms were developed for data reduction and spectral interpretation. Initially multivariate statistical methods were applied to the analysis of NMR biofluid data to classify ^{1}H NMR spectra according to their origin (e.g., healthy or diseased, control, or toxin treated) based on a number of scored or quantified metabolites. There are many different mathematical algorithms for establishing similarities between samples and for extracting variables or features that are important in discriminating between two or more groups of samples. These methods have derived from many academic fields including artificial intelligence, chemistry, economics, psychology, and biology and include linear projection methods [e.g., principal components analysis (PCA), projection to latent structure regression], clustering analyses, Bayesian probabilistic approaches,

genetic algorithms, and artificial neural networks.[67–70] Each of these methods has associated strengths and weaknesses. We illustrate the principle of PR here using basic PCA.

Principal component analysis reduces the initial variables (NMR descriptors) to a smaller set of variables termed principal components (PCs) by means of a linear transformation, which allows the main variation in the dataset to be visualized while reducing irrelevant noise. Principal components are new variables that are orthogonal to each other and explain progressively less variance in the data set. They can be displayed in two- or three-dimensional "scores plots," allowing visualization of the distribution and grouping of the samples in the new variable space. Since each PC is a combination of the original input variables, each original variable (or NMR signal) is given a weight (loading) that indicates the strength of influence that variable has on the overall distribution of samples. These weights can be displayed as loadings plots that indicate which spectral regions are dominating the differentiation between groups of samples (FIG. 1).

Chemometric modeling of spectroscopic data has, to a large extent, developed to accommodate the increased sensitivity and sample throughput of NMR technology. Sophisticated algorithms have been developed for metabonomic analysis that can use the full spectral resolution (typically 64K data points) and better accommodate the biological complexity in mammalian systems. While algorithms such as PCA have been used with considerable success in metabonomic analysis for data reduction and to allow the visualization of inherent structure in the data, spectral data from human urine or serum contains latent information on a range of diverse genetic and environmental influences, which inhibit derivation of the effects of single factors or stimuli. Data-filtering strategies and other preprocessing methods can be used to optimize models and to increase the interpretability of the models. Incorporating an orthogonal filtration step into methods such as projection to latent structure discriminant analysis (O–PLS–DA) can achieve separation of the systematic variation of interest from the systematic variation deriving from confounding sources, such as inherent physiological variation, but still retain the ability to interpret both types of variation independently to gain a better understanding of the variation in the data.[68,71] Statistical algorithms can also be applied directly to the identification of biomarkers in NMR spectra. Since each proton environment within a metabolite generates a separate signal of the NMR chemical shift scale, the calculation of a correlation matrix for a set of samples driven from the apex of a selected resonance will highlight correlated signals deriving from the same molecule and down weight other metabolite contributions to the spectra not correlated to the molecule of interest. Many
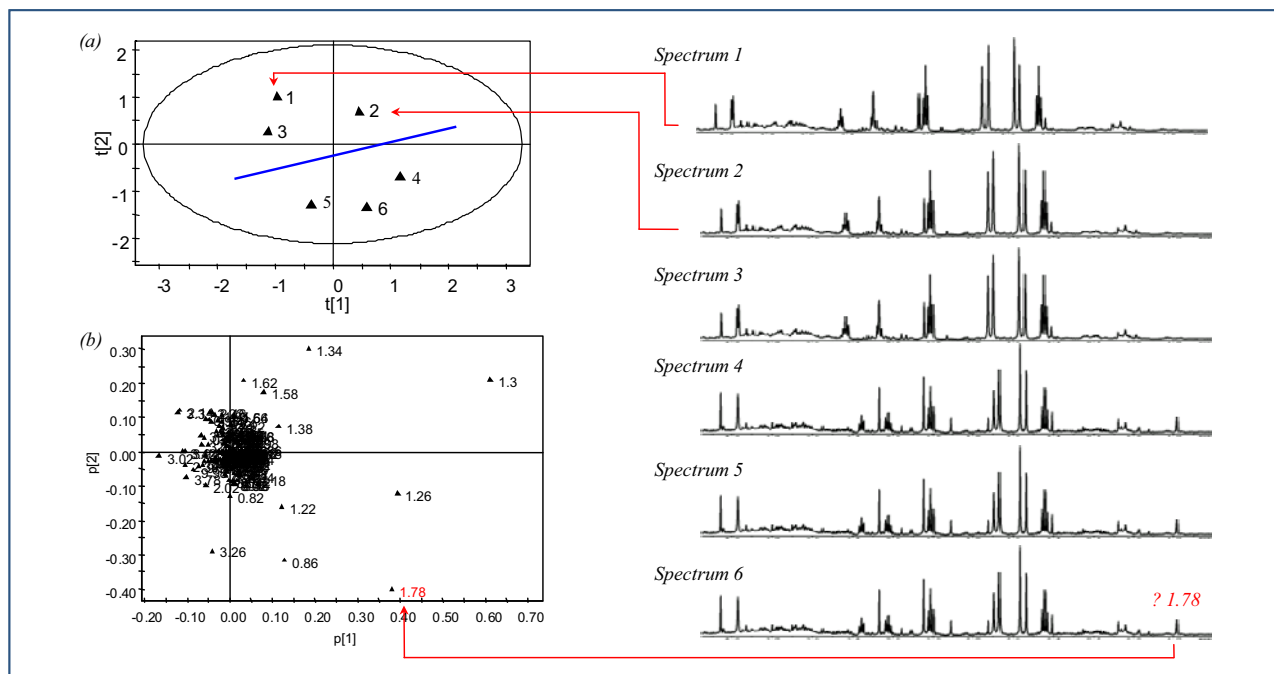
FIG. 1. An illustration of how ^{1}H NMR spectra (spectra 1-6) can be reduced and visualized in a 2D PCA scores plot. (a) Each spectrum can be viewed as an observation in PCA space (as exemplified by arrows for spectra 1 and 2) where the proximity of observations represents the similarity of the metabolic profiles of the biological samples. (b) The corresponding loadings plot presents the variables attributable to the spatial localization of the spectrum in the scores plot. In this example, spectra 1–3 are metabolically distinct from spectra 4–6 in the scores plot. Several variables contribute to the separation of clusters including variable δ 1.78. Cross-referencing back to the spectra reveals the presence of an additional peak that is only exhibited in spectra 4–6.

of the statistical methods developed for modeling NMR data can be applied equally successfully to modeling gene expression or proteomic data.[67] There is currently a drive toward integration of "omics" data sets with subsequent mapping of significant variables (genes, proteins, metabolites) onto biological pathways and networks to achieve a more holistic picture of the dynamic and interactive biological processes occurring in an organism (as demonstrated by Ma'ayan and coworkers[72] in the current issue).

APPLICATIONS OF HIGH-RESOLUTION NMR SPECTROSCOPY IN NEUROLOGICAL DISORDERS

The neurochemical profile of the mammalian brain has been well characterized by both MRS and high-field ^{1}H NMR spectroscopy.[73–75,148] Differences in structure and function between various distinct neuroanatomical regions have also been elucidated using both MAS of intact tissues and tissue extracts,[75] with the sensitivity and specificity of the analysis allowing differentiation between closely related structures such as the dorsal and ventral striatum.[75,76] As many neurodegenerative disorders exhibit neuronal loss in selective anatomical regions, e.g., caudate and putamen in HD and substantia nigra *pars compacta* in PD, NMR spectroscopic analysis requires the examination of specific localized regions. In

addition, the effect of development and maturation on brain structure has also been explored (FIG. 2). Metabolic profiles for neural cell lines (e.g., neuronal and glial) have also been established using high-resolution NMR spectroscopy.[77,78] Such information may allow the interpretation of metabolic perturbations observed by ^{1}H NMR spectroscopy, for example, in cases in which selective neuronal loss or abnormal development is expected or in patients with brain tumors in which changes in cell populations may occur. Although many changes observed in neurological disorders are associated with specific neuroanatomical regions and are disease specific, certain metabolic changes are globally indicative of degeneration. The most common of these changes is a decrease in *N*-acetylaspartate (NAA) in brain tissues, which is indicative of neural loss and has been reported following AD,[41,48] HD,[79–81] Rett's syndrome,[82] PD,[39,83] ALS,[36,37] multiple sclerosis,[23,84] Batten's disease,[85] and traumatic brain injury.[86] A summary of the key NMR spectroscopic-based studies in neurodegenerative disease and models of neurological disorders are listed in Table 1.

Preclinical metabonomic studies

Toxicological models of neurodegenerative disease. Toxicological studies into various neuroactive compounds have provided invaluable insight into the etiology of a number of neurodegenerative disorders. The

FIG. 2. (a) A PCA scores plot mapping ^{1}H NMR spectra of aqueous extracts of cerebellum, cortex, and brainstem samples from R6/2 transgenic and control mice. Visualization of the metabolite profiles from R6/2 transgenic and wild-type mice in PCA reveals a clear distinction at 8 and 12 weeks. (b) The corresponding loadings plot presents the variables attributable to the class separation. (red = frontal cortex extracts from wild-type mice; pink = frontal cortex extracts from R6/2 mice; black = cerebellum extracts from wild-type mice; gray = cerebellum extracts from R6/2 mice; dark blue = brainstem extracts from wild-type mice; light blue = brainstem extracts from R6/2 mice; ▲ = 8-week-old; ■ = 12-week-old); (c) A coefficient plot showing the metabolic differences associated with maturation (8–12 weeks) in wild-type mice.

administration of neurotoxins to experimental animals has produced many models with clinical phenotypes analogous to human disorders. Some toxins share the ability to selectively destroy specific neuroanatomical regions that are primarily associated with specific diseases.

Parkinson's disease. Both genetic and environmental factors have been implicated in the onset and development of PD. Twin studies,[87,88] case control studies,[89,90] and the identification of mutations in several genes in familial PD[91–97] point to there being a significant genetic contribution to the etiology of PD, in at least a proportion of patients. The association of environmental toxins with

parkinsonism derives from studies implicating carbon monoxide,[98] manganese,[99] industrial herbicides,[100] and, most importantly, 1-methyl-4-phenyl-1,2,3,6 tetrahydro-pyridine (MPTP).[101] In reality, it would appear that PD is a heterogeneous disease likely to be caused by more than one specific etiological factor. This is supported by the fact that the clinical and pathological syndromes associated with environmental agents are remarkably similar to those observed with familial PD cases.

Over the years, investigators have developed the use of agents that selectively disrupt or destroy catecholaminergic systems to produce animal models of PD. Several compounds such as reserpine,[102] 6-hydroxy-

TABLE 1. *Summary of Some Key NMR Spectroscopy-Based Studies in Neurodegenerative Conditions*

Neurological Disease	Study	Reference
	Identification and characterization of mammalian brain metabolites	Tsang et al.,[75] Tkac et al.,[148] Pfeuffer et al.,[74] Govindaraju et al.[73]
Spinocerebellar ataxia	Metabolic characterization of the SCA3 (Machado–Joseph disease) mouse model	Griffin et al.[1,15]
Parkinson's disease	Analysis of brain metabolite perturbations in toxin-induced primate model for PD	Brownell et al.[109]
	Characterization of metabolite perturbations in PD and neurodegenerative parkinsonism	Hoang et al.,[79] Luccetti et al.,[83] Seppi and Schocke[38]
	Metabolite analysis after thalamotomy as putative therapy for PD	Baik et al.[140,141]
Huntington's disease	Characterization of the metabolite perturbations and impaired energy metabolism in PD	Hoang et al.,[79] Jenkins et al.[28,81]
	Metabolic characterization of toxin-induced and transgenic animal models of HD	Tsang et al.,[76] Jenkins et al.,[121] van Dellen et al.,[120] Dautry et al.[113,149]
	Evaluation of creatine supplementation in HD models and patients	Bender et al.,[90] Verbessem et al.,[142] Matthews et al.,[150] Tabrizi et al.,[146] Kasparova et al.[39]
	Evaluation of striatal allografts in primate model of HD	Palfi et al.[114]
	Evaluation of ciliary neurotrophic factor in primate model of HD	Mittoux et al.[115]
	Metabolic profiling of blood sera from HD patients	Underwood et al.[122]
Alzheimer's disease	Metabolic characterization to improve the understanding and diagnosis of AD	Fernandez et al.,[40] Pfefferbaum et al.,[41] Cheng et al.,[48] Frederick et al.[127]
Schizophrenia	Metabolic characterization of human brain tissues from schizophrenic patients	Prabakaran et al.[17]
Bipolar disorder	Biomarker evaluation	Gallelli et al.[33]
Meningitis and ventriculitis	Evaluation of metabonomics as a diagnostic tool	Coen et al.[19]
Multiple sclerosis	Characterization of the metabolic consequences during relapsing–remitting episodes of MS	Kapeller et al.,[23] Karrenbauer et al.,[24] Tartaglia and Arnold,[25] Matthews et al.,[37] Tourbah et al.,[34] Mader et al.,[130] Arnold et al.[131,135]
Cushing's syndrome	Brain metabolite alterations in Cushing's syndrome	Khiat et al.[31,32]
Amyotrophic lateral sclerosis	Screening the efficacy of potential therapeutic agents for ALS	Kalra[36]
Human brain tumors	Characterize and differentiate types of brain tumors	Preul et al.,[42] Cheng et al.,[49] Barton et al.[51]
Rett syndrome	Characterization of the metabolic changes occurring in Rett syndrome	Horska et al.[82]
Batten disease	Phenotypic characterization of a mouse model for Batten disease	Pears et al.[85]

dopamine (6-OHDA),[103] and MPTP[101] cause the specific degeneration of the nigrostriatal cells, providing good pharmacological models of PD. Direct injection of 6-OHDA into the substantia nigra produced the first toxin model of PD.[103] The selective accumulation of this toxin in dopaminergic neurons leads to their death, pre-sumably mediated by oxidative stress.[104] Animals have a quantifiable motor deficit, but the characteristic Lewy bodies observed in human patients are not seen, and there is nonspecific toxin-induced damage to neighboring neu-rons. This model has been particularly useful for phar-macological screening but does not replicate many key

clinical features of PD. Parkinsonism in drug addicts caused by drugs contaminated with MPTP,[101] an inhibitor of complex I of the mitochondrial respiratory chain, led to the development of mouse and primate models of PD using this neurotoxin.[105] In nonhuman primates, MPTP causes tremor, rigidity, akinesia, and postural instability, which are all successfully treated with L-Dopa and dopamine agonists. Dopaminergic neurons are selectively lost in brain areas similar to idiopathic PD, including the substantia nigra. However, MPTP-induced subacute or acute onset parkinsonism lacks the typical electron microscopy features of Lewy bodies.[101] It is unknown whether chronic administration of MPTP may induce the formation of Lewy bodies. The MPTP model represents the best characterized model of PD and fulfills many of the criteria for the ideal model of the disease with the exception of Lewy body formation. It is the study of MPTP-lesioned animals that has led to most of the current surgical and pharmacological therapies for PD.

Relatively little analysis has been carried out on the metabolic profiling of toxin-induced PD models given the wealth of information from imaging studies performed.[106–108] However, metabonomic strategies have been applied in a primate model of PD.[109] Chronic low doses of MPTP were infused to selectively target subpopulations of dopaminergic neurons and present a stable Parkinsonian syndrome, increases in striatal choline and lactate together with decreases in NAA were described, which persisted months after the termination of toxin administration. Similar metabolite changes were also reported in feline PD models, although the choline: creatine ratios were observed to decrease after MPTP administration.[110]

Huntington's disease. HD is primarily associated with a progressive atrophy and selective neuronal cell loss of the medium spiny neurons of the striatum. However, neuropathological analysis of postmortem HD brain demonstrates a reduction of 300–400 g in total brain weight compared with normal controls, and, as the striatum only weighs ~20 g, then clearly ~90+% of the atrophy is extrastriatal in HD and marked cortical loss is observed. 3-nitroproprionic acid (3-NP) is a mitochondrial inhibitor used to create an animal model for HD. Unlike intrastriatal kainic acid (KA) injections, which exhibit a nonselective necrosis,[111] 3-NP has been shown to selectively destroy medium-sized spiny GABAergic neurons with a relative sparing of interneurons and afferents,[112,149] as is observed in the HD striatum. This, together with the striking morphological and behavioral similarities, supports the view that a deficit in energy metabolism may be involved in the etiology of HD. In primates subjected to 3-NP administration as two daily injections for 8–10 weeks, progressive decreases in NAA, creatine, and choline could be detected by *in vivo*

^{1}H-NMR spectroscopy before the detection of elevated lactate levels.[113] Changes in endogenous metabolite concentrations were demonstrable before the onset of striatal lesioning. Our own preliminary NMR spectroscopic studies have shown a global increase in succinate and decreased concentrations of NAA, *N*-acetylaspartylglutamate, and taurine, together with a decrease in GABA levels specific to the ventral and dorsal portions of the striatum (manuscript in preparation). Subsequent metabonomic studies have been performed to assess possible therapeutic strategies in toxin models of HD, including drugs to correct brain energy deficits,[39] striatal allografts,[114] and neurotrophic factors.[115]

Xenobiotic studies. NMR spectroscopy has also been used to explore potential neurotoxic effects of xenobiotics. Although hydrazine, a metabolite of isoniazid and hydralazine, is most commonly associated with hepatic steatosis, it is also known to cause CNS effects such as seizures. ^{1}H MAS NMR investigation of brain tissues identified increased alanine, indicative of transaminase inhibition, and decreased hippocampal concentrations of aspartate, NAA, and myo-inositol. Integration of these results with NMR profiles of liver and kidney tissue achieved a more global perspective on hydrazine-induced toxicity.[116]

It is debatable as to whether the use of neurotoxic compounds can be regarded as truly neurodegenerative. However, all animal models are limited by their capacity to mimic only specific aspects of the neurodegenerative condition, in that they simulate certain aspects of pathogenic, histological, biochemical, or behavioral features, but may be of use in furthering the understanding of neurodegenerative mechanisms.

Genetic models of neurodegeneration. Transgenic animal models of several neurological diseases have been generated that are neuropathologically and clinically similar to the clinical disorders. For example, transgenic mice overexpressing human α-synuclein, a major component in the formation of Lewy bodies observed in PD, not only show α-synuclein–positive cytoplasmic inclusions, but reveal loss of nigrostriatal dopaminergic terminals in the striatum and motor impairments.[117] A transgenic model containing a doubly mutated form of the human α-synuclein gene has been shown to exhibit age-related impairments in motor co-ordination and reductions in dopamine.[118] Nuclear magnetic resonance spectroscopy has been applied to characterize the metabolic effects of genetic modification in several models. Brain extracts from mice transgenic for Sandhoff disease, a lysosomal storage disorder resulting from the accumulation of *N*-acetyl–containing glycophospholipids in the brain, revealed the presence of *N*-acetylhexosamine–containing oligosaccharides that were not observed in matched control animals.[119] Additional *in vivo*

MRS studies identified signals from stored glycosphin-golipids, which increased with disease progression.

Several transgenic mouse models exist for HD with the most common being the R6/1 and the R6/2 mouse models containing 115 and 150 CAG repeats, respectively.[118] The R6/1 model is consistent with a less aggressive onset of HD than the R6/2 model. MRS studies report a 26% decrease of NAA in the corpus striatum of transgenic animals at 5 months of age.[120] Nuclear magnetic resonance–based metabonomic studies in the R6/2 mouse model have identified the evolution of characteristic metabolite profiles across several biological matrices (urine, plasma, skeletal muscle, striatum, cerebral cortex, cerebellum, and brain stem). Clear differentiation of transgenic from wild-type mice was achieved in mice as young as 4 weeks old.[76] The metabolic differences observed in the plasma and urine may yield useful biomarkers if they prove to be transferable to humans. The differentiation of choline species by high-resolution NMR spectroscopy[76] allowed the identification of glycerophosphocholine as the primary progressive neurochemical perturbation that was previously attributed to choline.[121] More notably, choline levels were observed to decrease in most of the neuroanatomical regions analyzed in the R6/2 mouse.[76] A recent study using GC in combination with time-of-flight MS analysis of serum reported changes in glycerol, mannitol, and several amino acids suggestive of a procatabolic phenotype in both the R6/2 mouse model and in a small number of HD patients that correlated with disease progression.[122]

The SCA3 mouse is a model for Machado–Joseph disease, a dominantly inherited spinocerebellar ataxia, also caused by expansions of CAG repeats. ^{1}H MAS NMR analysis of brain extracts from this model revealed a consistent increase in glutamine in both the cerebellum and cerebrum together with decreases in levels of GABA, choline, phosphocholine, and lactate.[15]

MONITORING DISEASE PROGRESSION

An important driver in current research into neuronal dysfunction is the identification of reliable biomarkers for disease progression and response to therapeutic intervention. To be truly useful, a biomarker must be quantifiable, reproducible, and analytically simple to measure. In addition, it is preferable that the biomarker is inexpensive to measure, has a limited variation in concentration or expression, and is unaffected by comorbid factors. Biomarkers that can indicate detection of neurodegenerative diseases in an early or even presymptomatic stage are essential. There is a paucity of suitable biomarkers for assessing disease progression in this group of disorders. Currently many potential disease-modifying therapies are being developed and evaluated at the preclinical stage and will lead to clinical trials in the near future for which biomarkers are urgently needed. For the evaluation of therapies the biomarker needs to change linearly with disease progression and closely correlate with established clinicopathological parameters of the disease.[123] Although there may be situations in which a single molecule or protein will fulfill this requirement, in practice most diseases are polygenic in origin, often with strong environmental influences, and consequently there will be a complex syndrome or fingerprint of molecular markers associated with disease state, and the efficacy or toxicity of drug that will evolve through time. Thus, in reality, combinations of biomarkers are far more likely than single entities to form the basis of a reliable diagnostic test. The capacity of metabonomics for generating a metabolic fingerprint is uniquely suited to identifying such combination biomarkers and can be placed alongside conventional clinical assessments encompassing specific cognitive and neurophysiological testing and supplemented with genomic and proteomic profiling. Many different approaches are being undertaken to identify biomarkers and include imaging and neurophysiological and cognitive testing in addition to newer technologies such as biochemical, proteomic, metabonomic, and gene array profiling of tissue and biofluids from patients. Since the metabonomic analysis of biofluids is a relatively noninvasive and rapid profiling tool, it is highly suitable for monitoring of disease progression that requires a multisampling protocol.

Alzheimer's disease

Alzheimer's disease is the most common form of dementia and is difficult to diagnose in the very early stages. However, in recent years, there has been interest in the clinical picture of "mild cognitive impairment" (MCI), which may represent a very early form of AD in some patients.[124] Currently there is no clinical method to determine whether a patient with MCI has incipient AD or has a benign form of MCI without progression; the conversion rate from MCI to AD with dementia is about 15% per year.[125] Thus there is a great need for diagnostic biomarkers to identify incipient AD in MCI cases so that early symptomatic (i.e., cholinsterase inhibitors) or disease-modifying treatments may be given early in the course of disease. MRS is used to measure relative amounts of metabolites in selected brain areas. Reduced NAA/Cr ratios have been suggested as a marker of subsequent conversion from MCI to AD.[126] Decreases in NAA levels in the temporal lobe have been reported to correlate closely with cognitive decline and to distinguish AD patients from a group exhibiting non-Alzheimer's dementia.[127] Elevations in choline levels together with a decline in NAA concentrations associated with AD were also observed in senescence.[41]

Parkinson's disease

Parkinson's disease also can be very difficult to diagnose in its early stages and may be mimicked by other diseases such as essential tremor, multiple system atrophy, and progressive supranuclear palsy. In addition, by the time a patient presents with the clinical features of PD, there is already a ~50% reduction in dopaminergic nigral cells,[128] and it appears that this preclinical phase of PD lasts for ~5 years.[129] Clearly treatment with putative neuroprotective treatments is likely to be most beneficial in this preclinical phase before marked neuronal loss has occurred. Magnetic resonance spectroscopy is used to measure relative amounts of metabolites in selected brain areas. Reduced NAA/Cr levels have been suggested as a marker of cognitive decline in nondemented PD patients relative to controls.[90]

Huntington's disease

Although there is a clear genetic test for HD, clinical trials for HD remain challenging and, to date, only a few double-blind phase III trials have been conducted. There is an urgent need to obtain biomarkers of disease progression and response to therapeutic intervention. The current method of assessment for clinical disease progression is the Unified Huntington's Disease Rating Scale (UHDRS), which lacks sensitivity and specificity particularly over short periods of time. Therefore, the identification of biomarkers that could also be used to track disease progression would be invaluable. In addition, markers capable of detecting disease related changes in presymptomatic HD gene carriers will be essential for the future detection and monitoring of treatments that can delay disease onset. A GC–MS profiling study by Underwood and coworkers[122] indicates that low-molecular-weight metabolites such as glycerol and amino acids may be useful in monitoring disease progression in HD patients.

Multiple sclerosis

Multiple sclerosis is characterized by the degeneration of the myelin sheath leading to neuronal damage and loss that ultimately results in the irreversibility of the debilitating disorder. Alterations in metabolite levels are described by NMR spectroscopy that are specific for axonal damage, demyelination, and inflammation and have been monitored during the remitting–relapsing phases typical of the disease.[23,84,130] Numerous *in vivo* ¹H MRS studies in patients with multiple sclerosis have revealed reductions in NAA in both lesions and normal white matter.[37,131–134] The recovery of NAA, which coincides with the improvement in disability,[135] appears to be related to diminishing edema and remyelination.[84,130]

DRUG DEVELOPMENT AND TOXICITY

Nuclear magnetic resonance spectroscopy generates profiles for molecules of both endogenous and xenobiotic origin and has a long history in the investigation of drug metabolism.[136,137] Both conventional and ¹H MAS NMR spectroscopic analyses have been used to characterize the toxicity profiles of toxins and candidate drugs and to generate information on putative metabolic pathways. Thus, NMR spectroscopy is a useful tool in drug development, which demands rapid resolution of safety issues with an emphasis on interspecies comparisons of mechanisms of toxicity to determine relevance to man. Furthermore, recent studies indicate that the predose metabolic profile can in some instances be used to predict the dominant metabolism of pharmaceutical compounds.[138]

THE USE OF METABONOMICS IN DEVELOPMENT OF THERAPEUTICS

Baseline metabolite profiles obtained from HD and PD patients have been acquired and characterized[79] from which to determine the clinical benefit of intracerebral stem cell transplantation.[139] In HD and PD patients receiving stem cell grafts, concentrations of NAA were not significantly different from controls. Furthermore, the reported replenishment of NAA concentrations in PD and HD patients may suggest the maturation of stem cells *in vivo*, since immature fetal transplant cells possess low concentrations of NAA; however, the infiltration of dendritic processes cannot be ruled out. In mice intoxicated with MPTP, the adoptive transfer of Copaxone immune cells prevented NAA depletion and revealed protective properties toward dopaminergic neurons in the substantia nigra *pars compacta*.[108] In PD patients receiving thalamotomy to control parkinsonian tremor, further reductions in NAA/choline ratios were reported in the substantia nigra after stereotaxic surgery.[140,141] Clinical improvements in motor function in the majority of patients after thalamotomy putatively arises from remote metabolic consequences beyond the lesion site.

In vivo and *in vitro* animal experiments and recent studies in humans have shown that oral creatine supplementation may delay the progression of HD.[90,142–146] Creatine has several potential neuroprotective effects, including buffering intracellular mitochondrial energy reserves, stabilizing intracellular calcium, and inhibiting activation of the mitochondrial permeability transition pore, which have all been linked to apoptotic and oxidative cell death. Initial pilot ¹H MRS studies have shown alterations in glutamate and glutamine ratios after creatine treatment that may be indicative of creatine enhancing the energy-dependent conversion of

glutamate to glutamine, which has been shown to be perturbed in HD.[76,90,121]

A recent study by Coen and coworkers[19] showed that, on the basis of NMR spectroscopic analysis of human CSF, viral meningitis could be differentiated from meningitis of bacterial or fungal origin. A range of metabolites, of both mammalian and microbial origin, were responsible for disease classification. A longitudinal evaluation of a patient with cryptococcal meningitis further showed that the metabolic profiles correlated strongly with response to therapy.

CONCLUSION

The potential of metabonomics in improving the early diagnosis and detection of neurodegenerative diseases has been reviewed, with particular emphasis on the contribution of NMR spectroscopic strategies. Clearly, the hope and expectation around the application of this technology, to the study of human disease, is that novel biomarkers will be discovered, which allow a deeper understanding of the molecular basis of disease processes, a better diagnostic for disease progression, and an efficient means of monitoring response to therapeutic intervention. Individually the "omics" technologies can be a useful addition to the armory of investigative tools used to define and monitor neurodegeneration. Although each of these technologies has room for maturation, there can be no dispute as to their potential value.[16] The integration of metabonomic data with results obtained from transcriptomic and proteomic studies will ultimately achieve a more global understanding of neurodegenerative mechanisms. Integration of "omics" data is a nontrivial task and, to date, few attempts have been made to systematically integrate multilevel data, although an elegant pioneer study in schizophrenia has shown good correlation in transcriptomic, proteomic, and metabonomic data in which evidence of oxidative stress and perturbed energy metabolism was generated by each technology.[147]

With the rapid developments in NMR hardware and computational analysis of spectra, metabonomic technology has the potential to impact strongly on the early diagnosis of the disease. Furthermore, the discovery of appropriate biomarkers will facilitate more effective management and care of patients with these neurodegenerative diseases.

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