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Power of a Metabonomic Approach to Investigate an Unknown Nervous Disease

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1. Introduction

The field of neurological disorders becomes now one of the most important investigation areas in clinical medicine, whatever the toxicological, genetic, degenerative or environmental aetiology they have. Because it involves the main complex organ as target tissue, because also of the intrinsic specificity of the biological network of the nervous system, or the technical difficulty to access such a composite organ, the nervous diseases remain particularly difficult to study. Certainly, the rapid development of transgenic animal models of neurological diseases and the expanding growth of imaging techniques to functionally and non-invasively access some specific brain regions constitute a favourable situation to study the basis and the progression of some nervous diseases. However, the use of such transgenic animals or spontaneous animal models needs that the clinical symptoms are reproducible and that a *prior* knowledge of the aetiopathology of these diseases may exist. These latter conditions are not always available, especially concerning toxicology. In this case, how can both pathophysiology and therapies be investigated? Indeed, classically, when considering a toxicological approach, clinical signs, similar to those ascribed on the target species, need to be reproduced on the animal model. But how to do with disease displaying no known aetiology or with an animal model, on which it is impossible to reproduce, at least partially, some clinical signs of the target species? Furthermore, because of evident ethical reasons added to practical ones, some neurological disorders in humans or in large animals remain scarcely explored. "Omics" approaches seem to be a good alternative in the clinical medical research, enabling to take advantage of the global living system and, simultaneously of the control of the toxicological factor. To illustrate such an original approach, a neurological horse disease, Australian stringhalt, which has been described for several centuries, but for which aetiology is still only partially known, was reassessed using metabonomics in combination with other classical techniques. This has led to show how powerful this method may stand for in clinical medical research and, particularly in neurological studies.

2. Current neurological investigations: Advantages and limits of routinely used approaches and techniques

2.1 Limits of classical studies

Up to now, the neurological investigations tended to reproduce a human disease using a convenient animal model. However, they laboured to give results. In fact, it may appear surprising to recreate all the metabolic complexity prevailing in the genesis of a given disease and, to work on it, before having any knowledge of the specifically involved metabolic pathways specifically involved. Before considering an animal model as a convenient model of a human disease, it seems more consistent to record and describe all the putative impacts of a controlled *stimulus* on a living system without any *a priori* hypothesis because of our ignorance of the inherent metabolic disruptions involved. Indeed, this may help to efficiently tackle a neurological disease.

2.2 Behavioural approaches

The use of animal models of human diseases, on which some behavioural tests are carried out, is fundamental to investigate nervous disorders. The field of psychopharmacology or behavioural pharmacology enables to test and to measure effects of drugs on behaviour. The toxicological studies test the short- or long-term exposure, the acute intoxication or the chronic effects following administration of subclinical doses and the associated effects of chemical compounds or contaminants. Each behavioural manifestation in animal model tends to reflect a specific human behavioural alteration or cognitive effect like depression, anxiety, fear or schizophrenia. This may be susceptible to reveal a disruption in some mean way of neurological transduction involving, for example, dopamine, acetylcholine, amphetamine or catecholamine's impairments. However, this approach has some limits. In case of the lack of any behavioural manifestation in animals, the conclusion isn't that there is a lack of effect but only that there is an impossibility to give an interpretation of this lack of effect because of an inadequate "observation window" as in delayed toxicity of some contaminants for example. Moreover, the putative link between a visible behavioural impairment and a putative mechanistic explanation requires going back to the cerebral metabolism to translate the observed behavioural variance and to confirm the pertinence of metabolic pathways specifically involved. Most of the time, such behavioural approaches are hardly self-sufficient; they need to be completed by other studies such as metabolic, histological, anatomical or immunologic ones.

2.3 Imaging techniques

A wide range of imaging techniques provides powerful tools for studying tumours (Cooper et al., 2011), congenital diseases (Toga et al., 2006), metabolic and infectious diseases (Kastrup et al., 2005), development of organisms (Davis et al., 2011) and for realizing preclinical or clinical studies, or for measuring a treatment effect (Song et al., 2011). These techniques can also be used in neurotoxicology (Pogge and Slikker, 2004) or for exploring neurodegenerative or psychiatric disorders (Masdeu, 2011; Stoessl, 2011). The choice of one of these techniques is made according to some awaited answers to a specific anatomical, metabolic or functional information question, some of imaging techniques being able to perform several specific assessments. They are well adapted to describe functions in the frame of non-invasive *in vivo* studies, some being planned with a longitudinal follow-up. Concerning some specific tissues analyses, some compromises have to be done between the

spatial or the temporal resolution according to what it has to be focussed on. Among these different techniques, anatomic or functional imaging techniques have to be distinguished. The first ones, tomodensitometry and magnetic resonance imaging or MRI (Griffith et al., 2007) provide highly detailed anatomic information. Their ability to give an access to *in vivo* biological information acquired non-invasively or to define a seemingly normal body composition and its perturbation in response to a pharmacological or a pathological event may facilitate exploration of nervous diseases (Frisoni and Filippi, 2005; Griffith et al., 2007; Tartaglia and Arnold, 2006). In parallel with the description of novel biomarkers coming from transgenic animal models developed for studying neurodegenerative diseases and more efficient therapies, the use of MRI and magnetic resonance spectroscopy (MRS) provide new information for *in vivo* neurochemistry, such as neuronal apoptosis, osmoregulation, energy metabolism, membrane function or signalling disruptions (Choi et al., 2007; Ross and Sachdev, 2004; Ross and Bluml, 2001). Most of clinical researches are based on the metabolites that are detectable using proton spectroscopy (Figure 1), which can quantify them in localized volumes in brain.
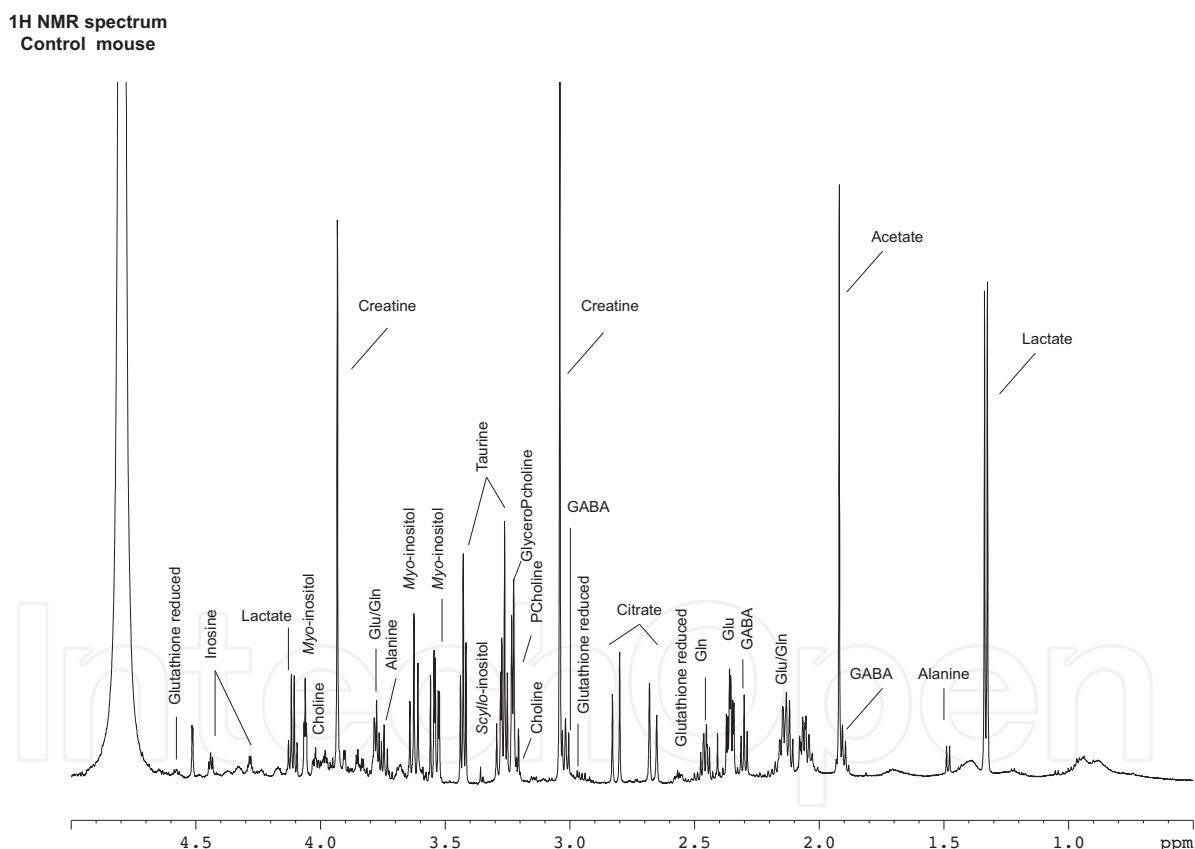

Fig. 1. 600.13 MHz 1H NMR spectra from aqueous extracts of brain in mouse (control animal) (from Domange, 2008)

Nuclear magnetic resonance (or NMR) methods (MRI or MRS) can be successfully used to reveal neurological markers like N-acetyl-aspartate (a neuronal and axonal marker associated with neuronal viability), *myo*-inositol (a cerebral osmolyte and an astrocytic marker), glutamate and glutamine (the first is a major excitatory neurotransmitter and the second can restore it), creatine (which plays a crucial role in ATP biosynthesis in astrocytes),

choline (its increased concentration theoretically means an alteration of myelin) and gamma-amino butyric acid (a main inhibitory neurotransmitter) (Martin, 2007). But MRS can also record intrinsic containing metabolites containing other atoms, as phosphorus, sodium, potassium, carbon, nitrogen and fluorine (this last atom often being a constituent of many drugs). Among the functional imaging techniques, positron emission tomography (PET) is a three-dimensional diagnostic imaging technology used in nuclear medicine that measures physiological function by looking at various functions of the body. It is a non-invasive diagnostic imaging tool enabling to follow some chemical neurotransmitters like dopamine in Parkinson's disease. Whatever the technique used to cover a specific neurological question, most of the time, it often requires laboratory animal and more particularly animal models of given human diseases.

2.4 Laboratory animals model contribution

The use of animal models in clinical research is crucial. As models, they usually display the same features and clinical signs as those observed in humans. So, they enable to establish some comparisons and extrapolations with the human physiology, to give access putative metabolic mechanisms involved in the progression of the disease and, hence, to identify biomarkers. A wide range of animal models has been used according to their origin. Animal models can be spontaneous, namely "mutant". Therefore, identification and characterization of novel laboratory animal lines carrying an interesting mutation combined with genotype-driven approaches are useful approaches to investigate some specific mechanistically-related molecules, to give new information about the function of the mammalian nervous system (Banks et al., 2011) or to study how genetic, environmental, toxicological or dietary factors can explain aetiology of a given disease. Animal models can also be created, using surgery, pharmacology or genetic interventions. These models are used to identify aetiological markers of disease or drug target and to test some new therapeutic drugs. The first cases have traditionally been induced by neurotoxins, acting selectively on neurons affected by human diseases. They are particularly useful for the study of the pathogenetic mechanism or to test new therapies for human neurological disorders (psychiatric or motor disorders) like obsessive-compulsive disorder or Parkinson's disease (Nowak et al., 2011). In parallel, the knockout technique, in which a gene is made inoperative leading to animals deficient in one specific gene, enables to evaluate the effects of the depletion of one protein in all the series of biological reactions within an organism and the putative followed consequences (Berman et al., 2011). More recently, the use of transgenic animals, constructed by inserting a human gene downstream into promoter, followed by microinjection in animal, ensures to indicate whether an over- or under-expression of a gene in one or several tissues should be susceptible to promote the pathogenesis and the development of a disease (Liu et al., 2011). The common point of all these animal models is the necessity of having some preliminary knowledge concerning a disease or the deleterious effect of a given xenobiotic. However, this information is not always available. Therefore, researchers have apace become aware of the necessity to access a wider range of knowledge in a living system and not only a specific molecular entity.

3. "Omics" approaches and their interest in clinical research

3.1 "Omics" approaches presentation

Similarly to imaging techniques, omics-based approaches appeared to be used according to the biological pool they consider (genes, proteins, lipids, metabolites) and the nature of

target they have to reach (gene, enzymatic mechanism, biomarker). The full range of metabolites synthesized by a given biological system corresponds to its metabolome. In the same way, the full range of genes is contained in the term genome, the mRNA and the proteins ones, respectively, in the terms transcriptome and proteome (Figure 2). All these systems can be defined according to the level of biological organization, *i.e.* organism, organ, tissue, and cell. Related to these biological levels, omics-based approaches, mainly genomics, transcriptomics, proteomics (Colucci-D'Amato et al., 2011), lipidomics (Li et al., 2007), and metabolomics are terms standing for various global molecular-oriented approaches to better understand the underlying mechanisms, as the physiological regulations and the networks involved on all levels of gene products (mRNA, proteins, metabolites) in their respective systems and, if possible, between different sub-networks. Indeed, the observable property of organisms, *i.e.* their phenotype, is issued from genotype submitted to the concomitantly interactive action of the environment. Most of the time, the association between some of these approaches can be beneficial, enabling to understand the temporal progression of a pathophysiological state or the functioning of metabolic networks (Fiehn, 2001). Interest of these methods is to apply a controlled disruption to a biological system, whatever its nature (genetic, toxicological, pathophysiological, dietary), under some
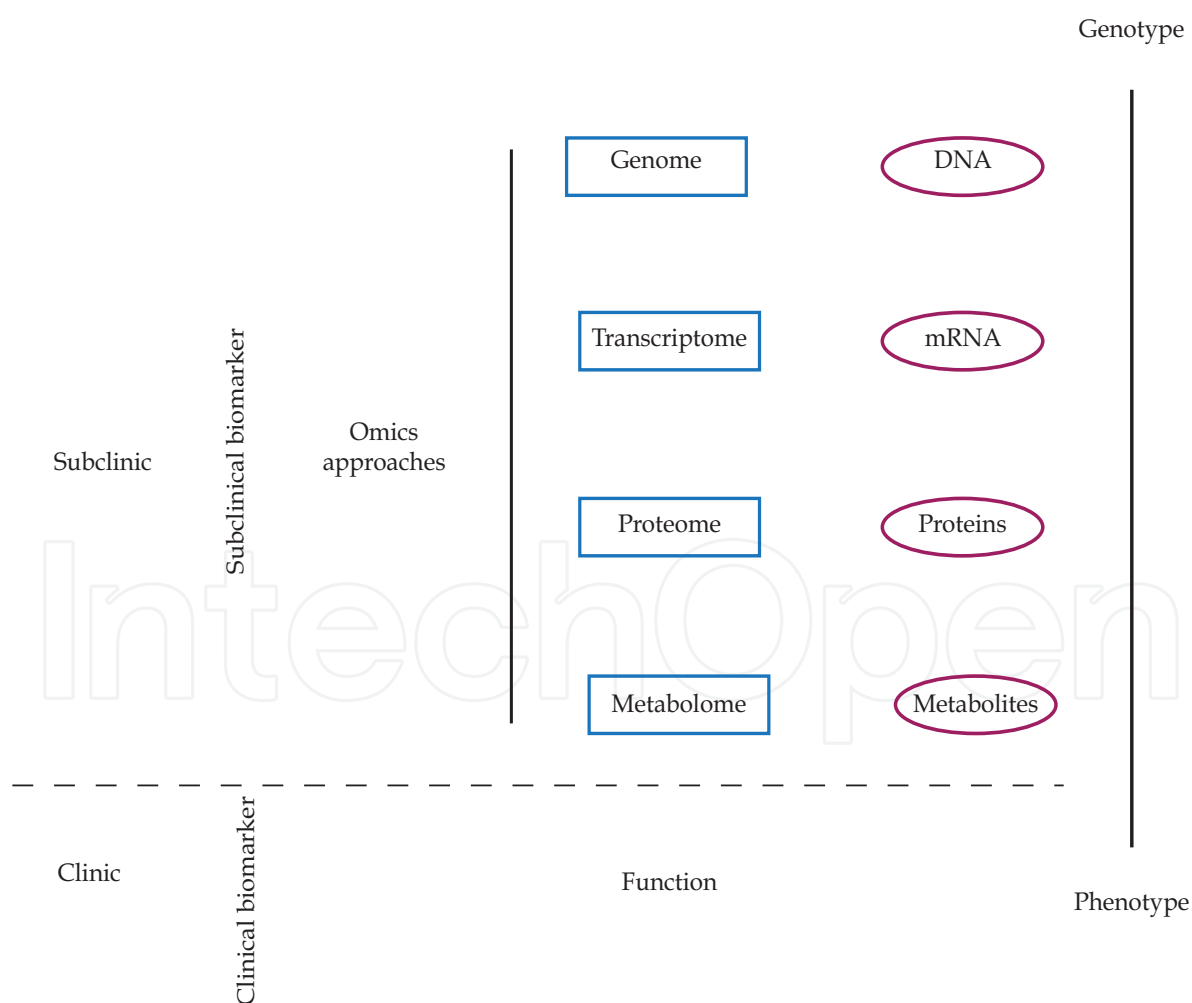

Fig. 2. "Omics" approaches and their different levels of biological living systems investigation

specific conditions (most of the time, the investigation is focused on an animal model), and to consider the subclinical consequences of such a disruptive perturbation. Therefore, "omics" approaches are widely used in biomedical research to make easier the understanding of disease mechanisms and to give access target tissues, to make easier the identification of biomarkers useful for therapeutic and diagnostic development and to predict clinical responses to treatments. The large amount of acquired data is as much an advantage as a hindrance. Indeed, the challenging subtlety is that all this information needs to be explored without any *a priori* hypothesis but by extracting only the interesting data. This fact partially explains why the real capacity of "omics" technologies stays in some instances rather limited because of the necessary requirement of some specific bioinformatics tools to efficiently mine multidimensional data but also the requirement of some specific analytical database to identify the candidate biomarkers at the gene, mRNA, enzyme, protein, or metabolite level. Moreover, transcriptomic studies require high-cost technologies and so, are less used than proteomic ones, which are based on two-dimensional gel-electrophoresis, which is cheaper and can be more easily used in many laboratories. However, analytical techniques related to the detection of large arrays of metabolites seem more robust, the resulting information being often easier to interpret because of the lower number of molecular entities, even though a rigorous identification of new metabolites still remains particularly fussy. According to the aim of studies and considering an increasing level of complexity of the analytical strategy used, investigation of metabolites may require either a metabolic profiling approach, which is focused on a small number of known metabolites (targeted metabolic profiling), or metabolomics including investigation of several classes of compounds (open metabolic profiling) or functional genomics, also called metabolic fingerprinting or metabonomics. This latter one is based on classification of samples according to their biological relevance to the studied disruption event and on identification of the fully informative markers detected at the statistical and functional sides and measured within the analyzed biological matrices.

Therefore, among these omics-based approaches, metabonomics stands for one of the most used holistic methods. Its emergence and its development mainly come from pioneering works of Pr J. Nicholson from the Imperial College of London. Because metabonomics enables to identify and quantify simultaneously low molecular weight compounds (metabolites) using spectroscopic methods such as nuclear magnetic resonance (NMR) or mass spectroscopy (MS), it gives access to a molecular level and may define the quantitative measurement of multiparametric metabolic responses of living system to pathophysiological stimuli. This can bring to the determination of some comprehensive metabolic signatures of biological matrices (Nicholson et al., 1999; Robertson, 2005). Metabonomics approach can be divided into successive steps. After the crucial step concerning the development of the experimental design, the choice of the animal model, the choice of the instrumentation used to quantitatively generate the metabolic information, the choice of samples of interest to be collected during the animal or the human experiment (biofluids such as plasma, urine, cerebral spinal fluid, saliva or faeces, tissues or organs), these biological samples are treated using appropriate analytical techniques. These latter ones enable to establish a metabolic fingerprint through the spectrum recording for every sample. All these fingerprints are summed up into datasets, in which metabolic information is subdivided and identified through coding variables. Each of them stands for either an integration bucket in NMR spectra corresponding to a defined chemical shift, or a relative or an absolute intensity of the

ionic current measured at a specific mass to charge ratio in MS. Datasets are then treated using sophisticated statistical tools, *i.e.* multivariate or multidimensional statistical analysis tools, to access the most suitable model able to discriminate the different groups of samples according to the studied factors and to reveal main variables explaining this segregation. These variables can be considered at this step as many putative biomarkers, which need to be fully characterized by convenient structural identification methods. Finally, a detailed map of regulation and interaction between identified metabolites, their disruptions and the putative explanation of the pathophysiological state according to all involved factors may be suggested. Among analytical techniques mainly used in metabolomics, MS spectroscopy coupled to liquid (LC-MS) or gas chromatography (GC-MS) and NMR spectroscopy are the most appropriate ones concerning analysis of biofluids or liquid samples, whereas high resolution magic angle spinning (HR-MAS) NMR and MRS are adapted to solid samples like tissue or to achieve *in vivo* studies, respectively.

3.2 An integrated and functional approach

As previously mentioned above, the major constraint the "omics" approaches have to answer to, is to give access to a global pool of information belonging to the living system without focusing on a specific molecular entity. Indeed, one of the main assets of these approaches is the property of data integration necessary to render it as functionally understandable as possible. These features can be revealed through three complementary characteristics, namely *i)* the global nature of living systems underlined by homeostasis, *ii)* the multifactorial nature of diseases with both intrinsic and extrinsic factors, and *iii)* the ability to access multiple biological levels in living systems, and then to compile them to reveal one of the most realistic progressions of a disruption within a complex organism. Let's go into details of these three points. i) Contrary to classical biochemical approaches, which are set out to study only a single or few metabolites or metabolic reactions at the same time, metabolomics provides quantitative data on a wide range of known and unknown metabolites. It enables to visualize an overall pattern comprehensively linked to a set of interactions between metabolites or metabolic pathways and, hence, to an intrinsic homeostasis defined in these specific conditions (Kaddurah-Daouk et al., 2008). Indeed, whatever the stress applied on living systems, some allostatic changes, defined as an adaptive process, lead to short-term corrective changes of the different relevant regulatory systems to maintain a metabolic homeostasis. Concept of homeostasis is fundamental in biology and in clinical medicine to understand pathophysiological processes. The current clinical medicine tends now to come back to a more global view and, at the same time, on a more individualized approach of every patient because each of them differently answers to the environment according to their own homeostatic specificity. Clinical and subclinical signs give personalized information for every subject and, hence, physiological "means" used to adapt for everybody the set of parameters of homeostatic control in response to a disruptive stimulus and so to avoid falling down in a pathological state. The understanding of overall adaptation requires a good knowledge of metabolic pathways and related biochemical networks involved in the efficient control of homeostasis. For example, the knowledge of the glucose metabolism and the different ways by which homeostatic control of the circulating glucose concentration is achieved is crucial in the investigation of the Type 2 diabetes (Fiehn et al., 2010). ii) From global approaches can emerge a more accurate understanding of a given disease considering it is not only a single functional entity which is

concerned, that is not only the consequence of a single causative explanation with a single mechanism involved in a single cell type in a given condition. Indeed, most of the pathophysiological disorders are not unique functional events but are resulting from complex interactions. These latter ones involve different concomitant actions in different biological compartments leading to different disruptions, which can be categorized according to the environmental conditions encountered and the inherent variability of subjects. Becoming aware of the importance of the environment and, more particularly, of the multifactorial nature of most of the disruptive events displayed by a living system is among the first pillars of the concept of global approach used in biological research in clinical medicine or in toxicology. iii) Finally, as a microscope could do it, omics-based approaches enable to focus on a specific level of a living system depending on the available analytical techniques used to generate data, but also to statistically integrate data coming from complementary fingerprinting techniques by using canonical analyses.

3.3 A metabolomic-based approach to reveal subclinical metabolic disruptions: A powerful tool in investigation of biomarkers

Besides the ability to define and to understand the aetiology of a disease, the discovery of novel biomarkers stands for a fundamental step to characterize and to manage it, especially to spot the homeostatic break down before appearance of the first clinical signs. Biomarkers, which are relevant indicators of disrupted biological processes in a given pathophysiological context, have to disclose features of disease (Moore et al., 2007; Nicholson and Lindon, 2008). The metabonomic approach is particularly interesting to explore subclinical disruptions of a living system before the outset of manifest clinical signs, and to identify biomarkers of disease risk and, if possible, to initiate prevention like in cancer (Roberts et al., 2011), diabetes (Wang et al., 2011), or nervous system illnesses (Kaddurah-Daouk and Krishnan, 2009; Nicholson and Lindon, 2008; Quinones and Kaddurah-Daouk, 2009). The identification of the metabolites requires the use of up-to-date structural databases of metabolites and metabolic pathway resources (Kouskoumvekaki and Panagiotou, 2011). As it has been previously mentioned, the use of complementary approaches stands for a wise way search of biomarkers displayed at different levels, namely biochemical, neuroanatomical, metabolic, genetic and neuropsychological ones, as it can be reported in the case of Alzheimer's disease investigation (Wattamwar and Mathuranath, 2010).

3.4 Examples of "omics" approaches in neurological investigation area

Use of metabolomics in neurological studies has been reported in many reviews (Choi et al., 2003; Rudkin and Arnold, 1999). It has been applied to a variety of biological samples for a better understanding of pathogenesis. This approach, because of its integrated and functional nature, stands for a powerful tool to study normal or pathological living systems, especially in central nervous system disorders through the use of specific animal models (Pears et al., 2005). Thus, it allows the identification of biomarkers of such diseases, but also of illness progression or response to therapy. In the drug discovery process, metabolomics brings some biochemical information about drug candidates, their mechanism of action and their therapeutic potential. In the field of neurosciences, the use of metabolomic approach can generate some questionings. Contrary to other organs in mammals, brain is isolated from the rest of organism by the blood-brain barrier, with consequences on the passage of some metabolites. Therefore, a metabolic fingerprint of brain predicted from a blood or

urine metabolomic analysis is not prone to reflect the real state of the subject, contrary to data coming from other organs like liver and kidney. Nevertheless, some first encouraging studies on neurological disorders performed using metabolomics have confirmed the interest of application of this approach in the field of neuroscience (Griffin and Salek, 2007). Analysis of blood or urine gives access to putative cerebral disruptions and can help to successfully reveal some biomarkers, as in the case of the manganese neurotoxicity, which is a significant public health concern (Dorman et al., 2008). So, because it reflects the presence of both extrinsic and intrinsic disruptive factors, metabonomics can define accurate biomarkers in neurology. Moreover, some specific metabolic pathways or some biological disruptions can be particularly interesting to study, because of their central or ubiquitous role in many pathological states. One example is the oxidative stress, leading to neuronal death, a mechanism that is found in early stages but also in secondary manifestations of many neurodegenerative states like Alzheimer's, Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis, and neuroinflammatory disorders (Sayre et al., 2008). Because of the pivotal role of a metabolite in many biological functions, a

better understanding of some metabolic pathways like the biosynthesis of the amino acid L-serine can be interesting to investigate (Tabatabaie et al., 2010). Metabolic profiles acquired on human or animal biofluids like urine, cerebrospinal fluid (Lutz et al., 2007b), plasma, serum or tissue extracts, using either NMR or MS techniques, can give some precious information concerning neurological disorders (Sinclair et al., 2009). For example, ultra performance liquid chromatography/mass spectroscopy (UPLC/MS) metabolic profiles from serum collected on cerebral infarction patients have been analyzed using a metabonomic approach (Jiang et al., 2011). Quantitative analysis of human cerebrospinal fluid using NMR spectroscopy has been performed in multiple sclerosis (Lutz et al., 2007a), to identify biomarkers in the early stages of the amyotrophic lateral sclerosis (Blasco et al., 2010). Plasmatic metabolic disruptions between healthy and old persons with Alzheimer's disease were investigated using UPLC/MS-based metabonomic approach (Li et al., 2010). CRND8 transgenic mouse, model of this disease, enabled to analyze brain extracts using ^{1}H NMR spectroscopy (Salek et al., 2010). The interest of brain extracts coming from an animal model has been also illustrated to investigate epilepsy, for which the pharmacologically-induced animal model was obtained using pentylenetetrazole, a drug that induces seizures (Carmody and Brennan, 2010). Plasma from an experimental animal model of the spinal cord injury (Blasco et al., 2010) has been analyzed by ^{1}H NMR to get fingerprint profiles of this pathology (Jiang et al., 2010). Other cerebral alterations like brain tumors (Tate et al., 1996; Tate et al., 1998), schizophrenia and meningitis (Holmes et al., 2006; Lutz et al., 2007a) have also been investigated.

Beyond the use of a unique "omics" approach, it seems that it is all the more interesting and powerful to call for several complementary approaches and to tend to integrate so-generated data to yield a more comprehensive understanding of many diseases. In this way, Caudle et al. have used "omics" to characterize and identify some biomarkers of Parkinson's disease (Caudle et al., 2010). As an example, the following part illustrates the power of such a use, in a rodent model, of a neuro-intoxication caused in the horse by a plant, *Hypochoeris radicata* (L.). Indeed, because of the lack of knowledge about a neurological disease described only in the horse, we have tempted to use a laboratory animal model of this intoxication by applying metabonomics combined to imaging or behavioural experiments to reveal, in brain, candidate biomarkers of this pathology.

4. Example of a metabonomic approach of a neurological horse disease, the Australian stringhalt or how to address a toxicological issue on a seemingly non-target species without referring to a known toxic molecule

4.1 Problem for studying such an animal disease

Australian stringhalt is the name of a horse disease described since the middle of the 19th century in Australia (Robertson-Smith et al., 1985). It is defined as a syndrome characterized by an abnormal gait and an involuntary hyperflexion of both hind limbs during movement (Figure 3).

Fig. 3. Horses displaying clinical signs of Australian stringhalt (grade IV on the left, grade V on the right) (from (Collignon, 2007))

Since this time, several other outbreaks had been reported in many countries such as New Zealand (Cahill et al., 1985; Cahill et al., 1986; Cahill and Goulden, 1992), Chile (Araya et al., 1998), United States (Gay et al., 1993; Huntington et al., 1989; Robertson-Smith et al., 1985; Slocombe et al., 1992), Italy (Torre, 2005), Brasil (Araujo), more recently in France (Domange et al., 2010; Gouy et al., 2005) and were suspected in Japan (Takahashi et al., 2002). According to most of the authors, a plant of the Asteraceae family (formerly Compositeae family), *Hypochoeris radicata* L. also named cat's ear, flatweed or capeweed was suspected to be responsible for this disease (Araujo et al., 2008; Gardner et al., 2005; Gay et al., 1993; Gouy et al., 2005). This rosette-forming herb with a yellow terminal flower has a deep taproot, giving it resistance to drought. This explains a growth achieved preferentially on poor-quality pastures after a prolonged dry period, mainly in late summer and early autumn. Such climatic conditions, associated with the aggressiveness and the dominance of *Hypochoeris radicata* L. on other species, enable it to colonize pastures and to become the major plant available as herbivore feeding. These favouring factors, in aggravation for many years because of the global change in climatic conditions, appeared particularly marked in 2003 in France, after a blistering and dry summer, leading to an epizooty with a few tens of recorded intoxicated horses (Domange et al., 2010; Gouy et al., 2005). These latter's showed a wide range of symptoms but mainly dominated by several severity degrees from grade I to grade V, (Huntington et al., 1989) with an involuntary exaggerated hyperflexion of hind limbs and a delayed extension of hocks during forward movement. A marked atrophy of the hind limbs musculature, especially in the distal muscles, is often associated with this gait in the most affected animals. Most of the time, this amyotrophy is related to neurological lesions of the hind limbs with a proximal-to-distal gradient in the intensity, *i.e.* a loss of fibres, a decrease of the number of large myelinated nerve fibres, in agreement with the supposed pathogenesis described as a distal axonopathy (Cahill et al., 1986; Domange et al., 2010). However, in spite of these rare epidemiological and pathological data, the link between this horse disease and the toxicity of *Hypochoeris radicata* (HR) has been poorly investigated in spite of a recent study, which tended to reproduce the disease on animals after a 50-day HR treatment (9.8 kg HR/animal/day) (Araujo et al., 2008). The lack of investigation of such a disease is further partially explained by the critical approach of the nervous system, especially the peripheral nervous system and by the only target species. Besides, we need to consider ethical and financial issues. Moreover, as Araujo and colleagues underlined, the plant material is susceptible to differ in toxicity depending on several factors, one being the geographical location (Araujo et al., 2008).

4.2 Concept of orthology and interest in metabonomics

Most of the time, investigating a disease often requires a convenient laboratory animal model enabling to reproduce clinical symptoms, to access pharmacological data, to reveal some biomarkers of the disease and, in the best cases, to suggest some therapeutic treatments. Because of the nervous nature of Australian stringhalt, the fact that this illness was only described in target species, and the difficulties to link the supposed plant (more particularly if a specific secondary metabolite present in the plant would be involved) to the pathogenesis, the assessment of such an induced intoxication using a "classical" neurological approach seemed not efficient enough to reveal valuable biomarkers. Data obtained until recently remained too scarce. The "omics" approach, more particularly metabonomics, appears to be the most suitable mean to obtain some pertinent information about the target organs and candidate metabolic biomarkers by using an *a priori*

"metabolically competent" animal model. The orthologous hypothesis considered in the case of an induction of a metabolic disruption in a rodent animal model of another animal species, here horse species, is crucial in characterizing a set of candidate metabolic biomarkers. Even though clinical signs may strikingly differ between the two species, some metabolic similarities may exist between their metabolic networks, particularly in their ability to be similarly disrupted by one or few toxicants. Among these latter's, plant secondary metabolites, for which nothing is known at the chemical and pharmacological sides, can be studied.

4.3 Use of complementary approaches: ^{1}H NMR-based metabonomics, MRI and behavioural tests

4.3.1 Metabolic fingerprints on biofluids and tissue extracts

Using the orthologous metabolic disruption assumption existing between two species, horse and mouse in the present case, metabonomics was used to investigate at the metabolic side this orphan neurological disease, Australian stringhalt. The purpose was to combine it with MRI as published elsewhere (Griffith et al., 2007) and with behavioural tests to improve the functional understanding of the metabolic data. Based on the orthologous hypothesis previously mentioned, the mouse was chosen as a "metabolically competent" laboratory animal model of horse intoxicated by HR, even though this rodent model of exposure to HR does not display any observable clinical sign. In a first time, metabonomic studies using male and female C57BL/6J mice fed for 21 days a diet containing 3 or 9% HR had been performed (Domange et al., 2008). ^{1}H NMR spectroscopy analyses have been done on weekly collected urine samples but also on tissue extracts prepared from liver and brain tissues collected at 0, 8, 15 and 21 days of treatment, after sacrifice of a subpopulation of the animals included in the experimental design. Urine and liver analyses were performed to detect the putative systemic disruption after the HR ingestion, and the brain analysis to access the nervous system disruption. All ^{1}H NMR spectra were acquired at 300 K on a Bruker DRX-600 Avance NMR spectrometer operating at 600.13 MHz for ^{1}H resonance frequency, using a cryoprobe and the 1D "Improved Watergate" sequence for suppression of water resonance. Multidimensional statistical analyses of fingerprint data were achieved on log-transformed variables. After removing redundant variables, linear discriminant analyses and partial least-squares regression-based discriminant analyses (PLS-DA) were performed on NMR data to maximize the groups' separation on a factorial map. Projection of these groups on every factorial axis enables to associate canonical ^{1}H NMR variables to the axis construction revealing thus the respective influence of the different factors of interest (gender, intoxication duration, toxicant dose). Therefore, the main part of the metabolic information related to urine ^{1}H NMR data and enabling the discrimination between the different groups of animals can be summed up in a factorial map (Figure 4). On this map, appears the temporal evolution between day 0 and day 21 of the metabolism of animals. This latter depends significantly on the gender of mice, through the 1st axis (this factor contains the main part of the variance explained by the statistical model used) and on the diet factor, through the second axis, covering from the bottom part of the factorial map diets without HR (named "control") to the middle part, diets with 3% HR (named "3%HR"), then to the top part, diets with 9% HR (named "9%HR"). By searching the first variables involved in the second axis construction, we reached the main metabolites, the concentration of which was influenced by a HR-induced metabolic disruption (Domange et al., 2008).
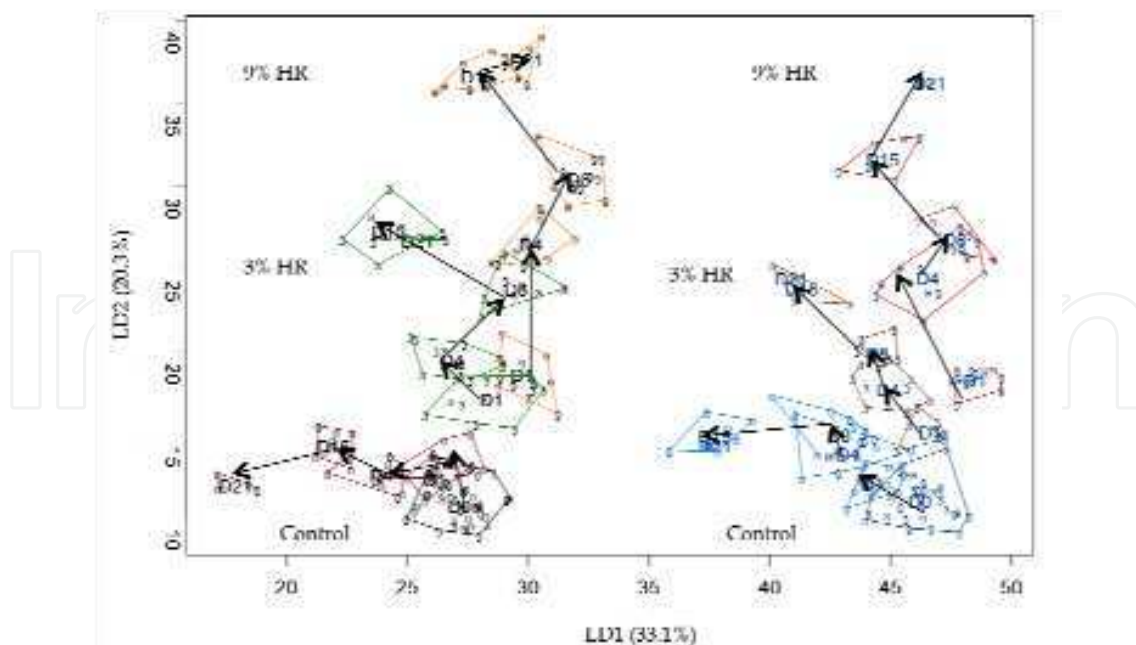
Fig. 4. LDA performed on 150 metabolic variables selected from fingerprints obtained by ^{1}H NMR performed at 600.13 MHz on 332 urinary samples (from Domange et al., 2008). The dummy variable selected is the « group » factor. A 61.5% amount of the total metabolic information is projected on the factorial plan LD1 x LD2. Arrows stand for metabolic trajectories throughout the study followed by every group fed either a control or a 3 or 9% HR diet. Barycenters give the dates of urine collection and correspond to the duration of HR intoxication (d8, day 8; d15, day 15; d21, day 21)
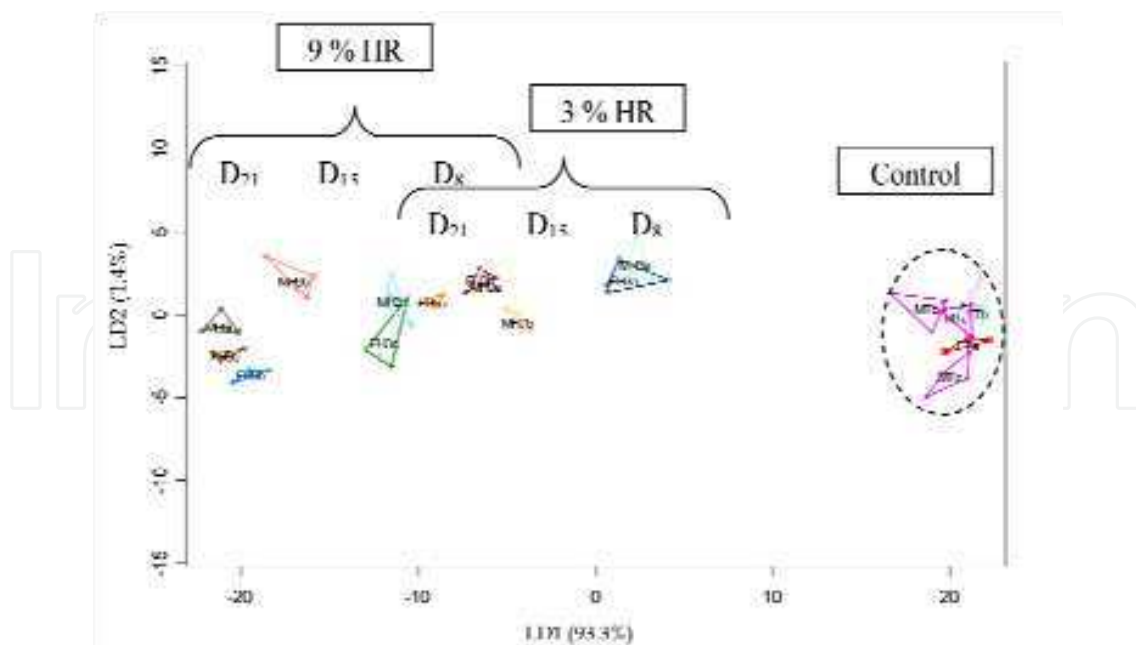
Fig. 5. LDA performed on 20 variables filtered from 600.13 MHz ^{1}H NMR data of brain aqueous extracts from male and female mice according to groups and in agreement with the two first components (from Domange, 2008). Barycenters give the date of brain collection and correspond to the duration of HR intoxication (d8, day 8; d15, day 15; d21, day 21)

In a same way, the main part of metabolic information contained in ¹H NMR data characterizing liver and brain hydrosoluble extracts and enabling the discrimination between the different groups of animals during the experiment could be summed up into a more complex factorial map (Figure 5). Firstly, is displayed the temporal evolution of the brain metabolism of mice orally exposed or not to HR, which holds almost all the part of the variance explained by the statistical model with, respectively from the right to the left side, a projection of the cerebral metabolisms of control animals, then the 3%HR-treated mice, and finally, the 9%HR-treated ones (Domange, 2008). The factor "time" is clearly revealed through every HR treatment with, respectively, from the right to the left side, an emphasis of the disrupted metabolism in a given direction all along the experiment duration. Given the fact that the two matrices of ¹H NMR fingerprinting data obtained on hydrosoluble brain and liver extracts were issued from the same individuals, a global correlation using a canonical analysis (PLS2 here) have been performed between them. A significant correlation between the two first PLS2 components has been revealed (Figure 6), in which, the gender factor is orthogonally projected to the diet one. Concerning the projection of variables involved in the variance calculation, *i.e.* the information explaining this construction, on the same plot, we can show that liver and brain ¹H NMR fingerprint data display close
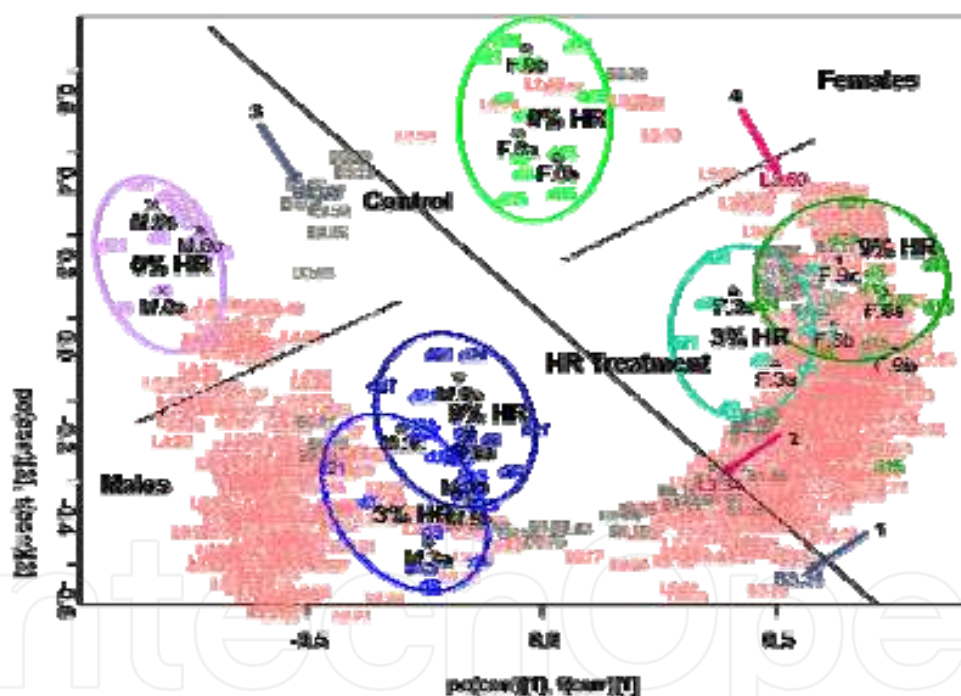

Fig. 6. Resulting biplot performed on the two first PLS2 components calculated between the hydrosoluble liver and brain extracts (from (Domange et al., 2008). Only the projection of the variables with contribution is above 0.5 is displayed (in grey for brain variables, in pink for the liver ones). Most of the variables containing the variance explained by the statistical model is spread according to the gender factor for the liver and according to the diet concerning the brain. The brain variable named B3.34 (arrow numbered 1) and the corresponding liver variable named L3.34 (arrow numbered 2) stand for the *scyllo*-inositol, detected at δ = 3.34 ppm. The brain variable named B3.60 (arrow numbered 3) and the corresponding liver variable named L3.60 (arrow numbered 4) stand for the *myo*-inositol detected at δ = 3.60 ppm
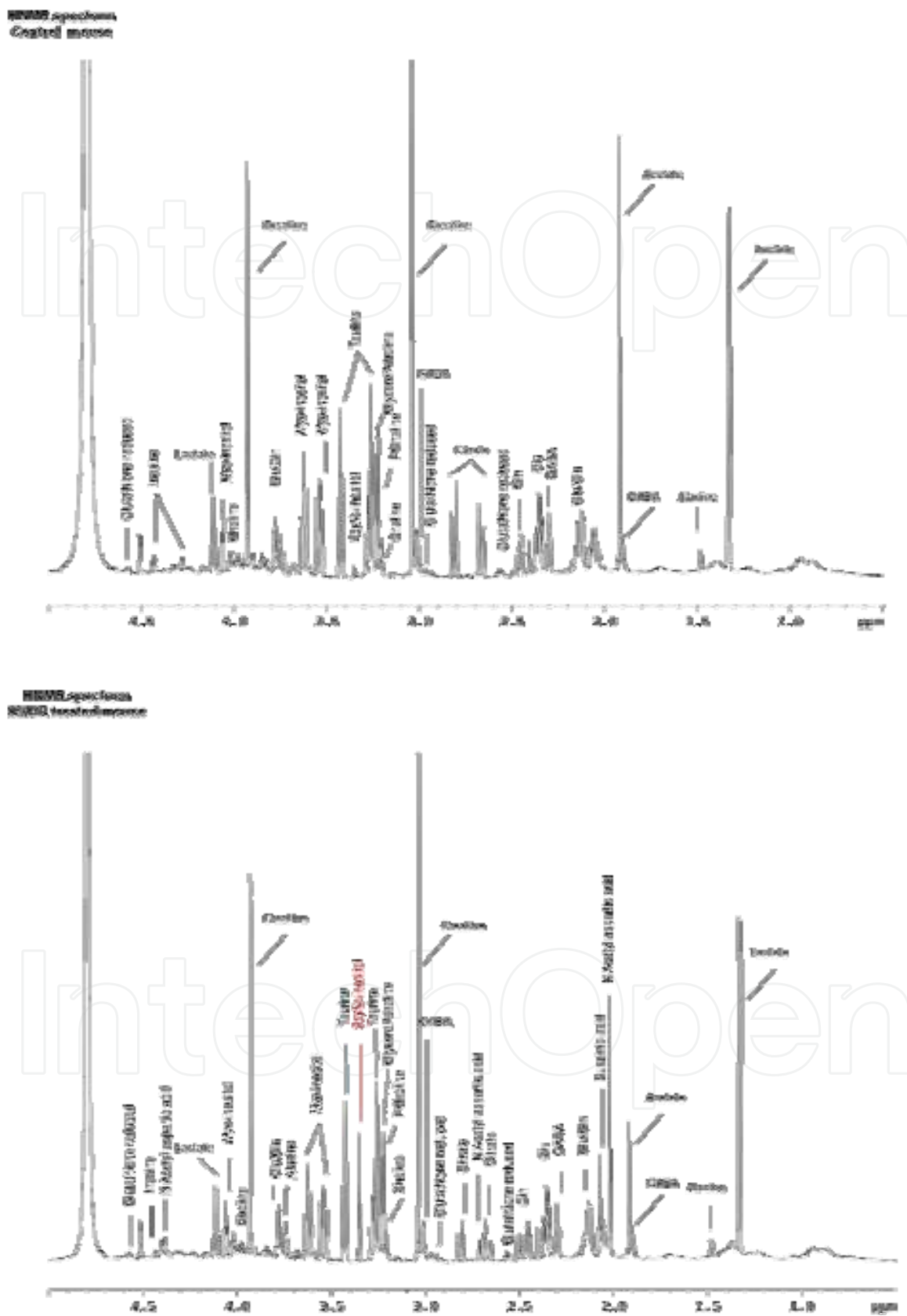
Fig. 7. 600.13 MHz ¹H NMR spectra from aqueous extracts of brain in 9% HR-treated mouse and control mouse (from Domange, 2008)

information. Among the main variables involved in the segregation of the groups of animals, *i.e.* which are related to the HR-treatment factor, and whatever the biological matrix analysed, the first identified variables correspond to the chemical shifts of the *scyllo*-inositol (δ = 3.35 or 3.36 ppm in urine and in liver extract fingerprints, δ = 3.34 ppm in cerebral and liver fingerprints), which are positively correlated to HR-treatment, when the *myo*-inositol ones (δ = 3.60 ppm) are negatively correlated to HR-treatment (Figures 7 and 8). Moreover, the comparison between ^{1}H-NMR metabolic fingerprints in control and HR-fed laboratory animals revealed a dose-dependent increase of the ratio *scyllo*-inositol/*myo*-inositol in urine, plasma, and hydrosoluble extracts of liver and brain of the HR-treated animals, enabling us to reveal some putative candidate metabolic biomarker(s) even though no aetiological factor was characterized, and no requirement of the target species was performed in this toxicological exploration.
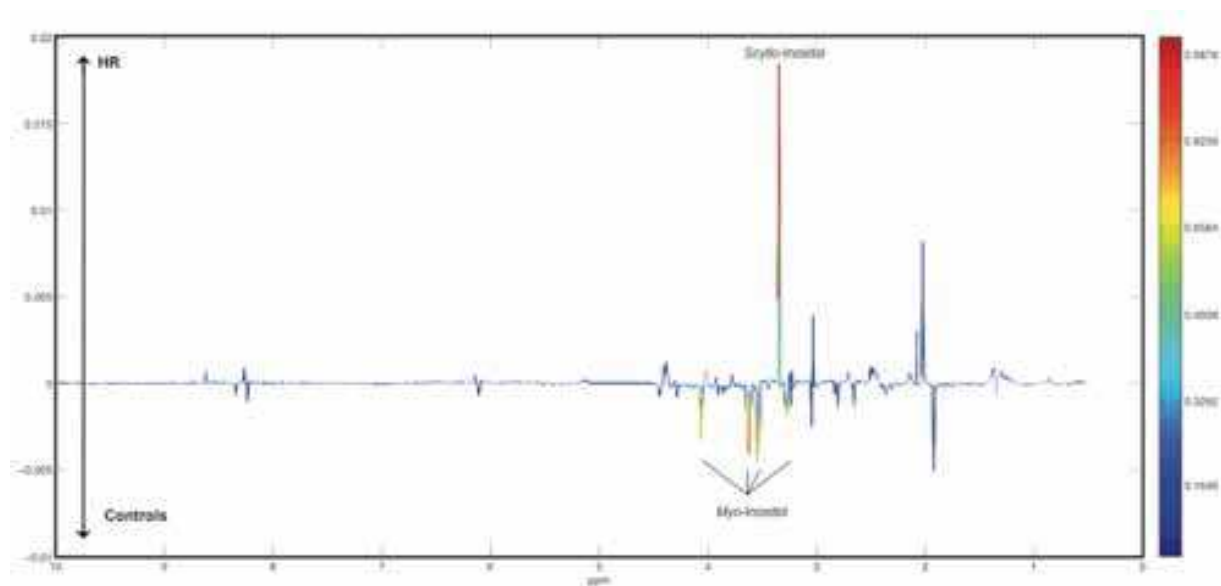

Fig. 8. Loading plot from O-PLS models performed from the aqueous extracts of brain in 9% HR-treated mice

4.3.2 Magnetic resonance imaging

To get access *in situ* to some potent cerebral metabolic changes thanks to a second spectroscopic technique, ^{1}H NMR localized spectroscopy, six male mice given a 9% HR diet and six control mice were used for *in vivo* metabolite quantification. All experiments were performed at 9.4 T on a Bruker Avance DRX 400 microimaging system with a wide-bore vertical magnet and a Micro 2.5 gradient system (Bruker Ettlingen, Germany). Because a preliminary experiment performed on a spectroscopic volume of interest (VOI) positioned in the cortex of mice was inconclusive, spectra have been performed from ^{1}H NMR data acquired by *in vivo* MRI using a VOI positioned in the thalamus of control and 9% HR-treated male mice (Figure 9.a). In this region, only the 9% HR-treated mice displayed a significant although minor signal found at δ = 3.34 ppm corresponding to *scyllo*-inositol (Figure 9.b). A one-way ANOVA performed for every other identified variables quantified at the same time from raw integrated spectra coming from *in vivo* MRI enables us to give significant results only for *scyllo*-inositol (p = 0.0013). As it could be described above, and as

one of the interests of metabonomic approach is to combine data generated by different techniques to get more powerful biomarkers, a PLS2-based canonical regression between the set of brain metabolites issued by *in vivo* MRI and the ^{1}H NMR fingerprints of hydrosoluble brain extracts performed on the same animals has been obtained after correction of the two data sets by an OSC-PLS-driven correction procedure. The canonical analysis obtained by the PLS2 analysis between these two corrected data sets showed that the ^{1}H NMR variable called B3.34, namely *scyllo*-inositol, was projected in the region where scores of HR-treated animals were also projected (Figure 9.c). Moreover, the relative contents of *N*-acetyl-aspartate (NAA), lactate and choline were increased ($p < 10^{-5}$, $p = 0.02$ and $p = 0.03$, respectively) whereas the glutamine one was decreased ($p = 0.04$) in response to the 9% HR treatment. MRI studies were also conducted in poisoned living mice and corroborated the abnormal higher presence of *scyllo*-inositol in the thalamus of poisoned animals. Even this result was unable to explain the exact pathophysiological mechanism involved and the outset of the illness, it confirmed that *scyllo*-inositol was a biomarker of interest in the central nervous system, particularly when it is related to some brain metabolic disturbances (Griffith et al., 2007; Jenkins et al., 1993; Viola et al., 2004). The increase in NAA, which has been previously revealed following MRI and ^{1}H NMR spectroscopy of hydrosoluble brain extracts was suggested to be linked to the enhanced locomotor activity observed in 9% HR-treated mice. Besides, NAA has been reported in epileptic seizures cases (Akimitsu et al., 2000).

This accumulation of NAA has also been shown in a rat model of the Canavan's disease, suggesting that NAA increase in brain should be linked to neuroexcitation and neurodegeneration (Kitada et al., 2000).

4.3.3 Behavioural testing

The two previous exploratory studies led us to consider in more details the role of inositols in the development of the Australian stringhalt. The location of such metabolic disturbances, the current knowledge of the metabolism and the pathways involved, such as neurotransmission, signalling system and regulation of many cellular functions, depending on the balance between *scyllo* and *myo* inositol needed to be rounded out by a complementary functional assessment as a large extent behavioural testing of HR-treated animals can provide it. Indeed, the administration of inositol (*myo*-inositol) is used as a therapeutic molecule in depression (Einat et al., 1999), panic disorder, obsessive-compulsive disorder (Cohen et al., 1997; Levine, 1997). It partially explains an enhanced locomotion (Kofman et al., 1998) and may be linked to a putative anxiolytic effect (Kofman et al., 2000) with possible involvement of serotoninergic (5-HT$_2$) receptors (Einat et al., 2001). Therefore, to investigate the functional consequences of such previous disrupted metabolic events, various behavioural aspects of C57BL/6J mice orally exposed to 9% HR for 3 weeks were performed in parallel with the ^{1}H NMR metabolomic exploration of the brain. Several behavioural tests related to locomotor activity (open-field test), motor coordination (Locotronic® apparatus, Wespoc test), learning and memory [Y maze, (Hughes, 2004), Figure 10.a and Morris water maze], anxiety [elevated plus maze, (Rodgers and Johnson, 1995), hole board (do-Rego et al., 2006; Takeda et al., 1998), Figure 10.b], and depression forced swimming test or test of Porsolt (Porsolt et al., 1977; Porsolt et al., 1979), social interaction (resident/intruder model), and addiction (place preference test) were carried out (Domange et al., submitted).
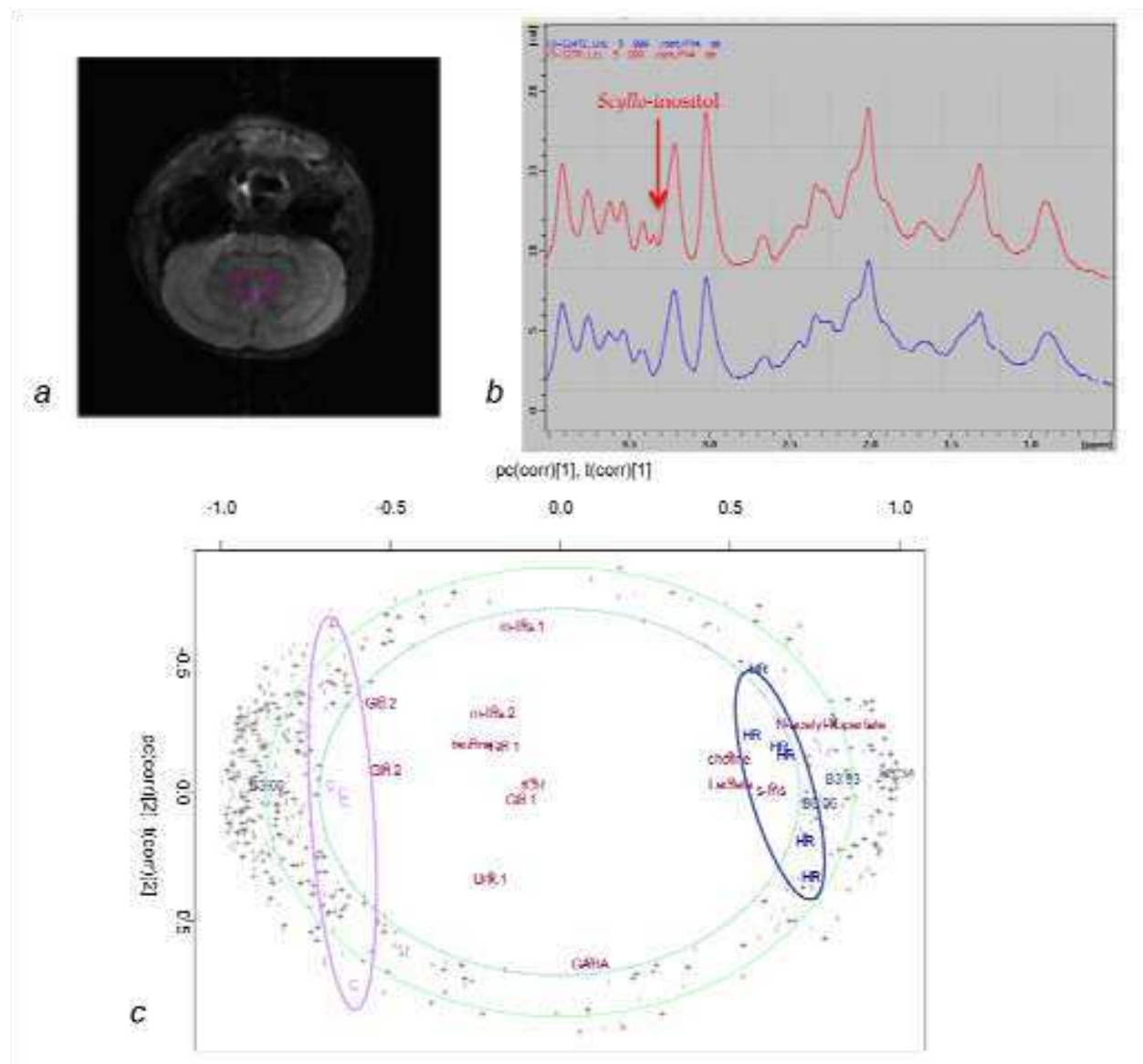
Fig. 9. a) MRI performed at 9.4 T on a Bruker Avance DRX 400 microimaging system positioned in the thalamus region with an *in vivo* parallel metabolite quantification using ¹H NMR localized spectroscopy (VOI, 12 mm³). b) Spectrum comparison between the sum of six ¹H NMR spectra acquired on control male mice and the sum of six ¹H NMR spectra acquired on 9%HR-treated male mice with the presence of *scyllo*-inositol (chemical shift detected at δ = 3.34 ppm). c) PLS2 between MRI quantitative data and ¹H NMR data. A loading projection is given for metabonomic variables having a norm above 0.5 (pale blue circle) or above 0.75 (pale green circle). The purple and the dark-blue ellipses, respectively, correspond to the scores of control and 9% HR-treated mice. Among the main MRI loadings having a positive correlation with HR treatment are *scyllo*-inositol (*s*-Ins), *N*-acetyl-aspartate, lactate and choline. For MRI variables having a negative correlation with HR treatment are glutamate (Glu.2, second chemical shift) and glutamine (Gln.2, second chemical shift). Uninformative MRI loadings: *myo*-inositol (*m*-Ins), glutamate, first chemical shift (Glu.1), glutamine, first chemical shift (Gln.1), GABA, taurine and unknown 1 are projected in the centre of the biplot (from Domange et al., 2008)
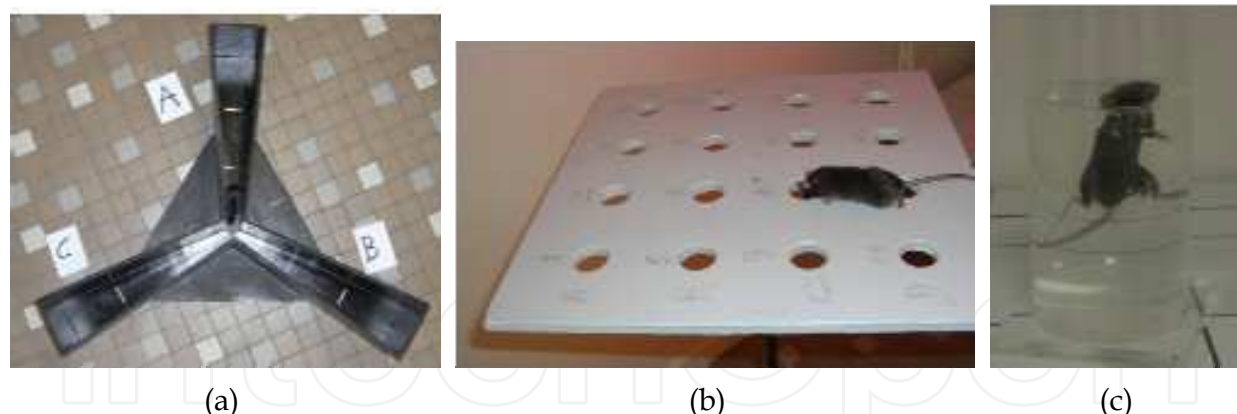
(a)   (b)   (c)

Fig. 10. Examples of behavioural tests used in mice: a) Y-maze or Y-maze spontaneous alternation that estimates the immediate working memory performance. b) Hole board test that evaluates the exploratory rate and the anxiety level. c) Forced swimming test defined to visualize a depression state (from Domange et al., submitted)

Although the lack of motor coordination impairment is commonly observed in the sick horses, 9% HR-treated mice displayed a motor hyperactivity, which is reflected by the decrease of immobility time in the forced swimming test, and the increased numbers of head dipping in the hole board test, of arms visited in Y-maze and of the number of entries in the upper quarter of the maze in the Morris water maze (Domange et al., submitted). This increased activity of treated mice, which is clearly observable at the end of tests, could be linked to a decrease in the resignation state or an enhanced motivation. Moreover, the 9% HR-contaminated mice seem to be addicted to the plant as indicated by results obtained in the place preference test. A regularized canonical analyses performed using mixOmics, an R package (Le Cao et al., 2009) to establish a canonical link between the two multidimensional data sets, *i.e.* the one containing the ^{1}H NMR fingerprints of hydrosoluble brain extracts and the one corresponding to the behavioural data set, which comprises nearly 100 variables, has revealed a clear relationship between some behavioural impairment variables (the motor hyperactivity and the addiction for the plant) and the main metabolic disruptions, *i.e.* the increase in *scyllo*-inositol in the brain of HR-treated mice and the relative decrease in *myo*-inositol. These results underlie the interest of such a dual and combined approach to characterize the functional end-points of a pathophysiological model of the horse Australian stringhalt in a seemingly metabolically orthologous murine species.

5. Conclusion

In this chapter, we underlined the interest of "omics" approaches and their recent introduction in the field of neuro-toxicological research. Indeed, metabonomics can especially be considered as a potentially powerful mean to explore the subclinical disruptions of an organism before the outset of clinical signs, and would particularly be useful in discovery markers of disease risk. This approach would help to prevent some risks in spite of the difficulty to detect some minor metabolites or molecules in tiny doses or mixtures, with the ability to access and explore some isolated and intricate tissues (like brain) *via* the general metabolism (urine, plasma) and to link statistically these subclinical metabolic changes with complementary data coming from other phenotyping approaches and across multiple physiological levels. Besides, these combined techniques have been

applied in some toxico-environmental assessments possibly aetiologically linked to some neuro-physiological diseases. Thus, coupling metabolomic and behavioural studies may help to functionally describe neurotoxicity resulting from ingestion of milk of lactating goats fed a hay contaminated with various persistent organic pollutants (POPs) like Polycyclic Aromatic Hydrocarbons (PAHs), PolyChloroDibenzo-p-Dioxins (PCDDs), PolyChloroDibenzoFurans (PCDFs) and PolyChloroBiphenyls (PCBs) (Schroeder et al., in preparation). Nevertheless, these "omics" technologies required new specific bioinformatics tools to mine multifactorial data and, in the case of metabolomics, some well-documented analytical databases to structurally characterize metabolites revealed as candidate biomarkers. Therefore, further progress needs to be obtained to improve at the statistical side these integration strategies and to reduce some still existing drawbacks. Nonetheless, such techniques have also the outstanding capacity to give some interpretation of the results in a larger biological perspective, given that this holistic approach stands for an emerging level of knowledge in clinical medical research.

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7. References

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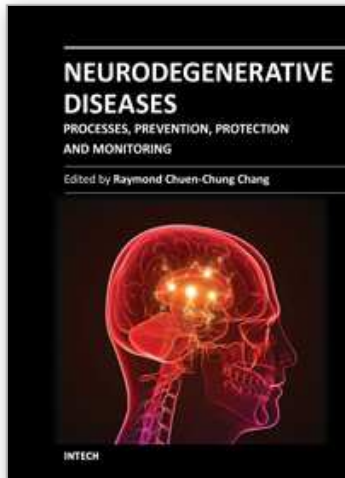
Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring
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Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring focuses on biological mechanisms, prevention, neuroprotection and even monitoring of disease progression. This book emphasizes the general biological processes of neurodegeneration in different neurodegenerative diseases. Although the primary etiology for different neurodegenerative diseases is different, there is a high level of similarity in the disease processes. The first three sections introduce how toxic proteins, intracellular calcium and oxidative stress affect different biological signaling pathways or molecular machineries to inform neurons to undergo degeneration. A section discusses how neighboring glial cells modulate or promote neurodegeneration. In the next section an evaluation is given of how hormonal and metabolic control modulate disease progression, which is followed by a section exploring some preventive methods using natural products and new pharmacological targets. We also explore how medical devices facilitate patient monitoring. This book is suitable for different readers: college students can use it as a textbook; researchers in academic institutions and pharmaceutical companies can take it as updated research information; health care professionals can take it as a reference book, even patients' families, relatives and friends can take it as a good basis to understand neurodegenerative diseases.

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