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The Microtubule-Dissociating Tau in Neurological Disorders

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1. Introduction

Around 24 million of people worldwide have some kind of dementia, and most of them are diagnosed to suffer from Alzheimer's disease (AD). In fact, every seven second a new case of dementia is identified, arriving to the rate of 4,6 new million cases per year. It is expected that by 2040 over 80 million of people will be affected. Neurological diseases are therefore a major public health problem due to the rise in the aging population, only in Europe these disorders cover approximately 35% of the burden of all diseases. In economical terms, brain diseases in Europe cost a total of 386 billion of euros per year, with an average of 829€ per inhabitant. AD and other dementias represent the second-leading cause of brain disorders after affective ones and equal with addiction diseases (Wittchen and Jacobi, 2005). Altogether dementias, and in particular AD represent a huge socio-economical impact, not only regarding the cost from the pharmacological point of view but also familiar cares which increase in an alarming rate in the last stages of the disease. Worthy to mention is the role of the family during the progression of this kind of diseases, relatives have to watch the patient every moment above all during the first lapses of memory and some of them need psychological help to assume the situation and the change in their lifestyle.

The incidence and prevalence of this group of diseases explain the need to understand mechanisms underlying dementia to uncover early and discriminative diagnostic markers as well as new therapeutic targets in order to improve the quality of life of these patients and the efficacy of the treatements. For these reasons research in AD is currently considered as a priority. At this time, the pharmacological treatments available aim to enhance the cognitive impairments once the disease is diagnosed, only cholinesterase inhibitors and one NMDA receptor antagonist are commercialized. Despite these products can alleviate the symptomatology, they are far away to constitute an effective remedy to cure or prevent the deleterious effect of the disease. In line of these observations, methods for improving diagnosis are needed, the search of biomarkers and neuroimaging techniques might help to support clinical diagnosis and detect the disease in the earliest stages. The identification of potential genetic and environmental risk factors as well as protective ones may provide a new window of action even if interventions at this level are more complex and controversial (Ballard C et al., 2011).

Despite AD covers between 60 to 80% of the causes of dementia, there are many other causes: vascular dementia, mixed dementia, dementia with Lewy bodies, Parkinson's disease, frontotemporal dementia, Creutzfeldt-Jakob disease, Huntington's disease and Wernicke-Korsakoff syndrome are some of them (http://www.alz.org). Current available diagnosis of AD is based mainly on the severity of cognitive impairments. However, even with the help of several neuroimaging techniques it is not simple to discriminate among AD and other age-related cognitive impairments. Unfortunately only an accurate diagnosis of AD can be reached after autopsy examination. Nonetheless, it is necessary and desirable to incorporate new biomarkers that are more sentitive, specific and may facilitate the diagnosis not only among the different disorders but also to discern the clinical progression (Seshadri S et al., 2011).

As it is described along this chapter the field of proteomics provides a powerful tool, which might enable to identify new proteins for early diagnostic and potentially therapeutic targets in AD. It is also remarkable the mandatory use of animal models in order to elucidate new pathways involved in the pathogenesis. Transgenic mouse models provide biochemical modulable approches where in a dependent or independent way several parameters can be studied (Sowell RA et al., 2009).

2. Historical input of proteomics to Alzheimer´s disease and other neurological disorders

AD is a progressive neurodegenerative disorder that leads to dementia. This pathology is characterized by two histopathological features: senile plaques and neurofibrillary degeneration (NFD) (Alzheimer A et al., 1907). Senile plaques are an extracellular accumulation of amyloid deposits formed by Aβ peptide. Aβ is a small 39 to 43 amino acid peptide produced by the complex catabolism of a type I transmembrane glycoprotein precursor named amyloid precursor protein (APP). Despite in AD only 1% of the cases have a familial history or inherited, most of the mutations described are related to APP, presenilin 1 (PSEN1), PSEN2 and SORL1 genes. Indeed the amyloid hypothesis of AD is considered almost like a dogma regarding the number of therapeutical research focused on this event (Hardy J and Selkoe DJ 2002). NFD has been consistently found in many neurodegenerative diseases among which the most prevalent is AD. Others include corticobasal degeneration (CBD), dementia pugilistica, fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), head trauma, Down syndrome, postencephalic parkinsonism, progressive supranuclear palsy (PSP), myotonic dystrophy (DM) and in Pick´s disease (Buee L et al, 2000). Nonetheless, the vast majority of studies have been performed in AD.

At the molecular level NFD corresponds to the aggregation of hyper- and abnormally phophorylated Tau proteins into filaments referred to paired helical filaments (PHFs) (Brion JP et al., 1985; Ihara Y et al., 1986). The spatiotemporal distribution of NFD in the diseased human nervous system is well correlated with the clinical expression of cognitive deficits (Delacourte A et al., 1999). However, there is a long and clinically silent period during which the lesions slowly developed and progress in several brain areas and are yet clinically silent. Neuropathological studies show that NFD is already detected in locus coeruleus of some people under 30. Moreover, the entorhinal cortex of non-demented individuals aged over 50 years, and the hippocampus are also often affected. During the earliest stages of AD with cognitive functions impairment, NFD is quite specific, spreading from the hippocampal formation to the anterior, inferior, and mid temporal cortex. NFD follows a

stereotyped, sequential and hierarchical pathway. The progression is categorized into ten stages according to the brain regions affected: transentorhinal cortex (S1), entorhinal (S2), hippocampus (S3), anterior temporal cortex (S4), inferior temporal cortex (S5), medium temporal cortex (S6), polymodal-association areas (prefrontal, parietal inferior and temporal superior) (S7), unimodal areas (S8), primary motor (S9a) or sensory (S9b, S9c) areas and all neocortical areas (S10). Up to stage 6, the disease can be asymptomatic (Figure 1).

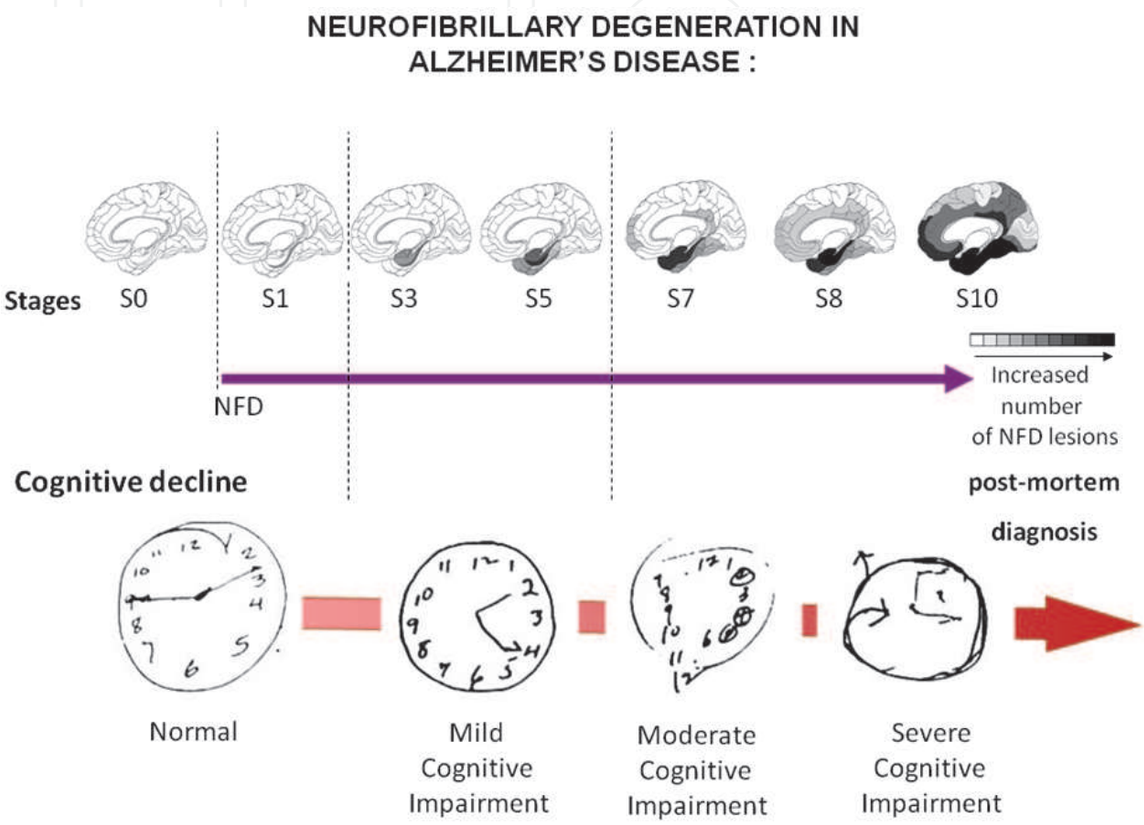

Fig. 1. NFD evolution in AD and cognitive decline. Watches represent the perception of the objects depending on the stage of the disease.

Despite tau proteins are heat stable, acid stable and very soluble in its native unfolded form (Cleveland DW et al., 1997), numerous methods have been used in order to dissect tau aggregates. First, PHFs in AD were initially observed by electron microscopy in 1963 (Kidd M. 1963). Then, in chronological order, Selkoe and collaborators described in 1982 a partial purification of PHFs from human brain tissue. PHFs showed a small solubility in urea, guanidine and detergents as sodium dodecyl sulphate (SDS), representing an example in neurons of a rigid intracellular polymer maybe as a consequence of covalent bonds that avoid a molecular separation by gel electrophoresis (Selkoe DJ et al., 1982). The first commonly used PHF preparation is that described by Nukina N and Ihara Y in 1985 and consists to have PHF in Sarkosyl insoluble fractions. Further purification of Sarkosyl pellets was described by Hasegawa and collaborators in 1992. Pellets were suspended in a small volume of 50 mM Tris-HCI (pH 7.6), and dissolved with 6 M guanidine HCI for further purification. The guanidine HCI suspension was centrifuged at 500,000 X g for 30 min on a TL100.3 microcentrifuge (Heckman). The supernatants were treated with iodoacetate after

reduction and fractionated on a TSK gel G-3000 SW column (7.8 X 600 mm, Tosoh) equilibrated with 6 M guanidine HCI in 10 mM phosphate buffer (pH 6.0), at a flow rate of 1.0 ml/min. The TSK fractions contain full-length tau with unusually slow mobilities in SDS-PAGE. The second commonly used preparation is that of Greenberg and Davies: about 50% of PHF immunoreactivity can be obtained in 27,200 x g supernatants following homogenization in buffers containing 0.8 M NaCl. Further enrichment was made by taking advantage of PHF insolubility in the presence of zwitterionic detergents and 2-mercaptoethanol, then removal of aggregates by filtration through 0.45-microns filters, and sucrose density centrifugation. PHF-enriched fractions contained proteins of 57-68 kDa that displayed the same antigenic properties as PHFs. The next stept was to develop an amino acid sequencing technique for PHFs combining a purification and solubilization procedure. After electrophoresis the insoluble fraction presented identical amino acid composition despite successive electrophoresis. Electron microscopy confirmed no changes in PHFs structures for the insoluble fraction even after electrophoresis. Moreover, this insoluble fraction displayed immunoreactivity against purified PHFs antibodies. Almost totally solubilization for the insoluble part was achieved by increasing the time of electrophoresis till almost 35 h showing one predominant band at 66 kDa and three additional bands between 50 and 70 kDa (Vogelsang GD et al., 1990).

Further studies based on the soluble and insoluble fractions after sucrose density gradient showed tau amino-terminal epitopes were more abundant in the soluble part and almost nonexistent in the insoluble one, in the other way around carboxy-terminal epitopes were observed in both fractions. These last observations pointed out the proteolytic degradation involved tau amino-terminal region and not in the carboxy-terminal part in the formation of PHFs in NFD (Ksiezak-Reding H et al., 1994).

Apart from characterization of PHFs from the solubility point of view, the development of additional approaches as electronic microscopy has definitely contributed to elucidate their ultrastructure. For instance, scanning transmission electron microscopy (STEM) provides accurate measurements of samples purified from human tissue and allows quantitave comparison between aggregated and dispersed population (Ksiezak-Reding H et al., 2005). Information regarding the filamentous conformation contributes to uncover the phosphorylation role in their formation. PHFs display ultrastructural different characteristics in AD and other neurological disorders. One possible classification is according to the straight or twisted filaments, based on the width of them along the length. Particularly twisted filaments are more abundant in AD and straight ones in PSP and both can be easily differentiated in CBD.

Along this section it has been described the main attempts to solubilize PHFs in order to clarify their composition, structure and their role in the aetiology in neurodegenerative disorders, mainly focused on AD. It can be considered that these were the first proteomics contribution to uncover the NFD progress involved in the cognitive impairments and loss of memory. In the next section we will discuss about the more modern and current proteomics methods and their application in the field of neurodegeneration.

3. Proteomic methods

Proteomics is the study of proteome, which are the whole set of proteins expressed by a genome of a cell, tissue or organism. So the analysis of a proteome is any study directed to level expression, degradation or post-translational modifications of proteins. Proteomics methods enable the identification and composition of these proteins from diverse biological samples.

Proteomics field may be divided into two main areas: protein profiling and functional proteomics. Profiling proteomics provides all the proteins of a sample, level of expression and global profile. At a functional level proteomics afford a lot of new and challenge pathways that may be related to disease aetiology and development of the symptoms. Identification of theses pathways and protein changes in expression or post-translational modifications might lead to a novel window of therapeutical targets. A better knowledge of the evolution in these proteins during the pathological process may also increase the accuracy for an early clinical diagnosis. In that sense, the most challenging discovery would be to find characteristic biomarkers of each disease and their modifications concerning the worsening of the symptoms during the progress of the illness. The study of the human brain proteome is one of the most challenging aspects in science during the last decades. Brain functions and their involvement in process like memory, behavior, and emotions in physiological as well as in pathological orchestration remain far from understood.

Independently where samples come from tissue, cells or body fluids as cerebrospinal fluid (CSF), the extraction of proteins is the *caput anguli* in all experiments. It is mandatory to establish the brain area, neuronal population or affected region, which is object of study. Moreover, thanks to the enormous protocols available for protein isolation, it is possible to achieve material enough from subcellular regions such as mitochondria or lipid rafts. Nowadays it is very useful and worldwide use the microdissection that enables to select a homogenous tissue or neuronal population, using a laser-dissecting microscope. Noteworthy that proteome analysis is not always reliable, not only because of changes in the expression profile as a consequence of genomic modifications, but also due to variability in extraction protocols and the quality of the sample after autopsy.

Proteomics analyses include two key steps, on one hand the separation and isolation of the protein to study and on the other hand the identification of proteins by mass spectrometry. In addition to separation and identification methods, there are also many well characterized technology to quantify protein as 2D differential gel electrophoresis (2D-DIGE), iTRAQ-Isobaric Tags for Relative and Absolute Quantification or SILAC-Stable Isotope Labeling by Amino Acids. Proteomics and bioinformatic developing technologies run in pararell since it is not possible to achieve hight standards in protein quantification and reliable identification if softwares do not allow discriminating among the possible variants and erasing the background that all the experimental conditions generate. Filters and integrators constitutes a general paradigm for signal detection in biology (Ideker T et al., 2011). In any case the researcher owns the most powerful weapon that is the capacity to assume the feasibility of a biological data, it means how the system is constructed and the functions carried out. Software enables to have update database easily accessible on internet including genome, transcriptome, metabolome, interactome and of course proteome (Brewis IA and Brennan P, 2010). There are several databases available for the research community dedicated to the analysis of protein sequences and structures, some of them are NCBI Peptidome, Expert Protein Analysis System (ExPASy), PeptideAtlas, the PRoteomics IDEntifications database (PRIDE) and Global Proteome Machine Database (GPMDB) (Vizcaíno JA et al., 2010).

3.1 Identification methods

Mass spectrometry (MS) is one of the most widespread developed analytical technique in biological sciences. Analysis of the amino acid sequence, tridimensional structure and characterization of post-translational modifications has allowed elucidating protein functions. Despite it is not the aim of this chapter it is useful to say that MS is also used in

DNA studies (Murray KK, 1996). MS is nowadays used in a large number of fields including from biochemistry to genome studies (Pandey A and Mann M, 2000). In combination with separation techniques, MS due to its sensitivity and speed may have an important role in identifying and monitoring biomarkers in physiological fluids as well as in drug discovery. This approach enables to identify therapeutic targets present at low concentrations in complex biological samples.

From the theorical point of view MS is not a measure of the mass, indeed it is a mass-to-charge (m/z) ratio of gas-phase ion. The values should be represented in terms of Daltons (Da) per unit of charge and the unit in the International System are Kilograms per Columb. In spite of the information obtained with this analysis is directly associated with the molecular weight and amount of protein, the results offered the possibility to acquire additional information as structural disposition (Zellner M et al., 2009).

MS are composed by three different parts: an ionization source, a mass analyser and a detector. The development of this technique is strengthly linked to the introduction of new and more sensitive components in these equipments.

Ionization source

Ionization can be defined as any process by which electrically neutral compounds are converted into ions (electrically charged atoms or molecules). Samples must be ionised and transferred to the gas phase, as a consequence of this step sample is destroyed. Classically ionization takes places in two separate steps, one in which the sample is volatilized and another one where it is ionized. The improvement in ionization methods permits to ionise large, non-volatile and thermally labile biomolecules and convert them into a gas phase without dissociation (Chait BT and Kent SB, 1992). The importance of these improvements was awarded in 2002 by the Nobel Prize in Chemistry "for the development of methods for identification and structure analyses of biological macromolecules" with one half jointly to John B. Fenn and Koichi Tanaka "for their development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules" and the other half to Kurt Wüthrich "for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution". Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the most worldwide ionization sources used nowadays.

In ESI the ion transfer from the solution to the gas phase ocurrs at atmospheric pressure (Zellner M et al., 2009). It is a process by which an aerosol is generated between two electrodes through a capillary held at a high potential (classically 3–4 kV), ions are separated of the solvent and get into the mass analyser. This method does not present a limit of size of the molecule to ionize and it can be easily coupled to MS and liquid separation techniques. Another variation of ESI is nanospray that owns a higher ionisation efficacy and it is less sensible to salt contamination. ESI might be the technique of choice for the design and development of quenchers against α,β-unsaturated aldehydes that are strongly associated with the oxidative stress (Beretta G et al., 2008), it has been used for instance to identify a human T-cell activation RhoGTPase-activating protein in a high frequency electromagnetic field irradiation model to induce AD features (Chang IF and Hsiao HY, 2005), to identify phosphorylation sites on tau (Reynolds CH et al., 2008) and analysis of phospholipids in CSF of AD patients (Kosicek M et al., 2010).

MALDI is maybe the most common ionization source used at the present time in proteomics era. Above all because it can be easily coupled to time-of-flight (TOF) mass analysers.

MALDI was introduced by Hillenkamp and Karas and currently is like ESI a suitable technique to the study of complex biological samples (Hillenkamp F and Karas M, 1990). MALDI produces mostly singly charged ions by a pulsed-laser irradiation. Moreover, MALDI owns a really high sensibility with almost no sample wasting and no desalting process is necessary since it works at physiological concentration of salts. In addition, MALDI requires relatively cheap equipment and quite easy to handle. MALDI TOF mass spectrometry is the technique of choice for protein identification separated by two-dimensional gel electrophoresis. MALDI TOF is widely used in the study of AD in different cellular compartment as synaptosomes proteins (Yang H et al., 2011), Aβ isoforms and their effect on tau phosphorylation in transgenic mouse model overexpressing Aβ1-40 and Aβ1-42 (Mustafiz T et al., 2011), evalutation of a vaccine specifically targeting the pathological amino-truncated species of Aβ42 that induces the production of specific antibodies against pathological Aβ products (Sergeant N et al., 2003), the possible role of heavy metal as copper (II) in the formation of PHFs (Zhou LX et al., 2007), identification of lipids containing in the PHFs from human brain as phosphatidylcholine, cholesterol, galactocerebrosides and sphingomyelin (Gellermann GP, et al 2006), identification of post-translational changes of proteins involved in AD as JNK-interacting protein 1 that is hyperphosphorylated following activation of stress-activated and MAP kinases (D'Ambrosio C et al., 2006), enrichment of more truncated glycans in PHFs (Sato Y et al., 2001) and decrease in the expression of M2 acetylcholine receptor (Zuchner T et al., 2005) are some examples.

Mass analyser

Once ions have been originated they are transported to the mass analyser region and separated according to their *m/z*. The election of one o the type of analyser will depend on their resolution, when more resolutive high capacity to defferentiate two close signals. Mass analysers available in the market are electric- and magnetic-field, depending on the way to separate the ions. The choise among them will depend on the application needed and the budget since each analyzer type has its strengths and weaknesses. Mass analysers systems are Quadrupoles, Sectors, Fourier transform cyclotrons and TOF. Quadrupole analysers are normally coupled to ESI ion sources and TOF analysers are often used with MALDI ion sources. Anyway, hybrid systems are also employed as ESI–TOF and MALDI–QTOF.

TOF spectrometer separates ions based on their velocity with a theorical mass gap unlimitated. TOF consists basically of a flight tube in high vacuum where ions are accelerated with equal energies and fly along the tube with different velocities. The flight time is related to the m/z values of the ions. The combination of high m/z range and compatibility with pulsed-ionization methods has made TOF the most commonly used analyser for MALDI experiments.

In Peptide Mass Fingerprinting approach gel-separated proteins are digested in the gel with a site-specific proteinase as trypsin (Hellman U et al., 1995). Then MS measurement of the cleavaged proteins is performed generally by MALDI TOF equipment. Finally Fingerprint peptides are compared to databases in which protein sequences have been already digested with the same proteinase. This is the method of choise for highthroughput identification of numerous samples. Moreover, robotic systems launched onto the market make possible the automation from detection spot in the gel till MS identification (Henzel WJ et al., 1993).

Tandem Mass Spectrometry (MS/MS) is another identification method predominantly suitable for analysing complex samples and a routine method used in research. This technique permits the identification of unkown proteins by sequencing their peptides.

MS/MS involved two steps of MS. In the first analyser ions with a desired m/z are separated (product ions) from the rest of the ions coming from the ionization source, and in the second type of analyser the mass spectrum is measured. Furthermore, MS/MS experiments improve the ratio signal/noise facilitating the resolution.
The product ions can be used to find out the primary structure of the peptide but nowadays most efforts are directed towards identification of post-translational modifications. In the case of tau protein is particulary special, since phosphorylation provides an additional negative charge to the sample. This fact complicates the analysis by MS because of detection of phosphopeptides is highly dependant on the equipment used as well as the software applied to analyze the spectra. Moreover, the existence of several adjacent serine or threonine residues allows MS/MS not to attribute the exact position of a phophate group as a result of the fragmentation of the peptide data.
The team of Hasegawa performed the earliest application for identification of Tau into PHFs. They used different fractions: purified PHF-tau, AD-soluble tau, or normal tau treated or not with alkaline phosphatase. The digests were applied to a Superspher Select B column (2.1 X 125 mm, Merck) and eluted with a linear gradient of 4-48% acetonitrile in 0.1% trifluoroacetic acid in 20 min at a flow rate of 0.2 ml/min. Amino Acid Sequence and Mass Spectrometric Analyses of the API Peptides-Fractionated peptides were sequenced on an Applied Biosystems 477A Protein Sequencer equipped with an on-line 120A PTH Analyzer or on an Applied Biosystems 473A Protein Sequencer. Mass spectral analysis was performed on a PE-SCIEX API 111 Hiomolecular Mass Analyzer (triple-stage quadrupole mass spectrometer) equipped with a standard atmospheric pressure ion source. Detailed comparison of peptide maps of PHF-tau and normal tau before and after dephosphorylation pointed to three anomalously eluted peaks which contained abnormally phosphorylated peptides, residues 191-225,226-240,260-267, and 386-438, according to the numbering of the longest tau isoform. Protein sequence and mass spectrometric analyses localized Thr-231 and ser-235 as the abnormal phosphorylation sites and further indicated that each tau 1 site (residues 191-225) and the most carboxyl-terminal portion of the protein (residues 386-438) carries more than two abnormal phosphates. Ser-262 was also phosphorylated in a fraction of PHF-tau. Modifications other than phosphorylation, removal of the initiator methionine, and Nu-acetylation at the amino terminus and deamidation at 2 asparaginyl residues were found in PHF-tau, but these modifications were also present in normal tau (Hasegawa et al., 1992).
NMR spectroscopy is an alternative to MS and it has been used to uncover physiological and pathological roles of tau protein. However, this is challenging since tau protein has 441 amino acids and an unfavorable amino acid composition. Quantification of phosphorylated tau samples is complex and studies are being performed in vitro using recombinant kisases (Landrieu I et al., 2010).

3.2 Separation methods

Analysis of a sample is always a challenge, it depends on the origin and of the aim of the experiment. Separation of the components of a sample offers the possiblility to establish a pre-selection and to perform a study concerning parameters as molecular weight (MW) and isoelectric point (pI). The separation methods available today have the enormous advantage that they can be coupled to other quantification techniques, including in this way not only the identification of the protein of interest, but also it relative amount compared to the control conditions. During this section we will converse abouth two separation approaches such as bidimensional electrophoresis and liquid chromatography.

3.2.1 Two-dimensional gel electrophoresis in AD brain

Two-dimensional gel electrophoresis (2D) is one of the most often-used separation methods in proteomics since first description by O´Farrell PH in 1975. This approach combines two electrophoretic methods: in the first dimension proteins are separated on an immobilized pH gradient strip with isoelectric focusing and migrate to the point on the strip at which their net charge is zero or pI, and in the second dimension or SDS-PAGE, proteins are separated according to their MW and thus isolating isoforms and isovariants of a certain protein.

This approache provides two kinds of information depending on the aim of the study. On one hand it can offer the global proteome profile with a high resolution containing nearly one thousand protein spots. However, the main limitation of the 2D is that several replicates of the same gel should be performed in order to reach statistically differences. The lack of a loading control makes complicated to rule out between differences in protein expression and loading variability among gels (Molloy MP et al., 2003). In addition, absence of an internal control for loading makes this approche very hand variable. On the other hand, this method is quite indicated if qualitative analysis is pointed out, ie if post-translational modifications are searched, the performance of a 2D western blot for two different conditions may supply changes in pI and /or MW. More specifically in the case of the tau protein, this method might give interesting data about the acidification or alkalinization as a consequence of phosphorylation process, which is the most common post-translational modification. For instance in figure 2 is shown 2D western blots for human total tau and phospho dependent AD2 antibodies in AD brain sample. Remarkably in the acidic part of the membrane it can be observed the characteristic triplet of phosphorylated tau (2A) in AD (60,64,69 kDa), while in the basic region all the tau isovariants dephosphorylated with postmorten delay are revealed (2B). Interestingly, in a recent study of our group it has been shown that the use of 2D may provide evidence that tau mutations dysregulate tau phosphorylation status. This event could be one of the first steps in the NFD cascade (Bretteville A et al., 2009).

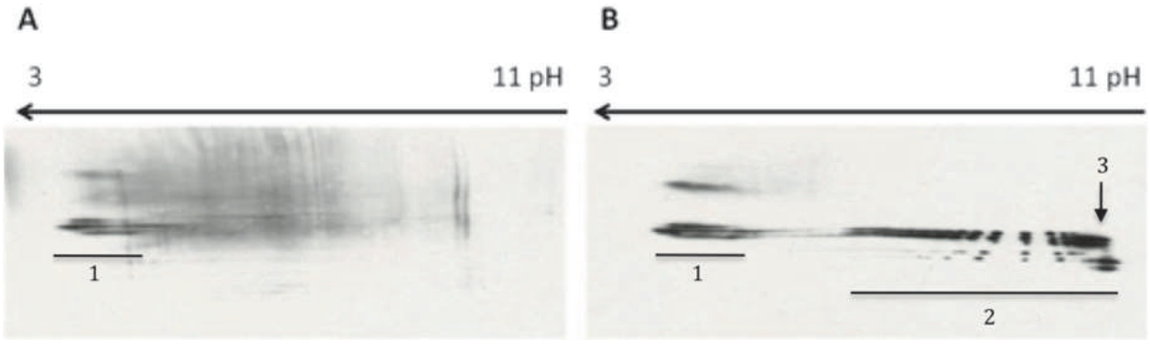

Fig. 2. 2D profiles of phospho-tau (A) and total tau (B) antibodies. Number 1 represents the hyperphosphorylated isovariants of tau while number 2 shows the low phosphorylated ones. Number 3 displays the native form of tau (Fernandez-Gomez FJ et al., personal unpublished data).

3.2.2 Quantitative proteomics by Two-Dimensional Differential Gel Electrophoresis (2D-DIGE)

2D-DIGE method is based on the same principle as "classical" 2D. The main differences rely on the fact that proteins are labeled with fluorescent dyes and all the samples are separated at

the same time in the same gel reducing spot pattern variability and the number of gels in an experiment. The reduction in number of gels during the manipulation increases the cost effectiveness and accurate spot matching. 2D-DIGE presents also the advantage that it is a quantitative approche since each protein spot has its own internal standard (IS), which ensure that the differences found are real and not due to a gel-to gel variation. Moreover, 2D-DIGE is a very sensitive technique with a detection threshold of around 1 femtomole of protein (Gong L et al., 2004). In the minimal labeling proteins are stained by cyanines, these dyes has a N-hydroxysuccinimidyl ester reactive group which forms a covalent bond with the epsilon amino group of the lysine in proteins via an amide connection. The single positive charge of the cyanine replaces the single positive charge of the lysine and the pI of the protein is not altered. This labeling reaction is minimal since only affects between 1-3% of the lysine residues. Using different cyanines dyes as Cy2, Cy3 and Cy5 covalently coupled to one protein sample each, then they can be mixed and loaded in the same gel (Viswanathan S et al., 2006) as it is shown in figure 3. A pool of all the samples is labeled with Cy2 and in this way the loading variability among gel is reduced to about 7% (Tannu NS et al., 2006). Differences will be observed after measurement of the intensity of the fluorescence for each cyanine. The 2D analysis software using the IS achieves a fast detection of less than 10% of differences between samples with more than 95% of statistical confidence (Gharbi S et al., 2002).

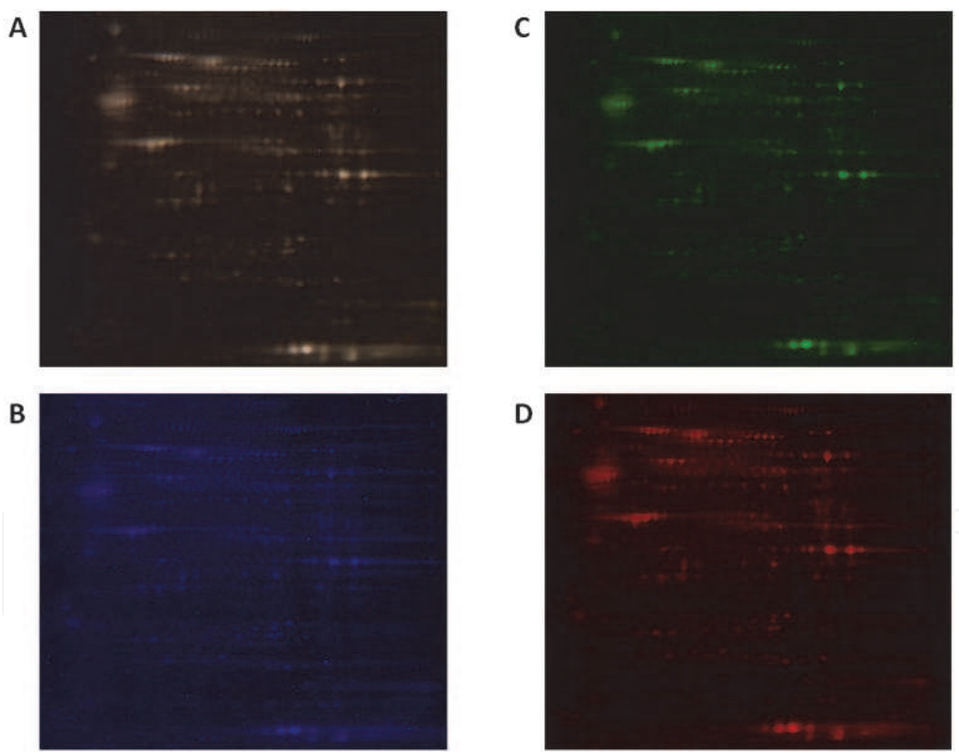

Fig. 3. Cy2, Cy3 and Cy5 merged (A) Cy2 labels IS (B) Cy3 pool of control (C) and Cy5 pool of AD samples (D). The software overlaps Cy2, Cy3 and Cy5 in order to establish the statistical differences among the replicates of the gels for each spot (Fernandez-Gomez FJ et al., personal unpublished data).

Despite the fact that it is far less used, there is in the market another 2D-DIGE method called saturation labeling where only two cyanines are used. Cy3 is the pool of samples and it

constitute the IS and Cy5 is the sample object of study. In this technique saturation dyes have a maleimide reactive group, which is designed to form a covalent bond with the thiol group of cysteine residues on proteins via a thioether linkage, and a high dye-to-protein labeling ratio is required. This type of labeling approach tries to label all available cysteines on every protein. This method has the main inconvenient that only one sample can be loaded in a gel apart from the IS and not two sample like in the minimal labelling. The big adventage is that cyanines offer great sensitivity with detection over 5 orders of magnitude (Shaw J et al., 2003).

The main limitation inherent to 2D method is that the gap of separation is among pH 3-10. As a consequence of this, poor solubilisation of highly acidic and basic proteins is reached. Proteins strongly attached to the biological membranes and samples with high concentration of salt own difficulty to be separated by isoelectric focusing, for this reason it is strong recommended to perform a purification step previous to the first dimension.

2D-DIGE application accomplishes one of the new perpectives in the medical research. This approach is been widely used for many studys in neurodegenerative disorders including AD. 2D-DIGE has been utilized in the search for biomarkers in CSF in amyotrophic lateral sclerosis (Brettschneider J et al., 2008), in Creutzfeldt-Jakob disease (Brechlin P et al., 2008) in AD patients (Maarouf CL et al., 2009), in frontal cortex brain samples of AD (Müller T et al., 2008) and in animal models.

3.2.3 Quantitative proteomics by Liquid Chromatography linked to Mass Spectrometry (LC-MS)

Liquid chromatography (LC) consist in separating proteins eluted from a LC column after the peptides are enzimatically digested, then they can be measured by MS. LC separation takes place when the sample components interact to a different extent with a mobile or stationary phase and elute at different times from this system. Normally several chromatographic systems are used in order to achieve a high resolution separation since only one system may not separate the complex mixture of peptides successfully. Then eluted fractions are undertaken to MS. The biggest adventage of LC coupled with MS is that this system presents a high-speed identification of the sample in an automatically way avoiding interindividual variability (Zellner et al., 2009). LC-MS is not a quantitative method *per se*, the peptide products coming from the proteolytic cleavage may alter the intensity of the signal in MS analysis due to their physicochemical characteristics. In order to discard this problem the use of stable isotopes has had a wide acceptance in the science community to achive accurancy in the quantification. The approach is based on the idea that a stable isotope-labeled peptide is chemically identical to its native counterpart and behaves identically during fractionation, digestion, chromatographic and MS analysis, but is distinguishable in a MS due to the mass diference. The ratio of signal intensities for the labeled and unlabeled peptide pairs provides an accurate measure of relative abundance of peptides from different samples. Stable isotopic tags can be introduced onto selective sites on peptides via metabolically, chemically, enzymatically, or provided by adding synthetic peptide standards to the sample. Strategies for isotope-based quantitative proteomics can be divided into two groups, depending on whether the isotopic tag is incorporated *in vitro* during sample preparation (iTRAQ, ICAT) or *in vivo* (SILAC) (Colucci-D´Amato et al., 2011).

Isotope Coded Affinity Tagging (ICAT) reagents consist of an affinity biotin tag for selective purification, a linker that incorporates stable isotopes and an iodoacetamide group that specifically reacts with free thiol of cysteines. Proteins from two different samples are

labeled with either light or heavy ICAT reagents obtaining a distinctive mass (eight or nine Da). To minimise the error, the labeled mixture of protein samples are combined, digested with protease to peptides and fractionated by multidimensional chromatography and analysed by LC-MS. The ratios of signal intensities of differentially mass-tagged peptide pairs are quantified to determine the relative levels of proteins in the two samples. An interesting application of this technique is for the redox proteomic since ICAT labels cysteine residues (Sethuraman M et al., 2004). However, this method is not suitable for quantifying proteins that do not contain enough residues of cysteine and it presents the limitation that only two samples can be done at once (Shiio Y and Aebersold R, R 2006). For this reason this approach is limited for studying of post-translational modifications and splice isoforms.

Another amino group-based isotope labeling approach is isobaric tagging for relative and absolute protein quantification (iTRAQ). Unlike ICAT this method allows identification and quantification as well as comparison of up to eight conditions at the same time. This strategy has been developed in order to overcome the limitations of the previous one, so this method targets the peptide N-terminus of the residues (Ross PL et al., 2004). The iTRAQ reagent consists of a reporter group that is a tag with a specific mass in each individual reagent and a balance group to ensure that the reporter and balanced groups remain invariant without changing the mass. After collision-induced dissociation reporter ions spectra is correlating with the protein-sequence database and relative quantification of proteins with high accuracy is reached (Gevaert K et al., 2008).

Stable Isotope Labeling by Amino Acids (SILAC) is a metabolic stable isotope labeling during cell growth and division in bacteria and afterwards was adapted to amino acids in cell cultures (Ong SE et al., 2002). SILAC is a simple procedure in which natural variants of essential amino acids are replaced by deuterated, carbon-13 or more currently by nitrogen-15. Using nitrogen-15 the number of incorporate labels is defined and not dependent of the number of carbons that constitute the peptide sequence, this facilitates the analysis of the results. The advantage of this method relies on it accurate quantification since stable isotopes are incorporated very early in the sample. The main inconvenient of this technique is that isotopes can only be incorporated during protein synthesis. This is a huge limitation for the study of CSF and human brain tissue taking into account that neurons are post-mitotic cells (Bantscheff M et al., 2007). Despite this handicap SILAC is a powerful tool to study cellular pathways as polyubiquitin involment in the aetiology of AD (Dammer EB et al., 2011), neuroinflamation (McGeer EG and McGeer PL, 2010), reactive microglia (Klegeris A et al., 2008), neurotrophin signaling (Zhang G et al., 2011), oxidative stress (Akude E et al., 2011), TDP-43 proteinopathy in frontotemporal lobar degeneration and amyotrophic lateral sclerosis (Seyfried NT et al., 2010) mitochondrial alterations in dopaminergic cells (Jin J et al., 2007) and modulation of ion channels by phosphorylation (Park KS et al., 2006).

Other methods for protein quantification are multiple reaction monitoring (MRM) that has been successfully used for low abundant proteins in plasma (Anderson L and Hunter CL, 2006) and phosphopeptides quantification (Lange V et al., 2008). The absolute quantification of proteins (AQUA) technology uses a known quantity of heavy isotope labeled peptides as IS added as soon as possible in the analytical process (Kettenbach AN et al., 2011).

3.2.4 Surface-enhanced laser desorption/ionization mass spectrometry

Surface-enhanced laser desorption/ionization mass spectrometry (SELDI) method combines retention chromatography with MS detection, and it can be used in biological samples such

as cancer cells, CSF and tissue lysates. A few microliters of a sample of interest are deposited on the chromatographic surface. The protein chip arrays are incubated and then washed with a suitable buffer. SELDI protein chip surfaces are uniquely designed to retain proteins from complex mixtures according to their specific properties using chromatographic-based selectivity. The proteins of interest are captured on the chromatographic surface by adsorption, partition, electrostatic interaction or affinity chromatography depending on their properties, and analyzed by MS. SELDI is frequently coupled to MALDI-TOF and possess the significative advantage that minimal amount of sample is consuming and consequently not destroyed.
The main application of this technique is in the search of biomarker in cancer as well as in neurodegenerative disorders. In the field of AD, SELDI has been used to find significantly higher levels of amyloid-beta peptides monomer and dimer in the blood of AD subjects compare to controls (Villemagne VL et al., 2010) and in CSF the enrichment in Aβ10-40 paralleled by depletion of the fragment Aβ1-42 seems to be a common event in familial AD (Ghidoni R et al., 2009).

4. Contribution of proteomics to Tauopathies classification

Classification and characterization of neurodegenerative disorders have been one of the biggest achievements in proteomic field. Proteomics enable to separate, identify and study protein-protein interactions within the different pathologies. Nowadays the term tauopathies includes more than twenty well-characterized diseases. The high resolution separations of tau proteins in electrophoretic profiles as well as the immunoreactivity with a wide range of antibodies provide substantial information to discriminate among the different diseases. Major post-translational modification in tau proteins is phophorylation. For this reason vast of studies are focused on the role of this modification in the structure, function, pI and signalling pathways of tau proteins during the progression of the diseases.

4.1 Tau proteins

Tau (tubulin associated unit) is the major component of PHFs. Weingarten MD et al. described this protein for the first time in 1975 as an essential factor for the organization, stabilization, and dynamics of microtubules (Weingarten MD et al., 1975). Tau is essentially a neuronal phosphoprotein located within the axonal compartment (Butler M and Shelanski ML, 1986). Tau is prone to modulate the axonal transport and neuronal plasticity (Sergeant N et al., 2005). Recently, it has been established that tau regulates the motility of dynein and kinesin motors proteins by an isoform-dependent mechanism. Indeed, the shortest tau isoform lacking exon 2, 3 and 10 impedes the motility of both kinesin and dynein whereas the longest tau isoforms with all exons less affects motor protein motility (Dixit R et al., 2008). Therefore, a modified pattern of tau isoform expression/ratio, due to tau aggregation for instance, may profoundly affect the axonal transport and could possibly lead to neurodegeneration (Crosby AH, 2003). Besides its known role as a microtubule-stabilizer and organizer, tau may exert several other functions as signalling pathway in neurons (Ittner LM et al., 2010 and Leugers CJ, 2010) and DNA protection under stress stimuli (Sultan A et al., 2011).
A unique human tau (MAPT) gene is located on chromosome 17 at the band position 17q21. The restriction analysis and sequencing of the gene shows that it contains two CpG islands, one associated with the promoter region and the other with the exon 9 (Andriadis A et al., 1992). The human tau primary transcript contains 16 exons and in the adult human brain,

alternative splicing of exons 2, 3 and 10 gives rise to six tau isoforms where exon 3 never appears independently of exon 2. Alternative splicing is regulated during development and differentially between tissues. A single isoform lacking the 3 alternative exons 2, 3 and 10 is expressed in the foetal brain. Exon 10 encodes an additional microtubule-binding motif numbered R1 to R4. Half of tau proteins contain three microtubule-binding motifs and the other halves have four microtubule-binding motifs (figure 4A). Constitutive exons are 1, 4, 5, 7, 9, 11, 12 and 13 and the start codon is located in exon 1. There are two alternate stop codons located either following exon 13 or inside exon 14 (Andreadis A, 2005 and Sergeant N et al., 2008). Human brain tau isoforms have a range from 352 to 441 amino acids and a molecular weigth between 45 to 65 kDa in polyacrylamide gel electrophoresis (figure 4B).
Primary sequence analysis of tau protein shows that it can be subdivided in four structural regions. The amino-terminal region is acidic and variable, depending on the presence or absence of exons 2/3 and a proline-rich domain follows it. The latter is followed by 3 or 4 imperfect repeat motifs (R1 to R4; see figure 4A) - depending on the presence or absence of exon 10 - and corresponding to the microtubule-binding domain of tau. Finally, a short carboxy-terminal region is found and it is the basic region of the protein (figure 4C).

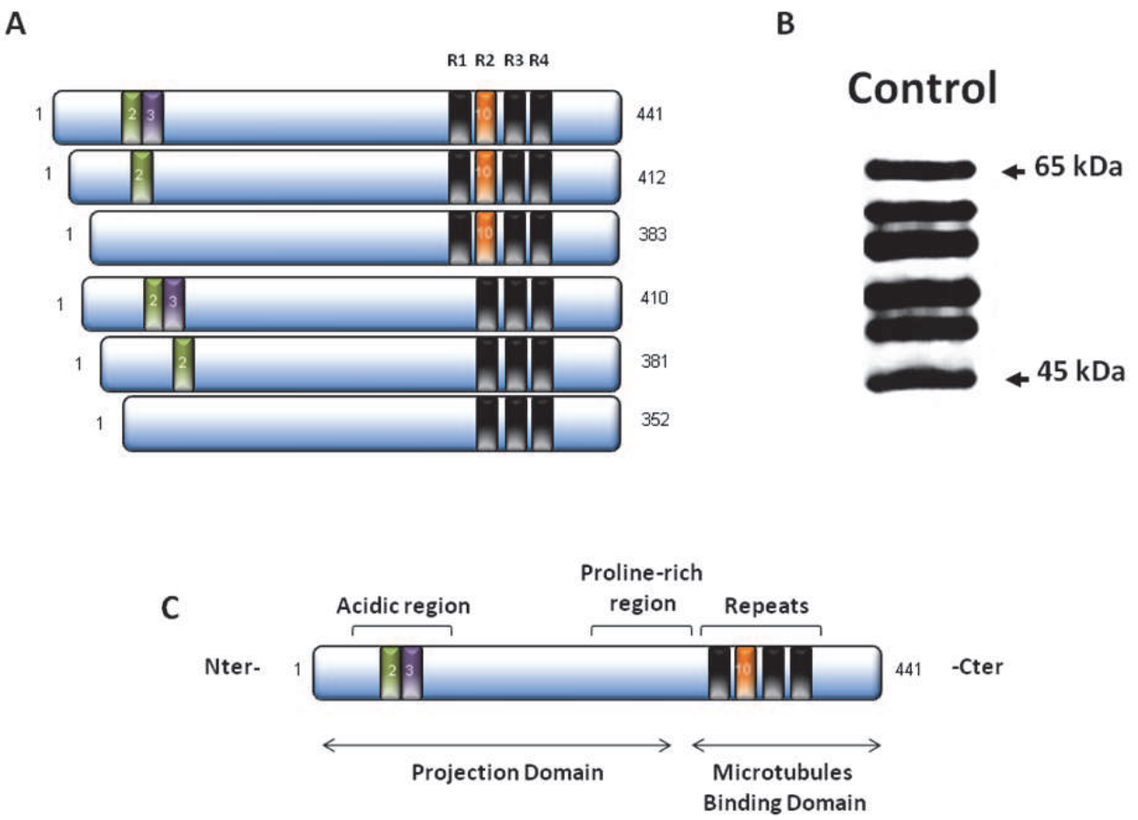

Fig. 4. Six tau isoforms are presented in human brain. These isoforms differ by the absence or presence of one or two 29 amino acids inserts encoded by exon 2 (green box) and 3 (violet box) in the amino-terminal part. Exon 3 is always incorporated with exon 2. R2 corresponds to the presence of exon 10 (orange box) that encodes an additional microtubule-binding motif numbered R1 to R4 in the carboxy-terminal part and they are represented as black boxes. (A). Molecular weight in mono-dimensional electrophoresis for the six isoforms of tau (B) and tau protein regions corresponding to the full-length isoform (C).

The amino-terminal region together with the proline-rich domain is referred to as the "projection domain". This unstructured and negatively charged region detaches from the surface microtubules (Hirokawa N et al., 1988) and can interact with the plasma membrane or cytoskeletal proteins (Brandt R et al., 1995). Tau may therefore contribute to spacing in between microtubule lattice and to the parallel ordered organization of microtubules in axons (Chen J et al., 1992). Amino-terminal region of tau also interacts with a growing panel of polypeptides including motor proteins such as kinesin-1 (Utton MA et al., 2005) and dynactin/dynein complex (Magnani E et al., 2007). All interacting polypeptides constitute the interactome of tau and indicate the functions in which tau may be implicated. The application of 2D gel electrophoresis method has been used to study tau (Janke C et al., 1996). The six main isoforms of tau are separated as several isovariants with isoelectric points comprised between 9.5 and 6.5 due to the alternative splicing and to post-translational modifications. The amino-terminal region has a pI of 3.8, proline domain has a pI of 11.4 and carboxy-terminal has a pI of 10.8. Regarding to the primary structure, the polypeptide sequences encoded by exons 2/3 add to tau acidity, whereas exon 10 encodes a positively charged sequence that adds to the basic character of tau. Thus tau is rather a dipole with two domains with opposite charge modulated either by post-translational modifications or tau proteolysis (Wischik CM et al., 1988).

Tau stabilizes oligomers of tubulins, it is partially folded while interacting with microtubules and it was shown to link laterally protofilaments made of tubulin (Santarella RA et al., 2004). NMR investigations showed that residues between Val226 to Glu372 are binding to microtubule surface involving the all four repeat binding motifs showing that amino- and carboxy-terminal domains do not participate in the binding properties of tau to microtubules (Sillen A et al., 2007). Tau mutations like in FTDP may impair the binding of tau to microtubules (Delobel P et al., 2002). Regarding the physic-chemical properties of tau protein it has been addressed that tau protein owns pro-aggregative motifs called PHF6 and PHF6* in its carboxi-terminal region at the level of R2 and R3. The amino acids sequence of these motifs (306)VQIVYK(311) and (275)VQIINK(280) are prone to promote aggregation by the formation of beta-structure (von Bergen M et al., 2001). This aggregation and accumulation of misfolded proteins might have a common cause and pathological pathway in several neurodegenerative disorders resulting in neuronal loss (Tyedmers J et al., 2010). Several studies have revealed that truncated tau drive NFD *in vivo* (Zilka N et al., 2006) and caspase activation lead to tangles formation (de Calignon et al., 2010).

4.2 Post-translational changes of Tau proteins

Phosphorylation of tau is instrumental to NFD and it is the main post-translational modification in tau isovariants as it was shown by 2D immunoblots (Butler M and Shelanski ML, 1986). These data shed light to the impact of tau protein for tau biology. There are 85 potential phosphorylation sites on the longest brain tau isoform. Phosphorylation sites were identified with proteomic approaches as MS, NMR, phospho-peptide mapping and the use of site-specific phosphorylation dependent tau antibodies (Hanger et al., 2007). Among them around 71 correspond to putative phosphorylation sites in physiological and pathological conditions. It is worthy to remark that most of the phosphorylation sites surround the microtubule-binding domains in the proline-rich region and carboxi-terminal region of tau. Phosphorylation regulates several functions of tau such as its binding to microtubules, the axonal transport of tau as well as its interactions with amino-terminal partners' particularly

SH3-containing proteins (Rosenberg KJ et al., 2008). For instance, tau transport along the axon is negatively regulated by its phosphorylation by GSK3β leading to a reduced binding to kinesin-1 (Cuchillo-Ibanez I et al., 2008). By phosphorylating amino-terminal serines 212 and 217, GSK3β also reduces the binding of SH3-containing proteins, such as Fyn, PLC-γ1, p85α (Reynolds CH et al., 2008). Once tau proteins are phosphorylated they cannot polymerize tubulin into microtubules and do not stabilize the latter.

Tau phosphorylation is mainly regulated through kinases and phosphatases, but other enzymes are also involved, such as Pin1 isomerase (Buee L et al., 2000). A total of more than 20 protein kinases can phosphorylate tau proteins (Sergeant N et al., 2008). This includes four groups of protein kinases. (a) Proline-directed protein kinases (PDPKs), which phosphorylate tau on serines or threonines that are followed by a proline residue. This group includes CDK1 and 5 (Hamdane M et al., 2003), MAPK and several SAPKs (Ferrer I et al., 2005). (b) The non-PDPK group includes tau-tubulin kinases 1 and 2, casein kinases 1 and 2, DYRK1A (dual-specificity tyrosine-phosphorylated and –regulated kinase 1A), phosphorylase kinase, Rho kinase, PKA, PKB/Akt, PKC and PKN (Sergeant N 2005). (c) The third group includes protein kinases that phosphorylate tau on serine or threonine residues followed or not by a proline. GSK (glycogen synthase kinase) 3*α* and GSK3*β* and AGC kinases (such as MSK1 (mitogen- and stressactivated protein kinase) belong to this group and have recognition motifs SXXXS or SXXXD/E and RXRXXS/T respectively (Buée L et al., 2010). (d) The fourth group corresponds to tyrosine protein kinases such as Src kinases, c-Abl and c-Met (http://cnr.iop.kcl.ac.uk/hangerlab/tautable). The principal role of tau phosphorylation is related to microtubule binding. However, phosphorylation or dephosphorylation of tau may also contribute to the cell localization of tau. For instance, phosphorylation of tau by GSK3*β* regulates its axonal transport by reducing its interaction with kinesin. In sharp contrast, dephosphorylated tau is located to the cell nucleus and is suggested to contribute to nucleolar organization and/or contribute to chromosome stability. Mutations in TAU gene lead to a change in the affinity of kinases that phosphorylate tau near the site of the mutation. Some mutations like R406W may reduce the phosphorylation of tau at Ser404, which is necessary for GSK3-β to phosphorylate tau at Ser396 afterwards (Tatebayashi Y et al., 2006). However, this priming putative phosphorylation site is not a prerequisite for JNK3 to phosphorylate tau at Ser396. These data provide evidence that tau mutations may potentially modify the global phophorylation state of tau.

Abnormal phospho sites on PHF-tau were identified on constitutive exons, such as Ser212–214 together and Ser422. These three new sites were identified on the alternative sequence encoded by exon 2. As tau isoforms expression may be different in subneuronal populations, these phospho epitopes would be of interest in identifying such subneuronal populations or the laminar distribution of NDF in AD (Delacourte A et al., 1996).

In normal brains the phospho-epitopes are rapidly dephosphorylate during postmorten delay, this effect may be due to the drop in ATP and inactivation of phophatases. However, in AD brains this dephosphorylatyon does not occur. Some of the hypotheses are that aggregation of tau proteins into filaments render them inaccessible to phosphatases, phosphatases are not activated any more or their activity is suddenly decreased.

Other post-translational modification of tau proteins is O-glycosylation. O-glycosylation results from the attachment of a sugar on the hydroxyl radical of serine or threonine residue in the vicinity of the proline-rich domain. Glycosylation decreases tau phosphorylation by CDK5, PKA and and GSKβ, probably due to a competition between phosphorylation and

glycosylation for the same sites. In fact, tau proteins from AD brains present abnormally glycosylation in comparison with controls. Using a recombinant O-GlcNAc modified tau, MS has mapped O-GlcNAc on tau at Thr-123, Ser-400 sites and a third one on either Ser-409, Ser-412, or Ser-413 (Yuzwa SA et al., 2011). The identification of these sites may provide evidence to elucidate the role of glycosylation in tau function.

The microtubule-associated protein tau is known to be post-translationally modified also by acetylayion. Recent studies reported that tau is acetylated and this acetylation avoids its degradation. Tau acetylation impares tau-microtubules interactions and facilitates tau aggregation. In fact, specific antibodies for acetylated tau showed an increase in acetylation in several Braak stages with the involment of histone acetyltransferase p300 and the deacetylase SIRT1 (Min SW et al., 2010). MS provides specific lysines within the microtubule-binding domain including lysine 280 (K280) that are main sites of tau acetylation. One model shows that K280 is exclusively acetylated in pathological conditions (Cohen TJ et al., 2011).

4.3 Tau as a bar code for neurodegenerative diseases

The most obvious pathological event in tauopathies is the presence of aggregates of tau isoforms into intraneuronal filamentous inclusions. The evolution in the proteomics era allows to establish different physiological and pathological electrophoretical patterns to distinguish among the diversity of tauopathies. Comparative biochemistry of tau aggregates differs in both isoform phosphorylation and content, which enables a molecular classification of tauopathies. In postmorten brain tissue tau proteins are resolved as six bands (figure 4B) whereas more acidic hyperphosphorylated isoforms present four bands between 60 and 74 kDa depending on the disorder (figure 5). The classification presented here is composed by five classes of tauopathies, depending on the type of tau aggregates that constitute the bar code for neurodegenerative diseases (Sergeant et al., 2005).

Class 0: frontal lobe degeneration non-Alzheimer non-Pick

Frontal lobe degeneration is the second more common presinile disorder that leads to dementia after AD. This class is genetically linked to mutations in the progranulin gene (Baker M et al., 2006 and Cruts M et al., 2006). Frontal lobe degeneration presents no specific neuropathological hallmarks, no tau aggregation and a loss of expression in tau proteins. The transactive response (TAR)-DNA-binding protein with a molecular weight of 43 kDa (TDP-43), encoded by the TARDBP gene, has been recently identified as a major pathological protein of frontotemporal lobar degeneration with ubiquitin-positive and tau-negative inclusions. It is the most common underlying pathology in frontotemporal dementias with and without motor neuron disease. In fact TDP-43 pathology is identified till the 50% of AD cases and it is the main component in the amyotrophic lateral sclerosis (Wilson AC et al., 2011). This pathology from the clinical point of view is quite similar to Pick´s disease. It is characterized by a frontal distribution of morphologic changes involves neuronal cell loss, spongliosis and gliosis mainly in the superficial cortical layers of the frontal and temporal cortex (Delacourte A et al., 1977).

Class I: all brain Tau isoforms are aggregated

Class I is characterized by a pathological tau quartet at 60, 64 and 69 kDa, and a minor pathological tau at 72/74 kDa (figure 5). This pathological tau quartet corresponds to the aggregation of the six tau isoforms (Sergeant N et al., 1997b and Goedert M et al., 1992). The pathological tau 60 is composed of the shortest tau isoform (2–3-10-). The pathological tau 64

and 69 are each composed of two tau isoforms: tau isoforms with either the exon 2 or exon 10 alone compose the pathological tau 64, while the pathological tau 69 is made of tau isoforms with either exon 2 + 10 or 2 + 3. The longest tau isoform containing exons 2, 3 and 10 (2 + 3 + 10) constitutes the 72/74-kDa pathological component, as determined by 2D gel electrophoresis coupled to western blotting using exon-specific tau antibodies (Sergeant N et al., 1997a). This typical tau profile was first characterized in AD, but now includes nine additional neurological disorders AD as cerebral aging (over 75 years), ALS/parkinsonism–dementia complex of Guam, Parkinson with dementia of Guadeloupe, Niemann–Pick disease type C, Postencephalitic parkinsonism, Familial British dementia, Dementia pugilisticia, Down's syndrome and FTDP-17. Using histochemistry, aggregates of this class can be observed with AD2 and antibodies against exon 2 and exon 10 (Buee L et al., 2000 and Sergeant N et al., 2008).

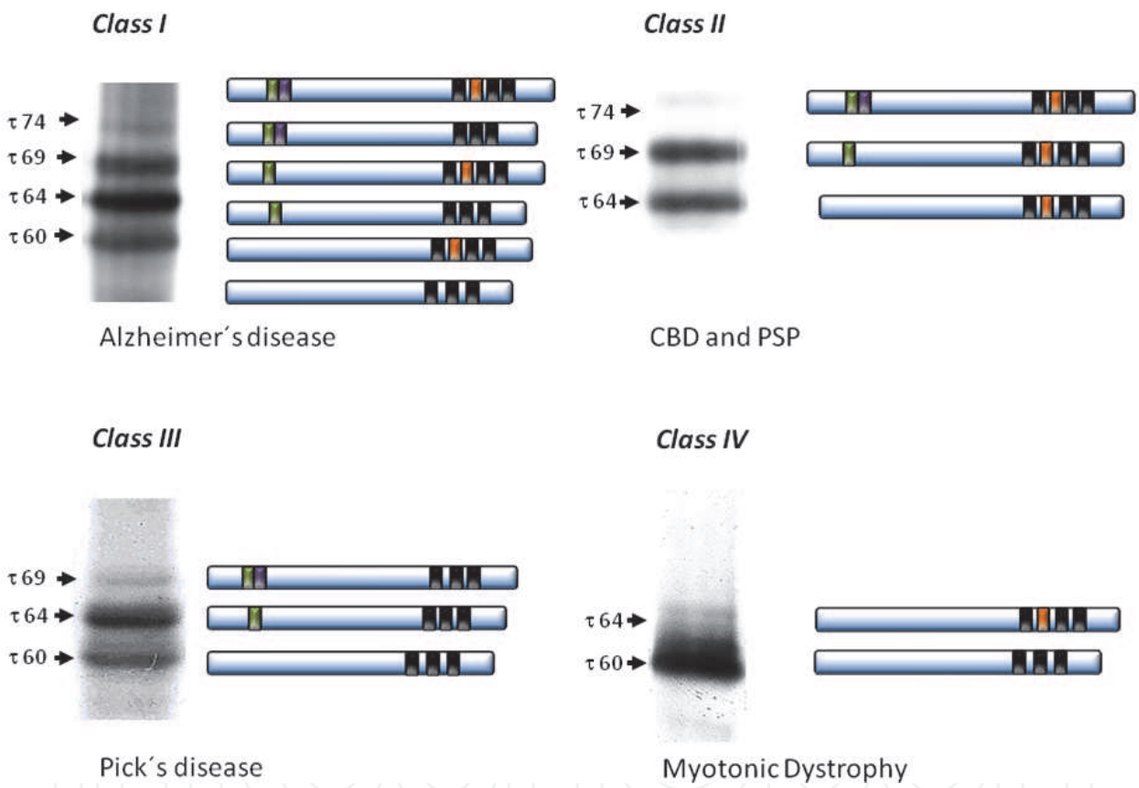

Fig. 5. Bar Code for neurodegenerative diseases. Schematic representation of the modifications leading to tau proteins aggregation in Tauopathies. Native tau proteins are detected as a triplet of bands ranging between 60 and 74 kDa by numerous phosphorylation-dependent antibodies. Tau proteins are shown by western blotting as three major bands between 60 and 69 kDa, and a minor band at 74 kDa. AD pattern is also found in Down's syndrome, post-encephalitic parkinsonism, ALS/parkinsonism–dementia complex of Guam among others (class I). The doublet tau 64, 69 represent the aggregation of hyperphosphorylated tau isoforms with exon 10 (orange box) typical for CBP and CBD (class II), the exclusion of exon 10 (only black boxes) in hyperphosphorylated tau aggregation lead to tau 60, 64 doublet characteristic for Pick's disease (class III). The aggregation of Tau isoforms lacking exons 2 (green box) and 3 (violet box) is found in myotonic dystrophy (class IV).

Class II: Tau isoforms containing the exon 10 encoding sequence aggregate

Aggregation of tau proteins with four microtubule-binding domains is the characteristic of class II (figure 5). This pathological tau profile is observed in CBD, argyrophilic grain dementia, PSP and FTDP-17 due to tau gene mutations (Sergeant N et al., 1999 and Tolnay M et al., 2002). PSP, CBD and argyrophilic grain dementia are rare atypical parkinsonism disorders.

Class III: Tau isoforms lacking the exon 10 encoding sequence aggregate

This class of tauopathies includes Pick's disease and autosomal dominant inherited FDTP-17 (figure 5). Pick's disease is a rare form of neurodegenerative disorder characterized by a progressive dementing process. Early in the clinical course, patients show signs of frontal disinhibition. Neuropathologically, Pick's disease is characterized by the presence of typical spheroid inclusions in the soma of neurons called Pick bodies. Pick bodies are labeled by tau antibodies, with a higher density in neurons of the dentate gyrus of the hippocampal formation than in the temporal and frontal cortices. The pathological tau profile of Pick's disease contrasts with that of class II tauopathies, with the pathological tau isoforms consisting essentially of the 3R tau isoforms.
Immunohistologic staining of these aggregates is positive for AD2 and exon 2 antibodies but negative for exon 10 antibodies. In addition, aggregated tau proteins in Pick's disease are not detected by the monoclonal antibody 12E8 raised against the phosphorylated residue Ser262/Ser356, whereas this phosphorylation site is detected in other neurodegenerative disorders. The lack of phosphorylation at Ser262 and Ser356 sites is likely to be related to either a kinase is not active in neurons that degenerate in Pick's disease or those neurons do not constitutively express these kinases within degenerating neurons (Mailliot C et al., 1998).

Class IV: Tau isoform lacking exon 2, 3 and 10 principally aggregate

This group is represented by a single neurological disorder: myotonic dystrophy (DM) of types I and II (figure 5). DM is the commonest form of adult-onset muscular dystrophy. Genetically it is an inherited autosomal dominant disorder caused by a single gene mutation consisting of expansion of a CTG trinucleotide motif in the 3V untranslated of the myotonic dystrophy protein kinase gene (DMPK), located on chromosome 19q. It is a multisystemic disease affecting many systems as the central nervous system (cognitive and neuropsychiatric impairments), the heart, the genital tract, the eyes, the ears, gastrointestinal tract, endocrine system, thus leading to a wide and variable complex panel of symptoms (Meola G, 2000). Cognitive impairments, as memory, visuo-spatial recall and verbal scale, cortical atrophy essentially of the frontal and the temporal lobe and white matter lesions are often described in both DM1 and DM2 (Sansone V et al., 2007).
Neuropathological lesions, as neurofibrillary tangles (NFTs), have been observed in adult DM1 individuals aged over 50 years. The pathological tau profile of DM1 is characterized by a strong pathological tau band at 60 kDa and, to a lesser extent, a pathological tau component at 64 and 69 kDa. This typical pathological tau profile is reflected by a reduced number of tau isoforms expressed in the brain of individuals with DM1, both at the protein and mRNA levels (Sergeant N et al., 2001). In addition, tau protein expression is also demonstrated to be altered in transgenic mice with human DM1 locus (Gomes-Pereira M et al., 2007). Using specific immunological probes against exon 2 and exon 3 corresponding amino acid sequences, the neurofibrillary lesions were shown to be devoid of tau isoforms with amino-terminal inserts (Maurage CA et al., 2005). An altered splicing of tau

characterized by a reduced expression of tau isoforms containing the amino-terminal inserts characterizes both DM1 and DM2. Overall, it demonstrates that the central nervous system is affected and that DMs are real tauopathies (Dhaenens CM et al., 2011). The direct relationship between the altered splicing of tau and NFD in DM remains to be established. Indeed, such an altered splicing of tau is commonly observed in FTDP- 17 and considered as reminiscent to NFD and tauopathies.

5. Use of proteomics to investigate the mechanisms leading to Tauopathies

Induction of tau fibrillization in cells remain unsatisfactory, this is a limiting factor since NFD cannot be totally reproduced *in vitro* (Sibille N et al., 2006). The development of *in vivo* models has provided an important tool to precise sequence of molecular events leading to tau aggregation. The use of proteomics in these transgenic animals has permitted to go further in the uncovering of the cellular and molecular pathways involved in NFD spreading within the brain and its relationship with the clinical expression of neurological disorders. In this section we will focus on the overexpresion either several isoforms of tau protein or mutated forms in animal models.

5.1 Tau models

Several animal models have been created to recapitulate the two main hallmarks of AD, refearing as amyloid plaques and PHFs. Despite the numerous models existing to mimic the features of this disease, none of them cover all the neuropathological, biochemical and behaviour alterations so far. There are models focus on overexpression of APP and/or presenilin containing one or more mutations linked to familial AD but they do not present NFD. Inspite tau mutations have not been described in AD patients, mutations in tau result in NFTs in an inherited form of FTDP and this dysfunction can lead to neurodegeneration and dementia. Taking into account that AD is a complex disorder and the perfect model does not exist, the large number of tau transgenic models with their strengths and weaknesses may allow for both understanding tau pathology and developing innovative therapeutic strategies. Nowadays there are several transgenic models which own combination of mutant APP, presenilin and tau (Chin J 2011). However, this triple model presents the "limitation" that tau pathology cannot be studied independently of the amyloid effects (Sergeant N and Buée L 2011).

5.1.1 *Caenorhabditis elegans*

The nematode *Caenorhabditis elegans* is widely being used to study neurodegenerative disorders despite the evolutionary difference. *C. elegans* has a short lifespan and it is easy to manipulate genetically. Modelling tauopathies is achieved through pan-neuronal overexpression either wild-type or mutated tau leading to a progressive uncoordinated locomotion which is directly correlated with the nervous system alterations in worms. This model is very useful to identify new genetic targets (Wolozin B et al., 2011). Recent data point out that tau pathology may lead to specific interference with intracellular mechanisms of axonal outgrowth and pathfinding (Brandt R et al., 2009).

5.1.2 *Drosophila melanogaster*

Another model used is the fruitfly *Drosophila melanogaster*. Regarding tauopathies, many groups developed fruitfly models by overexpressing wild-type and mutant forms of human

tau. Transgenic fruitflies showed key features of tauopathies as tissue- and temporal-specific effects as adult onset, progressive neurodegeneration, early death, enhanced toxicity of mutant tau, accumulation of abnormal tau and relative anatomic selectivity coupled with differential effects of distinct tau isoforms (Papanikolopoulou K and Skoulakis EM, 2011).

5.1.3 Zebrafish

The novel use of the vertebrate zebrafish as a model system for AD research offers a powerful platform for genetic and chemical screens as well as developmental studies (Tomasiewicz HG et al., 2002). The transgenic expression of the human tau mutation P301L in zebrafish neurons by Gal4/UAS–based vector system recapitulates most pathological features of tauopathies as abnormally phosphorylated reactivity with the epitopes AT180, AT270, 12E8, PHF1, 422, and AT8 in spinal cord neurons, aggregation and behavioral impairments (Paquet D et al., 2010). Application of inhibitors of human GSK3β reduced tau phosphorylation showing that zebrafish kinases are sufficiently conserved with respect to their human orthologues. Current evidence point out that zebrafish tau models recapitulate pathological and biochemical events that occur in tauopathies and therefore may be useful tools for further studies in the aetiology of dementia (Bai Q and Burton EA, 2011).

5.1.4 Tau knock out mice and transgenic mice with wild-type human Tau

Tau mouse models where tau expression is suppressed by MAPT deletion or invalidation present no major changes and animals are physiologically normal (Harada A et al., 1994). It seems other microtubule-associated proteins such as MAP1A probably compensate tau deficiency. Among the mice models available with wild-type human tau it is remarkable to note that overexpression of 3R tau isoforms lead to an accumulation of hyperphosphorylated tau proteins in spinal cord neurons and axonal degeneration as well as a reduction in axonal transport (Brion JP et al., 1999). Similar data were observed in transgenic mice expressing the longest human brain tau isoform under the control of the human Thy-1 promoter. Hyperphosphorylated human tau protein was present in nerve cell bodies, axons and dendrites (Gotz J et al., 1995). Furthermore, recent studies in transgenic mouse models that express the entire human MAPT gene in the presence and absence of the mouse Mapt gene show differences between mouse and human tau in the regulation of exon 10 inclusion during development and in the young adult. In addition, it was observed species-specific variations in the expression of 3R- and 4R-tau within the frontal cortex and hippocampus during the development as well as in cell distribution of the isoforms (McMillan P et al., 2008).

5.1.5 Transgenic mice with mutated human Tau

Mutated tau transgenes have been used under various promoters (2′,3′-cyclic nucleotide 3′-phosphodiesterase, CaMKII, PDGF, Prion, or Thy1.2) with or without inducible systems. The most common phenotype of transgene tau animal is the motor alterations. Tau transgenic mice rTg4510 present P301L mutation in an inductible way and develop NFTs, neuronal loss and behavioural impairments (Santacruz K et al., 2005). Nonetheless the suppression of the expression of this mutated tau revers behavioural impairements despite the NFTs formation keeps on, indeed it seems soluble tau rather than NFTs may be deleterious. These observations are in agreement with a recent report in which brain extract injection from mutant P301S tau expressing mice into brain of transgenic wild-type tau-

expressing animals induces assembly of wild-type human tau into filaments and spreading of pathology from the site of injection to neighbouring brain regions (Clavaguera et al., 2009).

Another transgenic mice model is TauRD/ΔK280 that expresses only the 4R tau domains and carry the ΔK280 mutation with a deletion of the amino- and carboxy terminal regions of tau protein. This mutation leads to tau aggregation followed by astrogliosis and neuronal loss. When the transgene is switched off the aggregation of the exogenous tau disappears within around one month and a haf and only aggregated murine tau proteins remain acting as a nucleation factor for tau aggregation (Mocanu MM et al., 2008). Other study suggest a "prion –like" propagation since aggregation continues even if the original tau species have disappeared (Sydow A and Mandelkov EM, 2010).

The K3 transgenic mouse strain expresses human tau carrying the K369I mutation under the Thy1 promoter (Ittner LM et al., 2008). This tau mutation was found in a family of patients presenting with Pick's disease without parkinsonism and amyotrophy (Neumann M et al., 2001). The transgenic mice present early-onset memory impairment and amyotrophy in the absence of overt neurodegeneration. Tau transgene is mainly expressed in the substantia nigra and such expression leads to an early-onset parkinsonism phenotype. Interestingly, motor performance of young, but not old K3 mice improves upon L-dopa treatment. Amyotrophy is probable to be related to tau expression in the sciatic nerve in the same way as in Tg30tau model where pathogenic mutations (P301S and G272V) are expressed in the forebrain and the spinal cord showing progressive motor impairment with neurogenic muscle atrophy besides the hippocampal atrophy (Leroy K et al., 2007). Moreover, transgenic mouse model overexpressing human 1N4R double-mutant tau (P301S and G272V) and invalidated endogenous TAU gene show an accelerated human mutant tau aggregation (Ando K et al., 2011) suggesting that murine tau proteins may act as inhibitors of tau aggregation.

Thy-Tau22 mouse transgenic line exhibits progressive neuron-specific AD-like tau pathology devoid of any motor deficits (Schindowski K et al., 2006). In addition to neurofibrillary tangle-like inclusions and mild astrogliosis, this model shows hyper- and abnormally phosphorylated tau on several Alzheimer's disease-relevant tau epitopes that accumulates within the somato-dendritic area in the hippocampus (Schindowski K et al., 2008). A progressive development of NFTs is observed in the hippocampus and amygdala, which parallels behavioural impairments as well as electrophysiological alterations (Van der Jeugd et al., 2011). These latter changes are observed despite any striking loss of neuronal/synaptic markers until 12 months of age in the hippocampus. Interestingly, at that time point, THY-Tau22 mice exhibit septo-hippocampal tau pathology accompanied by altered retrograde transport from hippocampus to medial septum (Belarbi K et al., 2009) with an accumulation of the nerve growth factor (NGF) levels in the hippocampus consistent with a decrease of its uptake or retrograde transport by cholinergic terminals (Belarbi K et al., in press). Recent data indicate that voluntary exercise prevented memory alterations in these transgenic mice and increased mRNA levels of genes involved in cholesterol trafficking such as NPC1 and NPC2 (Belarbi K et al., 2011).

6. Tau proteins as biomarkers of Tauopathies

Searching for biomarkers is one of the most challenges in current medicine. Biomarkers must be not only specific for a single pathology but also indicative of its progression

(Mayeux R et al., 2011). This is extremely complex in diseases concerning elderly since many symptoms are common and indistinguishable among them as the dementia sign. It is compulsory to find proteins and their post-translational modifications that may provide accuracy on the early diagnosis of the disease and eventually could serve as a therapeutic target. Successfully the development in neuroimaging techniques enables to facilitate and establish a preliminary diagnosis of different neurodegenerative disorders.

Focusing on tauopathies, the presence of tau in CSF was first described in 1993. In AD, tau inclusions in the brain associated with neuronal damages lead to the leakage of abnormal forms of tau in the CSF resulting in quantitative and qualitative changes in CSF-tau composition. Numerous studies demonstrated increased CSF total tau and phosphorylated tau levels in AD, with mean levels 2-3 times higher compared to healthy controls. Tau is now a validated biomarker for AD, it improves the clinical diagnostic accuracy and its assessment for AD diagnosis is now proposed (Dubois B et al., 2010). As the brain lesions develop very early during the disease course even before the first clinical symptoms appear, CSF tau is not only a useful diagnostic marker in the advanced stages of the disease but also a usefull predictive marker in the earliest stages when clinical expression is weak (Hertze J et al., 2010). However, for differential diagnosis of dementia, the actually available tests measuring tau and phosphorylated tau levels in CSF are not sufficient and the identification of more specific postranslational modifications of tau in AD by proteomic approaches is needed. In the future, for the use of tau as biomarker in large clinical trials or in clinical practice, one important goal will be to develop sensitive methods to detect the very low concentration of tau in the blood (<1 pMol). Therefore, sample pre-treatment and handling will be crucial in developing a reliable tau assay in blood/plasma.

7. Conclusion

Tau is a neuronal protein that promotes neuronal survival, it is essentially located within the axonal and indispensable for the organization, stabilization, and dynamics of microtubules. The interaction between tau and microtubules is regulated by phosphorylation. It is widely reported that abnormally and hyperphosphorylated tau proteins lead to insoluble aggregates. The presence of these aggregates is clinically correlated with cognitive decline in a process called NFD; this event common to more than twenty diseases is referred as tauopathies.

The development of the proteomics era has achieved to go further in the characterization of tauopathies and shed light to the mechanism involved in their aetiology. Proteomics approaches as chromatography, mono- and bi-dimensional gel electrophoresis have reached to separate proteins with a quite high resolution after fractioning precedures, selecting a concrete population of cells or organelle isolation. The use of additional reagents to the extraction buffer such as detergents and the evolution of concomitant technologies as microscopy have provided a broad spectrum to characterize the structure and size of a large number of biological complex samples. The combination of protein separation methods with fluorescence dyes and radioactive isotopes (ICAT, iTRAQ, SILAC) makes possible not only more sensitive and reproducible results but also provides a quantitative analysis among samples (2D-DIGE, LC-MS, SELDI).

The previous hallmark is extremely linked to the identification of the separated or isolated proteins. MS has provided the composition of the molecules and also their post-translational modifications since changes in amino acid residues may be identified and characterized by

MS/MS, Peptide Mass Fingerprinting and NMR. The utilization of the current available ionization sources as ESI and MALDI coupled to mass analysers mainly TOF allows almost any compound to be analysed by MS at low levels in complex mixtures. Furthermore, there are a large number of software tools dedicated to facilitate raw data processing, database-dependent search, statistical evaluation of the search result, quantitative algorithms and statistical analysis of quantitative data.

The generation of animal models helps to elucidate the genetic and proteomics aspects involved during the origin and development of tauopathies. Only by the knowledge of the different components of the disease and their contribution, it will be possible to proceed in the right way.

In summary, numerous proteomics approaches are available in order to accomplish new perspectives in the neurodegenerative disorders field. At the moment there are many studies focused on finding out the functions of tau protein thoughtout proteomics approaches. Proteomics methods allow to uncover the different signaling pathways involved in tau biology, proteomics data are intimately related to the protein state: post-translational modifications, cleavage, conformation, synthesis, degradation and activity (if it is known). The selection of the different techniques depends on the aim of the research: protein identification, de novo peptide sequencing, and identification of post-translational modifications or determination of protein-protein interactions. Understanding the human proteome and its variations in physiological and pathological conditions will be intimately related to uncover cellular and molecular pathways involved in the aetiology and progression of the tauopathies as well as to identify potential targets for drug design.

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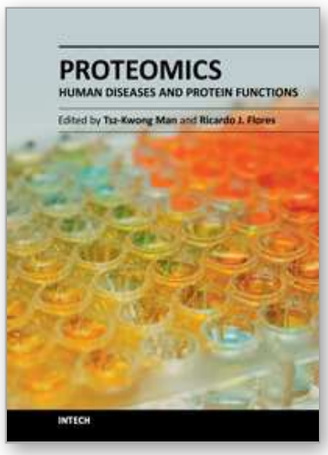

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Biomedical research has entered a new era of characterizing a disease or a protein on a global scale. In the post-genomic era, Proteomics now plays an increasingly important role in dissecting molecular functions of proteins and discovering biomarkers in human diseases. Mass spectrometry, two-dimensional gel electrophoresis, and high-density antibody and protein arrays are some of the most commonly used methods in the Proteomics field. This book covers four important and diverse areas of current proteomic research: Proteomic Discovery of Disease Biomarkers, Proteomic Analysis of Protein Functions, Proteomic Approaches to Dissecting Disease Processes, and Organelles and Secretome Proteomics. We believe that clinicians, students and laboratory researchers who are interested in Proteomics and its applications in the biomedical field will find this book useful and enlightening. The use of proteomic methods in studying proteins in various human diseases has become an essential part of biomedical research.

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