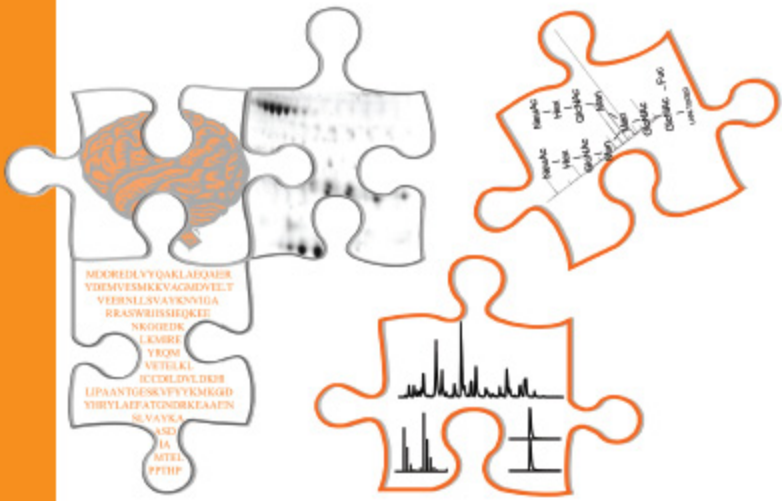
Mass spectrometry for comparative proteomics of degenerative and regenerative processes in the brain

Carina Sihlbom



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När vi leva, låtom oss leva.

While we live, let us live. "Let us enjoy life."

Dum vivimus vivamus.

ABSTRACT

Biological processes involve changes at the protein level which can be detected and quantified. Proteomics aims to determine protein changes from a normal state, for instance to measure the degree of recovery in a biological system or the state of disease progression. Mass spectrometry is the most important tool in proteomics for the identification of proteins and determination of post-translational modifications such as glycosylation. Glycoproteins were found to be altered in patients with Alzheimer's disease (AD), which is the most common form of dementia. Changes in glycosylation levels were quantified with a glycoprotein-specific stain after gel separation. Glycan structures were determined with mass spectrometry in this thesis. Protein quantification with mass spectrometric methods is based on stable isotope labeling of proteins and labeled cell cultures can be used as internal standards for tissue proteomics. Quantitative proteomics was applied to assess protein expression levels with mass spectrometry in the murine brain after specific neurosurgery to study regenerative processes.

In order to compare glycosylated proteins in cerebrospinal fluid (CSF) from individual AD patients with healthy control individuals, glycoproteomic methods were developed. To enhance the concentration of glycoproteins prior to gel separation, affinity chromatography of CSF was the most suitable prefractionation method for removing the most abundant CSF protein albumin. CSF proteins were separated with narrow pH-range two-dimensional gel electrophoresis followed by multiple staining for quantification of glycoprotein isoforms. Structural analysis of glycopeptides was performed with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), which provided very high mass accuracy and facilitated site-specific determination. A decreased glycosylation level for a protein localized in senile plaques, α_1-antitrypsin, was found in AD patients. No specific glycoform of the studied proteins could be assigned to AD, emphasizing that further studies should include a larger subject group and cover proteins in various pH intervals. Knowledge of the respective glycoprotein structures in relation to clinical disease parameters may assist in the elucidation of the pathogenesis.

In order to study proteins involved in the response of astrocytes and regenerative processes after neurotrauma, a quantitative mass spectrometric method was developed. Astrocytes, which are the most abundant cells in the central nervous system, react to neurotrauma by becoming reactive (reactive gliosis). Mice lacking the intermediate filament proteins GFAP and vimentin (*GFAP*$^{-/-}$*Vim*$^{-/-}$) show attenuated reactive gliosis and enhanced regeneration after neurotrauma. Comparative proteomic analysis showed upregulation of the adapter protein 14-3-3 in wildtype mice in denervated hippocampus, while this response was attenuated in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. Culture-derived isotope tags (CDIT) and nano-liquid chromatography FT-ICR MS showed that the 14-3-3 epsilon isoform was the major isoform upregulated in denervated hippocampus. Thus, the expression of the 14-3-3 epsilon protein is increased in neurotrauma appears to be linked to astrocyte activation. We demonstrated that the CDIT-based quantitative proteomic method is a highly useful approach to assess isoform-specific protein expression levels in defined parts of the brain after neurosurgical interventions.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Mass spektrometri kan användas för att identifiera och bestämma skillnader hos proteiner vid demens eller efter skada i hjärnan

Alla biologiska processer, vid hälsa eller sjukdom, innebär ständiga förändringar av proteiners koncentration eller struktur. Proteomik är ett vetenskapligt område som har till uppgift att identifiera alla proteiner i tex en kroppsvätska, ett organ, cell eller vävnad vid en viss tidpunkt. Funktionell eller jämförande proteomik skall hitta skillnader mellan ett normalt och icke normalt tillstånd vid sjukdom, efter skada, eller annat avvikande tillstånd. En vanlig analysmetod för att hitta dessa avvikande proteiner är gel-elektrofores, där proteiner kan separeras i två dimensioner med avseende på dess laddning och storlek, och separeras som skilda punkter i en polyakrylamidgel. Därefter färgas proteinerna med olika typer av färg som kan vara specifika för en modifiering eller som färgar hela proteinet. Sådana 2D-geler skannas för att digitalisera gelbilden och använda program för att bestämma mängden protein i varje punkt. Gelbilderna jämförs och de proteiner som har förändrats kan skäras ut från gelen, delas med enzym till peptider och extraheras med lösningsmedel och vatten. Vilket protein som är förändrat bestäms med en instrumentell teknik kallad masspektrometri och sökning i databaser för identifiering. I en analys med masspektrometri kommer först peptiderna i provet att joniseras, dvs få en positiv laddning, separeras med avseende på vikt och laddning, för att sedan detekteras. En joniserad peptid kan också skjutas sönder i masspektrometern för att bestämma massan på fragmenten som sedan matchas mot sekvenser i en databas.

Glykoproteiner är proteiner som har bundna sockergrupper dvs glykosylerade proteiner. Förändrade glykoproteiner har tidigare hittats vid den vanligaste demenssjukdomen kallad Alzheimers sjukdom (AD) i en mindre studie. Frågan var om mängden av ett glykoprotein var förändrad eller om det också var mängden bundet socker eller vilka typer av socker som var bundet. Proteomik med masspektrometri har använts i denna avhandlingen för att bestämma glykosylering av vissa proteiner hos AD-patienter. Ryggmärgsvätska (likvor) cirkulerar runt hjärnan och biologiska processer i hjärnan kan delvis reflekteras i likvor. Likvor är en genomskinlig vätska som kan tappas från patienter i nedre delen av ryggraden. Ett protein, albumin, är mycket förekommade i likvor och 80% av hela proteinkoncentrationen består av albumin. Den totala mängden protein som kan separeras på en 2D-gel är begränsad och för att kunna hitta förändringar eller nya proteiner så användes en metod för att ta bort albumin från likvor. Ett smalt pH-intervall användes i den första dimensionen till 2D-gel elektrofores och färgning specifikt för glykosylering samt för total mängd protein gjordes för att kunna detektera de olika glykanformerna på proteinerna.

FT-ICR MS är en unik typ av masspektrometri som ger en mycket noggrann bestämning av massa/laddning, vilket bidrar till att förenkla databassökning för bestämning av glykankomposition och position på peptiderna. Avhandlingen rapporterar resultat från en studie med AD-patienter och friska kontrollindivider där minskad glykosylering hos ett protein som finns i de senila placken vid AD hittades. Strukturell analys av alla glykoproteiner i likvor i det studerade pH intervallet genomfördes. Ingen specifik glykoform kunde hittas vid AD men ett större antal patienter och andra pH intervall måste studeras i

framtiden. Glykoproteomik av AD är ett område som ännu inte studerats tillräckligt och kunskap om de olika glykoproteinernas struktur i förhållande till sjukdomstiden och graden av sjukdom kanske kan ge förståelse för utveckling av demens såsom Alzheimer's sjukdom.

Masspektrometri är ingen kvantitativ metod men kan vara kvantitativ om de analyserade proverna är inmärkta med stabila isotoper såsom kol-13. Celler från hjärnvävnad kan odlas i vätska (medium) som innehåller en essentiell aminosyra märkta med kol-13. Alla nybildade proteiner i cellerna kommer då att bli inmärkta. Dessa märkta proteiner används som intern standard för kvantifiering av proteiner i ett specifikt område i hjärnan, hippocampus. En kirurgisk modell för skada i hippocampus används på möss för att studera vilka proteiner som är förändrade efter skada och som eventuellt ingår i processen för att läka skadan. Proteinet GFAP är specifikt för en celltyp kallad astrocyter och astrocyter är den vanligaste celltypen i det centrala nervsystemet. Genetiskt muterade möss som saknar proteinerna GFAP och vimentin har visat bättre läkning i nervsystemet efter hjärnskada. Funktionell proteomik användes och visade en stor ökning av proteinet 14-3-3 i normal mus efter hjärnskada och denna förändring var försvagad hos möss som saknar GFAP och vimentin. Vidare visade den kvantitativa masspektrometri analysen att en speciell variant av 14-3-3 svarade för proteinets uppreglering som kunde bestämmas med några unika peptider för den variant kallad 14-3-3 epsilon. Förändringen av 14-3-3 kan kopplas till astrocyter som blir reaktiva efter hjärnskada och troligen ingår proteinet 14-3-3 i en process som hämmar uppkomsten av nya nerver efter skada.

PAPERS INCLUDED IN THIS THESIS

This thesis is based on the following papers, which will be referred to by their roman numbers:

I. **Sihlbom Carina, Davidsson Pia, Emmett Mark R., Marshall Alan G., and Nilsson Carol L.**
Glycoproteomics of cerebrospinal fluid in neurodegenerative disease. *International Journal of Mass Spectrometry* (2004) 234, 145-152.

II. **Sihlbom Carina, Davidsson Pia, and Nilsson Carol L.**
Prefractionation of cerebrospinal fluid to enhance glycoprotein concentration prior to structural determination with FT-ICR mass spectrometry. *J Proteome Res* (2005) 4, 2294-2301.

III. **Sihlbom Carina, Davidsson Pia, Sjögren Magnus, Wahlund Lars-Olof, Nilsson Carol L.**
Structural and quantitative comparison of cerebrospinal fluid glycoproteins in Alzheimer's Disease patients and healthy individuals
submitted manuscript

IV. **Sihlbom Carina, Wilhelmsson Ulrika, Li Lizhen, Nilsson Carol L., Pekny Milos.**
14-3-3 expression in denervated hippocampus after entorhinal cortex lesion assessed by culture-derived isotope tags in quantitative proteomics
submitted manuscript

TABLE OF CONTENTS

ABBREVIATIONS

1D-GE	One-dimensional gel elctrophoresis (SDS-PAGE)
2D-GE	Two-dimensional gel electrophoresis
AD	Alzheimer's disease
AGC	Automatic gain control
CAD	Collision activated dissociation
CDG	Congenital disorders of glycosylation
CDIT	Culture-derived isotope tags
CID	Collision induced dissociation
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computerized tomography
DIGE	Differential gel electrophoresis
EC	Entorhinal cortex
ECD	Electron-capture dissociation
ESI	Electrospray ionization
FT-ICR	Fourier transform ion cyclotron resonance
Fuc	Fucose
Gal	Galactose
GalNac	N-acetyl-D-galactosamine
GFAP	Glial fibrillary acidic protein
Glc	Glucose
GlcNac	N-acetyl-D-glucosamine
GV	*GFAP*$^{-/-}$*Vim*$^{-/-}$
Hex	Hexose
HexNac	N-acetylhexosamine
ICAT	Isotope coded affinity tags
IEF	Isoelectric focusing
IPG	Immobilized pH gradients
IRMPD	Infrared multiphoton dissociation
LC	Liquid chromatography
LTQ-FT	hybrid linear ion trap-Fourier Transform
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
Man	Mannose
MCP	Multi channel plate
ML	Molecular layer (of the dentate gyrus of the hippocampus)
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional protein identification technology
MW	molecular mass
nanoLC	Nanocapillary liquid chromatography
NeuAc	N-acetylneuraminic acid
NFT	Neurofibrillary tangles
NMR	Nuclear magnetic resonance
pI	Isoelectric point
Q	Quadrupole
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	Stable isotope labeling by amino acids in cell culture
sol-IEF	Solution isoelectric focusing
SP	Senile plaques
TOF	Time of flight
Vim	Vimentin
WT	Wildtype

INTRODUCTION

1 PROTEOMICS

Biological processes involve dynamic changes at the protein level. To determine changes from the normal state protein quantification is needed and can be used for measuring the degree of recovery in a biological system or the state of disease progression. Most likely several proteins are associated with the onset and progression of a regulatory event or a disease, and they change in concentration and sometimes structure with time. Analytical tools that quantify proteins and determine post-translational modifications play an important role in life sciences.

The proteome is the protein complement of a genome (1). Proteomics is the direct qualitative and quantitative analysis of the proteins, or a subset of proteins, in a tissue or cell under a given set of physiological or environmental conditions at a given time. Global expression proteomics aim to identify all proteins present. Focused proteomics aim to identify or characterize and quantify proteins of a special biological interest due to function or role in a disease; this field could also be termed as functional proteomics (2). In 1997, Anderson presented a study of the overall level of correlation between mRNA and protein abundances in human liver and found a correlation coefficient of 0.43 between them (3), suggesting that post-transcriptional regulation of gene expression is a frequent phenomenon. Altered protein profiles of tissues or cells may be the result of altered protein modification rather than altered gene expression, and post-translational modifications are important to study in proteomics.

Mass spectrometry (MS) is the most important tool for protein identification and characterization in proteomics (4), (5), (6). Two-dimensional gel electrophoresis (2D-GE) combined with protein identification by MS is traditionally the core technology for proteomics. But it was not until the whole genome sequencing for an increasing number of organisms was completed, that 2D-GE together with mass spectrometry became important methods used in proteomics (7). Reports on the complete human genome was published in 2001 (8), (9). Mass spectrometric methods supply the needed information (partial information on sequence) to identify the protein by use of databases (10) at sensitivities below 10 fmol (~100 pg). Proteomics is a technology-driven science with the hallmark of analyzing many proteins at the same time in a possibly automated and large-scale mode. Recently developed methodologies offer the opportunity to obtain direct quantitative proteomic information by MS (11).

Protein mixtures are an analytical challenge because of the complexity and range of relative abundances (12) and there are several possible methods for proteomics, **Figure 1**. There are finite limits of hydrophobicity, isoelectric point and molecular weight range of proteins when using 2D-GE but the method is routinely applied for parallel quantitative expression lysates or body fluids (13). Complementary technologies such as multidimensional protein identification technology (MudPIT), also termed "shotgun proteomics" (14) and stable isotope labeling (15), have been developed. These methods are based on liquid chromatography (LC) MS/MS and perform analysis on peptides and therefore lose the information of protein mass, pI and post-translational modifications (isoforms), if not fully sequenced. Advantages of 2D-LC/LC are the possibilities to utilize physical-chemical properties for ion exchange and reversed-phase chromatography to achieve sharp separations. Disadvantages may be coelution of high and low abundance peptides prior to MS analysis and the huge data handling. Ionization of low abundance peptides can be suppressed and their spectra masked by high abundance peptides. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) provides higher confidence of identification in terms of mass accuracy and resolving highly protonated large biomolecules, than other MS instruments. The coupling of nano-LC to FT-ICR MS, has further improved proteomic applications (16,17,18).

One goal for proteomics in neuroscience is to find biomarkers for early detection and diagnosis of neurological disorders. A biomarker is an indicator of normal biological or pathogenic processes or pharmacological responses to a therapeutic intervention. Identification of a biomarker could facilitate the diagnosis of the disorder and/or improve the knowledge of the pathogenic mechanisms, which may provide new targets for therapeutic interventions. A biomarker can be primary or secondary to the disease and should be precise, specific and technically easy to observe. To discover biomarkers the measurements need to be reproducible in larger subject groups. Ideally, a study protocol associated with the collection of samples with clearly defined inclusion and exclusion critera designed in collaboration with medical expertise within the disease area would facilitate the discovery (19).

Proteomics has been widely used in neuroscience and particularly for comparison between healthy individuals and patients suffering from neurodegenerative or other diseases influencing the central nervous system (20), (21) as well as for mapping of proteins in hippocampus to study functions in the central nervous system (22), (23), (24).

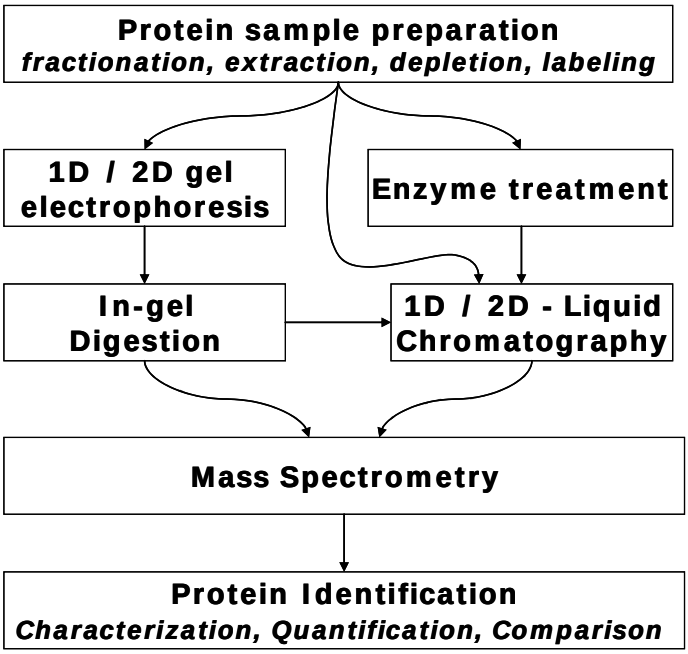


Figure 1. Schematic example of workflow in proteomics.

Aim of this thesis

The aim of the work presented in this thesis was to evaluate, develop and improve proteomic methods for protein quantification and characterization of post-translational modifications of proteins in neurodegeneration and neuroregeneration by mass spectrometry. Specific aims of the included papers were quantitative and structural analysis of glycoproteins in cerebrospinal fluid samples from patients with Alzheimer's disease and protein quantification in defined parts of the brain in mice showing enhanced regeneration after neurotrauma.

1.1 Classical gel-based proteomics

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis, 2D-GE, enables the separation of complex mixtures of proteins according to isoelectric point (p*I*) in the first dimension and molecular mass (MW) in the second dimension (SDS-PAGE) (25), (13). The initial publication on 2D-GE showed that post-translational modifications and single-site mutations could be detected (26). Protein solubility, aggregation, denaturation and relative abundance influence the separation. If a high protein load of unfractionated sample is used in attempts to detect low abundance proteins it will give rise to precipitation near the electrodes of the gel strip and to extensive smearing in the second dimension. Narrow immobilized pH gradients (IPG), in the first dimension, provide increased

resolution and in combination with prefractionation methods, enabled the detection of low abundance proteins (12), (27). After electrophoresis, the separated proteins are visualized by either silver, organic or fluorescent stains (28), or autoradiography of radiolabeled samples. A recently developed glycoprotein specific stain, Pro-Q Emerald, reacts with periodic acid-oxidized carbohydrate groups, generating a fluorescent signal on glycoproteins (29), (30). The same gel can be scanned and further stained for a total protein pattern. 2D-GE delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or posttranslational modifications. In differential gel electrophoresis (DIGE) up to three different samples are derivatized with Cy2-, Cy3- and Cy5-based chemistries, combined and separated in a single 2D gel. Proteins are detected with different excitation/emission filters in order to generate three separate images. The strongest feature of the DIGE technology is the possibility to use internal standard on each gel. Advantages and limitations of different detection technologies in gel-based proteomics has been discussed elsewhere (31).

One of the greatest strengths of 2D-GE is the capability to study proteins that have been modified, *e.g.* phosphorylated or glycosylated, because these appear as distinct spot trains on the gel. Glycoforms of glycoproteins often separate horizontally in the gel. This phenomenon is caused by a difference in the p*I* of the glycoproteins (32) and is believed to be caused by a variant sialylation of different protein glycoforms. Glycosylation or protein differences can be observed through comparative image analysis of 2D gels. It also permits the isolation of proteins for further structural analysis by MS.

1.2 Glycoproteomics

Glycoproteins

The aim of glycoproteomics is to define the different glycan compositions and structures at individual glycosylation sites in proteins. Glycoproteins are proteins that contain covalently bound oligosaccharides. Approximately one-half of all proteins from eukaryotic sources have been estimated to be glycosylated (33). Glycoproteins are present on the surface of all mammalian cells, and in the extracellular matrix with which they interact. Their abundance and large size have consequences in cell-cell and cell-matrix interactions. For a general review of glycostructures and determination with MS, see Dell and Morris, 2001 (34) and for a comprehensive description of glycoproteins, see Butters, 2002 (35).

Structural heterogeneity is an important characteristic of oligosaccharides and complicates the structural analysis of glycoproteins. In general, mammalian glycoproteins are the products of glycosyltransferases and glycosidases acting sequentially in the secretory pathway. A major contributor to glycan microheterogeneity is that the reactions involved in glycosylation and deglycosylation at specific sites in glycans are frequently incomplete. Many glycans of proteins in serum or cerebrospinal fluid are capped with sialic acid residues, generally linked to Gal or GalNAc. Examples of sialylated structures are given in the result section of Paper I. Sialylation is reversible and may be removed at some point in the life cycle of a glycoprotein. Sialic acid carries a negative charge at physiological pH and affects glycoconjugate conformation. The presence of sialic acid on the surface of a cell is a recognition determinant in cell-cell interactions (36), (37).

Common monosaccharides in human glycoproteins

Hexose:	Hex
D-Mannose	Man
D-Glucose	Glc
D-Galactose	Gal
***N*-acetylhexosamine:**	HexNAc
N-acetyl-D-glucosamine	GlcNAc
N-acetyl-D-galactosamine	GalNAc
Deoxyhexose:	
L-Fucose	Fuc
Sialic acid:	
N-acetylneuraminic acid	NeuAc

Two major types of covalent addition of oligosaccharides to proteins are found. These involve the modification of amino acid side chains: *N*-glycosylation of asparagine amino groups (*N*-linked) and *O*-glycosylation of serine or threonine hydroxyl groups (*O*-linked). Many glycoproteins will contain both *N*- and *O*-linked oligosaccharides and have more than one oligosaccharide chain per molecule. The consensus sequence for *N*-glycosylation is N-X-S/T/C, in which X cannot be proline. The requirements for *O*-linked glycosylation are less restrictive and no consensus sequence has been identified. All mammalian *N*-linked glycans share the same pentasaccharide core, two *N*-acetylglucosamine and three mannoses derived from a biosynthetic precursor $Glc_3Man_9GlcNAc_2$ that is added cotranslationally to polypeptides in the ER. *N*-linked glycans fall into three main classes, high mannose, hybrid and complex-type. Processing involves stepwise trimming by exoglycosidases and stepwise addition of new sugar residues catalyzed by glycosyltransferases. Individual protein molecules will carry a unique set of oligosaccharide structures and this subset of the population is termed a glycoform.

Aberrations in sialylation have been associated with disease. Increased sialylation on the surface of tumour cells is well known and is due to either increased sialyltransferase activity or increases in the number of termini available for sialylation as a result of an upregulation of branching in *N*-linked glycans. Glycans play key roles in processes such as protein folding, cell-cell recognition (37), cancer and for the immune system (38). In humans, congenital disorders of glycosylation (CDG) results in severe mental and physical disease. 2D-GE and MS analysis of plasma from CDG patients have revealed increased fucosylation and branching on transferrin and α_1-antitrypsin, relative to normal controls (39). Glycosylation changes in Alzheimer's disease (AD) have been studied with 2D-GE of frontal cortex samples and the quantitative analysis of the glycoprotein and total protein profiles revealed decreased glycosylation of collapsin response mediator protein 2 (CRMP-2) in AD brain (40). Because glycosylation and phosphorylation may interact and even compete for the same serine and threonine residues, aberrant glycosylation may participate in the intracellular signaling that mediates neurodegeneration in AD (41), (42).

Analysis of glycoforms

In 2D-GE, variant glycoforms of glycoproteins are seen as trains of spots of the same protein separating at different isoelectric points. The separation of the glycoproteins with similar composition is limited, and therefore a single 2D gel spot may contain more than one protein glycoform. Analyzing the glycosylation state is problematic because unglycosylated peptides outnumber the glycosylated ones. Glycoform heterogeneity is a sensitivity barrier to overcome, because 1 pmol of a glycoprotein on a gel will represent a mixture of many glycoforms present only in the low fmol range (43). Sample fractionation, purification or desalting may also be performed after gel electrophoresis. Recently, it was suggested to selectively purify glycopeptides from 1D band in-gel tryptic digested samples to increase glycopeptide signal by reducing the interfering unmodified peptides for analysis with electrospray MS/MS (44).

Affinity chromatography can be used to enrich glycoproteins that have a known carbohydrate epitope (45), (46). Lectins are proteins of non-immune origin that specifically bind complex carbohydrates either to terminal residues or as part of an extended sequence. Lectin-based affinity chromatography binds a subset of glycans and a range of lectins must be used to capture a diverse population, resulting in extensive sample preparation. However, a multi-lectin affinity column could be used to capture at least several glycoforms. In an approach to analyze glycoproteins from human serum, an affinity column with three lectins, were used with reproducible results (47). After depletion of the six most

abundant proteins, 50% of the remaining proteins were found to be glycosylated. Site-specific determination of glycosylation based on lectin affinity chromatography and isotope-coded tagging has been reported (48). A combination of labeling and a multi-lectin column may be promising for biomarker discovery in serum. Relative quantification of glycoproteins was also possible, but the lectin affinity capture did not identify very low abundant *N*-linked glycoproteins. Another type of glycoprotein isolation is the conjugation of *N*-linked glycoproteins to a solid support using hydrazide chemistry (49) which was recently combined with stable isotope labeling to also include quantification by mass spectrometry (50).

***N*- and *O*-linked oligosaccharides can be released** from glycoproteins before analysis. Determination of glycans in human plasma by enzymatic release of *N*-linked oligosaccharides using PNGase F, followed by the chemical release of *O*-linked oligosaccharides using reductive β-elimination, and analysis with LC-MS was recently described (51). The human plasma sample was depleted of serum albumin, IgG, and fibrinogen and the remaining proteins were separated by 2D-GE and electroblotted to PVDF membrane. The released *N*-linked oligosaccharides were aspirated from the membrane and desalted and then the remaining *O*-linked oligosaccharides of the protein were released. Another method based on deglycosylation with PNGase F after nonspecific proteolysis and solid-phase extraction combined with MALDI-FT MS provided a sensitive method for identification of glycosylation sites and oligosaccharide heterogeneity in glycoprotein from *Xenopus laevis* egg (52). With this release approach it is necessary to perform complementary analysis of the glycopeptide for determination of the glycosylation site, thus two MS analysis are required, **Figure 2**.

Glycoprotein analysis by ESI-FT-ICR MS and infrared multiphoton dissociation tandem mass spectrometry has been utilized in combination with 2D gel glycoprotein separation. Determination of differences in glycosylation from pooled samples of CSF glycoproteins was performed without glycan release (53). Extensive sample manipulation was avoided and sample loss was minimized. Efficient sample utilization is particularly important for scarce biological samples acquired for disease screening purposes. Deglycosylation results in loss of glycosylation site specificity. Analysis of intact glycopeptides allows assignment of glycan structures to specific sites of *N*-glycosylation, and their comparison between gel-separated glycoprotein isoforms.

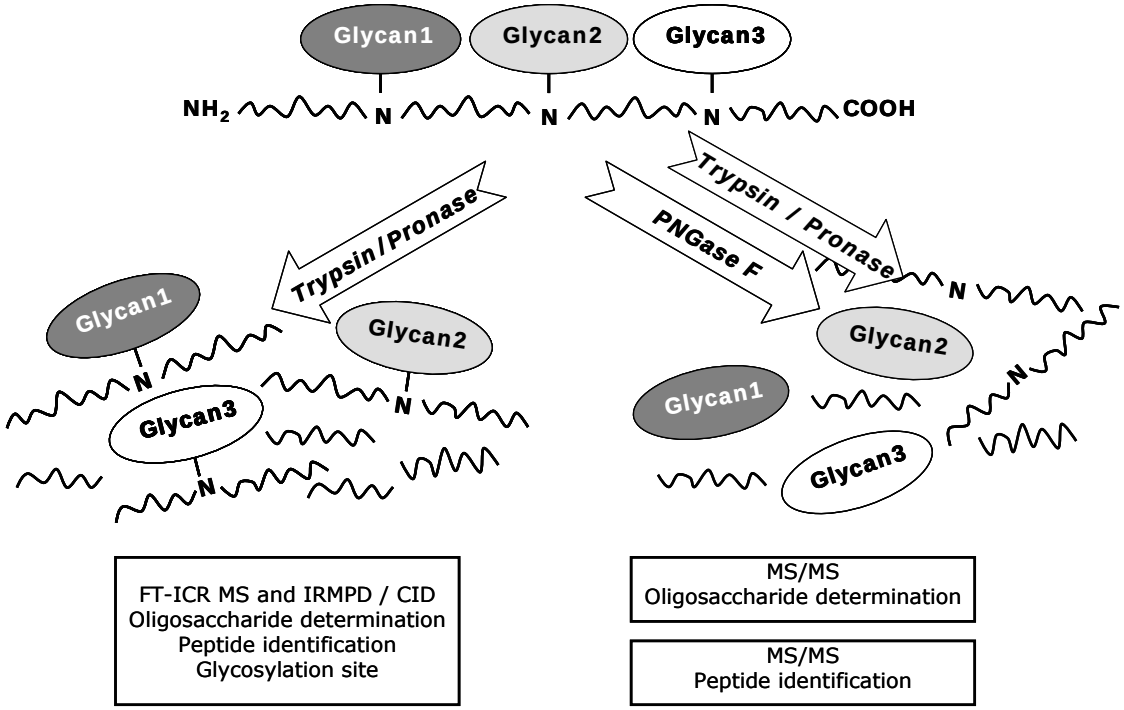


Figure 2. Two major strategies of glycoproteomics. Analyzing the glycopeptides with the glycan attached or the deglycosylation pathway.

Our workflow in glycoproteomics starts with proteolytic digestion of gel-separated glycoproteins followed by LC-MS and MS/MS analysis **(Figure 2).** Characteristic oligosaccharide fragment ions are used to search for glycopeptide precursor ions. Glycan structures can be assigned to the MS and MS/MS data through peptide sequence, oligosaccharide stoichiometry and biosynthetic consideration. For full structural characterization, nuclear magnetic resonance (NMR) spectroscopy and monosaccharide analysis with LC and exoglycosidase digestion must be used.

1.3 Quantitative MS-based proteomics

Stable isotope labeling

Quantitative proteomics has traditionally been performed by the combination of 2D gel electrophoresis (spot intensity and volume) with MS or MS/MS-based sequence identification of selected protein spots. The development of instrumentation for automated, data-dependent electrospray ionization (ESI) MS/MS, in conjunction with micro- and nanocapillary liquid chromatography (nanoLC) has increased the sensitivity and speed of identification of gel-separated proteins. Moreover, nanoLC-MS/MS has also been successfully used for the large-scale identification of proteins directly from mixtures, without gel electrophoretic separation. Mass spectrometry is not a quantitative

method because of the varying detector response, ionization efficiency for different peptides, condition of ion source and other factors. During the last years, mass spectrometric methods have, however, turned quantitative based on stable isotope labeling that is used for the simultaneous identification and quantification of complex protein mixtures. Observed peak ratios for isotopic analogs can be accurate when there is no chemical difference and they are analyzed simultaneously (54). Stable isotopes vary in their utility as labeling agents. Deuterium is inexpensive and easily incorporated into organic compounds but causes decreased retention during reversed-phase chromatography while coding samples with ^{13}C causes no observable difference in elution time (55), (56).

Metabolic incorporation of stable isotope ($^{15}N/^{13}C$) labeled nutrients in growth media of cultured cells is a method for global coding of proteomes. Metabolic coding has several advantages, but one drawback is that the number of heavy isotopes incorporated into a peptide will vary with amino acid composition and molecular weight and makes it difficult to recognize the coded isoforms. Instead, metabolic incorporation of stable isotopically labeled amino acids into proteins by growing cell cultures has been used for comparative proteomics.

ICAT, SILAC AND CDIT

Isotope coded affinity tags (ICATs) are chemical modifiers that covalently bind cysteine residues. Isotopic coding through chemical modification of cysteine in proteins (57) began with the use of deuterium isoforms of acrylamide to improve the identification of cysteine peptides in mass spectrometry. ICAT combines a biotin affinity tag and isotope coding in a single alkylating reagent (15), (58), (59). Each tag contains a cleavable linker attached to a biotin moiety so that labeled peptides can be purified, the biotin tag is then removed to generate a mass addition of 227 Da or 236 Da for the light or heavy tag, respectively. ICAT allows use of protein material from non-living sources and has been used in the study of postsynaptic signaling of proteins isolated from rat forebrain and cerebellum (60), **Figure 3**. The drawbacks of ICAT is the high cost and the required chemical modification and affinity steps which together with the relative low abundance of cysteines may compromise low level analysis and some proteins may not be detected. The benefit is that the selective enrichment of cysteine-containing peptides reduces the complexity of the peptide mixture.

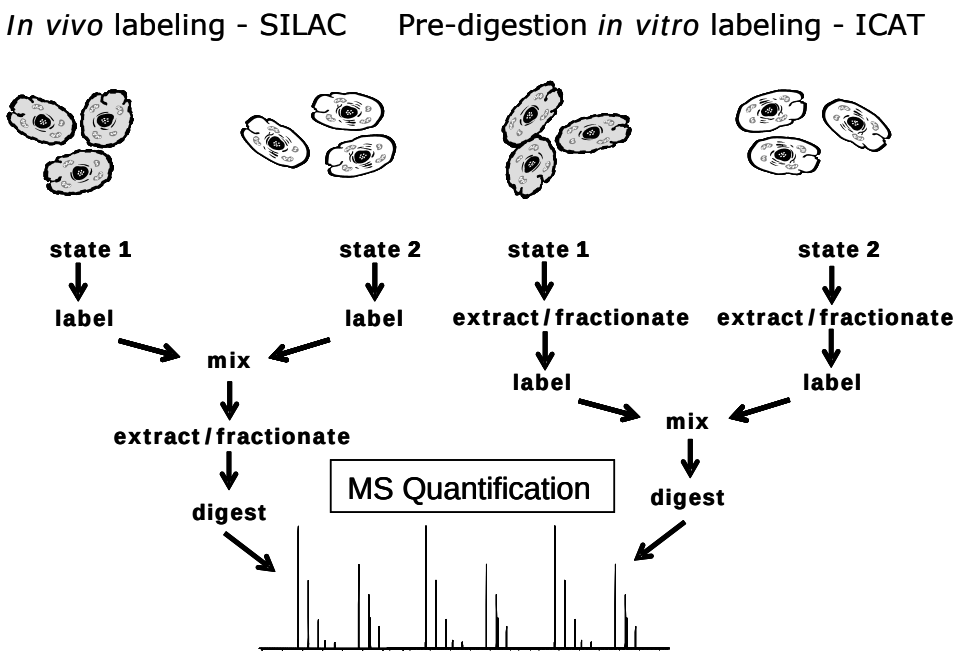


Figure 3. Schematic preparation and analysis for MS-based quantitative proteomics using SILAC or ICAT. The major differences between the methods are the point in the process where the label is introduced and the biological source to be analyzed.

SILAC, (**s**table **i**sotope **l**abeling by **a**mino acids in **c**ell culture) provides predictable mass shifts between peptide pairs (54), **Figure 4.** Cell cultures are grown in media lacking one amino acid but supplemented with a non-radioactive, isotopically labeled form of that amino acid, and cell cultures for comparison are grown in normal media. Protein populations are mixed directly after lysis and further analyzed together. The choice of labeled amino acid and its abundance will determine the number of tryptic peptides that are labeled. Lysine is relatively high abundant and proteases such as trypsin produce peptides with a single lysine residue. Leucine is another abundant amino acid that gives a broad labeling of a proteome. Quantification accuracy can be low if low abundance peptides only appear in one or two consecutive MS spectra (poor precision), if only one or two peptides are available (poor protein ratio) or if any of the labeled or unlabeled peptide partially coeluted with another peptide (isotope cluster overlap) (55). Five population doublings are required for isotopic equilibrium to be reached. SILAC is a simple, inexpensive and accurate procedure that can be used as a quantitative proteomic approach in any cell culture system for *in vitro* studies.

For quantitative tissue proteome analysis, the principle of SILAC was used to generate proteins to be used as internal standards and this approach was termed CDIT for **c**ulture-**d**erived **i**sotope **t**ags (61). An isotopically labeled amino acid, such as ^{13}C-leucine, is added to the cell

culture media lacking that amino acid, and will be incorporated into all newly synthesized proteins. An equal volume of the labeled lysate is added to each tissue lysate with equalized protein concentration, thus internal standard and tissue lysate are mixed in the beginning of sample preparation to reduce potential sources of experimental variation, **Figure 4**. The mixed lysate is separated according to molecular weight by 1D-GE. Corresponding light and heavy peptides from the same protein are co-eluted by chromatographic separation in the MS scan. Relative quantification is obtained from the ratio between intensity areas of the light (tissue peptide) and the heavy (cell culture peptide) peaks in the MS scan. To obtain a protein ratio, individual peptide ratios are averaged and compared between the groups.

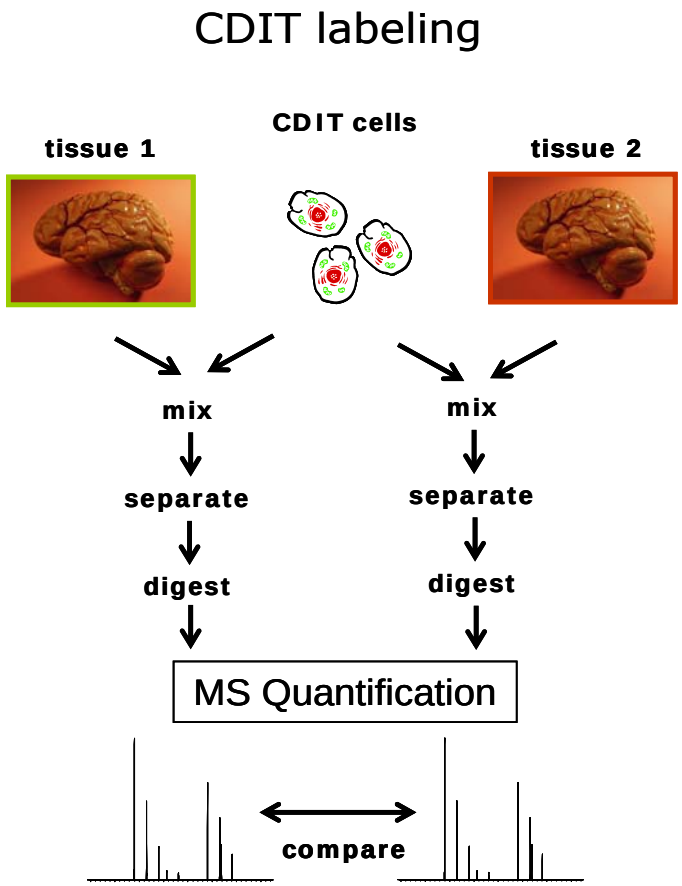


Figure 4. Strategy of quantitative tissue proteomics using culture-derived isotope tags as global internal standards. Tissue sample 1 and 2 are mixed with cultured cells early in the process to obviate the variations during sample preparation. After separation, digested proteins are analyzed with MS to identify and quantify proteins. The ratio between the two isotopic distributions (tissue versus cells) can then be determined from the MS spectra. Changes of protein level in two tissue samples are estimated (compared) by calculating the ratio of the two ratios, a procedure which cancels out the internal standards (cultured cells).

1.4 Prefractionation techniques

By performing a prefractionation step prior to 1D-GE, 2D-GE, LC or other separation, less abundant proteins can be enriched and detected. Chromatographic and electrophoretic fractionation methods have been developed to be compatible with 2D-GE (12), such as liquid isoelectric focusing (62), subcellular fractionation (63) and phosphoprotein enrichment (review in (64)). Sucrose gradient centrifugation and affinity purification using magnetic beads have been applied for fractionation of hippocampus (65). In cerebrospinal fluid (CSF), the very abundant protein, albumin, limits the amount of CSF that can be loaded on 2D-GE.

Solution isoelectric focusing, sol-IEF, is a separation technique based on electrophoretic prefractionation in liquid phase according to the pI of the protein. A multicompartment electrolyzer with isoelectric membranes has been developed by Righetti and coworkers (for a review see (12)) and further simplified by Zuo *et al.*,(66,67). These procedures are particularly useful if the prefractionated proteins are applied to narrow-range IPG gels (zoom gels) (68). This type of prefractionation allows higher protein load (6- to 30-fold) on narrow IPG gels without protein precipitation, and allows detection of low abundance proteins, because major interfering proteins such as albumin have been removed (66,67).

Depletion can be used for removal of a specific protein, for instance albumin (69), (70), (71), (72) but the limitation is that some other proteins might bind to albumin and would also be retained on the affinity column. Many commercial affinity albumin removal kits have been designed specifically for use with serum/CSF and for a minimum of unspecific binding. Among the albumin depletion columns, Cibacron Blue-Sepharose (73) media or monoclonal antibodies (74), (75), (76) are the most common affinity materials. The protein concentration is much higher in serum than in CSF and reducing the volume of CSF might be necessary before the affinity removal of albumin (77).

2 BIOLOGICAL PROCESSES

2.1 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of neurodegenerative dementia disorder. An early symptom is memory loss, and the disease progression finally leads to severe dementia with decreased intellectual functions and confusion. AD is characterized by the development of senile plaques (SP) deposits of amyloid beta peptide and neurofibrillary tangles (NFT), abnormally twisted forms of the protein tau, in the brain. SP and NFT are associated with neuronal degeneration. The amyloid beta peptide is a product from enzymatic processing of amyloid precursor protein. When tau becomes hyperphosphorylated, it is immobilized into paired helical filaments and the axonal transport is comprised, leading to loss of synapses and degeneration (78). Severity of dementia correlates with the number of tangles found in the brain (79). The diagnosis is based on clinical examination, memory tests, together with measurement of biochemical markers in blood and CSF, electroencephalography and structural brain imaging such as computerized tomography and magnetic resonance imaging (MRI) to exclude other disorders. Low levels of beta amyloid 1-42 and high levels of tau and phospho-tau in CSF taken together is a strong indication of AD (80), (81), (82). However, only post-mortem identification of SP and NFT definitely confirms the diagnosis of AD.

Lack of an objective biological measure for AD onset and progression also limits the ability to assess the potential of new therapies. This has delayed the development of AD treatments and preventatives. Current drugs improve symptoms, but do not have disease modifying effects (83). Acetylcholinesterase inhibitors are the recommended therapy for mild to moderate AD. Early diagnosis of Alzheimer's disease (AD) is needed to initiate symptomatic treatment with acetylcholinesterase inhibitors. New therapies targeted at the probable underlying pathophysiology of AD are currently in clinical trials. Their functions include inhibition of Abeta fibril formation, β- and γ-secretase inhibition, cholesterol lowering agent and anti-inflammatory agents. However, there is no clinical method to determine which patients with mild cognitive impairment will progress to AD with dementia (84). This increases the need for more specific biomarkers. An ideal biomarker should be able to detect a specific neuropathological feature, for example the tau or the synaptic pathology in AD.

2.2 Cerebrospinal fluid

Cerebrospinal fluid (CSF) contains a dynamic and complex mixture of small molecules, peptides and proteins. CSF circulates within the

ventricles of the brain, and surrounds the brain and spinal chord. CSF is secreted from several central nervous system (CNS) tissues; in particular, from the ventricular *choroid plexus,* and is directly connected to the extracellular (interstitial) fluid. The extracellular fluid surrounds the neurons and glia, therefore changes of protein and peptide concentration and modifications in CSF may reflect ongoing pathological processes in the CNS (77). The total volume of CSF in the human ventricular system is about 100-150 ml. CSF is produced, reabsorbed and replaced four times every day (85).

CSF contains a high salt concentration (>150 mM) and a low protein concentration, about 250 mg protein/ml, which is 200 times lower than in plasma. This high salt level interferes with the electrophoretic separation of proteins, because of the high electrical current that is carried by the salt load and thus reduces the efficiency of the 2D-GE. Some low abundance CSF proteins are difficult to characterize due to a low total protein concentration, a high amount of albumin and immunoglobulins. The amount of albumin is about 80% of the total CSF protein content. Precipitation of high-abundance proteins may occur during isoelectric focusing (IEF), and an increased number of horizontal and vertical streaks occur on a 2D-GE gel if too much sample is loaded in order to visualize the low-abundance proteins. Therefore, prefractionation of CSF is needed to enrich low abundance proteins (86). CSF contains many glycoproteins, such as prostaglandin-H2 D-isomerase, clusterin, apolipoprotein E and α-1-antichymotrypsin, and each glycoprotein has a variety of different glycosylated isoforms. An experimental example is about 60 gel spots that corresponded to the cellular prion glycoprotein revealed by immunoblotting in studies of CSF and brain (87).

Characterization of the CSF proteome (**Figure 5**) is necessary for the discovery of new biomarkers that may reflect the presence and progression of neurological diseases. The CSF proteome has been characterized in many studies, (72), (86), (88), (89), (90), (91), (92), (93) and recently reviewed (77) (24), (94), (95), (96). A highly reproducible 2D-GE separation of CSF resulted in identification of more than 480 spots including many isoforms using MS and MS/MS (97).

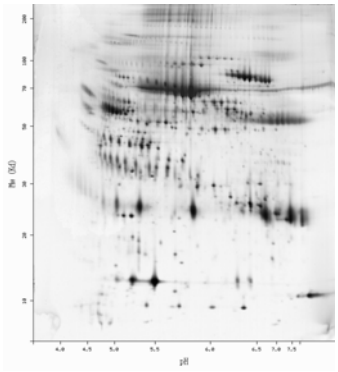


Figure 5. Example of CSF proteome map, 2D-GE pH 4-8 (www.expasy.org).

2.3 Reactive astrocytes

Astrocytes are the most abundant cells in the central nervous system (CNS). The importance of astrocytes in the maintenance of the homeostasis of the CNS, nutrition of neuronal cells and neurotransmitter recycling is well known. Astrocytes have been proposed to control both the number and the character of neuronal synapses (98), it was shown that astrocytes can themselves differentiate into neurons (99) and that neural stem cells differentiate more readily into neurons when co-cultured with mature hippocampal astrocytes (100). Activation of astrocytes accompanies many CNS pathologies including trauma, brain ischemia and neurodegeneration. These are situations in which astrocytes change both their appearance (**Figure 6b.**) and gene expression (101). Activation of astrocytes is referred to as reactive gliosis. Reactive gliosis may constitute a physical and biochemical barrier to neuroregeneration in the injured CNS, for a review see (102). The role of reactive gliosis in healing or recovery in various CNS pathologies is still incompletely understood.

The cytoskeletal system organizes the cell interior and determines the shape and many functions of a cell. It consists of actin filaments, intermediate filaments and microtubules. Intermediate filaments have a similar structure in all cell types, however their protein composition depends on the cell-type and often also on the functional state of a cell (103). Mouse transgenic models were developed to assess the role of reactive gliosis in CNS pathologies and regeneration, in which two astrocyte intermediate filament proteins, glial fibrillary acidic protein (GFAP) and vimentin (Vim) were genetically removed by gene targeting in vivo (104). *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice show slower wound healing and reduced glial scaring after brain or spinal cord trauma (105). The Pekny group and others have previously reported improved regeneration in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, specifically axonal regeneration (106), (107), survival and integration of neuronal grafts in the retina (108) or improved synaptic regeneration in the denervated dentate gyrus of the hippocampus (109), for a review see (102).

2.4 Denervated hippocampus

Entorhinal cortex lesion is an injury model for studying axonal degeneration and synaptic plasticity (110). A microsurgery lesion interrupts the axonal connections in the outer molecular layer of the dentate gyrus of the hippocampus, **Figure 6a**. This denervation triggers astrocyte activation, synapse remodeling and neurogenesis in the dentate gyrus, an area not directly affected by the trauma.

Mice lacking intermediate filament proteins, GFAP and vimentin, showed a more prominent loss of synaptic complexes in the denervated area at day four after lesion as compared to wildtype mice. Ten days later, there was a remarkable recovery with the number of synapses as high as prior to the injury. This and other evidence, reviewed in (111), suggests that wildtype reactive astrocytes are neuroprotective at an early stage after injury, but inhibit regeneration later on (109).

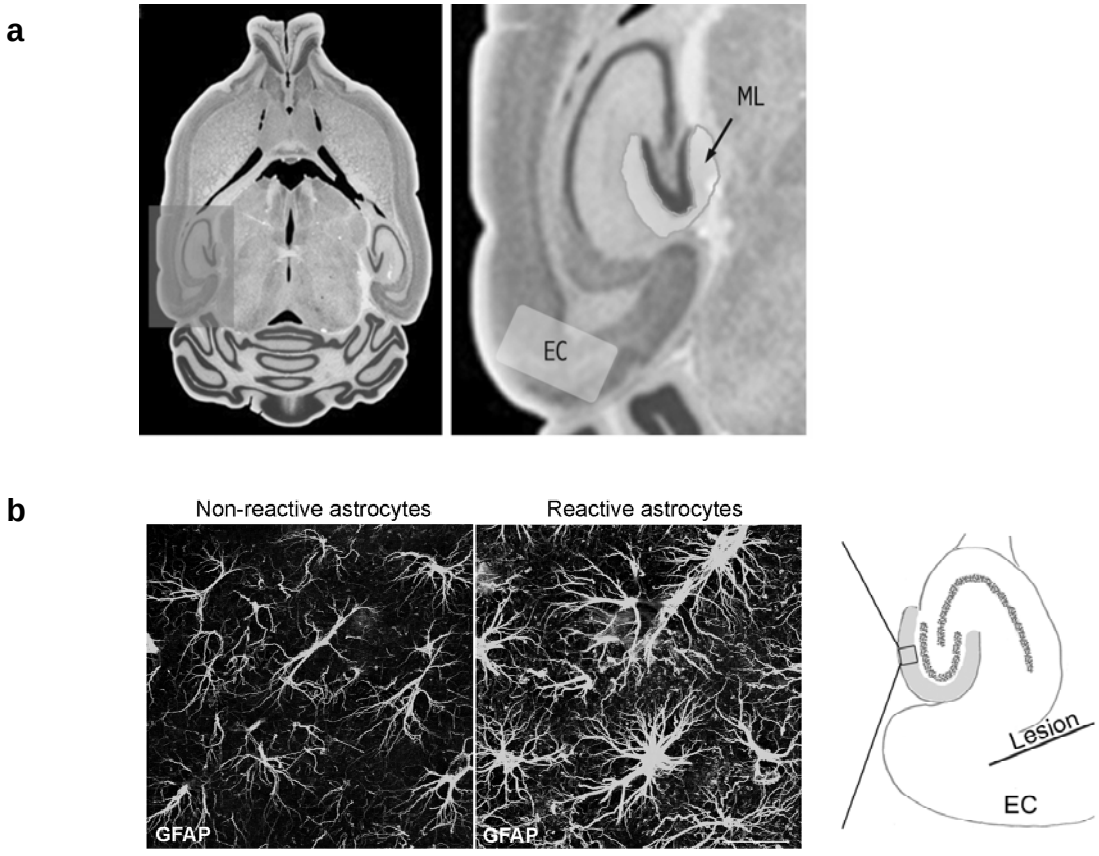


Figure 6. a) Entorhinal cortex (EC) lesion partially denervates the molecular layer (ML) of the hippocampus. **b)** Entorhinal cortex lesioning triggers reactive gliosis in the hippocampus. Astrocytes are activated in the molecular layer of the dentate gyrus (right in gray). *Panel b reprinted from Wilhelmsson et al. (112).*

3 MASS SPECTROMETRY

Principle of mass spectrometry

Mass spectrometry has played an increasingly important role in life science during the last decade and particularly for the identification and characterization of proteins. The principle of mass spectrometry is to ionize molecules, separate the gas phase ions and detect them according to their mass-to-charge ratio (*m/z*). There are several different types of ion sources, mass analyzers and detectors. The invention of electrospray ionization (ESI) (113) and matrix-assisted laser desorption/ionization (MALDI) (114), finally allowed sensitive and soft ionization of large biomolecules. Today, the two most common methods to generate gas-phase ions from a protein/peptide sample are MALDI and ESI. The ions are separated in a mass analyzer, for instance a magnetic sector, quadrupole (Q), time-of-flight (TOF), ion trap or Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Ions are finally detected by; conversion dynode/electron multipliers for sector-, ion trap- and quadrupole instruments, a multi channel plate (MCP) for TOF instruments or image current (frequency) for FT-ICR and Orbitrap instruments. The overall sensitivity of any mass spectrometer depends on the ionization mode, efficiency of ion transfer to the mass analyzer, the efficiency of the mass analysis and sensitivity of the detection. In this thesis, electrospray in combination with FT-ICR MS has been the analytical method, **Figure 7**, and the principles behind these techniques are described in more detail.

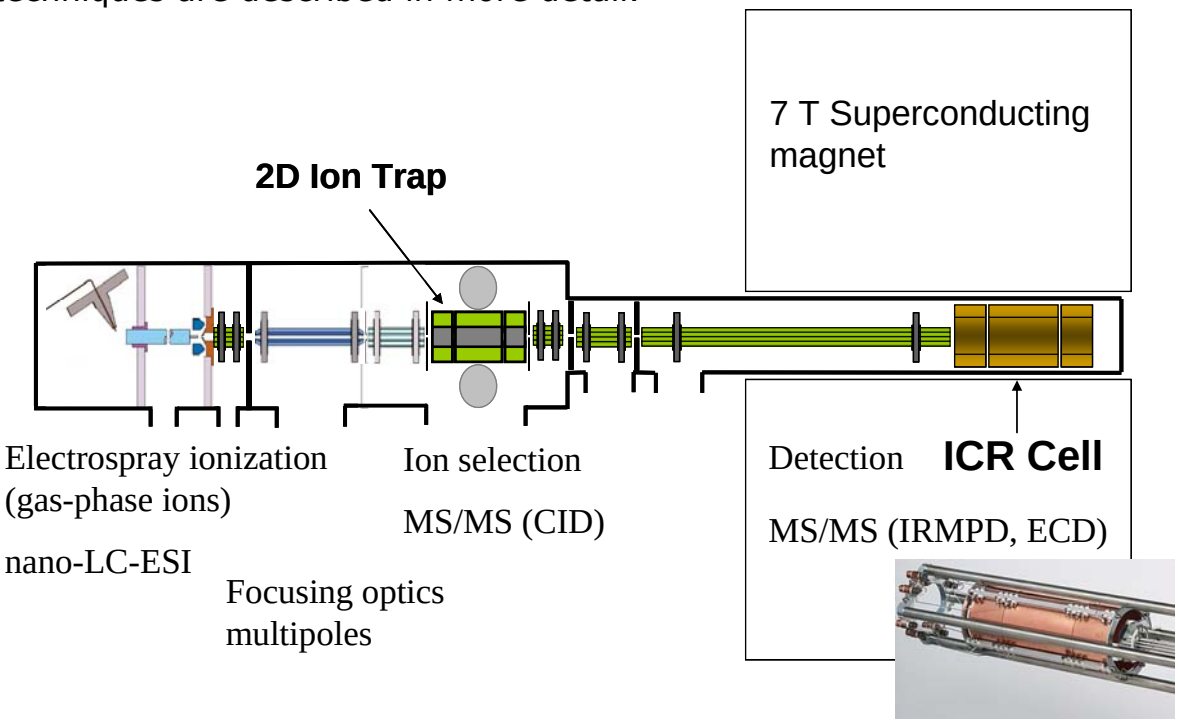


Figure 7. Schematic drawing of a hybrid linear ion trap-FT-ICR mass spectrometer.

3.2 Electrospray ionization and nano-LC

For analysis of large biomolecules in FT-ICR MS, electrospray ionization (ESI) is preferred (115) and can be in the form of nanospray (116,117) or microelectrospray (118), used in Paper I, for the formation of multiply charged ions. This allows the analysis of ions of several thousands Da in mass while retaining optimum FT-ICR performance with the typically observed low mass-to-charge ratios (m/z < 2000), (119). Combining on-line nano liquid chromatography (LC) with external ion accumulation in the FT-ICR mass spectrometer improves the duty cycle for analysis of continuously generated ions (120) and low fmol detection limits of peptides are possible (118). Nano-LC FT-ICR MS was used in Paper II-IV.

In positive mode ESI, the sample solution is sprayed from a thin capillary emitter at high voltage (+1-3 kV) and positive ions are formed at atmospheric pressure. Positively charged droplets will be formed from the extended liquid filament of the Taylor cone at the emitter tip and solvent evaporation take place through a heated capillary which leads to droplet shrinkage. The increasing electrostatic repulsion from the charges in the droplet will overcome the surface tension and droplet fission occurs (Rayleigh limit). This process continues until gas-phase multiply charged positive ions are formed by solvent evaporation from the very small droplets (< 10 nm) until each droplet contains only one ion, single ion in droplet model by Dole (121), **Figure 8**. Alternatively, positive multiply-charged gas-phase ions are formed directly from the surface of a droplet (≈ 10 nm), ion evaporation model by Iribarne and Thomson (122), (123).

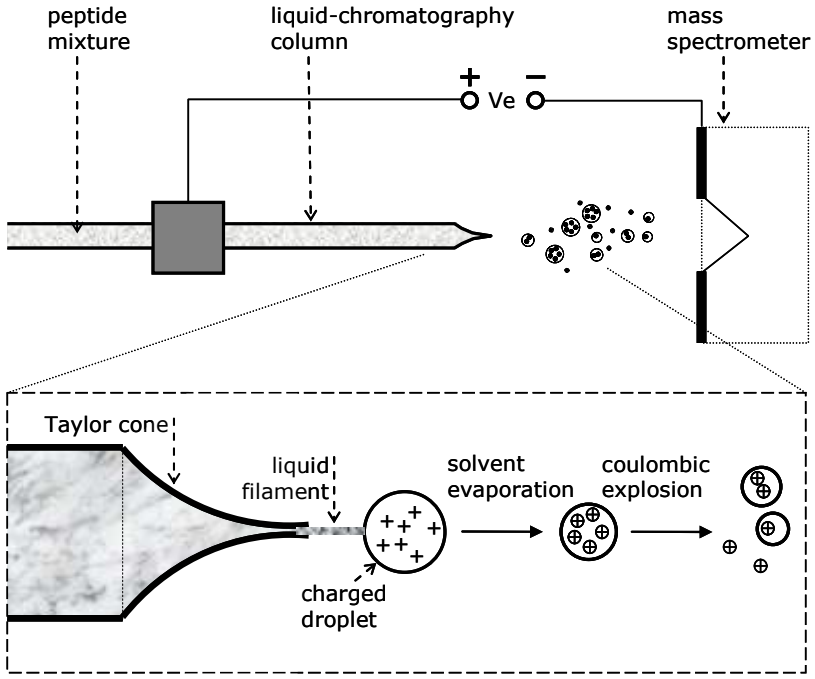


Figure 8. Electrospray ionization. *(modified from Steen et al. (124)).*

An ESI mass spectrum of a biomolecule [M] will contain ions in several charge states at $[M+zH^+]^{z+}$, which will be observed at different *m/z* values $[M+zH^+]^{z+}/z$. As ions become more highly charged, the mass difference between charge states decreases and the isotopic peak spacing becomes narrower. As a result, it becomes more difficult to resolve the isotopic peaks and the resolving power of the mass analyzer becomes more important making ESI combined with FT-ICR a powerful choice, because multiply charged ions can be more easily resolved by FT-ICR than by lower resolution MS techniques. Isotopic resolution allows a direct determination of the charge state, *z*, since the mass difference between the isotopes $\Delta m = 1/z$.

Nano liquid chromatography (nano-LC) with column i.d. of 75 μm or less opened a new pathway to high-sensitivity analysis of complex biological samples (125), Figure 9. Since ESI is a concentration sensitive technique, the best way to increase sensitivity in LC-ESI MS is to decrease the column inner diameter which in turn decreases the peak volume, as a result of decreased flow rate (20-400 nl/min), with increased concentration as a result. The sensitivity is important for proteomic applications with limited sample amount (125). Coupling of nano-LC in front of the mass spectrometer also improves sensitivity through preconcentration of the sample due to on-column focusing and desalting. Sample amounts of digested protein in the low fmol range loop-injected onto the nano-LC column have been demonstrated (126).

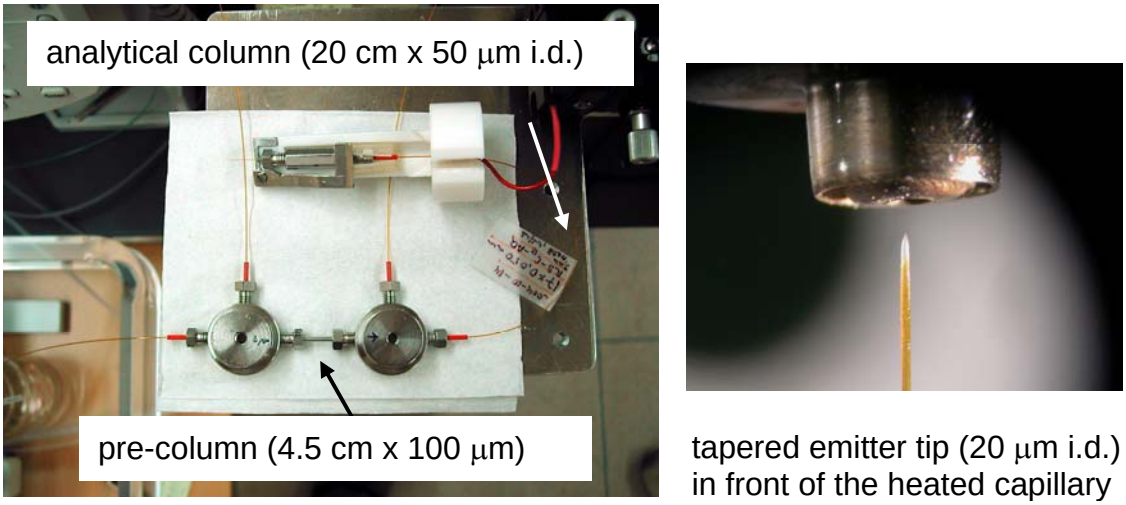


Figure 9. Nano-LC column setup used in Paper II-IV.

An example from our laboratory is nano-LC MS/MS of tryptic peptides from < 2 fmol of alcohol dehydrogenase (ADH 1 from yeast) separated on a fused silica column packed in-house with 3 μm RS-C_{18}-AQ particles coupled to a fused silica emitter through a stainless steel union and analyzed with a hybrid linear ion trap-FT-ICR mass spectrometer (Hasse Karlsson, unpublished data). The very fast scan rate of the linear ion

trap (LTQ-FT mass spectrometer), handles isolation, fragmentation and detection of up to six precursor ions during 1 s, making it highly compatible for coupling to nano-LC. A tryptic peptide can elute during less than 1 s and up to 10 s.

3.3 Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

Mass spectrometric analysis of larger biomolecules in complex mixtures requires that the mass analyzer have both high resolving power ($R=m/\Delta m$, Δm is the width at half maximum) and good mass measurement accuracy which can be provided by FT-ICR MS (127,128) and this technique is therefore an important complement to MALDI-TOF and ESI-Q-TOF instruments. The new generation of hybrid linear ion trap-FT-ICR mass analyzers have shown a great potential for proteomic analysis both at peptide levels (bottom-up approach) and for intact proteins (top-down approach) (129), (130). Ions can be measured in the ICR cell with high mass accuracy with simultaneous efficient fragmentation and detection in the ion trap. Unique fragmentation techniques for characterization of post-translational modifications such as glycosylation and phosphorylation, can be applied in the cell of the FT-ICR spectrometer (131), (132). FT-ICR MS applications and technological developments relevant to the field of proteomics were recently reviewed (133), (134).

Mass analyzer and detection

Mass analysis is performed at high vacuum, $\sim 10^{-10}$ torr for FT-ICR. The first FT-ICR mass analyzer was demonstrated by Comisarow and Marshall in 1974 (127), (128). In the FT-ICR mass analyzer, the ICR cell is a Penning trap in a strong permanent superconducting magnet, with a field strength of 3-14.5 T. Advantages of higher magnetic fields include improved mass resolving power, signal-to-noise ratio, dynamic range by increasing the upper limit for peak coalescence and mass accuracy (128). The ions are transferred into the cell by focusing optics such as radio frequency (RF-only) quadrupoles, hexapoles or octopoles. The cell, in which the ions are trapped, can be of different geometries but the most common design is cylindrical. To prevent the ions from leaving the cell along the axial direction, a trapping potential of 1-10 V is applied to the two end cylinders/endplates of the cell. Due to the trapping voltage the ions will oscillate sinusoidally back and forth between the end cap electrodes.

Ions entering the magnetic field of the FT-ICR mass analyzer experience a magnetic Lorentz force, $F_B=q(\mathbf{v}X\mathbf{B})$, which is balanced by the centrifugal force (mv^2 / r). As a consequence of this force, the magnet field in the z-axis direction bends the ion path into a circle in the xy-plane perpendicular to the magnetic field, **Figure 10**. The radius of the orbit will be, r = mv / qB. The angular velocity, ω, is given by

$\omega = v / r = q\ B / m;$
$\omega = q\ B / m;\ \omega = 2\ \pi\upsilon_c;$

$\upsilon_c = q\ B / 2\ \pi\ m$ (cyclotron equation)

υ_c is called the cyclotron frequency in Hz (128).

$\upsilon_c = 1.5356 \bullet 10^7\ B\ z / m$

v, ion velocity
r, radius of the ion cyclotron orbital
q, charge of the ion
B, magnetic field strength (Tesla)
m, mass of the ion
z, number of charges

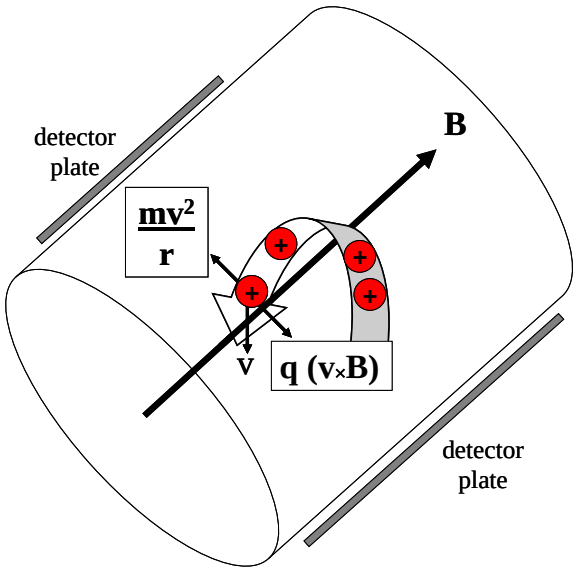


Figure 10. Cyclotron motion of a positively charged ion moving in the magnetic field.

Due to the trapping voltage, a radial force is produced, which direction is opposed to the Lorentz force, thus the center of the cyclotron motion will also orbit the axis of the cell in a circular path with a very small diameter. This extra orbital motion introduced by the trapping potential is called the magnetron motion. The magnetron frequencies (about 100 Hz) are much less than the cyclotron frequency (kHz to MHz). The cyclotron motion is also affected by the space charge built up when too many ions are present in the cell, which could shift the cyclotron frequency. A calibration mixture containing ions of several m/z values is therefore optimal for calibration (128).

In order to get a detectable signal from the ion cyclotron motion the ions need to be excited to larger orbits. This is done by a broad band excitation pulse. This waveform excites all of the ions to the ICR orbital radii (~ 1 cm), independent of *m/z*. Ions undergoing cyclotron motion at particular frequencies absorb energy and are excited as a coherent ion packet to higher kinetic energies so that their cyclotron radii increase, **Figure 11** (128). The coherent ion packets induce an image current on the two detector plates as they pass close to the plates. Thus, the principle of ion detection in FT-ICR MS is non-destructive in character.

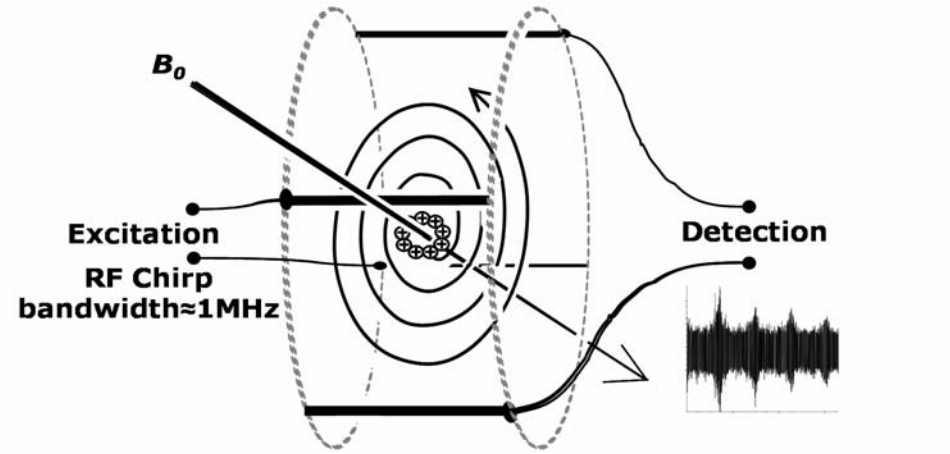


Figure 11. Excitation and detection of ions in the ICR cell.

If only ions of a single *m/z* were present, the signal in the time domain would be a slowly decaying sinusoidal wave (due to ion-neutral collisions). With ions of different *m/z* ratios, the time-domain signal is a sum of the frequencies of all ions present in the cell. The frequencies are measured by a stabilized quartz oscillator and the transient is digitized with a sampling rate that is at least twice of the lowest mass (highest frequency) according to the Nyquist theorem (135). The time-domain signal is converted to the frequency domain by applying a fast Fourier transform. Frequency is then converted to a mass spectrum through the cyclotron equation and the mass scale calibration, **Figure 12**. With this ultra-high mass accuracy, the mass of the electron (0.0005486 Da) must be taken into account m_H^+ = 1.007276 Da, when calculating the masses of the protonated ions.

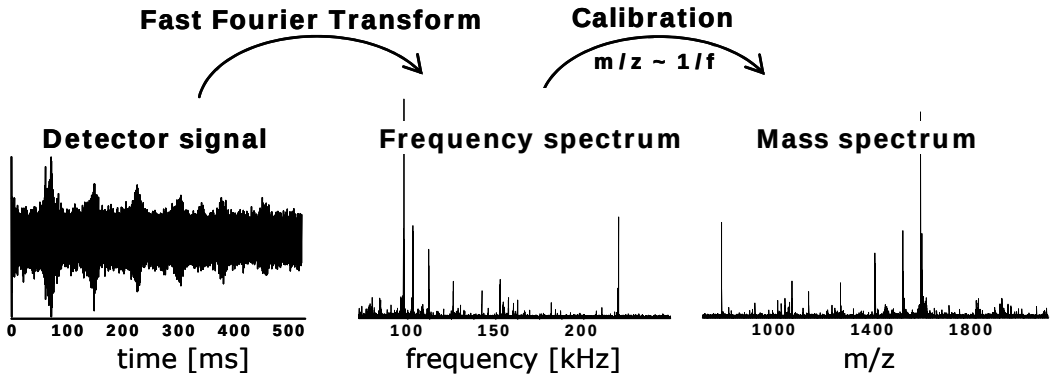


Figure 12. Converting the time-domain signal to frequency by Fourier Transform and frequency is then converted to a mass spectrum through the cyclotron equation and the mass scale calibration.

Mass accuracy and sensitivity

The physical quantity measured in FT-ICR MS is frequency. Because frequency can be measured very accurately, excellent mass accuracy is offered. Mass measurements as accurate as 1 ppm can be achieved on a routine basis for large biomolecular ions (136), (137), (138). High mass accuracy offers an alternative to tandem mass spectrometry for identification, an advantage if the amount of sample is limited. Accurate mass determination is necessary to characterize an exact modification, whereas high mass resolving power is necessary to distinguish between related forms of the peptide or protein with different degrees of modification. Two peptides differing in mass by less than 0.0005 Da (less than the mass of an electron) have been resolved (138). High mass accuracy is very helpful in distinguishing different isoforms of glycopeptides, because the number of possible combinations decreases with increasing mass accuracy. High resolving power enhances sensitivity by making it possible to distinguish between analyte species and chemical noise at or near the detection limit.

It is essential for ion traps to use automatic gain control (AGC) to estimate, with a very short prescan (10-30 ms), the optimal injection time for the ion gate in front of the octopole in order to fill the ion trap with the targeted number of charges. This gives a very reproducible ion population from scan to scan. The AGC controls the filling of the ICR cell to avoid space-charge effects caused by overfilling of the cell, that would lead to poor mass measurement accuracy (139), (140).

Tandem mass spectrometry – MS/MS

Isolation of a precursor, the parent ion, for tandem mass spectrometry (MS/MS) or fragmentation can be achieved either in the ICR cell or in an ion trap prior to transfer to the ICR cell. Both isolation and dissociation of precursor ions, peptides, can take place in the ion trap. The amount of the product ions compared to the parent ion is usually very low, thus, a limit will be reached at which the desired precursor ion amount is too low to produce observable fragment ions.

Collision induced dissociation

Ion accumulation in an ion trap outside the magnet permits isolation and fragmentation by collision induced dissociation (CID, also called collision activated dissociation, CAD) before the transfer to the ICR cell or with detection in the ion trap, **Figure 14c**. Dissociation (fragmentation of chemical bonds) occurs when the ions internal energy increase (vibrational excitation) after multiple collisions with the helium gas in the ion trap.

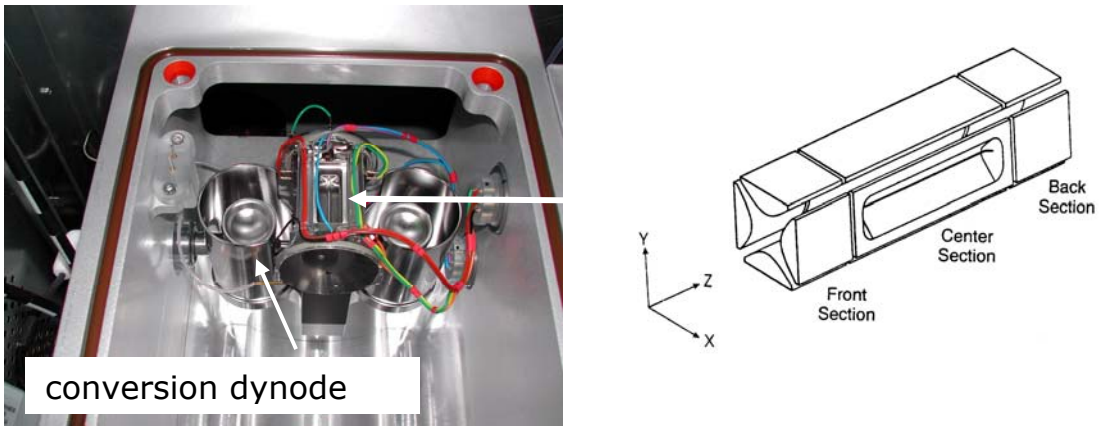


Figure 13. The 2D ion trap in the LTQ-FT MS. The ion gate controls the filling of the ICR cell, referred to as AGC. Octopoles transfer the ions both before and after the ion trap. Ions are scanned out through a thin slit in the center of the ion trap to the conversion dynodes and electron multipliers for detection.

The new hybrid FT-ICR mass spectrometers are often equipped with a linear ion trap in front of the Penning trap. Because a linear ion trap is an extremely fast scanning spectrometer (in the ms range) it can be used for detection of product ions while the precursor ions are mass measured in the ICR cell. In nano-LC MS/MS the two traps can perform parallel processing during the time the components elute from the nanocolumn.

The sequencing of peptides by MS/MS analysis depends on the formation of sequence ions by cleavage of the peptide bonds. There are different series of fragment ions, where *y*- and *b*-ions are the major ion series, nomenclature by Roepstorff and Fohlman (141), **Figure 14.**

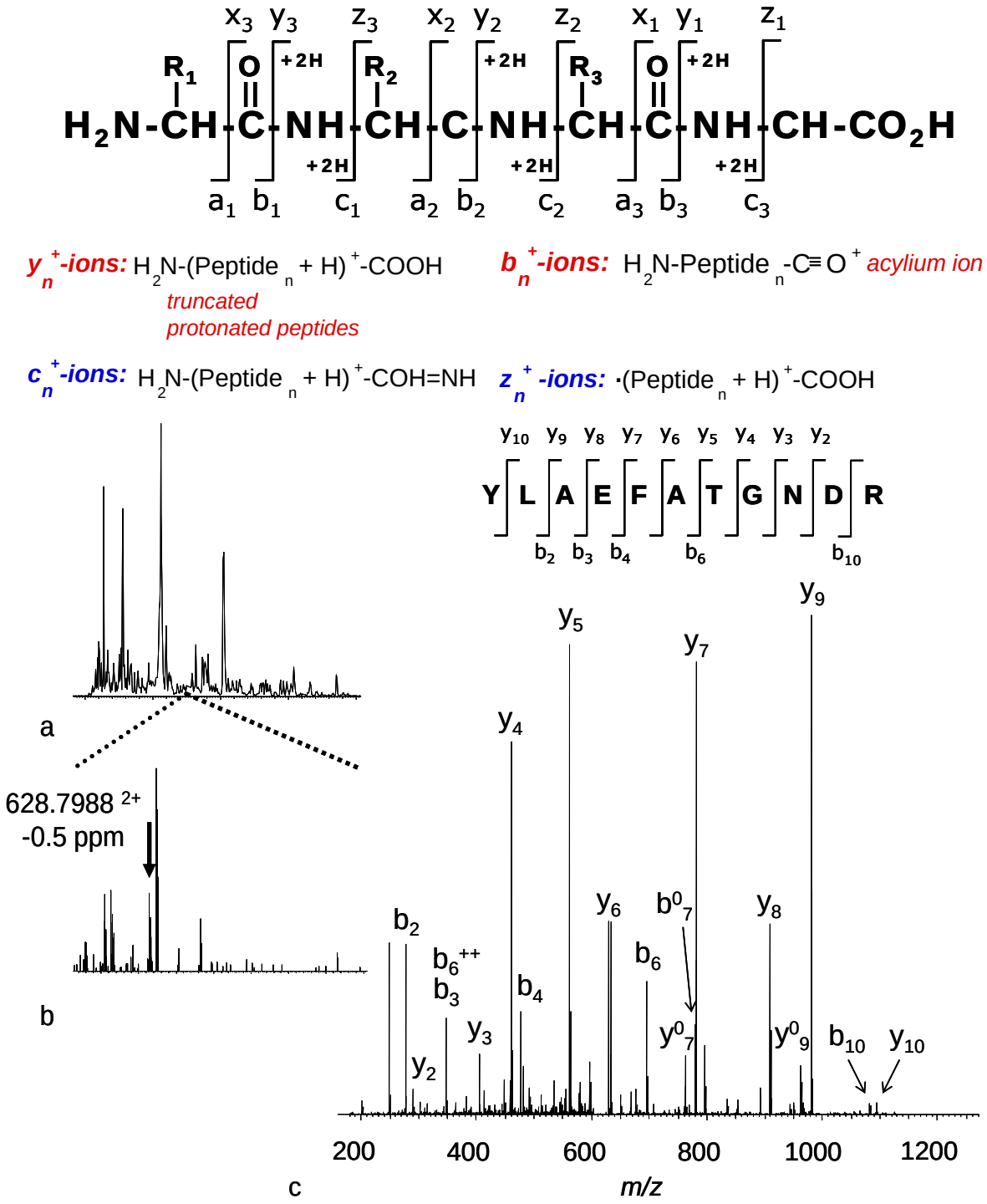


Figure 14. Nomenclature for fragment ions produced by MS/MS, such as CID of a peptide. Panel a) nano-LC-MS base peak chromatogram of a 1D gel band of mouse hippocampus, during 10-30 min; b) mass spectrum at 17.3 min, containing the doubly protonated peptide at *m/z* 628.800 [M=1255.585]; c) CID spectrum of 628.800^{2+}. The amino acid sequence matching to this MS/MS data was YLAEFATGNDR. b^0 is a b ion with loss of water, for further explanations see www.matrixscience.com/pdf/asms_tutorial_2005.pdf.

Infrared multiphoton dissociation

In IRMPD, infrared multiphoton dissociation, the isolated precursor ions in the cell, are irradiated with infrared photons that slowly (milliseconds) heat them to their dissociation threshold (142). A CO_2 laser (25 W) is usually employed to provide the high-energy photons. Ideal laser fluencies and irradiation times for glycopeptide fragmentation are lower than that observed for unmodified peptides, typically 30% of 25 W during 150 ms. For glycopeptides, IRMPD results mainly in cleavage of glycosidic linkages and determines the structure of the glycan. The advantage is that no release of the glycan is necessary prior to MS analysis (53).

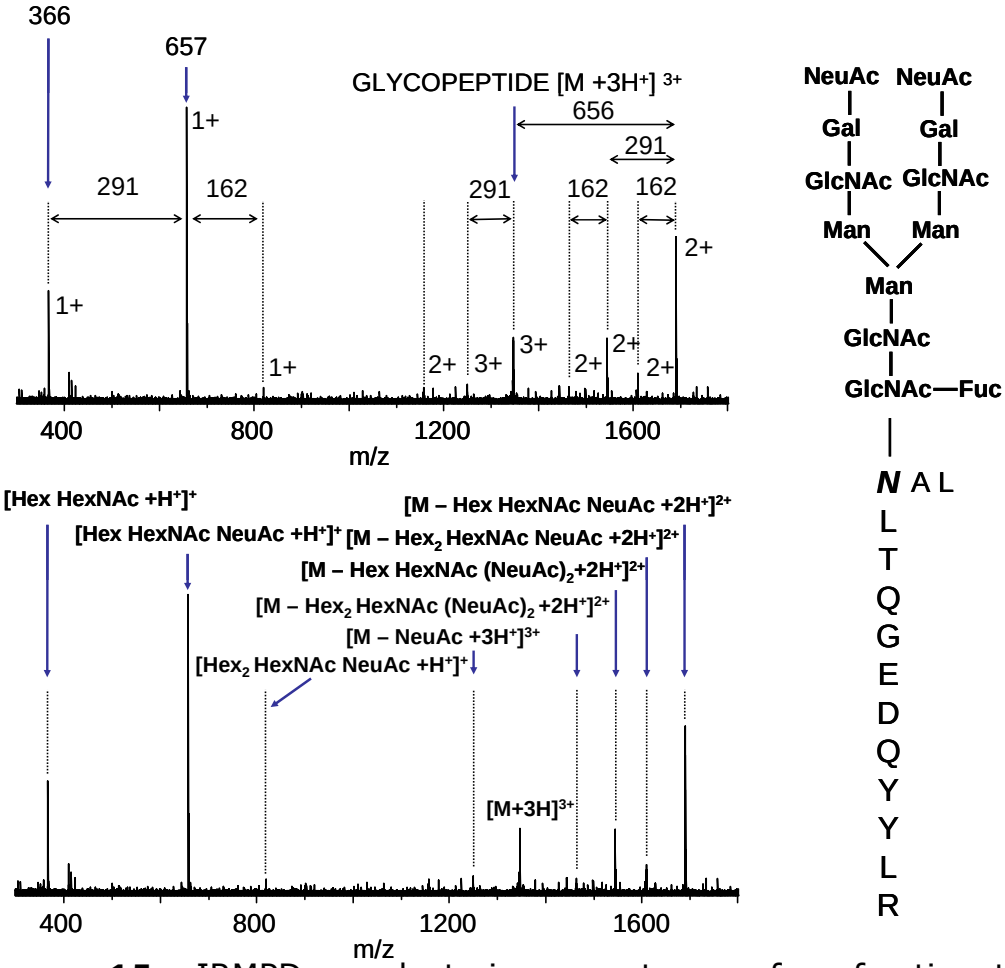


Figure 15. IRMPD product ion spectrum of unfractionated desalted tryptic glycopeptide at *m/z* 1345.56 $^{3+}$, from clusterin obtained by micro-electrospray ionization in a custom-built 9.4 T FT-ICR instrument. The fragmentation pattern confirms the *N*-linked glycoform $(Hex)_2(HexNAc)_2(DeoxyHexose)_1(NeuAc)_2(Hex)_3(HexNAc)_2$ to LANLTQGEDQYYKR predicted by GlycoMod. The product ions show different levels of losses of glycan residues as well as the parent ion $[M + 3H^+]^{3+}$. For example the most abundant product ion, at *m/z* 657, corresponds to loss of one antenna from the core in the glycan structure.

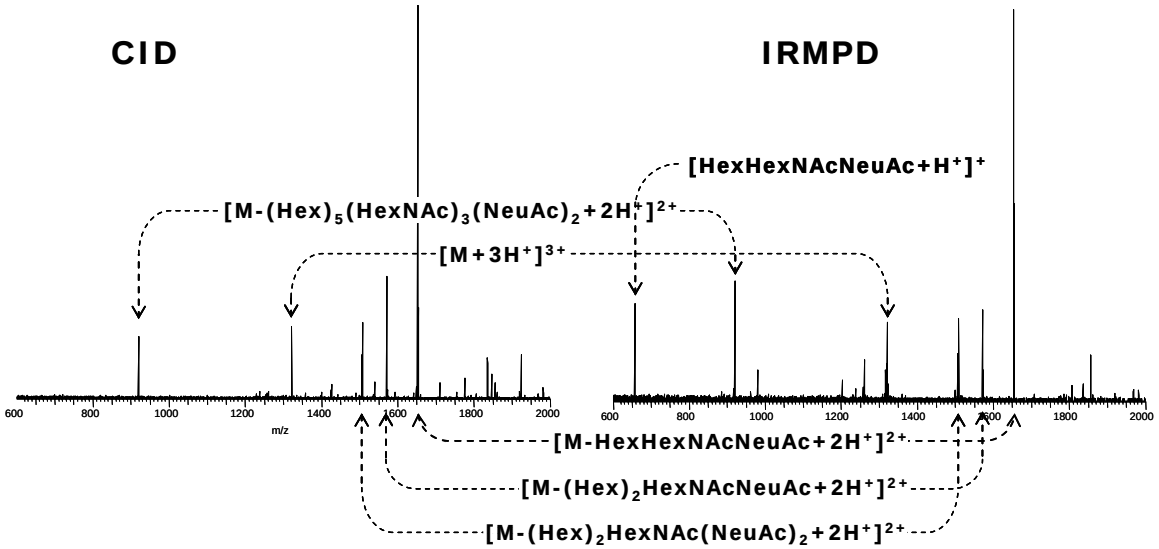


Figure 16. Comparison of fragmentation of glycopeptide by IRMPD in the ICR cell and CID in a linear ion trap, with detection of fragment ions in the ICR cell. The two methods produced equivalent spectra of the triply protonated glycopeptide at m/z 1320.893 with the attached glycostructure $(NeuAc\text{-}Hex\text{-}HexNAc)_2Man_3GlcNAc_2$ in a hybrid linear ion trap-FT-ICR mass spectrometer *[Sihlbom et al. ASMS 2005 poster]*.

CID is not practical to perform in the ICR cell but can be performed prior to detection in the ICR cell. A comparison of fragmentation pattern of a glycopeptide for CID and IRMPD is shown in **Figure 16.**

Electron-capture dissociation

ECD, electron-capture dissociation, is a fragmentation technique that offers unique advantages, with its abundant backbone N-C_α fragmentation of larger polypeptides while retaining labile post-translational groups (143,144). Mapping of post-translational modifications is expected to be the main application in protein research, with de novo sequencing and disulfide bond analysis as other important areas. ECD is performed by irradiating the ions trapped in the ICR cell with low energy (< 1 eV) electrons. Disulfide bonds and backbone peptide (N-C_α) bonds rapidly dissociate, except for N-terminally to proline, forming *c*- and *z*•- product ions. This result is in contrast to the more sequence specific *b*- and *y*- ions, which are typically formed in CID and IRMPD. Labile post-translational protein modifications such as glycosylation, dissociate readily in CID resulting in loss of structural information, but are retained in ECD (132,145), **Figure 17**. Because of the charge reduction, the sensitivity of ECD-based MS/MS is lower than in traditional MS/MS (144). The overall ECD efficiency for peptides is 20 to 50%, but can be higher for proteins (144). The use of dispenser cathodes with irradiation time in the millisecond range has enabled the combination of on-line LC and ECD (146), (147), (132) (148).

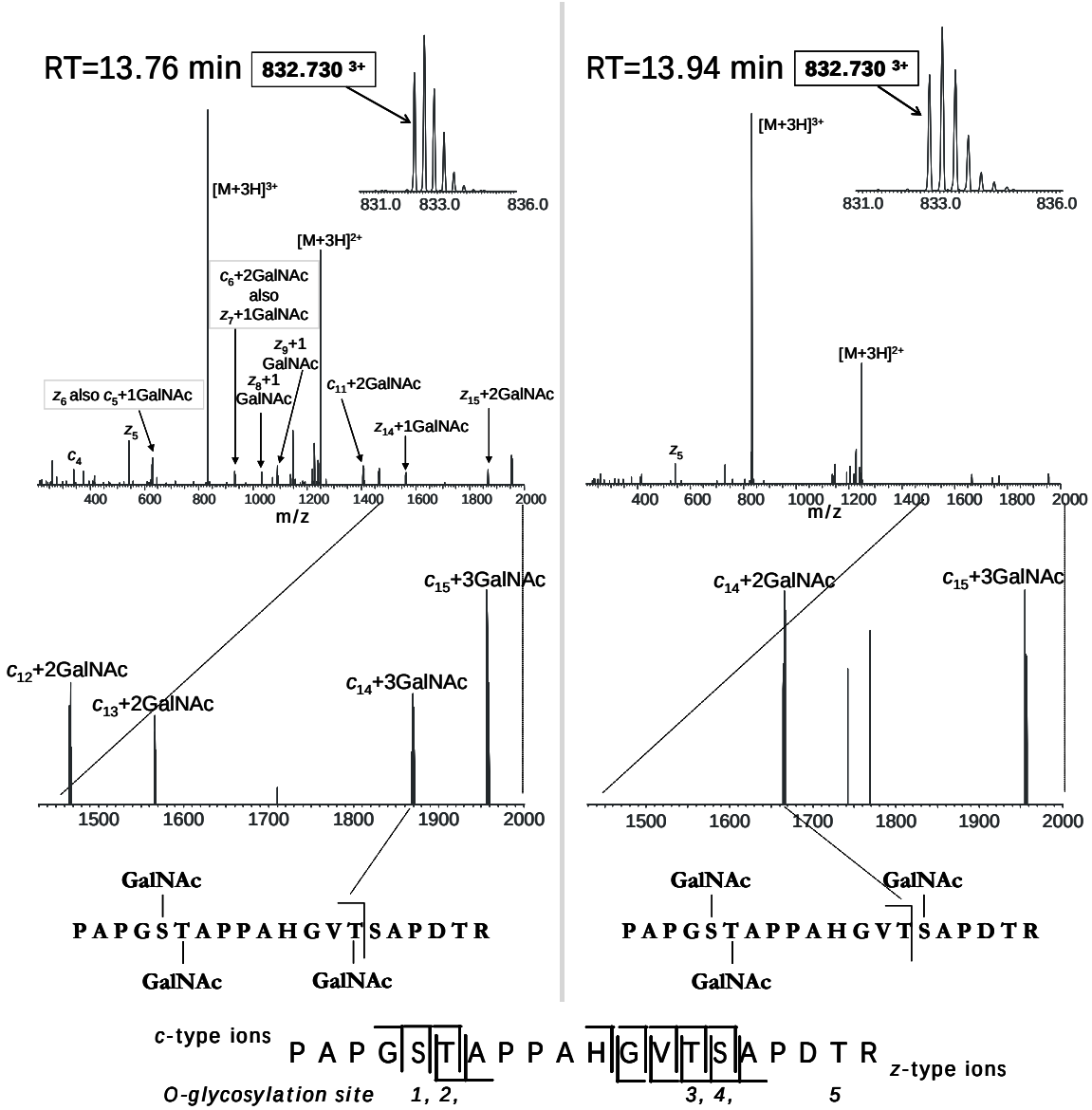


Figure 17. ECD spectra of digested recombinant MUC1 TR glycoprotein produced in a mutant CHO cell line, not extending the N-acetylgalactosamine (GalNAc) attached to each occupied serine or threonine site. Triply protonated ions corresponding to masses of glycopeptides with 3-5 occupied glycosylation sites and following ECD, c and z ions with or without the addition of one or several GalNAc were observed. For the MUC1 TR with 3 GalNAc, two ECD spectra collected only 10 s apart showing the same c ion with the addition of 2 and 3 GalNAc, respectively. The two TR glycopeptides with the variants of O-glycosylation at position 1, 2, 3 assigned as PAPGS-(GalNAc)T-(GalNAc)APPAHGVT-(GalNAc)SAPDTR and position 1, 2, 4, assigned as PAPGS-(GalNAc)T-(GalNAc)APPAHGVTS-(GalNAc)APDTR respectively, were confirmed. On-line LC with rapid switching between full MS scan to ECD of data-dependent precursor ions to be able to analyze heterogeneous site-specific glycosylation was demonstrated *[Sihlbom et al. ASMS 2006 poster].*

4 RESULTS AND DISCUSSION

4.1 Paper I

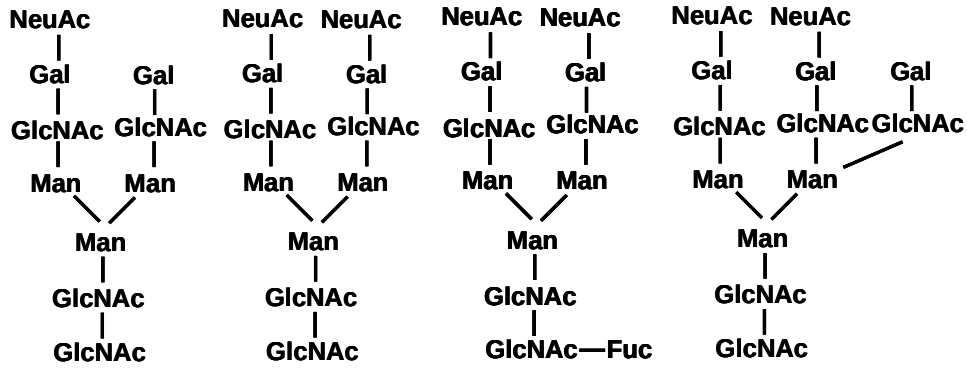
Glycoproteomics of cerebrospinal fluid in neurodegenerative disease

Structural determination of glycoproteins in CSF from AD patients may lead to identification of new complementary biomarkers. Sensitive and accurate MS and MS/MS methods are important to determine the glycosylation of low amounts of human proteins. The high mass accuracy reduces the number of possible glycoforms per peptide. Here, isoelectric focusing of proteins with a narrow pH range (one unit) improved the separation of several CSF glycoprotein isomers in 2D-GE. Image analysis of eight AD patients and eight control individuals highlighted nine isoforms of glycoproteins as significantly changed. A glycoprotein specific stain (Pro-Q Emerald) followed by a total protein stain was used to facilitate image analysis and spot excision of glycosylated proteins. Protein spots analyzed with MS were excised from a duplicate colloidal blue stained 2D gel.

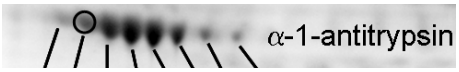
In this study, isoforms from two glycoproteins (α_1-antitrypsin and β-trace) in 2D-GE spots from three AD patients and three control individuals were extensively analyzed demonstrating the analytical capacity of FT-ICR MS for glycoproteomic investigations. Potential glycopeptides as predicted, by the database search program Glyco-Mod, have been fragmented by IRMPD for glycostructure confirmation. Characterization of α_1-antitrypsin glycoforms from gel-separated pooled CSF from control subjects was previously performed (53) on five of the eight isoforms that has been detected in plasma (149). All glycopeptides, from the three possible *N*-glycosylation sites of α_1-antitrypsin reported here, **Figure 18**, were also found in the previous CSF study. The plasma study reported additional glycoforms as compared to both CSF studies and includes several triantennary glycostructures. Another recent proteomic study of pooled reference plasma revealed tetraantennary structures that were not seen in the CSF studies (150).

Theoretically the negatively charged sialic acid would move towards lower pH in a gradient and the more sialic acids present, the lower the protein pI should be. The plasma study used 1D-GE and detected the tetra- and trisialoantennary glycoforms in the two most anodal (+) isoforms (150). Two glycoforms (here gel spots g and h), corresponded to the same glycoforms in to other spots (here d and e), but their protein N-termini were truncated (150) and started at position 30 instead of 25. Calculating the pI of the unmodified protein sequence, the truncated form (aa 30-418) has a higher pI of 5.51 as compared to pI of

5.37 for amino acids 25-418. Thus, protein truncation could be one of the underlying parameters for the migration behavior in pH gradients as well as other modifications.



Protein position	**N247**	**N247**	**N46**	**N83**
Gel spots	a, b, c, d, e, f, g, h	a, b, c, d, e, f, g, h	c, e	c, d, e
	N46 c, d	**N46** c, d, e, f		
		N83 b, c, d, e, f		



a b c d e f g h

Figure 18. Suggested glycoforms assigned for the three *N*-glycosylation sites in α_1-antitrypsin in individual 2D-GE of CSF found in both AD patients and control subjects. The circled isoform in the gel image showed increased protein expression in AD patients.

Four of the α_1-antitrypsin glycopeptides were found only in the individual AD samples, but they were present also in the pooled control CSF from earlier studies (53). Large glycopeptides are quite often found with different charge states in ESI-MS, which is the case for *m/z* 1437.17 $^{4+}$ being the same glycopeptide as *m/z* 1149.93 $^{5+}$, and a better overview of the present glycoforms would be given using the neutral glycopeptide mass. The number of glycopeptides of α_1-antitrypsin in total was larger for the AD patients, 58 *versus* 34, as compared to the control subjects.

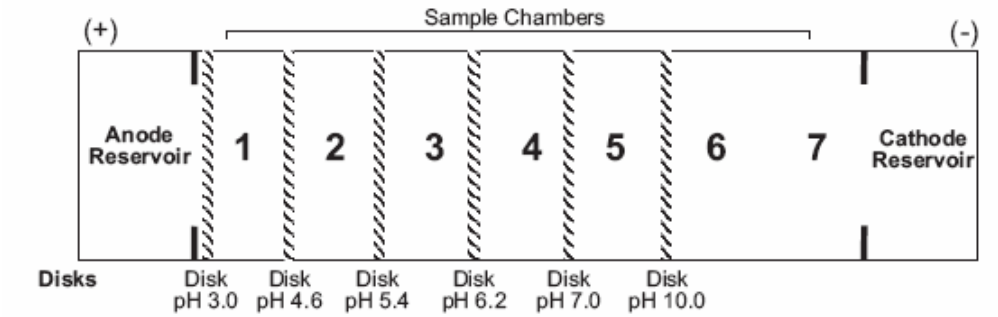
For β-trace, we found six different glycoforms distributed on the two possible *N*-glycosylation sites. Microheterogeneity in protein

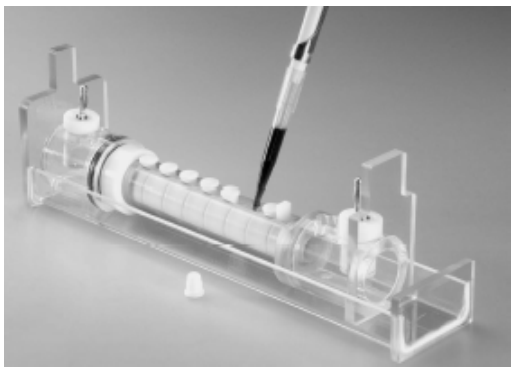
glycosylation is the relative site occupancy and complexity of glycan structures. There is a trend for greater microheterogeneity in the three control subjects shown by the total number of glycans found in the two spots of β-trace. **In summary**, no structural difference could be found for either α_1-antitrypsin or β-trace in this study. The other visible glycoproteins had very low signal intensity and further sample preparation would be necessary to fully characterize the different isoforms.

4.2 Paper II

Prefractionation of cerebrospinal fluid to enhance glycoprotein concentration prior to structural determination with FT-ICR mass spectrometry

CSF has a low total-protein concentration, high amount of albumin, high salt content and a wide concentration range of proteins which requires sample preparation to enable characterization of low abundant glycoproteins. We compared two sample preparation methods, micro-solution isoelectric focusing **(Figure 19)** and albumin affinity chromatography **(Figure 20)**, prior to 2D-GE and structural determination with FT-ICR MS.

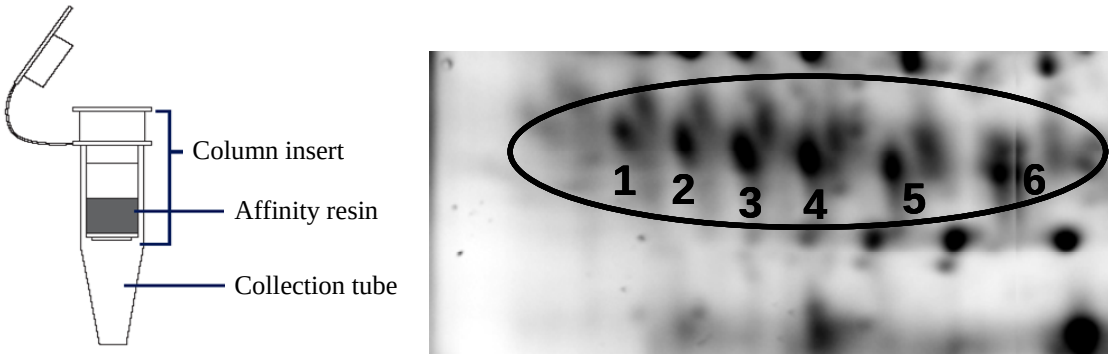




pH 4.7-5.9 gel, enlarged area for clusterin

Figure 19. Micro-solution isoelectric focusing of the volume corresponding to 1.8 ml CSF followed by separation on 2D-GE. Crude CSF was mixed with buffers and the fractions with the pH intervals of interest were removed from the chambers after focusing. One third of the fraction could be loaded on the gel.

2.0 ml CSF through 5kDa membrane
cut-off centrifuge tube (Amicon)
200 µL retentate for albumin depletion
in Montage columns



pH 4.7-5.9 gel, enlarged area for clusterin

Figure 20. Albumin affinity chromatography of 2.0 ml CSF which was a four-fold volume compared to the loading on a narrow pH-range gel without prefractionation of the sample. The total volume of the eluate from the column was directly loaded on the gel with minor buffer addition.

Albumin affinity columns that are commercially available are often produced for albumin depletion in plasma or serum and are therefore constructed for smaller volumes. To use these columns, the volume of CSF needs to be reduced.

We showed that it was possible to use a four- to eight-fold higher volume of crude CSF for narrow pH range 2D-GE after salt removal and albumin depletion. The glycoprotein spots showed improvement as demonstrated by better isomer detection and spot intensity. Analysis of the FT-ICR-MS data lead to the assignment of several potential glycopeptides in the six isoforms of clusterin from the 2D gel, compared to only one (and a few possible) glycopeptides from the unfractionated CSF in Paper I. Only a part of the solution IEF fractions can be loaded on 2D-GE, and therefore it was not possible to fully utilize the starting material. We concluded that the albumin depletion method was the best suited for structural determination of less abundant proteins for use with clinical CSF samples in the discovery of glycoproteins.

4.3 Paper III

Structural and quantitative comparison of cerebrospinal fluid glycoproteins in Alzheimer's Disease patients and healthy individuals

In order to address the question of disease-related alterations of glycosylation, we have used our focused proteomics approach including albumin depletion, narrow range iso-electric focusing and glycoprotein specific staining, followed by total protein staining. An individual quantitative comparison of gel-separated glycoprotein isoforms from twelve AD patients and twelve control subjects was performed by image analysis. For structural determination of glycoforms and protein identification by MS, pooled CSF was used representing the group of AD patients and the group of control subjects, respectively.

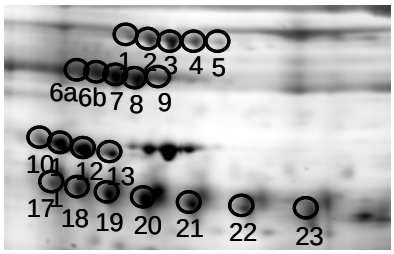


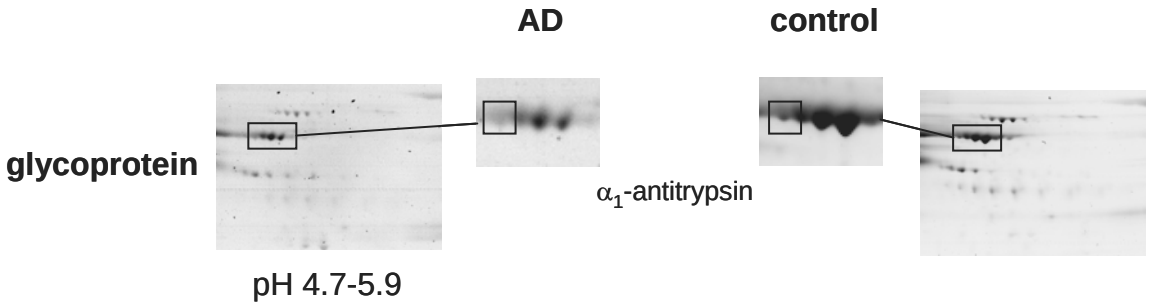
Figure 21. Protein gel spots in 2D-GE, pH 4.7-5.9, of 2 ml albumin depleted CSF from AD patients, stained for total protein pattern.

We examined all glycoprotein spots **(Figure 21)** with MS to find all possible glycoforms both in AD patients and control subjects. Some of the glycoforms from the pooled material turned out as possibly AD specific and this hypothesis was tested with an additional set of individual 2D gels. This final analysis showed that there is no glycoform structure that could be specifically assigned to AD patient CSF of the proteins analyzed in this study.

We compared the intensities of glycosylated protein isoforms and found decreased glycosylation in one isoform of α_1-antitrypsin in AD patients, **Figure 22.**

In the differential comparison of protein expression level in specific isoforms, clusterin and apolipoprotein E were both increased in the group with AD patients. None of the glycopeptides found in the 2D gel train spots of clusterin or apolipoprotein E were disease-specific.

It is very difficult to obtain CSF from age-matched control individuals. Thus, in this pilot study AD patients were compared to healthy individuals that were not age-matched. Discrepancies between proteomic studies may be explained by variations in sample size and handling of the samples may also contribute to divergent results.



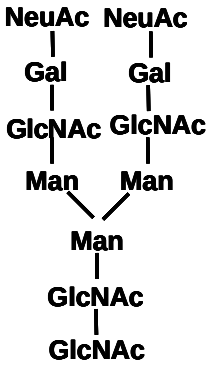


Figure 22. Decreased glycosylation level of α_1-antitrypsin in AD patients, $p<0.05$, using the average of twelve individuals in each group. The glycoform (to the right) found in the α_1-antitrypsin with decreased glycosylation was also found in the other following isoform spots and detected in both the group of AD patients and control subjects.

We demonstrated the feasibility of glycoproteomic profiling with a focus on CSF glycoproteins. The study included the pH interval of 4.7-5.9, in which some glycoproteins previously linked to AD, such as clusterin, apolipoprotein E, α_1-antitrypsin, α-1β-glycoprotein, could be detected and analyzed. Further studies could involve the AD- and tau-related protein cholinesterase with theoretical pI of 6.3. The study could also be extended to cover other narrow-range pH-intervals in order to include other glycoproteins. The field of glycoproteomics connected with Alzheimer's disease is still unexplored and knowledge of the respective glycoprotein structures in relation to clinical parameters, such as duration and severity of disease may also be of interest.

Comments on glycoproteomic methods

One alternative approach of glycoprotein analysis is based on isolation to enrich the glycoproteins from a complex mixture before MS analysis. Digestion of the protein and isolation of the glycopeptides can also be used. Hydrazide resin or lectin affinity columns are the two major isolation methods for enrichment of glycoproteins. A large-scale proteomic identification of more than 200 CSF glycoproteins using a combination of both deglycosylated peptides from hydrazide chemistry and tryptic digest of glycoproteins from lectin affinity chromatography followed by 2D LC-MS/MS were recently performed (151). This is an impressive study using a total volume of 12 ml pooled CSF, divided in 2 ml for each of the isolating methods run in triplicates. Within the pH-range of our study, the group was able to recover several glycopeptides from α_1-antitrypsin, haptoglobin, clusterin, α-1β-glycoprotein, beta-trace (prostaglandin-H2 D-isomerase), apolipoprotein E and zinc α-2-

glycoprotein and heterogeneity of the glycoproteins was shown. The majority of the identified glycoproteins were found to have *N*-linked glycosylation. However, no structural determination was performed, quantification was not possible and reproducibility and possible losses would require validation for use in comparative proteomics.

Quantification of lectin affinity captured *N*-linked glycoproteins through isotope-coded tagging of deglycosylated peptides is another approach that may be useful for disease-related screening with 2D LC-MS/MS (48). The isotope tag is located at the site of glycosylation which can be determined simultaneously. However, this method implies the use of various types of lectins to capture different glycoforms and low abundant proteins might not be detected.

Deglycosylation was applied for *N*-glycan profiling in a glycoproteomic study. All PNGase F-cleaved glycans from the tissue were analyzed by off-line LC-MALDI-TOF/TOF (152). Selection of affinity reagent for the parallel glycopeptide analysis was based on the *N*-glycan profiling. TOF/TOF spectra of glycopeptides are quite complex and there is no suitable software for the assignment of glycopeptides. For a faster identity and assignment, the deglycosylated peptides were also analyzed separately. This study also included determination of the relative quantities by derivatization of the glycoforms present at each *N*-glycan binding site. Although this was a very comprehensive study, it was focused towards high-mannose type glycoforms. Another focused study, compared *N*-linked sialic acid containing glycoforms with serial lectin affinity chromatography for changes in the degree of branching and glycan site heterogeneity (153). Human serum samples were digested with trypsin and two lectin columns selected sialic acid containing glycopeptides and divided them in fractions of bi- and tri-, tetra-antennary *N*-linked glycoforms. The glycopeptide fractions were isotope coded and Q-TOF MS/MS analysis was performed only on the labeled deglycosylated peptides. In this study, it was possible to determine the degree of branching in glycan structures but it was not possible to assign glycan position.

In-gel deglycosylation of *N*-linked glycans from gel-separated human plasma was applied in a study of congenital disorder of glycosylation (154). This method could be used for clinical diagnosis of aberrant glycosylation but the information of the site of glycosylation is lost and the strategy would require a second MS analysis for the protein identification of the gel spots.

Comments on albumin depletion

The performances of two methods for the depletion of human serum albumin and immunoglobulin G were compared (70). A higher proportion of albumin was removed using the antibody-based HSA/IgG-removal kit, and consequently more proteins could be identified using this approach. Both methods provided a significant reduction of albumin. However, for our purpose of glycoprotein analysis the albumin (only) affinity chromatography method is more suitable because clusterin was not detectable when both albumin and IgG were removed.

Different depletion methods for removal of abundant serum proteins have been compared, including both single-use columns and LC-columns (69). This comparison was based on reproducibility, specificity, efficiency and the resulting electrophoresis protein pattern. Among the disposable columns, a depletion column from Merck, which removes 6 plasma proteins (albumin, IgG, transferrin, IgA, haptoglobin, alpha-1-antitrypsin), displayed the best results. Maximum sample volume of this column is 60 μl and this volume was too small for use with CSF in our case. We therefore used centrifuge tubes with molecular weight cut-off 5 kDa to reduce 2 ml CSF and for final volumes less than 100 μl, there was significant target protein loss. If only a part of the concentrated CSF were used, we also observed target protein loss due to non-specific binding to the depletion column.

Evaluation of two protein depletion methods for analysis of glycoproteins in CSF was published at the same time as we finished our prefractionation comparison. One of the methods included removal of α_1-antitrypsin and were therefore not suitable while the other were based on IgY antibody microbeads in spin cups (155). Incomplete depletion was reported for the microbead method.

The advantage of prefractionation is the reduction of sample complexity, thus a larger volume or weight of the crude material could be used and would facilitate the detection of proteins available in very low amounts. By removing highly abundant proteins, the low abundant proteins that would typically be masked, become detectable.

Comments on glycoproteins as biomarkers of AD

A proteomic approach using micro capillary LC-MS of proteins labeled with ICAT quantified relative changes in the proteome of CSF from AD patients, and compared age-related changes. Almost 400 CSF proteins were identified and about 160 proteins were quantified. AD-related differences (defined as variation of 20%) were found in over 80 of the quantified proteins (156), (157). This is an arbitrary cut-off and can on its own not show significant changes between the AD and control group. Only 2 of the 30 proteins that showed age-related change also showed AD-related change, suggesting that processes of normal aging and AD are largely different. The ratio (AD/control) for some of the quantified glycoproteins in this study showed increased levels in AD; α_1-antitrypsin 1.6, α-2-HS glycoprotein 1.3 and haptoglobin 1.4. Clusterin and apolipoprotein were identified but not quantified which also illustrate one limitation with LC-MS based (shotgun) methods, in which only a subset of the separated peptides will be detected.

The mean level of the glycoprotein clusterin is significantly increased in AD patients compared to control subjects. However, it is not suitable as a diagnostic marker due to overlap between individual AD and control subjects of both native and deglycosylated clusterin (158). Clusterin expression levels were analyzed with an enzyme-linked immunosorbent assay. This study suggests an unaltered degree of glycosylation of clusterin in CSF from AD patients. Our study is a complementary pilot study of clusterin. No difference in glycosylation between AD patients and control subjects were found even when clusterin isoforms were analyzed. However, it must be kept in mind that this material represented only a small group of individuals.

A comparative proteomic study of CSF from neuropathologically confirmed AD and non-demented elderly subjects were performed with 2D-GE in pH range 4-7. This is a larger study with 43 age-matched subjects in each group, using post-mortem CSF (taken within 4 hours). Analysis was performed on pooled material and repeated five times (159). Five differentially-expressed proteins with potential roles in amyloid-beta metabolism and vascular and brain physiology were identified, apolipoprotein A1, cathepsin D, hemopexin, transthyretin, and two pigment epithelium-derived factor isoforms. Apo A-1, CatD and TTR were significantly reduced in the AD pool sample, while HPX and the PEDF isoforms were increased in AD. All of these proteins except Apo A-1 can be *N*-glycosylated. The complexity and heterogeneity of AD makes it difficult to find one single diagnostic marker but a reliable diagnosis may be obtained by statistical results from several protein markers. The differentially expressed proteins should be verified in

analysis of individual CSF samples and the possibility to use a characteristic protein pattern for AD diagnosis should be investigated.

Our glycoproteomic approach for discovery of disease-related glycosylation or glycoproteins is developed for human CSF collected for disease screening purposes. 2D-GE is used to separate glycoprotein isoforms and for quantification by specific staining. The advantage of 2D-GE over gel-free peptide-based approaches is that since protein modification can induce alteration in both mass and pI, different isoforms and post-translationally modified proteins can be resolved. However, one drawback of using narrow pH-range 2D-GE is the need for several gels to cover the full pH range from 3 to 11. Still, there are more advantages. Since no glycoprotein affinity method is used all present and detectable glycoforms can be analyzed without discrimination. The isoforms are in-gel proteolytically digested without glycan release. Sample manipulation is avoided and sample dispersal is minimal. When FT-ICR MS is used, the very high mass accuracy improves the specificity for protein database search results and prediction of glycoforms. IRMPD or CID provides valuable structural information of the glycan with very limited dissociation of the peptide backbone. Analysis of intact glycopeptides allows for assignment of glycan structures to specific sites of glycosylation.

4.4 Paper IV

14-3-3 expression in denervated hippocampus after entorhinal cortex lesion assessed by culture-derived isotope tags in quantitative proteomics

Culture-derived isotope tags (CDIT) were used as internal standard to hippocampal proteins and mass spectrometry was used for unique isoform-specific quantification, **Figure 23 and 24**. Classical proteomics, 2D-GE and mass spectrometry, was first applied to study the proteomic difference after entorhinal cortex lesioning in wildtype mice and *GFAP*$^{-/-}$ *Vim*$^{-/-}$ mice. Proteins were extracted from the injured and contralateral (i.e. in the opposite hemisphere) hippocampus four days after unilateral entorhinal cortex lesion. Among the differentially expressed ($p < 0.02$) proteins, the 14-3-3 protein was identified.

14-3-3 is an adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways. The 14-3-3 protein has seven isoforms and most 14-3-3 isoforms are present in the adult CNS, and show heterogeneous patterns of expression in different cell types and different anatomical locations suggesting specific functions for each isoform (160). The expression pattern is altered in CNS pathologies for instance increased expression of 14-3-3 proteins in reactive astrocytes was found in brain samples from patients with multiple sclerosis (161), Creutzfeldt-Jacob disease (162) or stroke (163). 14-3-3 proteins have been identified in neurofibrillary tangles in Alzheimer's disease (164).

The 2D-GE results showed a strong trend of 14-3-3 protein upregulation in injured hippocampus and suggested that 14-3-3 upregulation after injury was attenuated in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. The isoform specificity of 14-3-3 could not be determined. The selected 2D gel spots showed the presence of more than one component in each spot which makes it difficult to interpret the quantitative result from the image analysis.

The quantitative CDIT-based MS showed that 14-3-3 epsilon was the major isoform responsible for the upregulation of 14-3-3 in the denervated hippocampus, since the difference in the 14-3-3 amount was largely decreased when peptides common to all 14-3-3 isoforms were used for quantification, **Figure 24**. The MS data also showed that the injury-triggered upregulation of 14-3-3 epsilon was less pronounced in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. Consistent with this finding, immunohistochemical analysis showed only very weak 14-3-3 immunoreactivity in the injured hippocampus of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice in contrast to strong immunostaining in wildtype mice. This supports the conclusion that

14-3-3 expression, and in particular the expression of the 14-3-3 epsilon isoform, has a role in reactive gliosis.

As a validation step, the relative expression levels of actin were measured in the contralateral hippocampus of wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice, **Figure 24**. The relative standard deviation of five peptides in five hippocampus from wildtype mouse was 10% and similar within the group of *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice. There was no significant difference in actin levels between wildtype and *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice; the average peptide ratio was 1.10 compared to 1.03 for 14-3-3 epsilon, which also confirms an equal sample loading on the 1D gel.

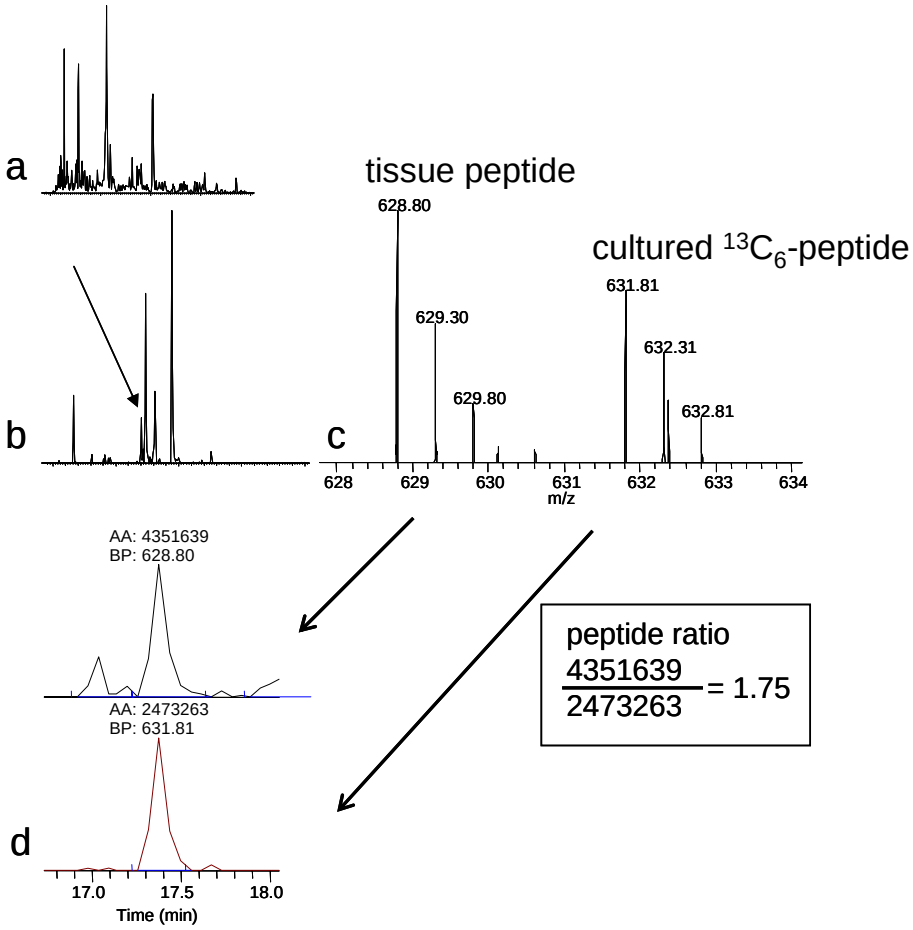
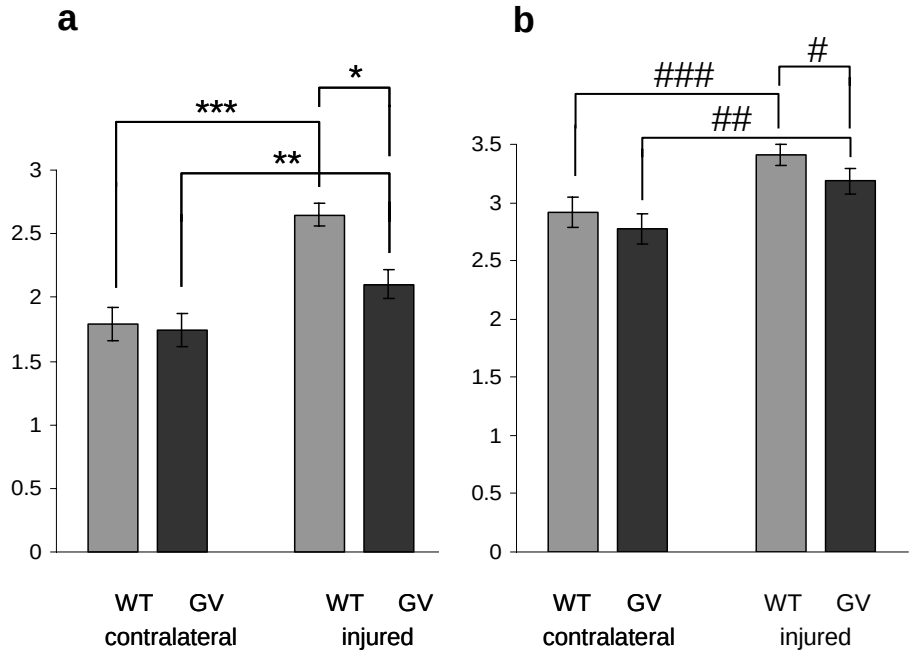


Figure 23. Culture-derived isotope tags, $^{13}C_6$-leucine containing peptides, used as internal standard in relative protein quantification of hippocampus. Same sequence peptides are used in calculation of ratios. 3-4 peptides in one sample gives the protein ratio. Average protein ratios are compared between groups. **a)** nano-LC MS chromatogram, **b)** mass chromatogram of 628.800 (extracted ion) over 10-30 min, showing the importance of sequence verification (Fig 14c), **c)** mass spectra at 17.3 min, (arrow in b), **d)** area integrated mass chromatogram of the light (tissue peptide) and the heavy ($^{13}C_6$-leucine containing) form of a unique peptide, YLAEFATGNDR, assigned to 14-3-3 epsilon.



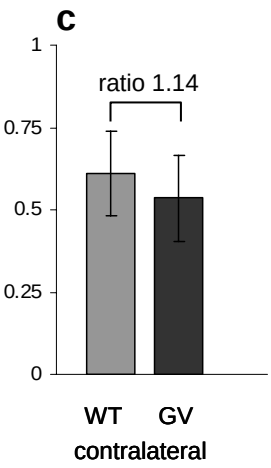


Figure 24. Protein quantification by the CDIT-based MS approach. Average peptide ratios (protein ratio) were compared between contralateral and injured hippocampus of wildtype (WT) and GFAP$^{-/-}$Vim$^{-/-}$ (GV) mice. The ratios between groups are shown in the table below.
a) 14-3-3 epsilon, **b)** all isoforms of 14-3-3, **c)** actin

Calculation with four peptides unique for the epsilon isoform of 14-3-3

	wildtype lesioned vs wildtype	***GFAP*$^{-/-}$ *Vim*$^{-/-}$ lesioned vs *GFAP*$^{-/-}$ *Vim*$^{-/-}$**	**wildtype vs *GFAP*$^{-/-}$ *Vim*$^{-/-}$**	**wildtype lesioned vs *GFAP*$^{-/-}$ *Vim*$^{-/-}$ lesioned**
ratio	1.48	1.21	1.03	1.26
ttest p-value	0.00007	0.036	0.706	0.002
	***	*		**

Calculation with three peptides common to all seven isoforms of 14-3-3

	wildtype lesioned vs wildtype	***GFAP*$^{-/-}$ *Vim*$^{-/-}$ lesioned vs *GFAP*$^{-/-}$ *Vim*$^{-/-}$**	**wildtype vs *GFAP*$^{-/-}$ *Vim*$^{-/-}$**	**wildtype lesioned vs *GFAP*$^{-/-}$ *Vim*$^{-/-}$ lesioned**
ratio	1.17	1.15	1.05	1.07
ttest p-value	0.107	0.196	0.648	0.429
	###	#		##

Calculation with five peptides for actin

	wildtype vs *GFAP*$^{-/-}$ *Vim*$^{-/-}$
ratio	1.14
ttest p-value	0.146

Comments on quantification of hippocampal and astrocyte proteins

The dynamic range of quantitative measurements is an important issue. Relative quantification relies on the fact that the mass spectrometer can detect large differences in peptide abundance. Signal-to-noise ratio and sufficient ion statistics must be considered in peak recognition and quantification. A MS detection limit of about 1 fmol protein (corresponding to 50 pg of a 50 kDa protein) is very low, but still not as low as the detection limit of antibody-based immunostaining methods (high fg or low pg level). However, the global large-scale relative quantification that can be provided by MS-based methods can hardly be solved with the laborious work that would be required by immunological methods.

Protein expression profiling of the mouse brain has been reported and more than 8000 protein spots were detected in three fractions by 2D-GE (165). While it was possible to detect even low abundance proteins there was a space limitation on the gel. One protein spot on a large pH-range in the first dimension can be at least five isoforms of the same protein when zooming in to the range between pH 4-7 (166). Some major cytoskeletal proteins such as tubulin were identified in 19 protein spots in astrocytes and actin were reported in 45 protein spots in astrocytes (167). If total protein quantification rather than post-translational modifications is the important question, then the MS-based quantitative proteomics is the analytical choice to avoid problems with over-lapping proteins in 2D-GE.

To accurately quantify specific proteins, the mass spectrometry-based quantification with CDIT was applied to assess protein expression levels in a defined part of the brain after microsurgery that induced astrocyte activation and reactive gliosis. Astrocytes were chosen as internal standard because they are the most abundant cells in the CNS and the activation of astrocytes is a hallmark of the used neurotrauma model. This approach allowed an accurate and efficient determination of the levels of specific isoforms of 14-3-3. The differential protein levels of a whole tissue proteome could be assessed directly with the use of appropriate software, for comprehensive global quantitative calculation of peptide area ratios of proteins present in the MS data set.

CONCLUDING REMARKS

Glycoproteomics of AD cerebrospinal fluid

Characterization of glycoprotein isoforms separated by 2D-GE of CSF from AD patients and control individuals demonstrates the analytical capacity of FT-ICR MS for glycoproteomic investigations. High mass accuracy and fragmentation of glycopeptides with CID / IRMPD provides information for glycostructures without the need for deglycosylation. Albumin depletion of CSF can be used to increase protein load on narrow pH-range 2D-GE which are sequentially stained, for quantification of both the level of glycosylation and protein expression. Decreased glycosylation level for α_1-antitrypsin in AD patients (localized in neurofibrillary tangles and senile plaques) were found but no specific glycoform of the studied proteins could be exclusively assigned to AD. To continue the discovery-type driven approach for disease-specific glycoproteomic profiling in connection with AD, further studies should include proteins in other pH intervals as well as larger subject groups of CSF samples.

Quantitative proteomics of denervated hippocampus

Classical comparative proteomics with 2D-GE may be used for screening purposes in the case of complex protein mixtures, such as hippocampal lysate, due to co-migrating proteins. Alternatively, the first dimension of 2D-GE should be divided in several pH intervals or prefractionation of the hippocampal lysate should be applied. Upregulation of the adapter protein 14-3-3 epsilon in denervated hippocampus and attenuation of this response in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice (less reactive gliosis) suggested that 14-3-3 may play a role in organizing astrocyte intermediate filament network in reactive gliosis. Culture-derived isotope tags and nano-liquid chromatography FT-ICR MS is a highly useful and accurate method for determination of protein isoform-specific expression levels. CDIT-based quantitative tissue proteomics emerges as a technique for the assessment of protein expression levels in defined parts of the brain after specific neurosurgical interventions. This study represents the first application of the CDIT approach in an experimental neurosurgery model with the aim to improve the understanding of a disease pathogenesis.

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