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Review

Recent advances in applying mass spectrometry and systems biology to determine brain dynamics

Enzo Scifo[1+], Giulio Calza [2+], Martin Fuhrmann[3], Rabah Soliymani[2], Marc Baumann[2] and Maciej Lalowski[2]

[+]these authors contributed equally

[1]Campbell Family Mental Health Research Institute of CAMH, Toronto, Canada

[2]Medicum, Meilahti Clinical Proteomics Core Facility, Biochemistry/Developmental Biology, Faculty of Medicine, FI-00014 University of Helsinki, Helsinki, Finland

[3]Neuroimmunology and Imaging Group, German Center for Neurodegenerative Diseases (DZNE), Bonn. Germany

Corresponding author:

Maciej Lalowski

Medicum, Meilahti Clinical Proteomics Core Facility, Biochemistry/Developmental Biology, Faculty of Medicine, FI-00014 University of Helsinki, Helsinki, Finland

maciej.lalowski@helsinki.fi

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Abstract

Introduction: Neurological disorders encompass various pathologies which disrupt normal brain physiology and function. Poor understanding of their underlying molecular mechanisms and their societal burden argues for the necessity of novel prevention strategies, early diagnostic techniques and alternative treatment options to reduce the scale of their expected increase.

Areas Covered: This review scrutinizes mass spectrometry based approaches used to investigate brain dynamics in various conditions, including neurodegenerative and neuropsychiatric disorders. Different proteomics workflows for isolation/enrichment of specific cell populations or brain regions, sample processing; mass spectrometry technologies, for differential proteome quantitation, analysis of post-translational modifications and imaging approaches in the brain are critically deliberated. Future directions, including analysis of cellular sub-compartments, targeted MS platforms (selected/parallel reaction monitoring) and use of mass cytometry are also discussed.

Expert Commentary: Here, we summarize and evaluate current mass spectrometry based approaches for determining brain dynamics in health and diseases states, with a focus on neurological disorders. Furthermore, we provide insight on current trends and new MS technologies with potential to improve this analysis.

Key words: brain, mass spectrometry, Laser capture microdissection, Liquid chromatography mass spectrometry, LC-MS^E, MS imaging, neurodegeneration, neuropsychiatric disorders

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1. Epidemiology and societal impact of neurological disorders

Alzheimer's Disease (AD), the leading cause of dementia is characterized by a progressive cognitive decline that culminates in withdrawal from society, agony and eventually death [1]. The pathological hallmarks of AD include extracellular accumulation of misfolded amyloid ß (Aß) peptides into senile plaques in the brain, which are frequently surrounded by dystrophic neurites and intraneuronal neurofibrillary tangles. In 2016 approximately 63,000 and 241,000 new AD cases were reported in the U.S. among people 65 to 74 years old and those 85 years old or more, respectively [2]. The number of people with AD is predicted to triple over the next four decades [3]. Between 1-5% of all AD cases are early onset (EOAD), including familial and sporadic forms which translates to 50,000 – 250,000 people in the U.S. [4]. Despite decades of research, the molecular causes of AD are still poorly understood.

Parkinson's Disease (PD) is the second most common neurodegenerative disorder affecting mainly the motor system with degeneration of midbrain dopamine neurons [5]. The molecular mechanisms leading to neuronal loss in PD are not completely understood, although alterations in mitochondrial functions and protein aggregation are thought to play a central role [6]. A meta-analysis of seventeen prevalence studies on PD predicted that the number of individuals over age 50 with PD in the most populous nations is expected to increase from 4.1 million in 2005 to 8.7 million in 2030 [7].

Neuronal Ceroid Lipofuscinoses (NCL) represent the most prevalent class of childhood neurodegenerative diseases [8,9]. They are mostly rare autosomal recessive disorders characterized by early accumulation of autofluorescent storage material in lysosomes of neurons or other cells [10]. Clinically, the NCL diseases are subcategorized into infantile, late-infantile, juvenile or adult forms. Several NCL types share distinct clinical features such as deterioration of motoric functions, seizures, visual failure as well as cognitive decline with dementia, which eventually lead to blindness and early death [11]. The prevalence of NCL is highest in the Nordic countries, for example, in Finland the estimated incidence of CLN1 is 9.4 per million whereas in the USA and United Kingdom it is estimated to be at 1.37 and 1.6, respectively [12].

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Major Depressive Disorder (MDD) which affects 6.7% of all American adults represents one of the most common mental disorders in the US and is predicted to be a leading cause of global disability by 2030 [13]. The underlying molecular mechanisms of MDD are still to be clarified and even upon successful therapy, depressive disorders impose a considerable strain on the patient, with remission rarely accompanied by a total disappearance of all symptoms [14].

The societal burden of the above mentioned neurological disorders and poor understanding of their underlying molecular mechanisms argues for the necessity of prevention strategies, early diagnostic techniques and novel treatment options to reduce the scale of their expected increase [3,7,14]. It is particularly important to consider that the diagnosis of these complex disorders usually takes place after onset of the first symptoms, enabling often only symptomatic and non-disease-modifying strategies.

In this review we depict various workflows for isolation and enrichment of brain-specific cell populations, both in health and disease conditions. Various aspects of sample processing, mass spectrometry technologies for differential proteome quantitation, analysis of post-translational modifications and imaging approaches in the brain are also critically discussed.

2. Collection and treatment of brain samples

Human post-mortem brain tissues for the investigation of cellular mechanisms associated with neurological disorders are obtained from a brain bank. Sample collection is done during routine autopsies with oversight from ethical committees that comprise experienced clinicians and pathologists. Findings from human postmortem studies may then be validated in appropriate animal models (typically mouse or rats) for purposes of probing causal links between a detected neurological pathology and affected molecular pathways. Mice may be euthanized by CO_2 asphyxiation and brains quickly extracted from the skull followed by brief washes with cold PBS to remove excess blood and gentle drying with soft tissue. The brains are placed on an aluminum foil strip and transferred to an empty, large tube (to preserve brain morphology), which

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is lowered into a liquid nitrogen bath and kept there until the whole tissue is frozen. Brains are then stored at -80 °C until sectioning.

Human brain tissue samples are especially challenging for proteomic studies due to the variation in postmortem interval before autopsy is performed (usually several hours), during which rapid increase in protein degradation is observed. A timely proteomic analysis is critical for proper assessment of protein dynamics, various proteoforms [15] and to target labile Posttranslational Modifications (PTM), which are usually lost or altered by the time of sample collection [16], thereby increasing the risk of artifacts. In order to facilitate proteomic and genomic investigations on post-mortem tissues, special *rapid autopsy* (also known as *"short postmortem interval autopsy"*) *programs* have been developed [17,18] to preserve the proteomic and genomic characteristics of samples before significant post-mortem degradation occurs.

Fresh-frozen specimens are the primary choice for brain proteomics analysis. Alternatively, Formalin-Fixed Paraffin Embedded (FFPE) samples, which are the standard method for tissue preservation in most hospitals, can be utilized upon specific treatments and enable multi-center retrospective studies with large sample cohorts. FFPE samples represent a particular challenge for proteomic analysis; formaldehyde-induced inter and intra-molecular cross-linking [19] poses a significant challenge to proteomic investigations, whereas the cleavage of methylene bridges by Heat-Induced Antigen Retrieval (HIAR) [20] requires incubation at high temperatures which might hamper sample integrity. Despite immense efforts, there is still a need to further develop protocols for sample processing towards downstream proteomics analysis. In spite of these limitations, recent methodological developments have facilitated quantitative analyses by parallel reaction monitoring on FFPE breast cancer tissues [21] and PTM detection [22,23]. For a complete review of the proteomic developments in the analysis of FFPE tissues we refer to [24].

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3. Laser capture microdissection

The cellular complexity and heterogeneity of brain tissue layers renders standard tissue homogenization and lysis protocols inadequate to isolate disease-specific cell populations of interest from predominant subsets (e.g. glial cells) [25,26]. Laser Capture Microdissection (LCM) is a technique for region or cell type specific sample enrichment of the proteome in health or disease states. This method, first introduced in 1996 [27], is of particular interest in neurological disorders characterized by “selective vulnerability”. For a detailed survey of the LCM technique for proteomics sample procurement we refer to [28] and [29].

Tissue sections of (5-15 μm thickness) from frozen brains are typically collected on polyethylene naphthalate (PEN) membrane slides using a cryostat and stained to visualize cellular architecture. Nissl based stains are the most commonly used for histological staining of tissues. Briefly, slides are fixed and washed in a series of ethanol dilutions, and subsequently stained with 0.2-2% Nissl based stains for 10 min followed by rinsing in ultrapure water. The slides are then dehydrated twice in a graded ethanol series (e.g. 50% ethanol, 75% ethanol, 95% ethanol and 100% ethanol for 30 sec each) and lastly placed in xylene for 3 min. For FFPE specimens, a deparaffinization step (usually 30 min. at 60° C followed by xylene washes) is required before proceeding with the standard LCM procedure [30,31].

Two main types of laser systems are used for LCM: near-infrared (IR) capture [27,32] and ultraviolet (UV)-cutting [33,34] lasers. The former are based on activation of a thermo-sensitive polymer (ethylene-vinyl acetate, EVA), which is then attached to the cells on the slide underneath prior to embedding and capturing them in the polymer [35]. The transmitted laser heat is rapidly applied to the tissue (in milliseconds), resulting in dissections with minimal heat damage to the tissue [36,37]. A comparison of UV and IR laser systems [38] points to higher speed and precision of the UV laser, especially when the collection of small discrete areas is required, whereas the advantage of the IR laser rests in its lower energy, which is less abrasive to the tissue.

Glass or membrane slides can be utilized for IR capture laser with the Arcturus XT LCM system which additionally has a UV-cutting laser. In the latter, a high energy

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laser is used to dissect target cells in tissues embedded on PEN membrane slides. Dissected cells may be retrieved into collection caps with the aid of photonic pressure from a second laser shot, gravity or glued onto a sticky cap after LCM [36]. Guidelines for manipulation of the various LCM systems are presented in their respective manufacturers' manuals.

4. Cell/tissue homogenization and protein solubilization

Sample preparation of brain tissue requires good solubilization of proteins with detergents, which in turn creates challenges in downstream LC-MS analysis. Different methodologies were developed to accommodate the requirement for good solubilization and MS compatibility. One strategy involves the precipitation of the protein content and subsequent resuspension in MS-compatible buffers containing e.g. anionic, acidic labile-cleavable surfactants [39]; however the precipitation step may result in sample loss due to incomplete recovery during the resolubilization step. Another approach to extract proteins from cultured cells and tissue lysates (fresh frozen, and formalin-fixed, paraffin embedded) is the Filter Aided Sample Preparation (FASP) [37,40,41]. Solubilized protein homogenate is applied to an ultra-filter for reduction/alkylation, detergent removal, enzymatic digestion and buffer exchange [37]. Application of FASP to the processing of limited clinical brain samples offers several benefits. First, previous reports have suggested that 50−70% of the protein in the lysates processed with FASP is converted to peptides with a minimum number of missed cleavages [41–44]. We and others have also demonstrated that the technique ensures cleaner sample preparation and better peptide yields than the traditional "in solution" and "in gel" digestion protocols, respectively [37,40,45]. Moreover, the use of multi-enzymes e.g. trypsin and Lys-C for digestion (in up to 4M urea, maintaining good solubility conditions) improves the quality of proteomic analysis [37]. Finally, the sequential elution of peptides in FASP shifts the cleavage reaction equilibrium and therefore favors digestion of less abundant proteins or lower affinity enzyme sites [46]. Modified FASP protocols are also well suited for good recovery/enrichment of PTM, including phosphorylations (in combination with strong-cation exchange chromatography and/or titanium dioxide bead chromatography

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and to capture phosphopeptides) [47] and glycosylations (N-Glyco-FASP utilizing lectin affinity chromatography for capture of N-glycosylated peptides) [48]. For example, by utilizing FASP methodology, 12,035 phosphorylation sites (8446 novel) on 4579 brain proteins were identified. Functional annotation revealed that 23% of the identified phosphosites were located on plasma membrane proteins, i.e. ion channels and transporters, facilitating better prediction/correction of brain proteins topologies [47].

5. Liquid chromatography tandem mass spectrometry analysis

Mass Spectrometry (MS)-based proteomics provides a sensitive and quantitative platform that is widely used to identify key protein players in neurological disorders [49–51]. The merits of the technique include automated high-throughput differential protein determination and quantitation from low sample amounts (sub-microgram levels), high dynamic range and resolution for analysis of post-translational modifications [52] or complex protein-interaction networks [53]. Altered protein amounts between control and disease states are typically either analyzed by unbiased global proteomic profiling (shotgun proteomics) or targeted MS, which relies on *a priori* knowledge to select a subset of proteins for quantitation. In both approaches, quantitation of proteins of interest is based on a comparison of signal ion intensities across sample groups of interest. A comprehensive survey of ion-intensity based label-free proteomics and two label-based approaches using isobaric tags incorporated at the peptide and protein levels (Tandem Mass Tags, TMT), revealed that the label-free approach outperforms label-based, TMT methods regarding proteome coverage (up to threefold more proteins were reproducibly identified in replicate measurements). These methods exhibited similar reproducibility concerning protein quantification, but slightly differed in terms of accuracy, with the label-free method being less accurate than both TMT approaches [54]. Importantly, the comparison of TMT and label free quantitation (LFQ) platforms should be considered with the following caveats. For example, the latest Proteome Discoverer v2.1 (Thermo Scientific) offers vast improvements for TMT analysis over the earlier v1.3 software that was used in the previous survey [54]. Moreover, developments in instrumentation have optimized MS3 TMT quantitation and multi-notch MS2 fragment

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collection yielding improved MS3 reporter ion intensity [55]. Given the interference of non-selected precursors in complex samples, gradients and pre-fractionation steps should be performed in TMT "single shot" analyses to optimize identifications and quantification accuracy [56]. LFQ analysis has also been greatly improved with the continued development of Maxquant freeware (http://www.coxdocs.org/doku.php?id=maxquant:common:download_and_installation), which is developed for protein identification and quantification from large-scale MS datasets. The software package is equipped with normalization algorithms, allows for peak detection/matching across replicate runs and utilizes its own search engine (Andromeda) for protein identification, quantifies identified proteins and provides summary statistics. In addition to LFQ, Maxquant also supports analysis of MS data from other main labelling techniques, including: SILAC, Di-methyl, TMT and iTRAQ. It allows for processing of MS spectra obtained by MS systems from various vendors, such as - Thermo Fisher Scientific, Bruker Daltonics, AB Sciex and Agilent Technologies [57,58].

Label-free and label-based MS approaches have been utilized to analyze brains from either human cohorts [49,59,60] or rodent animal models [61–63] of neurological disorders. In recent years, the application of label-free methods in brain research has increasingly gained in popularity, since they provide more precise quantitation for lowly abundant proteins [64] and at lower operational costs (e.g. costs of ^{15}N labelling of food or metabolic labelling in mouse disease models) in comparison to labelled approaches. A focused in-depth review of the benefits and shortcomings of the available techniques for addressing neuroscience-related questions has been published [65]. Molecular knowledge at the protein level is potentially more relevant to pathophysiological processes associated with the neurological disorders in comparison to findings from transcriptomics or molecular genetics.

6. MSI- measurements of molecular distributions in the brain

Mass Spectrometry Imaging (MSI) allows for region-specific, non-targeted label-free, multiplexed molecular measurements from biological specimens directly on

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tissues, yielding an image representing the distribution of both endogenous and xenobiotic compounds. The tissue spatial distribution of each MS peak can be utilized for "virtual microdissection" of areas corresponding to specific molecular profiles. After completion of the MSI analysis, removal of the matrix by ethanol washes allows histological staining of tissue thereby co-registering the two levels of information.

This technique is capable of detecting analytes in the low pM concentration [66,67] and shares a feature of spatial resolution with Immunohistochemistry (IHC) and capacity for tissue micro-extraction(s) and multiplexing with Liquid Chromatography (LC)-MS [68–70]. MSI is an important tool for imaging especially small molecules, such as peptides or drugs to which specific antibodies are difficult to generate.

Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI) is the most recent MSI approach, introduced in 2004 by Cook's group [71]. DESI is an ambient ionization technique where a high-voltage spray capillary generates an electrically charged plume which is directed towards the sample [72]. As the electrospray droplets impact the sample surfaces, the ion analytes are desorbed and carried towards the inlet of a mass spectrometer; placing the sample in a moving stage which allows a rapid analysis of the tissue surface. DESI excels in the analysis of lipids and small molecules, and is characterized by minimal sample preparation, high-throughput and ease of operation due to the ambient analysis and basic instrumentation requirements.

In Matrix-Assisted Laser Desorption Ionization Mass Spectrometry Imaging (MALDI-MSI), a tissue section is coated with a thin layer of matrix prior to irradiation by a laser in the MALDI source that ablates discrete spatial positions in a grid manner and generates a mass spectrum for each point (Figure 1). The complexity of sample preparation for MALDI-MSI requires careful optimization during each phase (i.e. *sample handling*, *tissue treatment* and *matrix application)* in order to maintain the spatial arrangements of molecular compounds and avoid delocalization of the analytes as well as morphological artefacts [73–76]. It is necessary to minimize the time between harvesting and preservation steps (usually freezing in liquid isopentane or nitrogen) to

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ensure attenuation of endogenous enzymatic reactions which could hamper the analysis [77]. Tissue preparation usually involves washing steps (e.g. cold acetone or EtOH), which are critical in removing any unwanted molecules and salts from the surfaces of the tissue which might suppress ionization [78,79]. In contrast, DESI-MSI requires minimal sample preparation (i.e. it does not require matrix deposition or conductive glasses to perform the analysis [71]).

High spatial resolution MALDI-MSI analysis requires generation of a homogenous coating of small matrix crystals on the tissue section with analyte incorporation [80–84], which is achieved by the repeated spray cycles deposition of small volumes of matrix solution.

Different studies have leveraged MALDI-MSI to detect and localize Aß deposits in transgenic mice and human brains. The distribution of signal arising from the deposits was linked to small anatomical structures and the composition of the amyloid deposits was investigated without disrupting tissue morphology [78,85–89]. MSI has also been used to characterize the correlation between Aß plaques and phospholipids changes in frontal cortex, hippocampus and subiculum [90].

Analysis of 6-hydroxydopamine (6-OHDA)-treated rat brains by MALDI-MSI indicated the distribution and altered levels of different proteins (e.g. calmodulin, cytochrome c, and cytochrome c oxidase) in the dopamine-depleted regions and an increase in unconjugated ubiquitin in the dorsal striatum of the 6-OHDA treated hemisphere, highlighting the involvement of the ubiquitin-proteasome system in PD [91]. Shariatgorji *et al.* applied a derivatization method to investigate dopamine, GABA, glutamate and acetylcholine without the need for matrix: the analysis enabled 15 µm spatial resolution and showed decreased striatal levels of DA and increased GABA, mirroring the findings in human PD [92].

In a recent study, MALDI-MSI on brain sections from a palmitoyl thio-transferase 1, *Ppt1* knockout mouse (NCL, CLN1 disease model) at different stages (pre-symptomatic, symptomatic and advanced) showed the spatial distribution of several protein species, which were assigned to Myelin basic protein (Mbp) isoforms,

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cytochrome c and its functional complex components, and subsequently validated by IHC and nano-Liquid Chromatography tandem mass spectrometry (nano-LC-MS^E) [39].

Advances in technical aspects of MALDI-MSI have enabled in-situ detection and analysis of histone PTM given its specificity and multiplicity, and due to the absence of suitable antibodies for such investigations [93]. The ability of MALDI-MSI to detect and distinguish metabolic fragments and PTM in a region-specific manner is one of the key aspects that render it appealing for investigating early changes in brain disorders, where only a small part of the tissues is affected.

For an extensive review of the application of MALDI-MSI in neurodegenerative and psychiatric disorders (i.e. PD, AD, schizophrenia and amyotrophic lateral sclerosis) we refer to Schubert *et al.* [94] which recapitulates the advancements of this technique in the last decade.

DESI-MSI has been successfully applied to detect neurotransmitters in wild type rat brain slices [95] and 6-hydroxydopamine-treated (6-OHDA) rat brains [96], and to quantify small molecule neurotransmitters in rat brain sections by incorporating deuterated internal standards in the nano-DESI [97]. Most previous studies that leveraged DESI-MS for the imaging of brain lipids were conducted for the purposes of method development or to evaluate sample preparation protocols rather than to answer neurological questions [98]. However, the technique has recently been utilized in a pilot study to investigate phospholipid dysfunctions in schizophrenia [99], linking the molecular distribution of lipid species to an increase in phospholipase A_2 in schizophrenia patients [100,101].

Analysis of current literature on MSI experiments points to the prevalence of MALDI-based approaches, likely related to the compromise between speed, spatial resolution (100 µm for conventional DESI, 10 µm for nanoDESI and 20 µm for the current high-end MALDI imagers), mass range (0-2,000 Da for DESI and nanoDESI; 0-100,000 Da for MALDI), and signal stability during the experiment [102]. The prevalence of singly charged ions also gives MALDI an additional advantage over the spray-based technologies due to the simplified spectral interpretation. Recent developments in MSI

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techniques (e.g. DESI-MSI and secondary ion mass spectrometry, SIMS-MSI), however, have enabled researchers with complementary tools to study different aspects of tissue dynamics on the same tissue section, given the non-destructive nature of such analyses.

7.1 Dynamics of processes in neurodegenerative disorders

Different studies have leveraged the combination of LCM with proteomics to investigate brain dynamics in pathophysiological conditions; in 2007, Lewy body (LB) inclusions were isolated from human temporal cortex neurons of dementia patients with cortical LB disease by LCM and subjected to proteomic analysis [103]. This in-depth characterization of cortical LBs enabled a specific enrichment of LB-associated proteins, highlighting 156 proteins of which only 17 had previously been associated with cerebral cortical or brainstem LBs utilizing conventional proteomics techniques. Targeted proteomics approaches (in particular immunoprecipitation methods), were also employed to study the different isoforms of alpha-synuclein in PD brain tissue homogenates [104]. Another study investigated the relationship between metabolic dysregulation induced by diabetes and the levels of parkin, PINK1 (phosphatase and tensin homolog-induced putative kinase 1) and DJ-1, highlighting a significant parkin dysregulation in the *substantia nigra* of a diabetic mouse model which was restored by treatment with metformin, one of the most commonly used anti-diabetic drugs [105].

Label free quantitative proteomics approaches were used to investigate the *cortex* [106,107] and *substantia nigra* [108] of AD patients, pinpointing differentially expressed proteins in pathological specimens compared to the normal aging brain.
Analysis of temporal cortex neurons from FFPE AD human samples [109] identified 78% neuronal proteins of which 50% were associated with AD. The high specificity of the results validates the strategy of combining LCM and proteomics to extract accurate meaningful data from a very small amount of tissue. Given the high precision of modern LCM apparatus it is also possible to isolate single amyloid plaques [110]; extraction of Aß deposits from patients with the Arctic (E693G) mutation enabled targeted characterization of Arctic Aß neuropathology. Different proteomic studies have

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examined the composition of morphologically different Aß plaques, identified many proteins co-localizing with the plaques [111] and showed that each morphotype contains different Aß isoforms, with the predominant forms being Aß1-42 and Aß1-40 [112,113] Based on the Aß-induced oxidative stress hypothesis of AD progression, various studies have examined the effect of oxidative stress on the proteome of AD brains (reviewed in [114]).

Proteome alterations in the brain of a *Ppt1* knockout mouse model of CLN1 disease and its age-matched counterpart at different stages were investigated using LCM- based label free nano-LC-MS^E and MALDI-MSI [39]. The goal of the study was to quantify/visualize changes in protein expression of disease-affected brain thalamus and whole brain tissue slices, respectively. Alterations in various metabolic processes and inhibition of neuronal functions, including neuritogenesis were revealed at the pre-symptomatic stage thalamus. The symptomatic stage of the disease was characterized by disturbances in mitochondrial functions, synaptic vesicle transport, myelin proteome and signaling cascades, such as RhoA signaling. These changes were more pronounced at the advanced stage of the disease with considerable myelin sheath breakdown (Figure 2) and elevated RhoA/Huntington's Disease (HD) signaling cascades, linking CLN1 to other neurodegenerative disorders. The identified changes in protein levels were further validated by bioinformatics and network approaches, linking it to PPT1 and CLN3/CLN5 protein interactomes [40,115], IHC on brain tissues and literature mining from other NCL models, thus identifying various functional modules affected in CLN1 disease which can be targeted therapeutically [39]. Another, proteomic study combining global profiling and Acyl-resin-assisted affinity capture (Acyl-RAC) biochemically linked two NCL-associated proteins, DNAJ5/CLN4 (co-chaperone CSPα, responsible for autosomal dominant form of NCL, ANCL) and PPT1/CLN1 [63]. In CLN4 patient brains, the abundance of PPT1 protein was profoundly elevated, misfolded and present in neuronal aggregates. Interestingly, it was demonstrated that CLN4 serves as a substrate for the depalmitoylating reaction of PPT1, which was severely affected in the ANCL brain. Thiopropyl-sepharose chromatography combined with LC-MS allowed for the enrichment and quantitation of 850 endogenously palmitoylated proteins [63].

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7.2 Dynamics of processes in neuropsychiatric disorders

In this review, neuropsychiatric disorders are restricted to anxiety, depression, bipolar disorder and schizophrenia which encompass most of the known symptoms in this group of diseases. Accurate diagnosis and treatment of these disorders is hindered by their etiological and clinical heterogeneity [116], hence the need for more reliable biomarkers as well as therapeutic interventions. Genomic studies have dominated neuropsychiatric research in the last decade and improved our understanding of mental diseases [117–122]. However, the increased output of new molecular data has thus far not led to any significant improvements in diagnostic and therapeutic outcomes for the patients [123].

Investigations of anxiety disorders using MS-based proteomics are recent and mostly focused on rodent animal models. Quantitative MS analysis of the cingulate cortex synaptosome proteome [124] and metabolome [125] from high and low-anxiety-like mouse models implicated alterations in mitochondrial transport/import, amino acid metabolism, pyruvate metabolism, oxidative stress and apoptosis in anxiety-like behaviour [124,125]. Two-Dimensional Electrophoresis (2-DE) coupled to MALDI-Time of-Flight MS (MALDI-TOF MS) analysis of hippocampus from a female rat model of anxiety identified 44 differentially expressed proteins, including dihydropyrimidinase-related protein 2 (DRP-2/CRMP2), dynamin-1 protein and glial fibrillary acidic protein beta [126].

Most proteomic analyses of brain dynamics in depression involved rodent models [127–131] with a few human postmortem studies [60,132–134]. Studies of brains from mouse models of depression by 2-DE combined with MALDI-TOF indicated cytoskeletal damage, inhibition of anti-oxidation protein machinery as well as dysregulated energy metabolism and neurogenesis [127,129]. LC-MS/MS analysis and Selected Reaction Monitoring (SRM) assays (Figure 3) of post-mortem prefrontal cortices from depression patients highlighted dysfunction in cytoskeletal, mitochondrial, energy metabolism and synaptic related proteins [132–134]. Evaluation of prefrontal cortex and hippocampal synaptic junction preparations from rat models of depression by 2-DE and isobaric labelling coupled to MS analyses respectively, showed significant alterations in energy

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and glutathione metabolism, as well as revealed association of synaptic junction proteins with stress vulnerability or insusceptibility [128,135].

In bipolar patients, MS analysis of the dorsolateral prefrontal cortex (DLPFC) pinpointed changes in cell metabolism, signaling cascades, regulation of gene transcription, protein and RNA chaperoning functional groups [136,137]. Two-dimensional difference gel electrophoresis (2-D DIGE) and MS analysis of postmortem hippocampus from bipolar and schizophrenia patients revealed similar proteomic changes in: cytoskeletal rearrangements and cellular trafficking, oxidative stress response, mitochondrial function, protein-endocytosis, -degradation, and –ubiquitination [138,139].

The few proteomic studies in schizophrenia mostly involved analysis of post-mortem prefrontal cortices [139–144]. MS analysis of post-mortem prefrontal and auditory cortices from schizophrenia patients suggested neuritic, synaptic and glutamate signaling dysfunction, as well as abnormal phospholipid distributions [140–142]. Peptidomic analysis of postmortem temporal lobe and corpus callosum from schizophrenia patients identified an intracellular peptide (PepH) with potential cytoprotective activity [143]. Quantitative MS assays of SNAP-25 isoforms in ventral caudate samples from schizophrenia patients were consistent with a greater effect of the SNAP-25A isoform in the observed reduced levels of SNAP-25 [144].

8. Expert commentary

Global proteomic profiling has been extensively utilized to investigate differentially expressed proteins in human postmortem or animal models of neurological disorders [133,145,146]. Studies in postmortem brains are better suited for investigating disease associated alterations in the neural circuitry, at a cellular and molecular level in comparison to the complementary *in vivo* animal models [147]. However, human postmortem studies are challenging due to confounding variables and limitations in sample availability [147,148]. Consequently, animal models which usually do not accurately mimic complex neurological disorders are widely used to probe for plausible pathophysiological mechanisms [149–151].

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Application of MS-based proteomics in studies of neurodegenerative diseases is well established. For instance, MS analysis and confocal microscopy of postmortem cortex and hippocampus from AD patients indicated lysine methylation of tau in neurofibrillary lesions [152] as well as dysregulated: ROS/stress responses, oxidative phosphorylation, organellar acidification and the cytoskeleton [153]. Western blot assays, co-immunoprecipitation and MS analysis of postmortem PFC/mouse primary cortical neurons suggested that changes in c-Abl expression, activation and/or c-Abl-mediated phosphorylation of Y39 play a role in regulating α-synuclein clearance and contribute to the pathogenesis of Parkinson's disease [154]. LCM has been used to isolate subsets of cells or tissue structures of interest (e.g. amyloid plaques from brains of AD patients) in neurological disorders, for subsequent MS analysis [39,49,155]. In contrast, the application of mass spectrometry in neuropsychiatry research is relatively recent [123]. Another emerging technique used to assay dynamic changes in neurological disorders is MALDI-MSI [94]. The technique has widely been employed to study rodent models of AD and PD, with the goal of validating the models, investigating localization of protein aggregates, mapping differential proteomes/lipidomes associated with disease states or spatial distribution of novel drugs in the brain [94,156].

Current limitations in MSI relate to the inability to perform purification and separation steps (which translates to detection of only a small fraction of the signals observed in LC-MS/MS method [69]), mass limit of usually 30 kDa, sample-volume-limited nature of the analysis and the requirement to validate the results with other targeted techniques (e.g. IHC). Despite these intrinsic limitations of MSI, rapid technical and methodological developments in the technique will quickly expand its capability in terms of both spatial and mass resolution.

Strategies for MS-based analysis of PTM have mostly involved using enrichment of modified peptides from cellular models [157,158]. Literature knowledge about the role of PTM in disease states is therefore biased towards findings from *in vitro* and animal models, for which sample material is relatively abundant [159]. Optimization of peptide enrichment and sensitivity for analysis of PTM in postmortem brains therefore presents the ultimate challenge for validation of known biomarker candidates and identification of

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novel ones. Insight from cancer research indicates that validation of known biomarker candidates remains the critical bottle neck in MS-based PTM analysis [160].

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) in combination with confocal fluorescence microscopy was recently used to image the spatial distributions of lipids, Aß deposits and neuronal/glial markers in two mouse models of Alzheimer's disease [161]. Although brain regions with the Aß deposits could not be identified or localized by TOF-SIMS alone, the application of fluorescence staining and confocal laser scanning microscopy to an adjacent tissue section allowed for the generation of images that were used as navigation maps in subsequent TOF-SIMS analysis. This work highlighted accumulation of cholesterol in hippocampal areas undergoing Aß deposition in two mouse models of AD, in agreement with previous reports linking the former to AD pathogenesis [162–164]. The combination of techniques facilitated parallel imaging of cholesterol/lipids by TOF-SIMS and Aß deposits/glial cells using fluorescence microscopy at submicron spatial resolution in two transgenic mouse models of AD. This study provides a framework for the analysis of spatial distributions between lipid species and neuronal/glial markers in a single tissue section from healthy and diseased mouse brain tissues.

9. Five-year perspective

Given the successful application of MS technologies to neurodegenerative studies [152,153,165], similar strategies should readily be adopted to probe for biomarkers and putative therapeutic targets in psychiatric disorders. In our view, several MS platforms for proteome investigation in neurological disorders especially with a focus on quantitation, will gain a major impetus in the near future and are critically discussed here.

Thus far, the majority of neurological studies employed global proteomic profiling towards identification of differentially expressed proteins between health and disease states [153,165]. In this respect, shotgun proteomics was particularly well suited for qualitative studies of complex proteomes, but it is limited in reproducibility and sensitivity [166,167]. In comparison, targeted MS platforms using Selected/Parallel

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Reaction Monitoring (SRM/PRM) enable sensitive quantitation for a selected subset of peptides and therefore proteins [168–171]. Although SRM is the reference method for targeted MS experiments, it has a few shortcomings. For instance, it is limited by the actual number of transitions to be monitored and co-isolation of interferences along with the target precursor ion [168]. PRM has similar acquisition features as SRM, except that it substitutes the second mass analyzer used in SRM (quadrupole) with a high resolution Orbitrap. Benefits of PRM include: the ability to monitor in parallel all transitions of a given precursor in a single MS/MS spectrum and more selective measurements, i.e. the separation of ions with close m/z values (i.e., within a 10 ppm range) [170,172].

Co-expression analysis, which is widely used to identify genes involved in the same processes [173,174] may provide insight into the relative contributions of specific cell types in disease pathogenesis of individual brain proteomes. In a recent report, protein specific co-expression analysis identified 10 modules that were correlated with the AD phenotype, a subset of which included modules associated with neurons and various astroglial cells [175]. It is likely that such methods, together with other -omics approaches (e.g. [39]) will play a major role in validation of quantitative MS experiments in the future.

The precise roles of PTM, e.g. phosphorylation, acetylation, glycosylation, fatty acylation, ubiquitination and nitrosylation (either individually or in combination) on neuronal physiology and pathology are poorly understood. Technological advances in MS instrumentation and *in silico* prediction tools should allow for more sensitive assays of PTM (including previously uncharacterized ones) in the brain, under health or disease states [176]. Therefore, better protocols that allow for enrichment and preservation of PTM during sample preparation are needed to achieve higher sensitivity in their detection.

Mass cytometry is another recent technology that can be leveraged for simultaneous detection and quantitation of dozens of markers in individual neuronal cells [177,178]. Individual cells are stained with antibodies using metal isotopes as reporter groups [179] and introduced into Inductively Coupled Plasma (ICP) by

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nebulization for subsequent mass spectrometry analysis [180,181]. Molecular signatures of single cell populations at a transcriptomic level, have already been successfully characterized and utilized to infer cell lineages [182], identify subpopulations [183], and highlight cell-specific biological features [184]. Similar approaches at the protein level will be critical in future diagnosis, identification of diseased cell subpopulations and evaluation of drug resistance.

In comparison to the unexpected number of unique neuronal populations recently discovered by single-cell transcriptomics experiments [185], the proteomic information obtained by simple homogenization protocols or even LCM isolation of targeted brain regions still yields proteomic signatures arising from mixed cellular populations. Especially, the different population of glial cells and neurons induces high variability in such experiments preventing the measurement of small changes. Moreover, subcellular compartments of neurons are more difficult to access due to the high differentiation of neurons. However, some long-standing subcellular fractionation methods like the preparation of synaptosomes [186], enrichment of post-synaptic densities [187] or the isolation of mitochondria and endosomes [188], in combination with Fluorescent-Activated Cell Sorting (FACS) may be used to address subcellular proteomes, by establishing specific cellular populations derived from defined brain structures. The FACS-MS approach, despite being challenging, has already been established for proteomic analyses of dendritic cells isolated from the spleen and V-ATPase-rich cells from kidney and epididymis [189,190].

Future studies should develop methods addressing the protein dynamics in distinct cellular populations making use of cell type specific *Cre*-driver mouse lines in combination with specific reporters. Furthermore, the possible developments of MACS (Magnetic-Activated Cell Sorting), [191] which similarly to FACS is able to isolate specific cellular populations utilizing magnetic sorting and targeted binding of antibodies to surface proteins in order to establish specific cell populations (i.e. neurons) [192], will enable in-depth proteomic investigations. Fluorescence activated nuclei sorting (FANS) coupled to LC-MS/MS analysis has also been utilized to identify and quantify proteins across NeuN-positive and NeuN-negative nuclear populations from human postmortem

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frontal cortex [193]. Moreover, as the dynamics of the processes in neuronal cells appears to be distinct in different subcellular locations (e.g. synaptic buttons, axons, neurites and dendritic spines [194–197]) subcellular region-specific proteomic tools in combination with fractionation methods need to be further developed in order to uncover the high spatial complexity of these processes. Alterations in subcellular locations are implicated in pathological conditions (e.g. dendritic spines aberrations in HD, ataxias, AD and PD [198,199]), therefore these specific analyses will yield more detailed information about distinct/shared disease processes and pathogenesis.

Undoubtedly, there is renewed interest within the scientific community for performing in-depth analysis of biological mechanisms by combining different –omics strategies (e.g. metabolomics, proteomics, transcriptomics) (Figure 4). An obvious benefit of such multi-omic approaches is the ability to increase confidence in acquired data from individual techniques and provide a global perspective on putatively affected pathways that could be targeted for pharmacological intervention towards the clarification of disease pathogenesis. Furthermore, given the rapid methodological and instrumental advancements in MSI, it is foreseeable that these –omics data could also be linked to the subcellular spatial localization of analytes in tissues [200] by automatic annotation of the high-resolution MSI data [201], thereby providing a complete profile of the physio-pathological state of biological tissues.

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10. Key Issues

- The societal burden of neurodegenerative and neuropsychiatric disorders, which is exacerbated by the poor understanding of their underlying molecular mechanisms, poses a significant challenge for society.
- Targeted and non-targeted proteomic techniques are capable of specifically tackling the dynamic changes in protein expression, proteoforms and the post translational modifications in both pathological and physiological states.
- Laser Capture Microdissection (LCM)-based approaches enable enrichment for specific disease-affected brain regions, enhancing the specificity of various –omics investigations.
- Mass Spectrometry Imaging (MSI, e.g. MALDI-MSI and DESI-MSI) allows for the investigation of protein/peptide spatial distributions in tissue sections in a non-targeted manner, in fresh frozen and formalin-fixed paraffin embedded specimens.
- Pros and cons of current methods for LCM-proteomics and MALDI-MSI are also discussed in this review.

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Legends

Figure 1. Scheme of MALDI-MSI on tissues. Schematic outline of a typical MSI workflow for fresh frozen (blue) or FFPE (orange) brain tissue samples. Analytical steps comprise tissue sectioning, paraffin removal (FFPE), washing, enzymatic digestion (FFPE mainly), matrix deposition and MALDI MSI analysis. The tissue is covered by a matrix compound and irradiated by the laser (which ablates discrete spatial positions in a grid manner), generating multiple mass spectra that are linked to a specific *x* and *y* coordinates. The spatial distribution of any given ion is depicted through a heat map, which represents the relative abundance levels of the selected ion in the tissue. ROI-Region of interest; HE – Hematoxylin/Eosin; LFB – Luxol fast blue; ITO – Indium tin oxide; IHC – Immunohistochemistry; PCA – Principal component analysis; ROC – Receiver operating curve. The image of MSI data elaboration was adapted from reference [202] with the permission of © *IOP Publishing*. The other images are from *Wikimedia Commons*.

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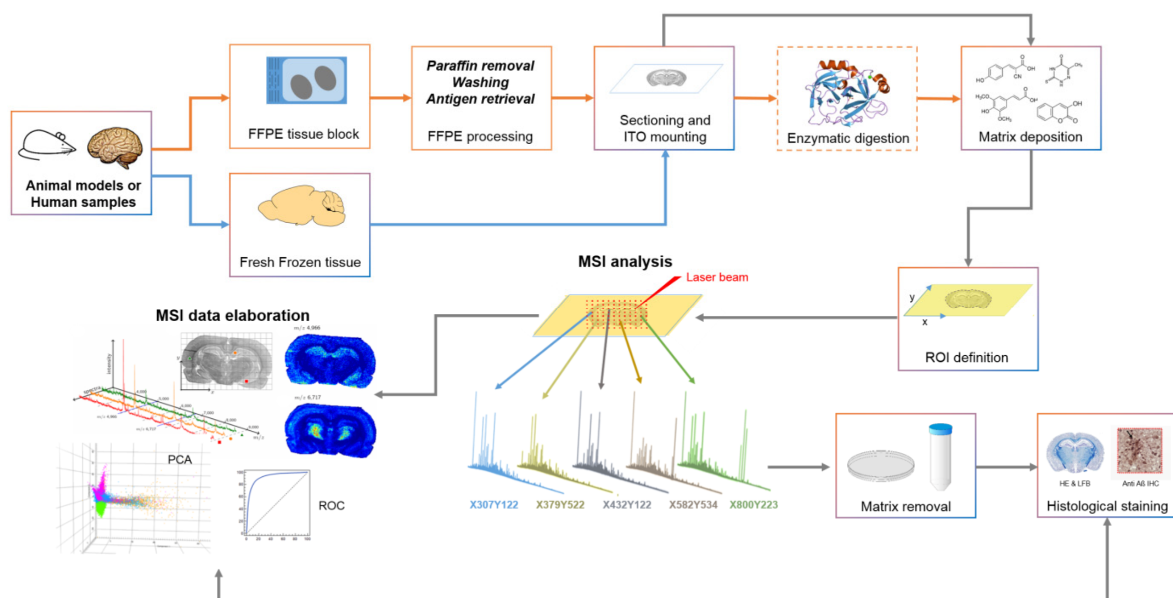

Figure 2. Down-regulation of myelin basic proteins in the *Ppt1*$^{-/-}$ brain. A) Ion density distributions of an average *m/z* 14145 Da ($[M+H]^+$) corresponding to myelin basic protein isoform 8. The increase in down-regulation of Mbp-8 isoform over time can be observed in the *Ppt1*$^{-/-}$ brain. The zooming of averaged spectra focusing on *m/z* 14145 peak region at 1 month (pre-symptomatic), 3 months (symptomatic) and 5 months (advanced stage), respectively. The mean of peak intensity is indicated. Relative intensity: dark blue- 0% intensity, red- 100% intensity. The maximum peak intensity in each image was set at 100%. T- Total ion current normalization (TIC). Scale bar- 5 mm. **B)** Immunohistochemical analysis of myelin basic protein immunoreactivity on consecutive slides. The area boxed in A) and corresponding to barrel field 1 of somatosensory cortex, SB1F, which has projections to thalamus, is indicated. A strong down-regulation of Mbp immunoreactivity in myelin layer at the advanced stage is shown. Scale bars- 200 µm and 50 µm respectively. **C)** A down-regulation of *m/z*, corresponding to myelin basic protein isoforms 5 (Mbp-5) and 8 (Mbp-8) is shown. **D)** Two different isoforms of Mbp detected in MALDI-MSI and nano-LC-MS^E experiments at the advanced stage. The brain tissue slices were digested with trypsin, and the digests from selected brain regions, pinpointed by immunohistochemistry were resolved by nano-LC-MS^E. The sequences of Mbp peptides detected in these experiments are underlined. Phosphorylated Tyrosine (T) measured in the nano-LC-MS^E experiments is shown in orange. Boxed sequences-peptides detected in MALDI-MSI experiments. In magenta- Mbp peptide (HGFLPR, *m/z* 726.67) measured in MALDI-MSI experiments of tryptic peptides on tissues. Adapted from reference [39], with the permission of the publisher, © *Springer*.

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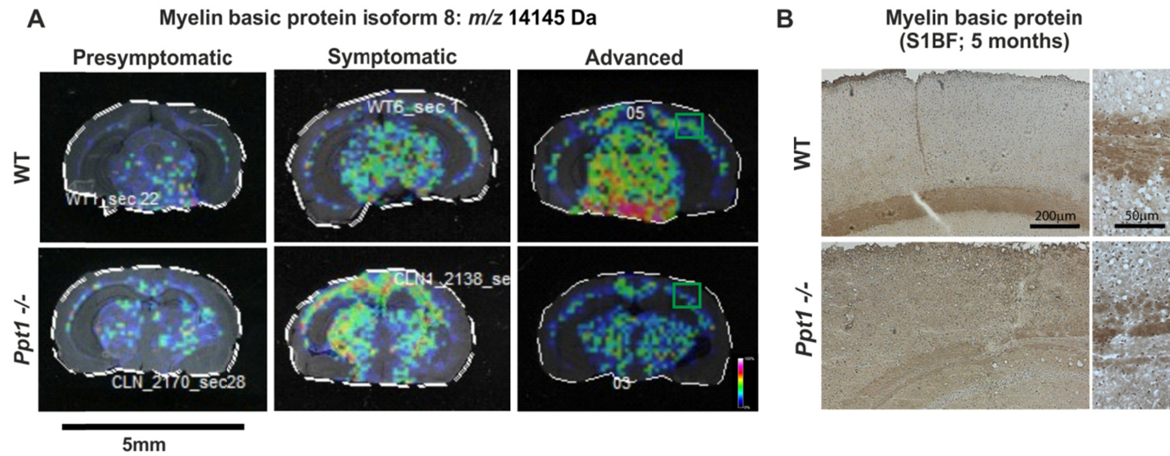

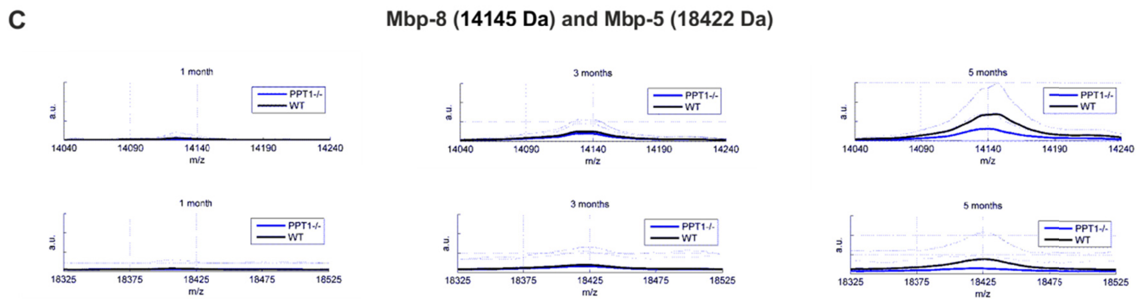

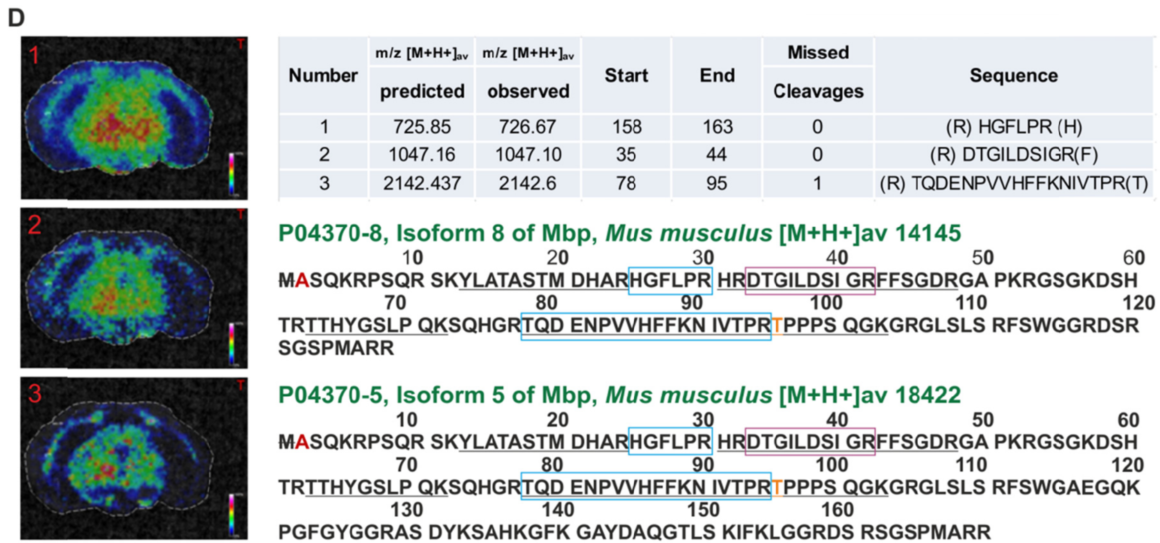

Number	m/z [M+H+]av predicted	m/z [M+H+]av observed	Start	End	Missed Cleavages	Sequence
1	725.85	726.67	158	163	0	(R) HGFLPR (H)
2	1047.16	1047.10	35	44	0	(R) DTGILDSIGR(F)
3	2142.437	2142.6	78	95	1	(R) TQDENPVVHFFKNIVTPR(T)

Figure 3. Targeted proteomics investigations in major psychiatric disorders. A) Schematic overview of the study design. Selected targets from various –omics and small-scale investigations are chosen for validation. Upon selection of initial 200 proteins, methods development phase, followed by synthesis of isotopically labeled peptides, 55-plex SRM assay to screen samples from major psychiatric disorders is established. **B)** Left, chromatographic Single Reaction Monitoring (SRM) profile of the sample's endogenic tryptic peptide AIFTGYYGK and of the spiked heavy labeled reference peptide. Right, chromatographic SRM profile of the transitions of the light peptide. Adapted from reference [134], with the permission of the publisher, © *Oxford University Press*.

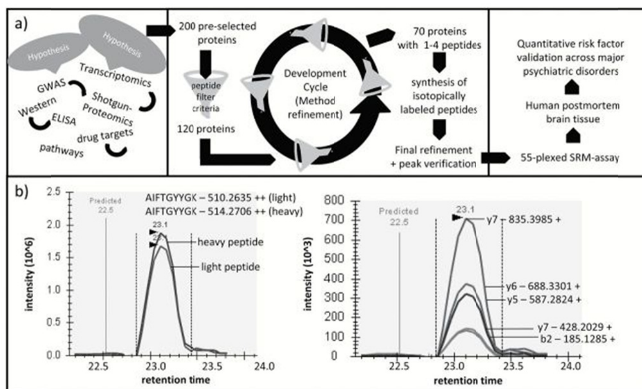

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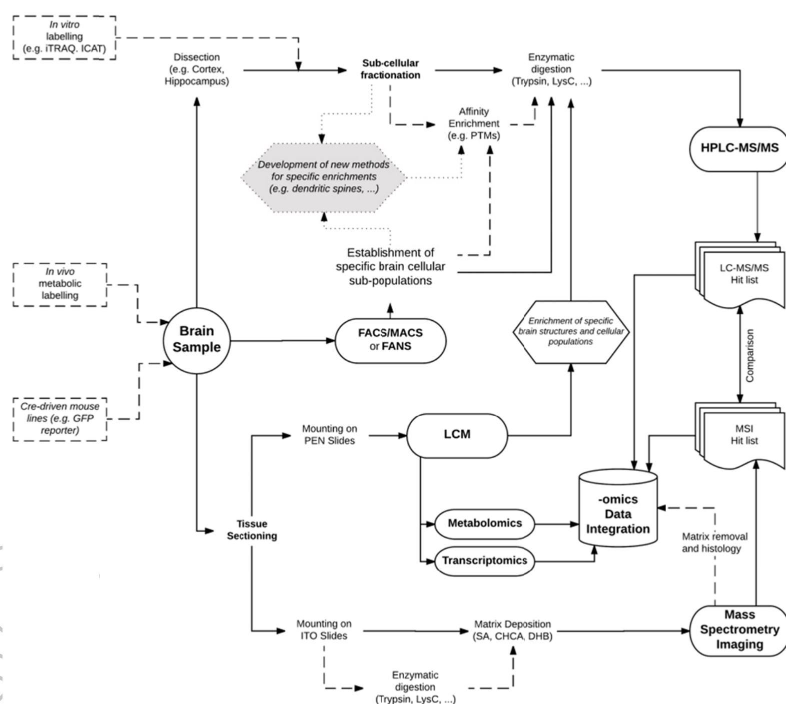
Figure 4. Outline of proposed -omics investigations in the brain. The flowchart depicts a proposed scheme of –omics approaches towards the complete analysis of the brain dynamics, encompassing proteomic, imaging, metabolomic and transcriptomic approaches in a single experimental setting, to yield a complete view of the pathophysiological state of the analyzed samples. The establishment of *Cre*-recombinase driver mouse cell lines allows for targeting specific brain populations (e.g. GABAergic/glutamatergic/cholinergic neurons, microglia, astrocytes and oligodendrocytes). Dashed lines depict optional steps in the analysis whereas the different techniques are represented as ovals. In grey- proposed methodological developments. PEN- polyethylene naphthalate; GFP- Green fluorescent protein; FACS- Fluorescence-activated cell sorting; FANS- Fluorescence activated nuclei sorting; MACS- Magnetic-activated cell sorting.



In the Review by Enzo Scifo, Giulio Calza, Martin Fuhrmann, Rabah Soliymani, Marc Baumann and Maciej Lalowski, 'Recent advances in applying mass spectrometry and systems biology to determine brain dynamics', which appeared in the June 2017 issue of Expert Review of Gastroenterology & Hepatology ([Epub ahead of print]; http://www.tandfonline.com/doi/full/10.1080/14789450.2017.1335200), it has been brought to our attention that the following inaccuracy appeared in the Funding and declaration of interest sections of the paper:

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Declaration of interest

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