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Master Thesis Report

"Comparative proteomic analysis of the MAM mouse model of schizophrenia"

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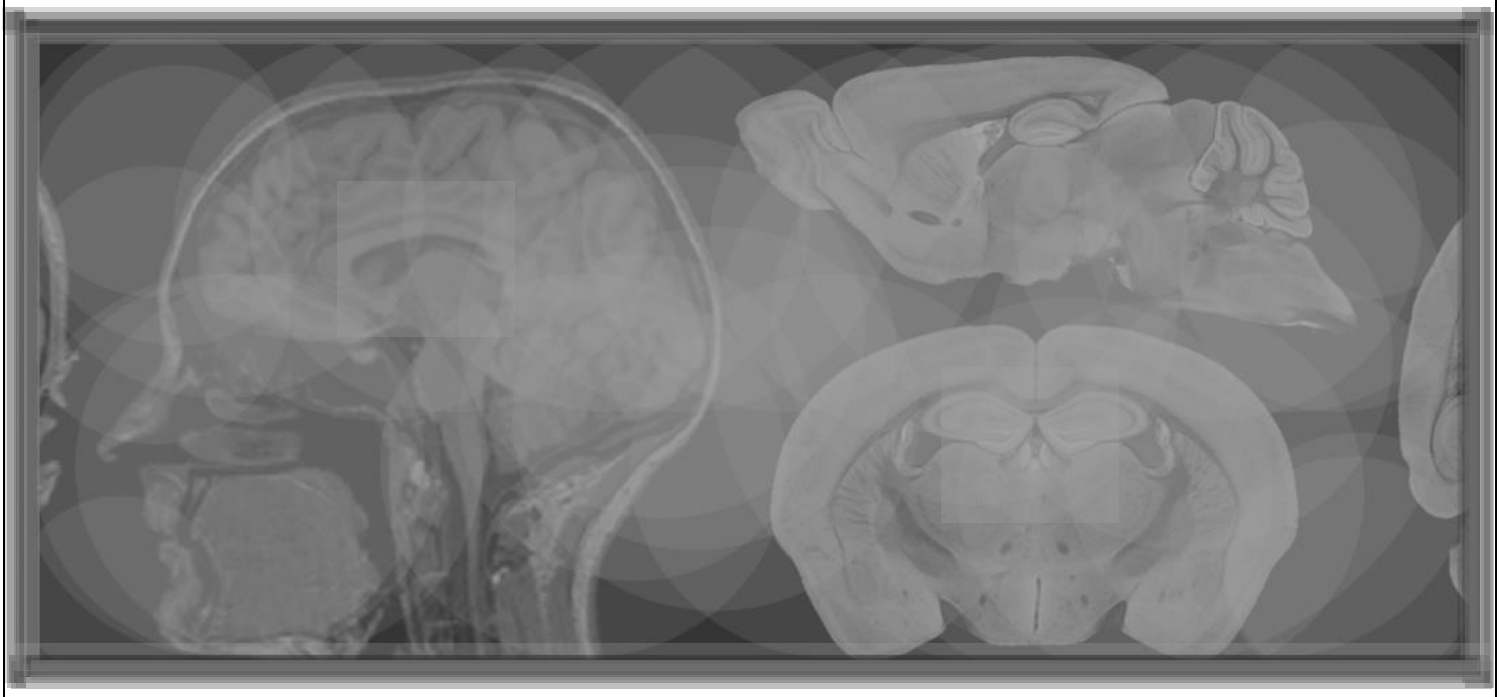

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Synopsis

Schizophrenia constitutes a chronic neuropsychiatric disorder, in the group of psychotic disorders, estimated to affect 1% of the world's population. Although the causal biological basis of the disease remains largely undetermined, variations in gene and protein expression, possibly in early developmental stages of the nervous system, have been presented as an important aspect of its pathogenesis. The proteomic characterization of neurodevelopmental models of schizophrenia, in this regard, comprises an intriguing direction towards understanding its neurobiological basis and the molecular effects of abnormal/perturbed neuronal development and aberrant synaptic physiology, in general. The present work focuses on a comparative mass spectrometry based proteomic analysis between the "prenatal MAM" (methylazoxymethanol acetate - exposed) mouse model of schizophrenia (induced at embryonic day 16) and saline animals with an emphasis on gender variations. In this respect, protein extracts of a distinct brain region, namely the prefrontal cortex, were fractionated by one dimensional SDS-PAGE into multiple protein fractions that were separately analyzed by mass spectrometry-based bottom-up proteomics and specialized bioinformatics, involving identification and relative protein quantitation. Significantly enriched and underrepresented proteins and pathways (over control animals) were determined for each gender as a means to characterize the animal model, in protein level. Substantial differences were observed between male and female MAM-treated animals illustrating a differential response to the gestational MAM administration. A prominent upregulation of various metabolic proteins in MAM-exposed males established a case for a functionally perturbed prefrontal cortex, suggesting a more intense "schizotypal" phenotype in males compared to MAM-treated females. These observations may provide an early framework towards investigating potential mechanistic molecular correlations with the human condition while highlighting the importance of incorporating gender into translational neuropsychiatric research.

Σύνοψη

Η σχιζοφρένεια συνιστά μία χρόνια νευροψυχιατρική νόσο, που υπάγεται στις ψυχωσικές διαταραχες, και υπολογίζεται πως πλήττει περί το 1% του παγκόσμιου πληθυσμού. Αν και τα ακριβή βιολογικά αίτια της νόσου δεν έχουν προσδιοριστεί, διαφοροποιήσεις στη γονιδιακή και πρωτεϊνική έκφραση, πιθανότατα σε πρώιμα αναπτυξιακά στάδια του νευρικού συστήματος, έχουν παρουσιασθεί ως σημαντικές συνιστώσες στην παθογένεση της διαταραχής. Ο πρωτεωμικός χαρακτηρισμός νευροαναπτυξιακών μοντέλων της σχιζοφρένειας αποτελεί μία ενδιαφέρουσα κατεύθυνση, προς κατανόηση του νευροβιολογικού υποβάθρου της ασθένειας και τη διερεύνηση διαταραχών στη φυσιολογική ανάπτυξη του νευρικού συστήματος και της συναπτικής φυσιολογίας, σε ένα μοριακό πλαίσιο. Η παρούσα εργασία εστιάζει σε μία συγκριτική πρωτεωμική μελέτη του «MAM» μοντέλου της σχιζοφρένειας (επάγεται με χορήγηση methylazoxymethanol acetate κατα την 16η εμβρυική μέρα) δίνοντας έμφαση σε διαφοροποιήσεις που σχετίζονται με το φύλο. Η ερευνητική εργασία περιλαμβάνει απομόνωση προμετωπιαίου φλοιού, κλασματωση πρωτεϊνικών εκχυλισμάτων του ιστού με ηλεκτροφόρηση σε αποδιατακτικές συνθήκες (SDS-PAGE) και ανάλυση των πρωτεϊνικών κλασμάτων με φασματομετρία μάζας, σε μία «bottom-up» προσέγγιση. Εξειδικευμένη υπολογιστική ανάλυση, ανίχνευση πρωτεϊνών και ποσοτικοποίηση τους επέτρεψε τον προσδιορισμό σημαντικά εμπλουτισμένων και υποεκπροσωπούμενων μορίων και μοριακών μονοπατιών, σε μία προσπάθεια διερεύνησης του μοντέλου. Σημαντικές διαφοροποιήσεις μεταξύ των φύλων πρότειναν διαφορική απόκριση στην χορήγηση της νευροτοξίνης κατά την κύηση. Ενδείξεις για αυξημένη ρύθμιση ποικίλων πρωτεϊνών του μεταβολισμού στα αρσενικά που είχαν εκτεθεί στη «MAM» υπέδειξαν διαταραχή της φυσιολογικής λειτουργίας του προμετωπιαίου φλοιού, υποστηρίζοντας έναν πιο έντονο «σχιζοτυπικό» φαινότυπο σε σχέση με τα θηλυκά πειραματόζωα-μοντέλα. Οι παρατηρήσεις αυτές, πιθανώς, να αποτελέσουν ένα πρώιμο πλαίσιο εργασίας προς, περαιτέρω, διερεύνηση μοριακών μηχανιστικών συσχετίσεων με την ανθρώπινη πάθηση. Παράλληλα, καθίσταται εμφανής η ανάγκη επανεξέτασης της σημασίας του φύλου στη μεταφραστική νευροψυχιατρική έρευνα.

Contents

1. Introduction

A

1. Schizophrenia; a complex disorder

Schizophrenia (SCZ) constitutes a chronic, highly detrimental, neuropsychiatric disorder, in the group of psychotic disorders, which is estimated to affect 1% of the world's population (more commonly males). It is characterized by a substantial heterogeneity of causal factors and symptoms that coincide in a disturbed perception of reality, as expressed through a wide spectrum of persistent deviant behaviors, typically manifesting in adolescence or early adulthood[1-3]. SCZ symptoms have been widely classified in positive (hallucinations, disordered speech/behavior) and negative (flat affect, aboulia, alogia). Intense introversion and distorted psychology of the self are often described as fundamental components of schizophrenic behavior patterns[2,3]. Although the causal biological basis of SCZ remains largely undetermined, variations in gene and protein expression, possibly in early developmental stages of the nervous system, genetic predisposition, along with a plethora of environmental factors (viral infections, inflammatory conditions, pregnancy complications, chemical components/pharmaceutical products) have been presented as important aspects of its pathogenesis[4,5]. Existing prevention strategies are considered inadequate, while the predominant use of anti-psychotic medication is associated with low efficiency rates (10%-30% of patients exhibits limited remission of the symptoms after administering multiple anti-psychotics and 30-60% moderate improvement) and increased risk of severe side-effects[6]. An apparent lack of sufficient prognosis and treatment schemes for the disease reflects, to a great extent, perturbing gaps in understanding its neurobiological background, despite copious research effort towards that direction[2,3].

The mere complexity of SCZ and its underlying molecular aspects has led to a significant number of research work and etiological interpretations addressing the onset and progression of the disorder. Characteristic examples include the neurodevelopmental hypothesis, ascribing a causal relationship to developmental abnormalities of the nervous system, the disconnection hypothesis presenting anatomical/functional alterations at the level of synaptic physiology, neurotransmitter hypotheses involving dopaminergic, serotonergic and glutamatergic, GABA-ergic pathways, as well as, immunological hypotheses exhibiting direct connections with proinflammatory response mechanisms[7-13]. Moreover, various studies suggest positive correlations with oxidative stress, glucose metabolism, folic acid, vitamin B12 levels and DNA methylation processes, among others[14-17]. Most reports converge at the multifactorial nature of the disease and the simultaneous involvement of functional derangements in multiple molecular signaling pathways in the brain and the periphery[18]. The implication of different brain regions i.a. the prefrontal/orbitofrontal cortex, hippocampus, hypothalamus, thalamus, nucleus accubens, anterior cingulate cortex, entorhinal cortex, substantia nigra and amygdala, based on various works adds to the complexity of the disease[19-28].

2. Genetic and epigenetic background

A strong hereditary component in SCZ (heritability estimated at 81% based on meta-analysis of twin studies) suggests potential genetic traits linked to the disease[29]. However, its genetic dimensions have not been clearly defined, despite extensive genomic research work. Genome-wide association studies (GWAS) already portrayed small scale chromosomal abnormalities (copy number variants, CNVs), involving deletion or duplication events, as factors that may increase susceptibility to the disorder[30]. The observed aberrations may be heritable, occur de novo, or involve both processes. The presence of CNVs at the 22 q 11.2 locus has established some genetic connections associated with the occurrence of complex psychiatric diseases, including SCZ, through mechanisms that possibly include multiple genes and environmental determinants[31,32]. Large scale genomic studies have indicated significant overlap of specific single nucleotide polymorphisms in five major psychiatric diseases (schizophrenia, bipolar disorder, autism spectrum, attention deficit/hyperactivity disorder, depressive disorder) implying molecular correlations between these disorders and SCZ[33]. Particular genetic loci, such as DISC1, that plays an important role in neuronal development and synapse formation, have been promoted as candidate genes for SCZ[34]. More than 70 genes involved in neurodevelopment, neuronal plasticity, neuroendocrinology and immune reactions have been associated with the disease, without perspicuous contributions to its etiology[35]. Despite important technological progresses in functional genomics, the genetic basis of SCZ remains largely elusive given no direct causal relationships with distinct genetic loci, as in monogenic pathological conditions, following mendelian inheritance.

The traditional simplistic notion of a solely genetic etiology for SCZ, involving alterations in particular genes, seems to be greatly afflicted by accumulating evidence pointing towards a systems disorder, implicated with functional and structural anomalies in large-scale neuronal networks, where altered function and spatiotemporal interactions of key brain regions is of great significance[36]. Epigenetics, moreover, provides new insights into the importance of differential regulation of gene expression in the establishment of higher-order organization that accompanies brain development and neurophysiological function, in general[37]. Epigenetic mechanisms, at the interface of intrinsic and environmental stimuli, are highly relevant to the pathogenesis of a wide array of psychiatric disorders, where strong correlations between the epigenome and physiological derangements have highlighted the requirement of strict epigenetic control pathways in maintaining healthy neuronal function[38]. In this regard, it has been suggested that part of the “missing heritability” links in liability to SCZ may be attributed to altered gene expression, due to epigenetic DNA and chromatin modifications[39]. Epimutations have been presented as an important source of maladaptations in the brain, often accompanied by notable behavioral changes/deviant acts, that are observed in SCZ, among other psychiatric conditions[40,41]. The majority of epigenetic studies focuses on DNA methylation changes, which have been noted in several genes involved in brain development, as well as, GABAergic and glutamatergic neurotransmission[42,43]. However, it is not clear whether, the described in SCZ patients, epigenetic changes reflect stably maintained/ disease-linked traits, since little is known about the dynamic turnover of epigenetic markings in the human brain[44].

3. Animal models of schizophrenia: the MAM-induced paradigm

The use of animal models offers significant advantages in investigating molecular mechanisms that underlie a wide array of psychopathological conditions. Developing realistic/credible models of complex neuropsychiatric disorders, like SCZ, in rodents, comprises a necessary requirement in understanding their neurobiological basis and evaluating novel therapeutic strategies[45]. Several cross-species translational tests (such as spontaneous or psychostimulant-induced locomotor activity, prepulse inhibition of the acoustic startle response, working memory and long-term explicit memory trials) have been proposed as to assess schizophrenia-like behaviors in animals[46]. Most SCZ models in rodents mimic positive symptoms of the disease (such as hyperactivity which may be associated with enhanced function of the mesolimbic dopamine system), while some exhibit dysfunctions of the cortical dopaminergic system as well as sensorimotor gating deficits which may be related with variations in the development of the prefrontal cortex and the limbic system[45]. Despite substantial difficulties in modeling negative symptoms of SCZ, assays that focus on social behavior, testing for anhedonia and avolition may hold great promise[47].As in any multifactorial/complex human pathological condition, the main purpose of developing animal models for SCZ is to reproduce major characteristics of the disorder (etiological factors or behavioral phenotypes akin to the human condition), since manifesting the entire spectrum of psychiatric phenomenology in animals would be considered an impossible task[48]. However, animal models of SCZ provide an indispensible framework for studies of circuit-phenotype relationships, at a systemic level, offering important information on the molecular effects of abnormal/deficient synaptic physiology and disrupted neural circuit formation, to a degree that is unattainable in humans[49,50].

Various methodological approaches (e.g. affecting development, genetic engineering, psychostimulant administration, lesions) have been utilized in an effort to model SCZ in rodents. Since SCZ is often presented as a neurodevelopmental disorder, a substantial number of the most highly regarded models focuses on directly perturbing neuronal development. The "MAM E17" model induced in rodents at embryonic day 17, through administration of a DNA-alkylating agent, methylazoxymethanol acetate (MAM), is characterized by analogous behavioral and histopathological patterns to those observed in humans, providing ample evidence for construct validity. The neurotoxin (causing DNA methylation/exhibiting antimitotic action), that targets neuroblasts selectively, without affecting glial cells, has been shown to reduce the thickness of the hippocampus and thalamus and various cortical subregions, leading to decreased brain mass (approximately 11%), without affecting striatal cells. The model appears to exhibit enhanced kinetic response to amphetamine and increased dopamine release in the nucleus accumbens (rather than the prefrontal cortex), supporting intense activity in the subcortical dopamine system, that is suggested to be implicated in human psychosis[45]. Various experimental data have denoted, moreover, disinhibition of the ventral hippocampus as a salient mechanism associated with the MAM phenotype, a finding that is consistent with imaging studies pointing towards a hyperactive hippocampus in SCZ patients[51,52].

B

4. Proteomics; an important toolkit for Molecular Biology

The development of two-dimensional gel electrophoresis (2D electrophoresis,2DE) in 1975, offered a fundamental methodological approach in protein detection and large scale molecular analysis of complex biological samples, that, eventually, led to the establishment of the field of Proteomics[53]. The term "Proteomics" was first described, in the 1990s, so as to define the large-scale characterization/analysis of the entire protein complement expressed by the genome of a cell line, tissue or organism[54,55]. Since then, it has been broadened to include the general study of proteins, their function and structure, as well as, their expressional profiles in diverse biological contexts/environments[56]. Research work focusing in the proteome, the sum of the expressed proteins of a genome, cell or tissue, has been established as a decisive step in investigating a wide spectrum of biological questions, having a complementary role with Genomics[57,58].

The main disparity between Proteomics and Genomics, the highly dynamic character, the great diversity and increased complexity manifested by the proteome (in contrast with the relatively static nature of the genome), implies the necessity for comparative studies, within the scope of both fields, as a means to attain a more comprehensive understanding of gene function[59]. Elaborate, moreover, mechanisms governing gene expression/information propagation may result in multiple proteins deriving from distinct genetic loci. The strictly defined, spatially and temporally, interactions between the expressed molecules, at the interface of environment-genome confer increased levels of variability in gene expression patterns (influencing gene regulation), in the overall composition of the transcriptome, as well as, at the levels of the protein species/populations of discrete biological systems. Moreover, the relative abundance of the alternative spliced variants, the differential degradation of messenger RNA and post-translational modifications (PTMs), such as proteolysis, phosphorylation and glucosylation, add an additional layer to the complexity of the proteome. It should be stated, for the sake of comparison, that a rough estimate of the human genome includes around 30.000 genes, whereas the proteome is suggested to consist of around 1.000.000 proteins[60,61].

Proteomics includes a wide spectrum of individual fields. Among the most notable sub-disciplines, “Structural Proteomics” aims in the characterization of the three-dimensional structure of protein molecules and complexes and the large scale identification of proteins and PTMs, “Translational Proteomics” employs quantitative protein analyses and studies focusing in apprehending differential protein expression relationships and “Functional Proteomics” establishes functional protein correlations while inquiring molecular interactions and mechanisms of enzyme function. In this regard, the isolation of specific protein subspecies (e.g. via affinity chromatography) comprises a common practice offering invaluable information on molecular mechanisms underlying pathological condition and physiological function, while permitting the addition of spatiotemporal dimensions in distinct molecular networks[62]. Functional proteomic approaches, moreover, may be utilized in detecting protein-drug

interactions in clinical research[63]. In all cases, proteomic analyses depend largely upon computational means of interpreting biologically relevant data in a cohesive manner[64].

Research in Proteomics benefits from various technological applications. Large-scale studies of gene function at protein level and PTM identification require specialized fractionation methods for cellular extracts, protein isolation techniques and subsequent selection schemes. Multidimensional electrophoresis, along with methodologies based in liquid chromatography, comprise the most common analytical approaches in protein fractionation and could be considered as complementary, given the particular characteristics of each method[65].

Proteomic analysis, typically, involves the identification of molecules through mass spectrometry configurations. Mass spectrometry (MS) is primarily based on the ionization of chemical species of a biological sample and ion separation according to the mass-charge ratio (visualized in mass spectra), that enable the accurate identification of specific proteins or peptide populations. The more common "bottom-up" proteomics direction achieves protein detection via identifying protein fragments/peptides (after a proteolytic digestion protocol, trypsin is a rather widespread enzyme for this purpose), whereas the "top down" proteomics identifies entire protein molecules. Mass spectrometry is also utilized in the quantitative analysis of proteins, on the basis of relative intensities of ion signals or with the aid of isotopic tracking technologies[66]. Accurate protein quantitation based on mass spectrometry, though, has been presented as one of the major challenges of proteomic research[67].

5. Proteomics in schizophrenia research: a cross-species endeavour

Taking into consideration the importance of the "operators" of genetic information, protein molecules, in understanding molecular mechanisms that govern biological systems, proteomic research of pathological conditions emerges as an imperative need, especially in complex disorders, like SCZ. In this direction, proteomic analysis, accompanied by substantial advantages over genomic studies, may offer significant insight in the understanding of the neuropathology and pathophysiology of the disease. A growing body of research work in both human subjects and animal models has been based on diverse proteomic technologies and experimental pathways. The most extensive part of this research effort concentrates in human patients. Analyses of human postmortem brain tissue, tissue from the periphery and bodily fluids have demonstrated differential gene/protein expression that may accompany SCZ. Work in animal models, moreover, has provided useful information on the dynamics of neurodevelopmental disorders, offering important molecular details on synaptic physiology and response to antipsychotics[68]. The existing comparative human studies have, already, been utilized in identifying potential SCZ biomarkers leading towards the development of novel diagnostic tools. A characteristic example includes a diagnostic platform of 51 markers (with an estimated sensitivity of 83%) that is based on blood samples[69]. The study of SCZ, following technological advances in Proteomics, Molecular Biology and clinical Neuropsychiatric Research may constitute a crucial step in improving disease prognosis, diagnosis and treatment strategies.

The first proteomics-based human studies of SCZ, involve the use of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and later "2D-DIGE" (Two-Dimensional Difference Gel Electrophoresis), in combination with mass spectrometry (MS), though "MALDI-TOF" (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) arrangements. The "2-D PAGE" platform achieves protein fractionation via isoelectric focusing, through a pH gradient, "SDS-PAGE" (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) separation based on molecular mass, protein staining, isolation of gel fragments and proteolytic trypsin digestion, followed by MS analysis. Peptide identification is attained by subsequent detection of specific patterns of peptide combinations, trough reference databases ("peptide mass fingerprinting") [70,71]. The introduction of "2D-DIGE" technology, which allows the analysis of multiple samples in one gel, by utilizing different fluorescent markers (differential excitation wavelength values) has proved useful in increasing the reproducibility and overall sensitivity of proteomic analyses[72]. Gel based technologies, however, despite substantial advantages (reduced cost, ability to detect post-translational modifications, increased reproducibility, among others) are accompanied by noted limitations in protein detection (decreased possibility of analyzing hydrophobic/transmembrane, highly acidic or basic proteins, exclusion of high molecular weight proteins, difficulties in identifying molecules in lower abundance)[73]. Novel technological adaptations attempt to overcome these problems. "SELDI TOF-MS" (Surface-Enhanced Laser Desorption Ionization Time-Of-Flight Mass Spectrometry), a variation of "MALDI-TOF", where chemically altered surfaces are employed in an effort to reduce the complexity of biological samples, prior to MS analysis, simplifies the sample preparation procedure. Reduced reproducibility and low resolution analysis (especially in high molecular weight molecules) have hindered the wide application of the technique[74].

"Shotgun Proteomics", that is usually considered as non-gel based, constitutes another, widely applied, methodology in human research on SCZ. The "shotgun" approach aims in identifying the sum of proteins that are included in a biological mixture/sample, in a large scale investigation of protein expression, that is characterised by increased accuracy and simplicity in preparation. The proteolytic digestion of the "intact" proteome, in "Shotgun Proteomics", is followed by high resolution separation via liquid chromatography and MS/MS mass spectrometry ("tandem mass spectrometry", which involves extensive fragmentation, selection and identification, of the MS-analysed ionized peptides). Various protein separation steps (such as numerous types of chromatography, isoelectric focusing, "SDS-PAGE") prior to "shotgun" analysis may increase the overall performance/resolution of the technique[75]. Furthermore, labelling technologies, such as "iTRAQ" (Isobaric tags for relative and absolute quantitation) and "ICPL" (isotope-coded protein labeling) have been a popular choice in establishing quantitative dimensions to different proteomic approaches in SCZ, but have been accompanied with significant disadvantages (high cost, accuracy challenges, increased sample requirements etc.)[76]. Instead, quantitation may be based on proteomic data of non-labelled samples, based on the conjecture that the height of chromatographic (peptide) peaks, at the m/z spectrum, corresponds to the relative concentration of the identified peptides[77].

Analyses of postmortem human brain tissue, comprises the most direct, possible investigation of the SCZ proteome, providing important information on the molecular aspects of the disorder. In this context, a large body of work based on various brain regions (i.e. prefrontal cortex, hippocampus, mediodorsal thalamus, anterior cingulate cortex, neocortex, temporal lobe) has denoted potential proteomic differences between healthy individuals and SCZ patients. The existing data, despite being generated from multiple technological resources/platforms, converge, mainly, in physiological perturbations at the level of metabolism, that extend to several brain regions, as indicated by altered levels of proteins that participate in glycolysis (e.g. Aldolase A&C, GAPDH, TPI1), marked differences in proteins that contribute to mitochondrial function (e.g. ATP5A1, ATP5B, ATP5H), oxidative stress (e.g. PRDXs, GSTs), Krebs cycle (e.g. ACO2, CS, MDH1), as well as, other energy metabolic pathways. Additionally, variations at actin isoforms (beta-, gamma-), tubulin (alpha-, beta-), molecules interacting with tubulins (e.g. MAP1A MAP2 MAPT DNM1) and intermediate filament proteins (e.g. VIM, DES, GFAP, NEFM, NEFL) may point towards cytoskeletal defects. Further, changes in immune/inflammation related pathways (differential levels of DEFA1, DEFA2, DEFA3, DEFA4, IL-1ra, IL-8, IL-10, IL-15, IL-16, IL-17, IL-18 etc.) in the blood of SCZ patients, establish, an immune perspective in the disease. Finally, downregulation of calcium signaling molecules (e.g. CALM1, CALM2 CAMK2B, CAMK2D, CAMK2G VDAC1, VDAC2) synaptic function and plasticity (NMDA and AMPA receptors, MAPK3, SYNPO, CYFIP2, VDAC etc.) may evidence dysconnectivity (non-canonical functional integration of brain processes, potentially due to abnormal functioning of neurotransmitter systems)[78,79,80]. It is important, though, to note that the use of postmortem brain tissue, may hinder, to some extent, the understanding of disease development and progression (in a dynamic framework)[81]. The biological heterogeneity between patients, age differences, medication use, as well as, the death process itself, may drastically alter the brain proteome. Astonishingly enough, even death as a biological occurrence and potential variations, during its pass, have been shown to pose substantial challenges in accurately mapping the "living" brain metabolome[81,82,83]. It is reasonable to consider that an analogous phenomenon may be observed in the brain proteome, as well.

Proteomic analysis of SCZ models may be considered as a necessary step in investigating the dynamics of SCZ and testing future or existing therapeutical pharmacological approaches. However, proteomic studies focusing in animal models of SCZ are sparse (compared to human research efforts) and limited to specific brain regions that are highly associated with SCZ. Analysis on the prefrontal cortex of postpubertal neonatally, neurotoxin-induced ventral hippocampal (nVH) lesioned rats, based on membrane enriched fractions/organelle purification and two-dimensional electrophoresis (2-DE), revealed decreased levels of neurocalcin delta and dysregulation in proteins participating in various neurotransmitter systems, in membrane receptor functions as well as vesicle exocytosis and recycling pathways (such as CLTB, STXBP1 and VSNL1). Besides being involved in neurotransmission, neurocalcin delta, STXBP1 and VSNL1, also play various roles in feedback mechanisms (such as formation of the SNARE complex and interaction with the M2 muscarinic ACh autoreceptor (M2 mAChR) at resting potential and dissociation at higher cellular polarization). The noted downregulation in the PFC of the model is suggested to predict altered feedback inhibition of acetylcholine (ACh) release (since ACh binding to the M2 mAChR promotes SNARE complex-M2 mAChR interaction)[84].

Proteomic research in the PCP (phencyclidine, a non-competitive NMDA receptor antagonist) model of SCZ, one of the most widely used preclinical models, that is closely resembling specific symptoms and abnormalities, observed in the human condition, has been a rather intriguing direction. A LC-MS/MS profiling study of the acute PCP rat model, in the frontal cortex and the hippocampus, identified significant molecular changes. Specifically, in the hippocampus, were more notable alterations were observed, 32 out of 449 proteins were found to be strongly altered (19 proteins had fold changes greater than 10%). Ingenuity Pathway Analysis (IPA), at an in silico approach, revealed that four of these proteins (excitatory amino acid transporter 1, microtubule-associated protein 6, dynamin 2 and calcium-/calmodulin-dependent protein kinase type II) were directly associated with an interaction network participating in molecular transport[85]. Excitatory Amino Acid Transporter 1 (EAAT1) bears significant clinical importance in SCZ research which has exhibited dysregulation of glutamate transporters in postmortem brain analyses, supporting the construct validity of the PCP model[85,86,87]. Additionally, it was shown that the top disease category linked with the altered proteins, found in the hippocampal extracts, was neurological disorders, and the highest-ranked canonical pathway was clathrin-mediated endocytosis signaling. Interestingly, transketolase, that connects the pentose phosphate cycle to glycolysis, was found highly increased in the hippocampus of the "acute PCP" model[85]. This finding may indicate hippocampal hyperactivity (that is already observed in SCZ patients) since network activation has been demonstrated to trigger a significant energy metabolism increase, a considerable part of which corresponds to glycolysis pathways[51,52,85,88].

Moreover, work on the "chronic PCP" model has portrayed frontal cortex alterations in glutamate-mediated calcium signalling (Ca2+-/calmodulin-dependent protein kinase II, PPP3CA and VISL1), energy metabolism and mitochondrial function (GOT2 and PKLR), as well as cytoskeletal remodeling (ARP3). A proposed decrease in calcium, induced by PCP treatment (as a result of blockade of NMDA receptors) is suggested to cause alterations at the levels of calmodulin-dependent protein kinase II (CAMK2) subunits, PPP3CC and VSNL1 and neurocalcin delta (NCALD) that are thought to lead to cytoskeletal remodeling (ARP2/3) and changes in long-term potentiation, synaptic transmission, and endocytosis. The disturbed intracellular calcium levels may affect energy metabolism through modulation of glycolytic and tricarboxylic acid (TCA) kinase activities and alterations in amino-acid metabolism. Morphological adaptations and changes in energy balance, in this respect, might finally lead to abnormal neural activity[89]. An extra study of the effects of chronic PCP administration in models of SCZ to the hippocampus, has denoted a greater number of significant changes in protein levels (than those observed in the frontal cortex). The authors have detected prominent protein level alterations of 22 enzymes in the frontal cortex, out of which 10 (45 %), catalyze a metabolic reaction. Observed alterations of 139 enzymes in the hippocampus, of which 94 (68 %) are involved in metabolic processes, moreover, have suggested extensive abnormalities in energy metabolism. Bioinformatic pathway analysis, in addition, presented potential perturbations in NMDA-receptor associated pathways in both brain regions, as well as alterations in other neurotransmitter systems (such as AMPA and GABAergic signaling) in the hippocampus and in proteins that have been implicated in neurodegeneration. Changes in the relative abundance of the superoxide dismutase enzyme (SODC) in the frontal cortex and the hippocampus, which indicate alterations in oxidative stress, further affirm apoptotic pathway anomalies[90].

A recent exhaustive study of the same group, that focuses on the anterior prefrontal cortex, has attempted a comparison of four different hypo-glutamatergic, well validated, rodent models (treatment with acute PCP, chronic PCP and ketamine and the NMDA receptor knockdown model) in relation to the human condition. A total number of 47, 84, 93 and 80 proteins were described as altered in the acute PCP, chronic PCP and ketamine models and the knockdown model, respectively. In silico analysis found partial overlap in protein-protein interaction networks across the four models which resulted in the identification of five functional domains that were present in all models (intracellular signaling and regulation, development and differentiation, intracellular transport and organization, biosynthetic processes and energy metabolism and nucleic acid metabolism and ATP/GTPase activity). An overall conclusion that the chronic PCP model represents SCZ to a greater extend (compared to the other models) was made, on the basis that the frontal cortical protein expression patterns of the model were closely linked to the latter four functional domains (presented above). An observed overlap of these common protein expression profiles with protein expression alterations of the brain of SCZ patients is presented as evidence for the validity of these animal models[91]. The described common patterns may indicate mechanisms that are not exclusive of individual models and may underlie fundamental neurobiological alterations of SCZ. Abnormalities in glycolysis and mitochondrial energy metabolism, between the observed functional enrichments, may affect multiple neuronal processes that depend to a great extent on the availability of cellular energy (such as presynaptic neurotransmitter release, recycling and intracellular transport mechanisms). An immediate result of these potential changes would be impaired information network processing accompanied by non-canonical/ambiguous behavior[92].

Research, in proteomics terms, has proven useful in investigating another important SCZ model, the “MAM E17” rat model. Proteomic/ metabonomic studies have shown that the MAM treatment on E17 results in deficient hippocampal glutamatergic neurotransmission, that has been observed in SCZ patients and further described via electrophysiological recordings. Use of two molecular profiling platforms provided a significant number of proteins (38 out of 673 identified molecules) found at altered levels in the hippocampus, out of which six (AMPA1, ARP3, MARCKS, HPCA, PMCA1, and SYNJ1) showed False Discovery Rate (FDR) q values of less than 0.05. The differentially regulated proteins, in the hippocampus, were presented as being part of an interaction network, suggesting notable effects of embryonic MAM treatment on neuronal signal transduction. The results also point towards altered synaptic neurotransmission and/or postsynaptic signal transduction pathways, along with functional influences of clathrin-mediated receptor internalization and calcium signaling. The frontal cortex, however, according to the authors, did not present any significant molecular effects. Out of 743 identified proteins there weren’t any pronounced differences in expression between the MAM-treated and the control animals[93,94]. This result would be considered as a rather unexpected occurrence, given substantial evidence for neuroanatomical changes in this region[95]. Previous research work has showed strong effects of MAM exposure in the frontal cortex and the hippocampus[93,96]. The underlying mechanisms of potentially subtle changes may be difficult to capture, on the basis of existing methodologies[93].

6. Objective of this study

The present work focuses on a comparative mass spectrometry based proteomic analysis between the "prenatal MAM" (methylazoxymethanol acetate) mouse model of schizophrenia, induced at embryonic day 16, and saline animals with an emphasis on gender variations at the prefrontal cortex (PFC), a rather unexplored research direction. It should be stated that MAM treatment at embryonic day 17 has been observed to cause milder schizotypic phenotype in mice compared to rats (which are the primary focus of interest). However, based on research efforts of Dr. Sidiropoulou and co-workers (Chalkiadaki et al, in preparation), exposure to the neurotoxin at embryonic day 16 yields a more intense phenotype, in relevance to SCZ. These findings may be further supported by studies that have presented brain maturation time differences between rats and mice (1-2 day deviation)[97,98].

The main aim of this study is to investigate already observed (in other animal models and human patients) anomalies of the prefrontal cortex while addressing the importance of incorporating sexual dimorphism into translational neuropsychiatric research as noted by multiple literature references[99,100].

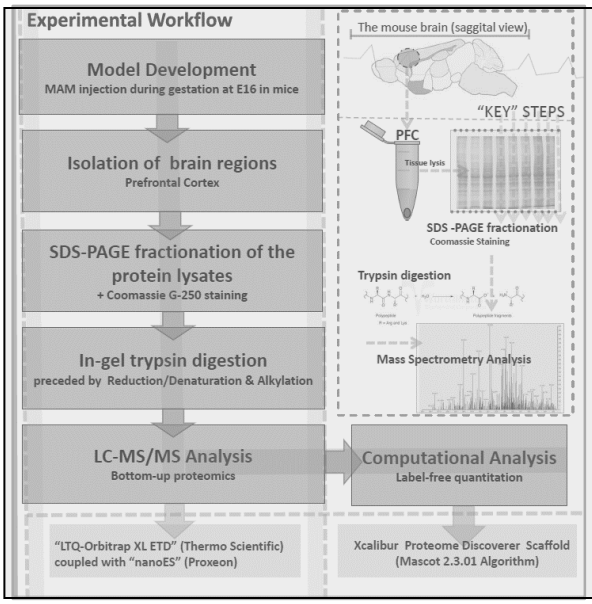


Figure 1.1: Scheme highlighting the main steps of the analytical procedure.

2. Materials and Methods

During the experimental procedure, protein extracts of the prefrontal cortex were fractionated by one dimensional SDS-PAGE into multiple protein fractions that were separately analyzed by mass spectrometry-based bottom-up proteomics and specialized bioinformatics, involving identification and relative protein quantitation (a brief summary of the utilized workflow is given in Figure 1.1). Significantly enriched and underrepresented proteins and pathways (over control animals) were determined for each gender as a means to characterize the animal model, at protein level, while providing a framework for elucidating potential mechanistic connections with the human condition.

1. Model development

Samples derived from adult (>3 months old) C57BL/6 male and female offspring of timed pregnant females treated with either saline or MAM injections. The experimental animals were housed in groups (3-4 per cage) and provided with chow and water ad libitum, under a 12 h light/dark cycle (light on at 7:00 am) at controlled temperature (23 +/- 1 oC). The pregnant dams were subjected to intraperitoneal injections (between 01.00-03.00 p.m) of MAM (26mg/kg) or saline (1ml/kg) on gestational day (GD) 16. Pregnant BALB/c dams were utilized as foster mothers, until weaning (day 25).

2. Protein fractionation protocol (Lysis/Bradford assay/SDS PAGE)

Mice were sacrificed via cervical dislocation. The prefrontal cortex (PFC) was isolated on ice. The samples in tubes were placed on dry ice and later homogenized on regular ice using a custom made Lysis buffer (Appendix, Table 1) and a plastic pestle (200µl of lysis buffer was added on each sample twice while homogenizing with the pestle). The lysates were further homogenized by passing their total content through an insulin-type syringe 2-3 times (the utilized lysis buffer volume was adjusted in accordance with the extracted tissue size and the desired protein concentration). After centrifugation at 4oC, 10.000 rpms for 20 minutes, the supernatant was collected on ice, constituting the original sample stock solution. The sample stock solution was stored long-term in -80oC, while aliquots were kept in -20oC.

Lysate protein content was estimated via Bradford assay. For this purpose, 300µl of undiluted Bradford solution was thoroughly mixed with 3µl of sample (in tubes through vortexing) and 200µl were loaded into a 96-well plate in duplicates. Absorbance measurements at 595 nm, for each duplicate, determined an average protein concentration based on a standard curve (OD value/BSA concentration, produced with sequential, known BSA dilutions at the same Bradford reagent/protein solution volumes).The sample volume containing 40 µg of protein according to the Bradford measurements (Appendix, section 2) was mixed with an equal volume of Sample Buffer (Appendix, Table 2) and Dithiothreitol, DTT (4/5 of the mix volume that was added to the samples consisted of Sample Buffer 2X and the remaining 1/5 of DTT 1M). Vortexing, spinning down, boiling of the samples in hot water for 3 minutes spinning down and placing on ice were followed by the loading of the samples in the wells of the gel. Adjusting sample protein solution volume to 10 µl by adding sterile ddH2O and mixing it with 10µl of Sample buffer/DTT mix ensured equal volumes of samples were loaded in all wells.

A typical polyacrylamide gel (10% acrylamide) was utilized for protein separation according to size through electrophoresis. A 30% acrylamide solution is of use in most recipes so as to achieve the desired pore size/gel density. (Appendix, Table 3) The gel was hand-cast (1 day prior to the analysis/stored at 4oC to prevent drying out) by sequentially adding the separating (pH:8.8) and the stacking phase (pH:6.8) at a purpose-built commercial glass support (recipes for the gel buffers and the gels at Table 4 A-C, Appendix). The separating gel phase was equilibrated by the addition of ethanol immediately after the addition of Tetramethylethylenediamine, TEMED before the polyacrylamide gel polymerization. The removal of ethanol after the polymerization was followed by the addition of the stacking

portion along with the placement of specialized plastic combs that form the wells of the gel. The polyacrylamide gel was submerged in a 1X running buffer (Appendix, Table 5). A molecular weight ladder along with the samples were, then, loaded into the wells. The electrophoresis device was set at 80 Volts until the samples reached the separating phase (~30 minutes/sample movement was traced via the pigment that is included in the loading buffer), where the settings changed at 120 Volts (~2 hours).

3. "Blue silver" Coomassie colloidal staining protocol

The gel was incubated in a fixation solution for an hour (30% methanol, 10% acetic acid added to ddH_2O) followed by 4 (15-minute) washes with ddH_2O. The utilized working colloidal "blue silver" solution (0.12% Coomassie Blue G-250 dye, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol) was prepared by sequentially adding phosphoric acid, ammonium sulfate, Coomassie Blue G-250, ddH_2O and anhydrous methanol (under constant stirring), as previously described[101]. The dye solution was preserved in a brown bottle at ~ 4oC for subsequent use (up to 6 months after production). The staining solution was removed after an overnight incubation (at ~ 4oC). The gel was, finally, destained with ddH_2O (4X 15-minute washes).

4. Gel excision/ In-gel digestion/ Sample preparation for MS analysis protocol

The protein bands of the Coomassie stained gel (Appendix, section 3) were excised and subdivided in smaller pieces using a disposable blade (at room temperature) prior to an in-gel digestion protocol[102]. The gel pieces of interest were separately stored in tubes (at ~ 4oC) and later incubated in 100 µl 50% Acetonitril,ACN/ ddH_2O (the sample tubes were shaked for 15 minutes, room temperature). Removal of the solution was followed by addition of 100 µl 50 mM Ammonium bicarbonate solution, AB (15-minute shake, room temperature). Subsequently, the AB solution was discarded and the previous steps were repeated twice (ACN solution addition-removal/ AB solution addition-removal). The gel pieces were later covered with 100 µl 10 mM Dithiothreitol, DTT and placed in a shaker for 15 minutes at 56°C (15.4 mg DTT were dissolved in 10 ml 50 mM AB). DTT was removed and the gel pieces were, then, covered with 100 µl 55 mM Iodacetamide, IAA (101.7 mg IAA dissolved in 10 ml 50 mM AB) and shaked for 45 minutes (at room temperature). The IAA solution was, later, removed and the gel pieces were washed in three cycles consisting of ACN solution addition-removal/ AB solution addition-removal (100 µl 50% ACN /100 µl 50 mM AB, 15-minute shaking, at room temperature). The AB solution was discarded and the gel pieces were covered with 25-30 µl diluted Trypsin solution (260x in 50 mM AB solution) and incubated overnight at 37°C (the original trypsin solution stock is kept in a storage buffer: 50 mM acetic acid/water). The next day, the supernatant was transferred into a new "LoBind Eppendorf vial". The gel pieces were later covered with 50 µl ddH_2O and shaked for 20 minutes at room temperature. The supernant was transferred into the "LoBind Eppendorf vial" and the gel pieces were covered with 50 µl 50% ACN solution and shaked for 20 minutes (room temperature). Acetonitril was, then, transferred into the "LoBind Eppendorf vial" and the gel pieces were incubated in 50 µl of 0.1% Trifluoroacetic acid, TFA in

50% ACN/water (shaking for 20 minutes, room temperature). The supernatant was transferred into the "LoBind Eppendorf vial" and the gel pieces were discarded. Speed Vac centrifugal concentrator was utilized so as to evaporate the frozen supernant (via liquid nitrogen) until dry powder remained. The dried peptide mixture was subsequently re-constituted in a 0.5% formic acid aqueous solution (100 µl final volume), prior to the LC-MS/MS Analysis.

5. LC-MS/MS Analysis

Protein identification and relative quantitation by nLC-ESI-MS/MS was performed on a LTQ-Orbitrap XL coupled to an Easy nLC (Thermo Scientific). The sample preparation and the LC separation protocol were based on a previously described laboratory workflow[103]. More specifically, the tryptic peptide mixtures of the samples were fractionated on a reversed-phase column (Reprosil Pur C18 AQ, Dr. Maisch), of fused silica emitters, 100 mm long, with a 75 µm internal diameter, (Thermo Scientific) packed in-house, using a pressurized packing bomb (Loader kit SP035, Proxeon). The separation and elution of the tryptic peptides was conducted in a linear water-ACN gradient prior to the MS injection.

6. Computational Analysis

The nLC-MS/MS raw data were originally acquired through Xcalibur (LTQ Tune 2.5.5 sp1, Thermo Scientific) and then further analyzed in Proteome Discoverer 1.3.0.339 (Thermo Scientific) utilizing the protein identification algorithm Mascot 2.3.02 (Matrix Science, London, UK) against the *Mus musculus* theoretical proteome, via a reference database from the Universal Protein Resource (UniProt)[104]. Protein identification was based on the following parameters: precursor error tolerance-10 ppm, fragment ion tolerance-0.8 Da, trypsin-full specificity, maximum number of missed cleavages-3, variable modifications-methionine oxidation. Relative label-free protein quantitation was performed using Scaffold_4.4.8 (Proteome Software Inc.) through the MS intensities of the identified peptide populations (the Total TIC method was employed, an estimation of the sum of all the Total Ion Current values of all spectra assigned to a specific protein). An over 99.9% identification probability score was applied as to confine the results. Contaminants were excluded from the final list and all available biological replicates and runs were merged/integrated, in an effort to reduce biological variability. The collected data were, then, processed in Microsoft Excel (2016). Protein interaction charts were constructed through String Database[105]. KEGG pathway enrichment was investigated via the data-mining environment of the Database for Annotation, Visualization and Integrated Discovery (DAVID)[106]. Heatmaps were generated with Heatmapper web server (average linkage clustering and euclidean distance measurement were applied)[107]. The visualization was based on log2(Fold change) values (over saline) for each gender, separately. Unique proteins for saline or MAM-treated animals were taken into consideration (saline-only proteins were presented as strongly downregulated and MAM-only as strongly upregulated).

3. Results

1. Comparison via Venn diagrams

An initial analytical step involved characterizing the protein sub-populations corresponding to each condition, as well as the overlap between different "subsets". In this regard, Venn diagrams were constructed based on a total of 1159 identified proteins (excluding contaminants, while integrating the available biological replicates/ >99.9% identification probability) offering a brief comparison between genders and different experimental conditions (Figures 3.1,3.2,3.3,3.4). Interestingly, a wider protein subset in saline female animals is observed compared to saline males (896 proteins-components in saline females and 756 in saline males/Figure 3.2). However, the MAM-treated male subset was estimated to be considerably larger than the MAM-treated female subset (886 proteins in MAM males and 725 proteins in MAM females/ Figures 3.3 and 3.4), where significantly less unique proteins for the MAM condition (compared to saline) were observed, in contrast with the higher number of unique proteins identified in MAM-treated males (88 unique proteins for MAM females and 299 for MAM males, when comparing saline and MAM-treated animals for each gender separately/ Figures 3.3 and 3.4). These findings may, collectively, indicate a more "complex" molecular/proteomic phenotype in MAM-treated males compared to MAM-exposed females, as observed by the utilized experimental means, a hypothesis that was further explored via the construction of protein interaction maps (sections 3 and 4).

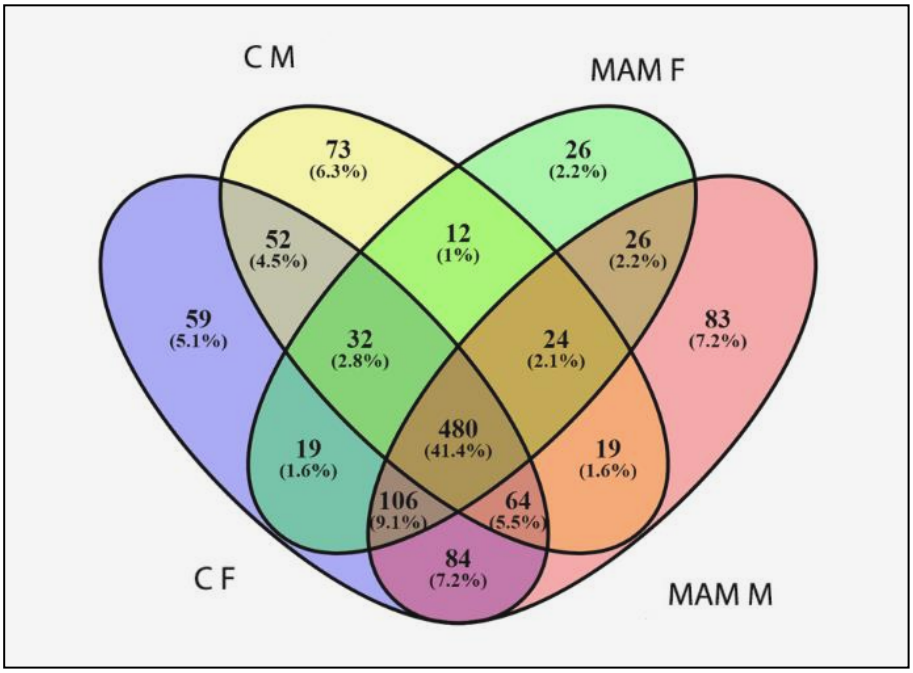


Figure 3.1: Venn diagram for both conditions [Saline-Control (C) & MAM-treated (MAM) animals] and both genders (F:Female & M:Male).

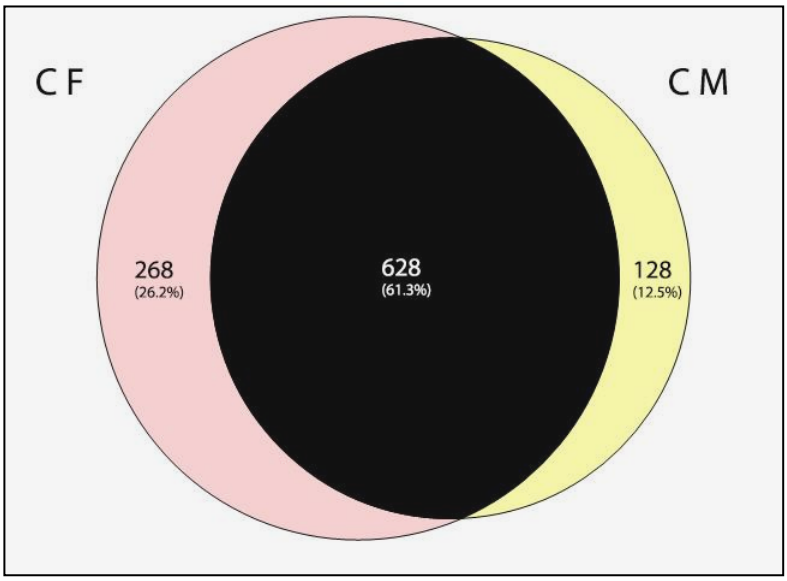


Figure 3.2: Comparison of identified proteins in Saline-Control (C) animals between genders (F:Female & M:Male).

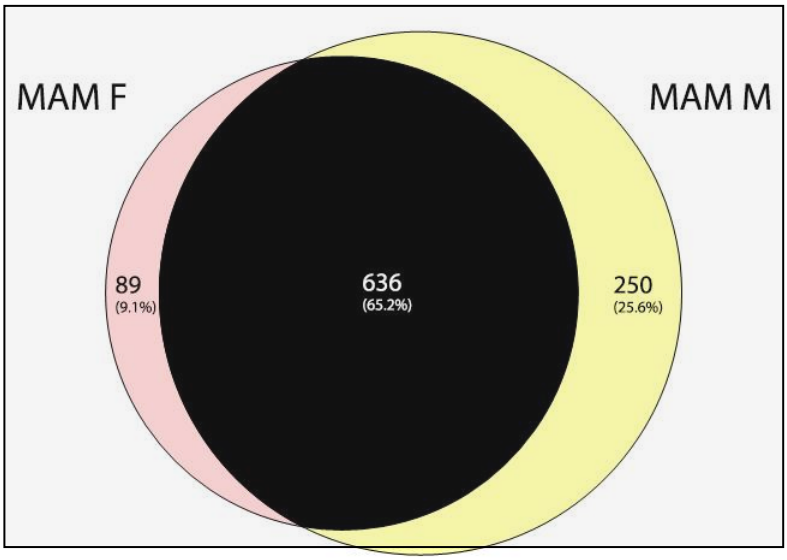


Figure 3.3: Comparison of identified proteins in MAM-treated (MAM) animals between genders (F:Female & M:Male).

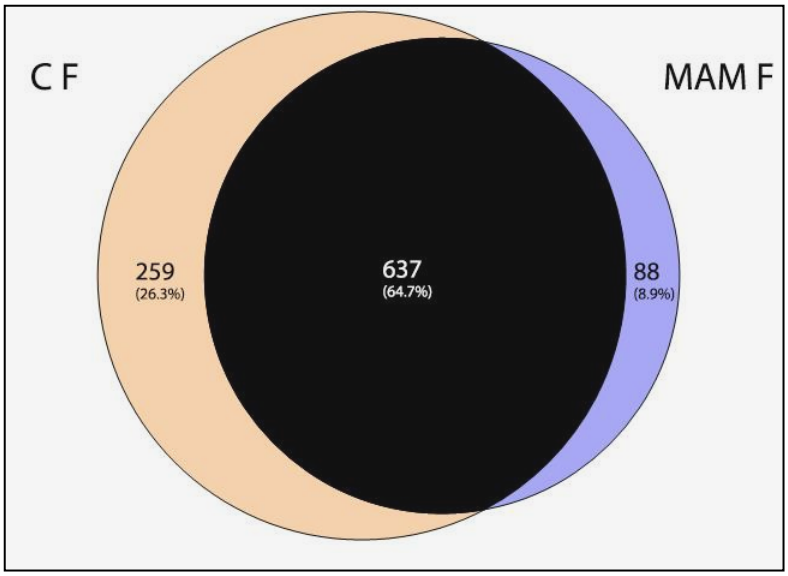


Figure 3.4: Comparison of identified proteins in both conditions [Saline-Control (C) and MAM-treated (MAM)] for Female animals (F:Female).

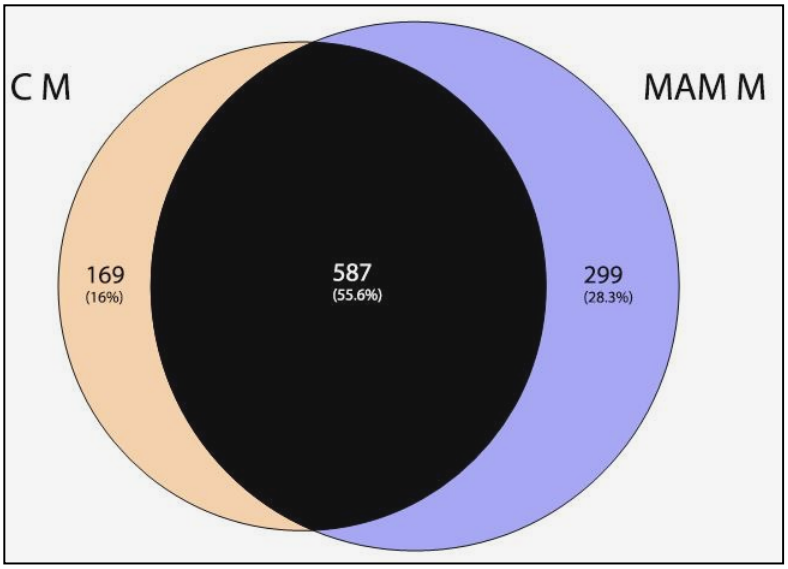


Figure 3.5: Comparison of identified proteins in both conditions [Saline/Control (C) and MAM-treated (MAM)] for Male animals (M:Male).

2. Trends among genders (upregulation or downregulation)

In an attempt to get an overview of the relative abundance of the identified molecules, proteins that exhibited the same (270 proteins in total) or different trends (215 proteins) in both genders (upregulation or downregulation) in comparison to saline animals were isolated and presented in graphs (Figure 3.6 and Figure 3.7). As evident, males exhibited, in overall, higher log2(Fold change values) positive and negative values in a substantial number of proteins (319 proteins exhibit over 2-fold change in males/202 in females), an observation that implies more pronounced quantitative differences in MAM-treated males over saline animals compared to MAM-treated females.

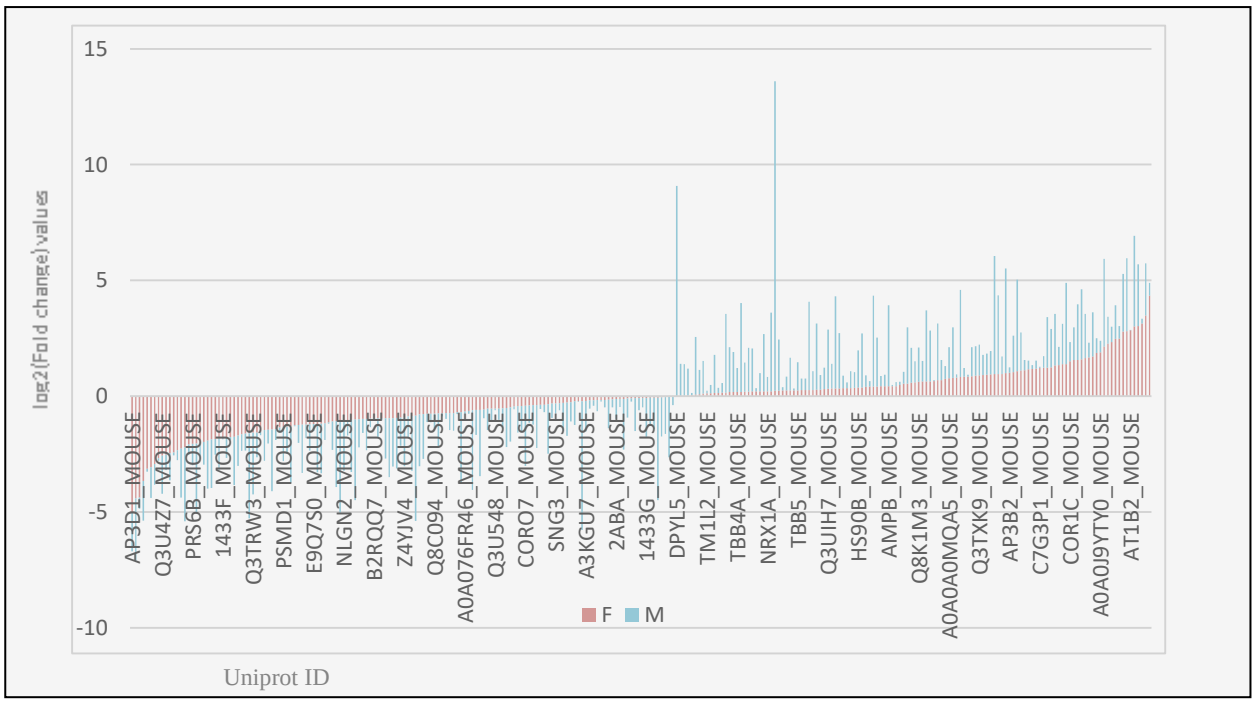


Figure 3.6: Chart, based on normalized (upon Median) log2(Fold change) values for the identified proteins exhibiting the same trend among genders (upregulation or downregulation, F:Female & M:Male).

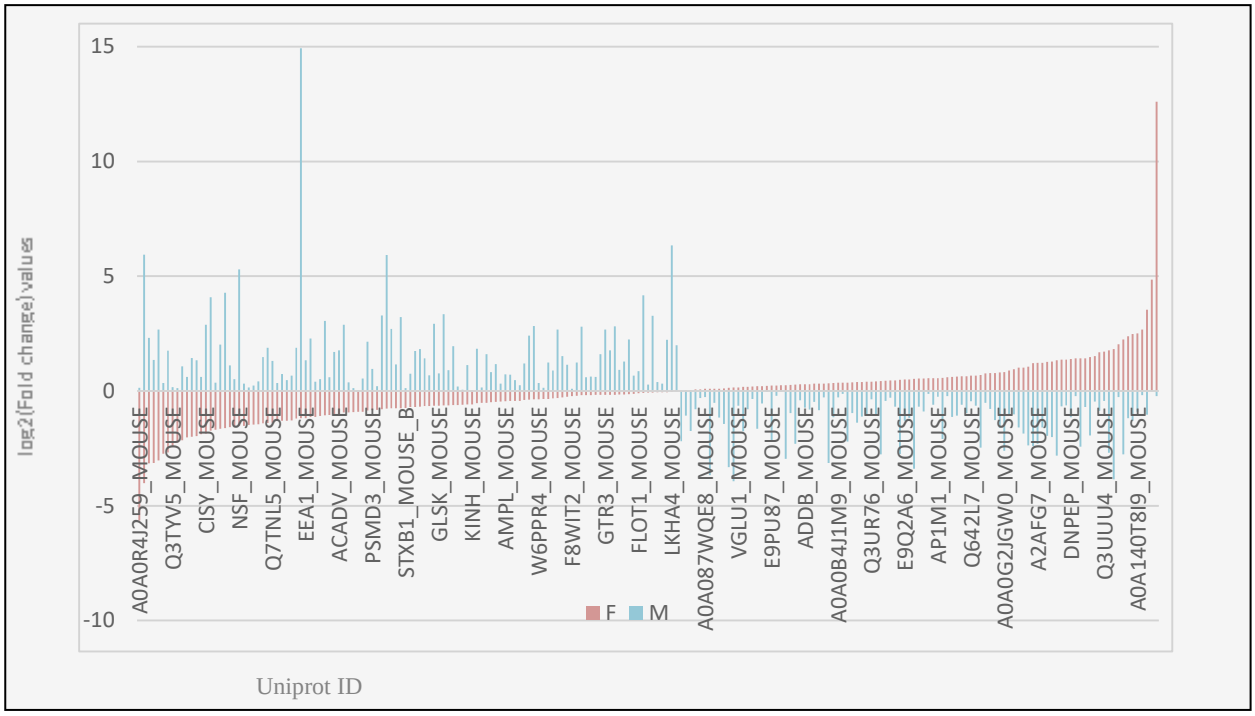


Figure 3.7: Chart, based on normalized (upon Median) log2(Fold change) values for the identified proteins exhibiting a different trend among genders (upregulation or downregulation, F:Female & M:Male).

3. Unique proteins in MAM-treated animals (compared to saline)

In an effort to highlight proteins that may be associated with the MAM phenotype and begin to investigate the model in functional terms unique identified proteins for MAM-treated animals (over saline) in each gender were presented in interaction maps and further tested for KEGG Pathway enrichment. Only one KEGG pathway: aminoacyl-tRNA biosynthesis (involving VARS, LARS, RARS2, MARS/ false discovery rate:0.00662) was found to be enriched in MAM-treated females (Figure 3.8), whereas, metabolism and endocytosis KEGG pathways (strongly enriched, among other pathways/false discovery rate: 2.57E-06 in metabolism and 0.000415 in endocytosis) represent a substantial part of the unique proteins identified in MAM-treated males (presented in Figures 3.9 and 3.10), a result that may point towards increased endocytic activity and metabolic function in the MAM-treated male PFC. An, overall, increased network complexity in MAM-treaded males (number of nodes: 297, number of edges: 596, average node degree: 4.01, avg. local clustering coefficient: 0.349) over MAM-treated females (number of nodes: 88, number of edges: 42, average node degree: 0.955, avg. local clustering coefficient: 0.339) may substantiate the previously suggested, more pronounced, molecular phenotype of the MAM-treated males.

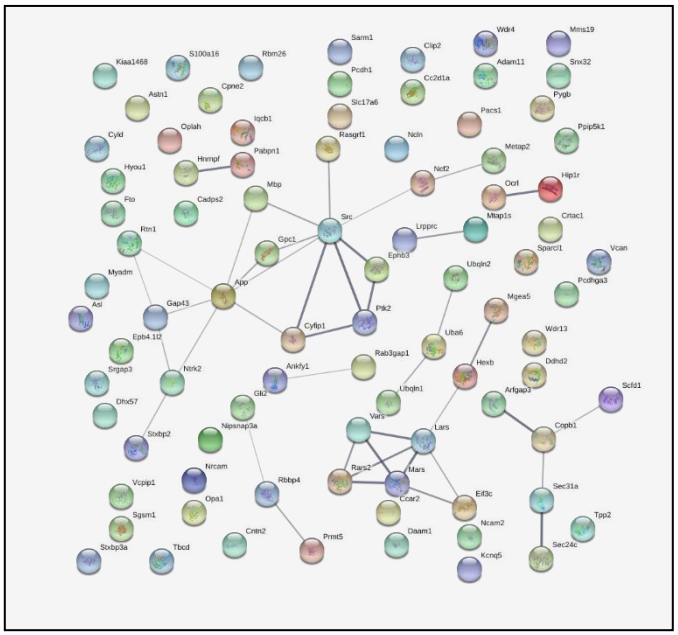


Figure 3.8: Protein-protein interaction network visualization for the unique proteins identified in MAM-treated females.

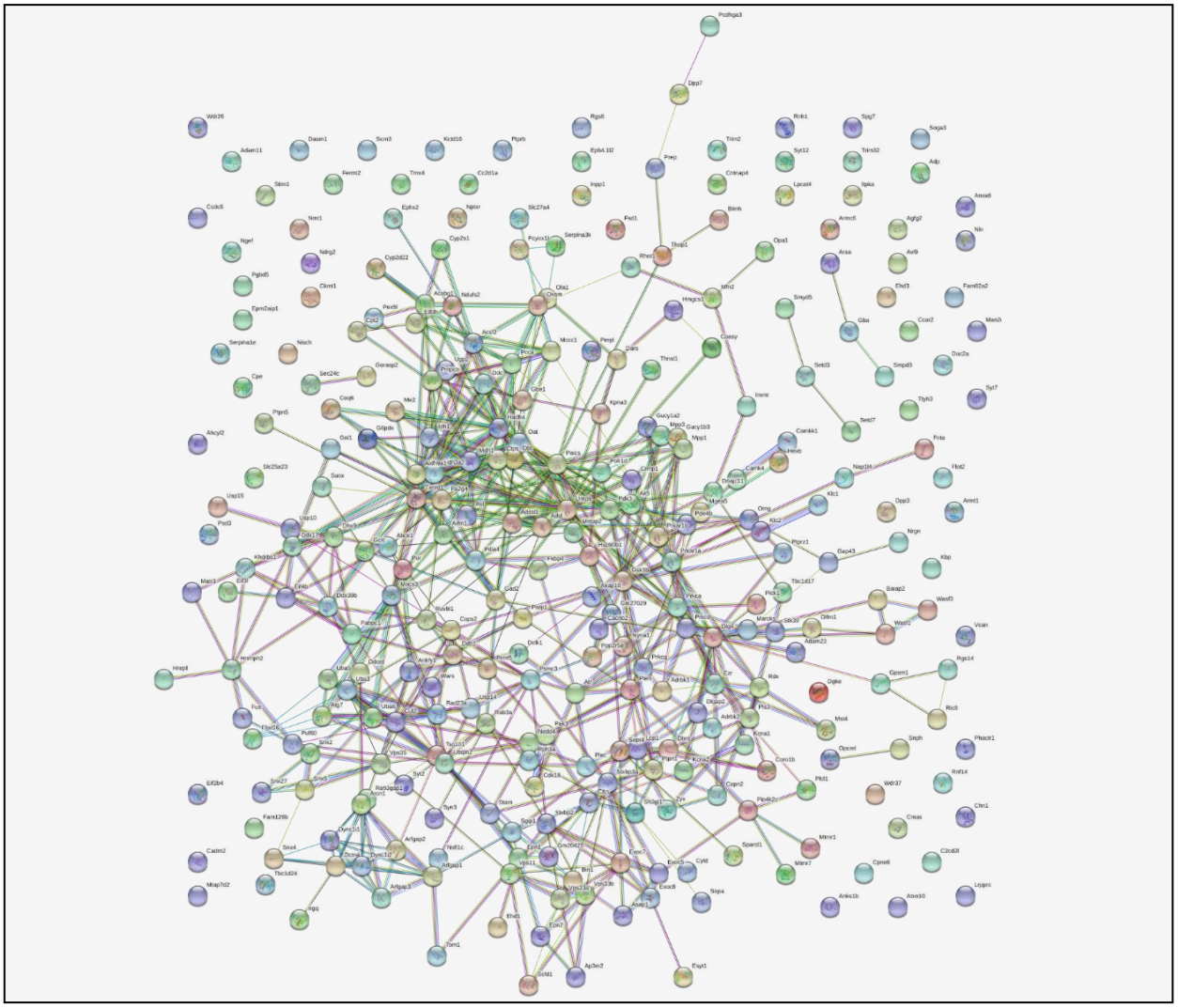


Figure 3.9: Protein-protein interaction network visualization for the unique proteins identified in MAM-treated males.

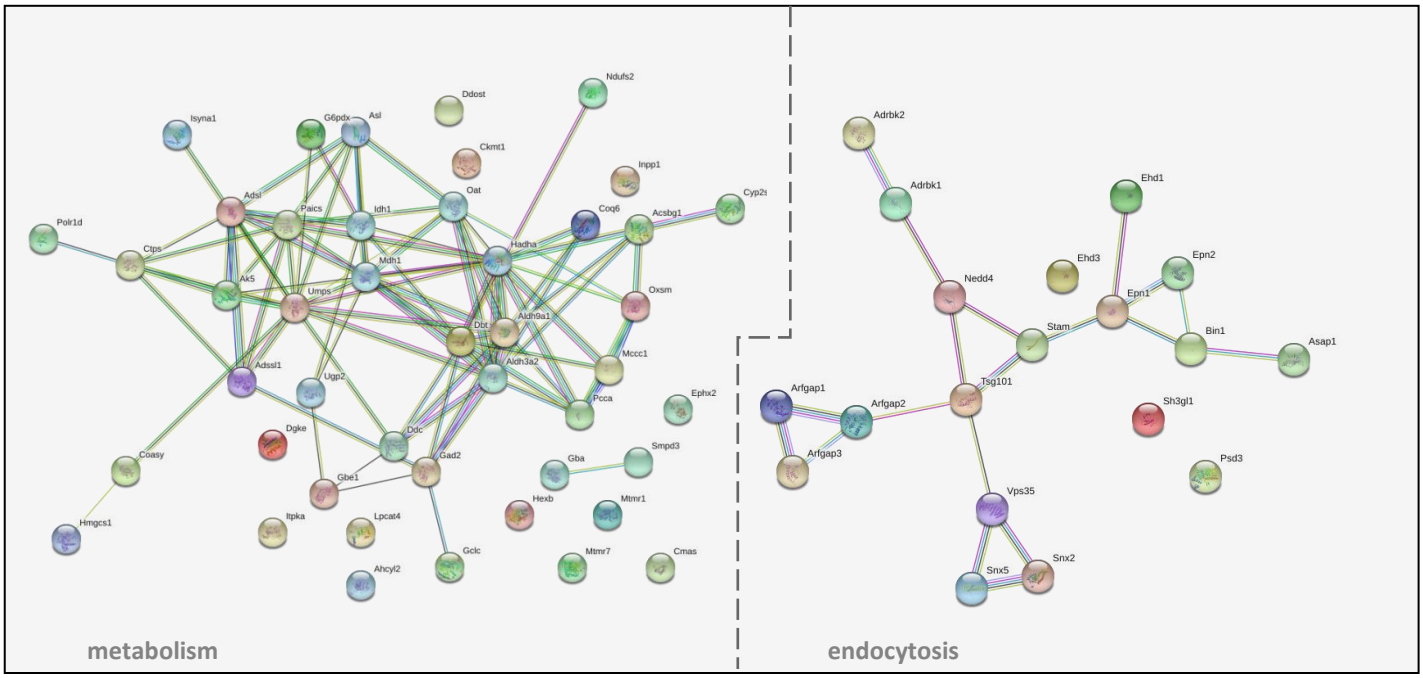


Figure 3.10: Protein-protein interaction networks for the unique proteins identified in MAM-treated males that correspond to metabolism and endocytosis.

4. KEGG pathway enrichment (over the sum of identified proteins)

A subsequent analytical step included a functional investigation by testing for KEGG Pathway enrichment based on the sum of all identified proteins, in a more unbiased approach. This procedure yielded lists of significantly enriched KEGG pathways which provided the basis for a deeper analysis through heatmaps/protein interaction maps (section 5). A table with all significantly enriched pathways (including the total number of proteins:Count, the percentage over the sum of identified proteins:Per.%, P-Value, and Benjamini Value) was constructed (Table 1). A significant protein overlap between multiple pathways/groups (as in, for example, biosynthesis of antibiotics and metabolism) was observed. However, each set of proteins corresponding to a distinct functional group/KEGG pathway may indicate changes/trends (upregulation, downregulation), in large-scale molecular network events, that are biologically relevant. The ensuing inquiry in selected pathways provides with a confined, yet promising, early direction towards characterizing gender differences in the MAM mouse model.

Table 1: KEGG pathway enrichment analysis

KEGG Pathway	Count	Per. %	P-Value	Benjamini Value
Biosynthesis of antibiotics	67	6	6.10E-23	1.60E-20
Carbon metabolism	46	4.1	2.00E-20	2.70E-18
Pyruvate metabolism	21	1.9	1.70E-12	1.50E-10
Citrate cycle (TCA cycle)	19	1.7	2.80E-12	1.80E-10
Glycolysis / Gluconeogenesis	26	2.3	1.40E-11	7.30E-10
Metabolic pathways	165	14.8	9.30E-11	4.00E-09
Biosynthesis of amino acids	26	2.3	8.90E-10	3.30E-08
Endocytosis	54	4.8	2.10E-09	6.80E-08

Dopaminergic synapse	32	2.9	5.70E-08	1.70E-06
Alanine, aspartate and glutamate metabolism	16	1.4	6.20E-08	1.60E-06
Valine, leucine and isoleucine degradation	19	1.7	1.50E-07	3.50E-06
Gap junction	24	2.2	1.90E-07	4.10E-06
Synaptic vesicle cycle	19	1.7	1.10E-06	2.20E-05
Endocrine and other factor-regulated calcium reabsorption	17	1.5	1.80E-06	3.40E-05
Amphetamine addiction	19	1.7	3.80E-06	6.50E-05
Arginine and proline metabolism	16	1.4	4.20E-06	6.80E-05
Inositol phosphate metabolism	19	1.7	7.40E-06	1.10E-04
Protein processing in endoplasmic reticulum	32	2.9	1.00E-05	1.50E-04
Aminoacyl-tRNA biosynthesis	18	1.6	1.30E-05	1.80E-04
Phosphatidylinositol signaling system	22	2	2.40E-05	3.10E-04
Proximal tubule bicarbonate reclamation	10	0.9	2.70E-05	3.30E-04
Salivary secretion	19	1.7	3.00E-05	3.60E-04
Phagosome	31	2.8	5.40E-05	6.10E-04
Fatty acid degradation	14	1.3	9.70E-05	1.00E-03
Glutamatergic synapse	23	2.1	1.10E-04	1.20E-03
Adrenergic signaling in cardiomyocytes	27	2.4	1.50E-04	1.50E-03
Gastric acid secretion	17	1.5	1.60E-04	1.50E-03
beta-Alanine metabolism	11	1	1.90E-04	1.70E-03
Tryptophan metabolism	13	1.2	2.70E-04	2.40E-03
Glyoxylate and dicarboxylate metabolism	10	0.9	3.20E-04	2.80E-03
Oxytocin signaling pathway	27	2.4	3.60E-04	3.00E-03
Histidine metabolism	9	0.8	4.00E-04	3.30E-03
Pentose phosphate pathway	10	0.9	4.30E-04	3.40E-03
Arginine biosynthesis	8	0.7	4.70E-04	3.60E-03
Propanoate metabolism	9	0.8	9.80E-04	7.30E-03
Butanoate metabolism	9	0.8	9.80E-04	7.30E-03
Morphine addiction	18	1.6	1.10E-03	8.10E-03
Thyroid hormone synthesis	15	1.3	1.20E-03	8.60E-03
Calcium signaling pathway	28	2.5	1.20E-03	8.40E-03
Bacterial invasion of epithelial cells	16	1.4	1.30E-03	8.40E-03
Insulin secretion	17	1.5	1.30E-03	8.30E-03
GABAergic synapse	17	1.5	1.50E-03	9.20E-03
ErbB signaling pathway	17	1.5	1.50E-03	9.20E-03
Vasopressin-regulated water reabsorption	11	1	1.90E-03	1.10E-02
Lysine degradation	12	1.1	2.50E-03	1.50E-02
Glucagon signaling pathway	18	1.6	2.50E-03	1.50E-02
2-Oxocarboxylic acid metabolism	7	0.6	3.00E-03	1.70E-02
Long-term depression	13	1.2	3.10E-03	1.70E-02
Fatty acid biosynthesis	6	0.5	3.70E-03	2.00E-02
HIF-1 signaling pathway	18	1.6	3.90E-03	2.10E-02
Insulin signaling pathway	22	2	4.10E-03	2.20E-02

Central carbon metabolism in cancer	13	1.2	4.70E-03	2.40E-02
Circadian entrainment	17	1.5	5.10E-03	2.60E-02
Long-term potentiation	13	1.2	6.10E-03	3.00E-02
Pancreatic secretion	17	1.5	6.20E-03	3.00E-02
Fatty acid metabolism	11	1	6.90E-03	3.30E-02
Fc gamma R-mediated phagocytosis	15	1.3	7.10E-03	3.30E-02
Axon guidance	20	1.8	7.50E-03	3.50E-02
Tight junction	21	1.9	8.10E-03	3.60E-02
Leukocyte transendothelial migration	19	1.7	8.30E-03	3.70E-02
GnRH signaling pathway	15	1.3	1.10E-02	4.60E-02
Cell adhesion molecules (CAMs)	23	2.1	1.10E-02	4.70E-02
Starch and sucrose metabolism	8	0.7	1.20E-02	5.20E-02
Cysteine and methionine metabolism	9	0.8	1.30E-02	5.50E-02
AMPK signaling pathway	19	1.7	1.30E-02	5.40E-02
Cocaine addiction	10	0.9	1.50E-02	6.10E-02
Fructose and mannose metabolism	8	0.7	1.70E-02	6.70E-02
Proteasome	9	0.8	2.60E-02	9.90E-02
Adherens junction	12	1.1	2.90E-02	1.10E-01
Oocyte meiosis	16	1.4	3.10E-02	1.10E-01
Glutathione metabolism	10	0.9	3.10E-02	1.10E-01
Glioma	11	1	3.50E-02	1.20E-01
Proteoglycans in cancer	25	2.2	3.80E-02	1.30E-01
Thyroid hormone signaling pathway	16	1.4	3.80E-02	1.30E-01
Regulation of actin cytoskeleton	26	2.3	3.90E-02	1.30E-01
Aldosterone-regulated sodium reabsorption	8	0.7	3.90E-02	1.30E-01
Galactose metabolism	7	0.6	4.10E-02	1.30E-01
cAMP signaling pathway	24	2.2	4.70E-02	1.50E-01
Salmonella infection	12	1.1	4.80E-02	1.50E-01
Purine metabolism	22	2	5.30E-02	1.70E-01
Bile secretion	11	1	5.90E-02	1.80E-01
cGMP-PKG signaling pathway	21	1.9	6.00E-02	1.80E-01
One carbon pool by folate	5	0.4	6.20E-02	1.90E-01
Choline metabolism in cancer	14	1.3	6.30E-02	1.90E-01
Adipocytokine signaling pathway	11	1	6.30E-02	1.90E-01
Retrograde endocannabinoid signaling	14	1.3	7.10E-02	2.10E-01
Focal adhesion	24	2.2	7.40E-02	2.10E-01
Huntington's disease	23	2.1	7.90E-02	2.20E-01
Aldosterone synthesis and secretion	12	1.1	8.60E-02	2.30E-01
Cardiac muscle contraction	11	1	9.10E-02	2.50E-01
RNA transport	20	1.8	9.30E-02	2.50E-01
Glycerolipid metabolism	9	0.8	9.40E-02	2.50E-01
Estrogen signaling pathway	13	1.2	9.70E-02	2.50E-01

5. Functional analysis (based on KEGG pathway enrichment)

KEGG pathway enrichment enabled further investigation of relative quantitative changes in a functional context. Relative protein abundance as expressed by log2(Fold change) values, in a comparison with saline animals, was visualized for both genders in the form of heatmaps (upregulation is denoted by red color, downregulation by green and lack of identification with grey). A limited number of KEGG pathways which may offer invaluable insight into the schizotypic phenotype of the MAM-treated mice, at the PFC were selected and presented, under the same formatting/analytical principles. The majority of identified pathways that exhibited sufficiently high enrichment values was not explored. The primary emphasis of this section was given to the following KEGG pathway entries: dopaminergic synapse, glutamatergic synapse, GABAergic synapse, calcium signaling, synaptic vesicle cycle, endocytosis and metabolism. Heatmap visualizations (Figure 3.11 up to Figure 3.17) and protein-protein interaction maps (not shown) were constructed as a means to extensively study each functional group.

Dopaminergic synapse

Proteins clustered under “dopaminergic synapse” KEGG pathway (Figure 3.11) exhibit an overall tendency for downregulation (over saline) which is, to a small degree, stronger in males (65% of regulated proteins exhibit downregulation in males/ 63% in females).Interestingly MAOB, an enzyme with an important role in dopamine metabolism, is found to be downregulated in both genders (compared to saline), an observation that may indicate decreased dopamine synthesis in the MAM-treated PFC. MAOA, however is presented as upregulated in females and downregulated in males. In addition TH, another important molecule involved in dopamine synthesis is downregulated in both genders. DDC, a protein with an essential role in the enzymatic synthesis of dopamine, is exhibiting strong differences between genders (downregulation in females, upregulation in males).GSK3B, an enzyme which a much broader role, is strongly upregulated in both genders (GSK3A is found to be downregulated in both genders). The majority of clustered proteins are involved in multiple pathways (MAOA, MAOB, DDC, TH may be considered as more specific in dopamine transmission). Taking into consideration the increased tendency for downregulation in the majority of proteins, associated with dopamine transmission, it is reasonable to assume that the MAM-treated PFC is characterized by decreased dopamine levels. However, this statement is not evident and may be compromised by the observed increased levels of MAOA (in females) and DDC (in males).

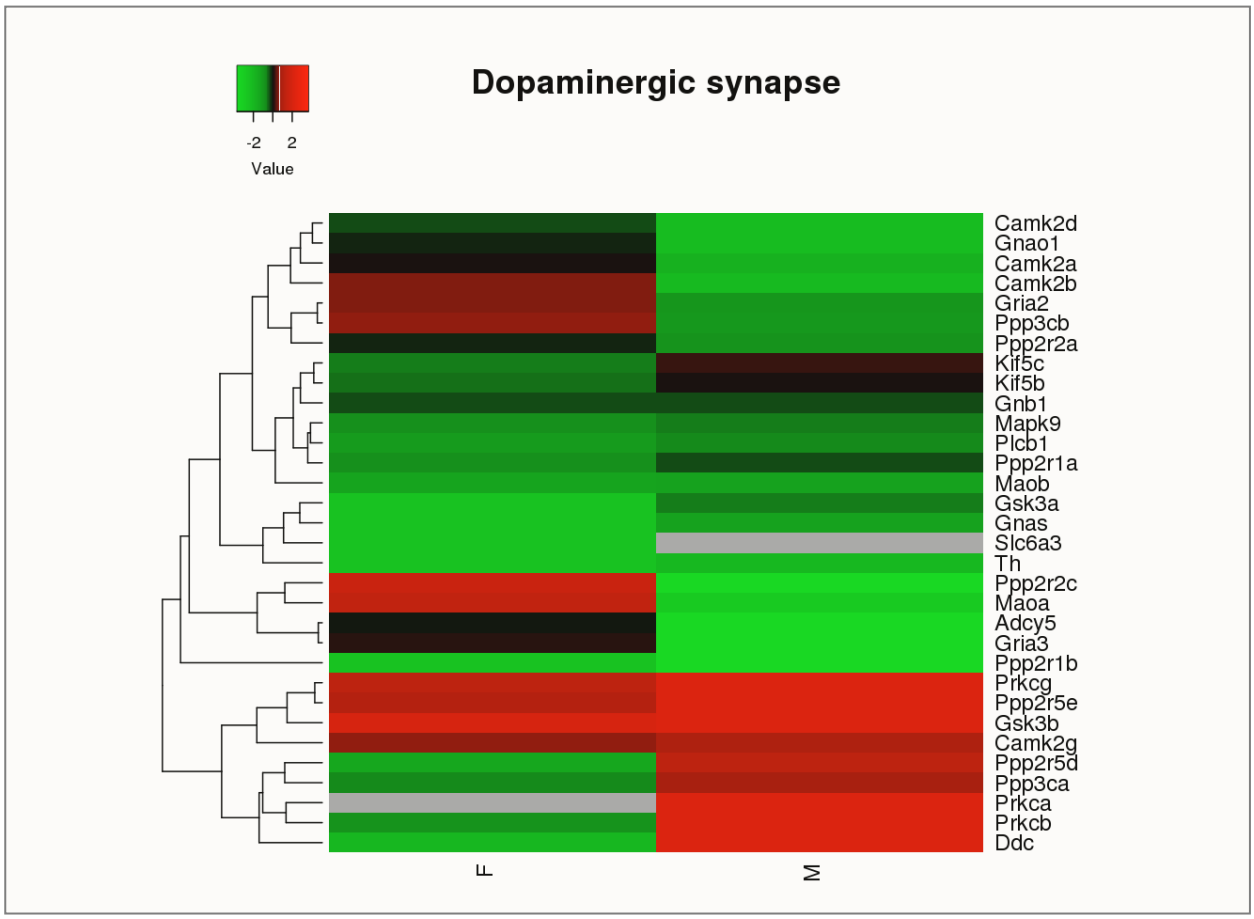


Figure 3.11: Heatmap visualization of identified proteins involved in the "Dopaminergic synapse" KEGG pathway, constructed via log2(Fold change) values, in a comparison of MAM-treated animals with saline for both genders (F:Female & M:Male).

Glutamatergic synapse

A significant number of proteins grouped in the "glutamatergic synapse" KEGG pathway (Figure 3.12) are characterized by downregulation which is stronger for MAM males (61% of regulated proteins in males are downregulated and 53% in females). Glutamate receptors GRM5, GRIA2, GRIA3 are all found downregulated in the male MAM-treated PFC (in females: GRM5 is not found, GRIA2 is upregulated, GRIA3 is upregulated to a small extend). SLC17A6 and SLC17A7, proteins that mediate the uptake of glutamate in synaptic vesicles at the presynaptic terminals, are both found to be downregulated in males and upregulated in females.SLC1A2 and SLC1A3, which are involved in terminating the postsynaptic action of glutamate are both downregulated in the MAM male PFC (in females, SLCA2 is also found to be downregulated while SLCA3 is upregulated). These observations may jointly point towards a more prominent impairment in glutamate transmission in MAM-treated males, compared to females. Paradoxically, GLS, that plays an active role in regulating the levels of glutamate is upregulated in males and downregulated in females. Another interesting finding is the potentially elevated levels of DLG4 in MAM males, that is not identified in females.

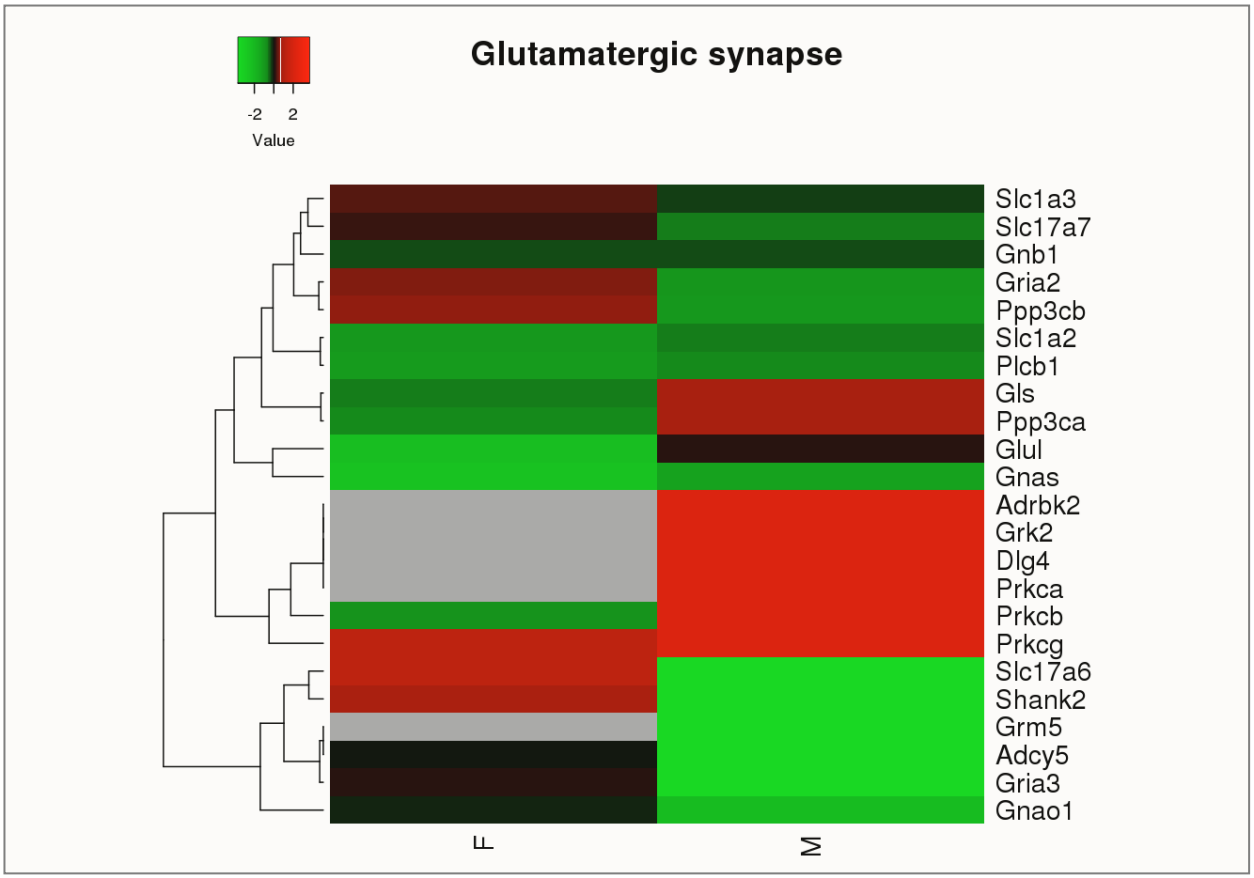


Figure 3.12: Heatmap visualization of identified proteins involved in the "Glutamatergic synapse" KEGG pathway, constructed via log2(Fold change) values, in a comparison of MAM-treated animals with saline for both genders (F:Female & M:Male).

GABAergic synapse

A stronger tendency for upregulation is observed in MAM males (Figure 3.13/ 69% of regulated proteins are upregulated in males, 38% in females). GABBR2, GABRG2, components of a heterodimeric G-protein coupled receptor for GABA are found to be, respectively, non regulated and downregulated in MAM males, However, in MAM females, GABBR2 is observed to be upregulated and GABRG2 is not identified. GAD1 and GAD2, that catalyze the production of GABA, are both found to be upregulated in MAM males and regulated to a smaller degree in MAM females, a result that may indicate more pronounced regulation in the levels of GABA in the MAM-treated male PFC compared to saline and the MAM-treated female PFC. SLC6A1, which terminates the action of GABA is found to be upregulated in the MAM female PFC and non-significantly regulated in the MAM male PFC.

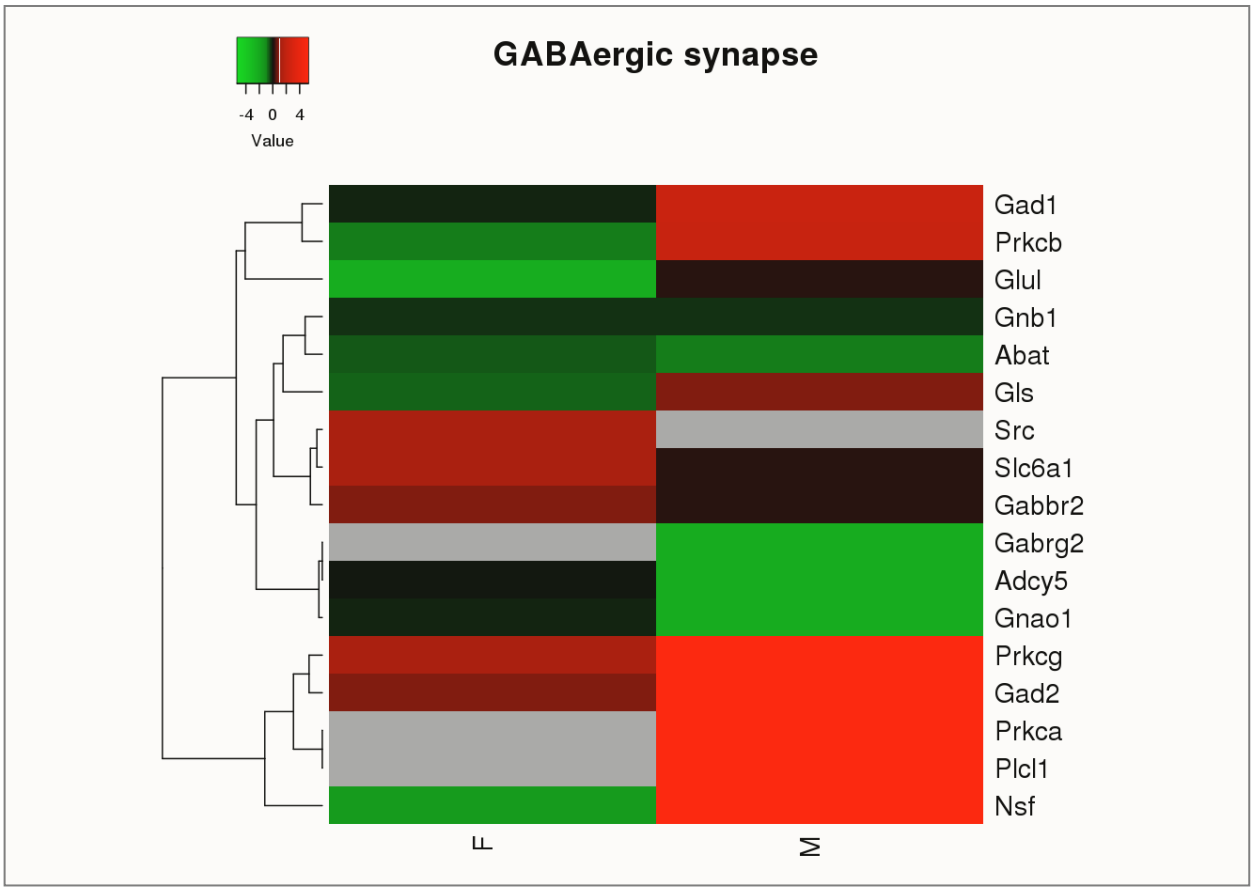


Figure 3.13: Heatmap visualization of identified proteins involved in the "GABAergic synapse" KEGG pathway, constructed via log2(Fold change) values, in a comparison of MAM-treated animals with saline for both genders (F:Female & M:Male).

Calcium signalling

Several Calcium/calmodulin-dependent protein kinases (CAMK2A, CAMK2B, CAMK2D) are found to be highly downregulated in MAM-treated males (Figure 3.14/not as strongly in MAM females, CAMK2B though appears to be upregulated in the MAM treated female PFC). CAMK2G is upregulated in both genders and CAMK4 is downregulated in MAM females and upregulated in MAM males. Moreover, calcium-activated, phospholipid- and diacylglycerol (DAG)-dependent serine/threonine-protein kinases, PRKCA, PRKCB, PRKCG are found to be upregulated in MAM-treated males. PRKCG is also observed to be upregulated in MAM-treated females, whereas PRKCB appears to be downregulated, while PRKCA is not identified. In addition, ATP2B1, ATP2B4, enzymes that catalyze the hydrolysis of ATP coupled with the transport of calcium, are found to be downregulated in both genders (more evidently in MAM males), whereas ATP2B3 appears to be upregulated in both genders (stronger upregulation in males). ATP2B2 is characterized by upregulation in MAM females, while it does not appear to be regulated in males. SLC8A1, that participates in the regulation of cytoplasmic calcium levels, is observed to be downregulated in both genders (to a greater degree in MAM males), a finding that may suggest decreased calcium trafficking in the MAM-treated PFC (possibly more pronounced in males).

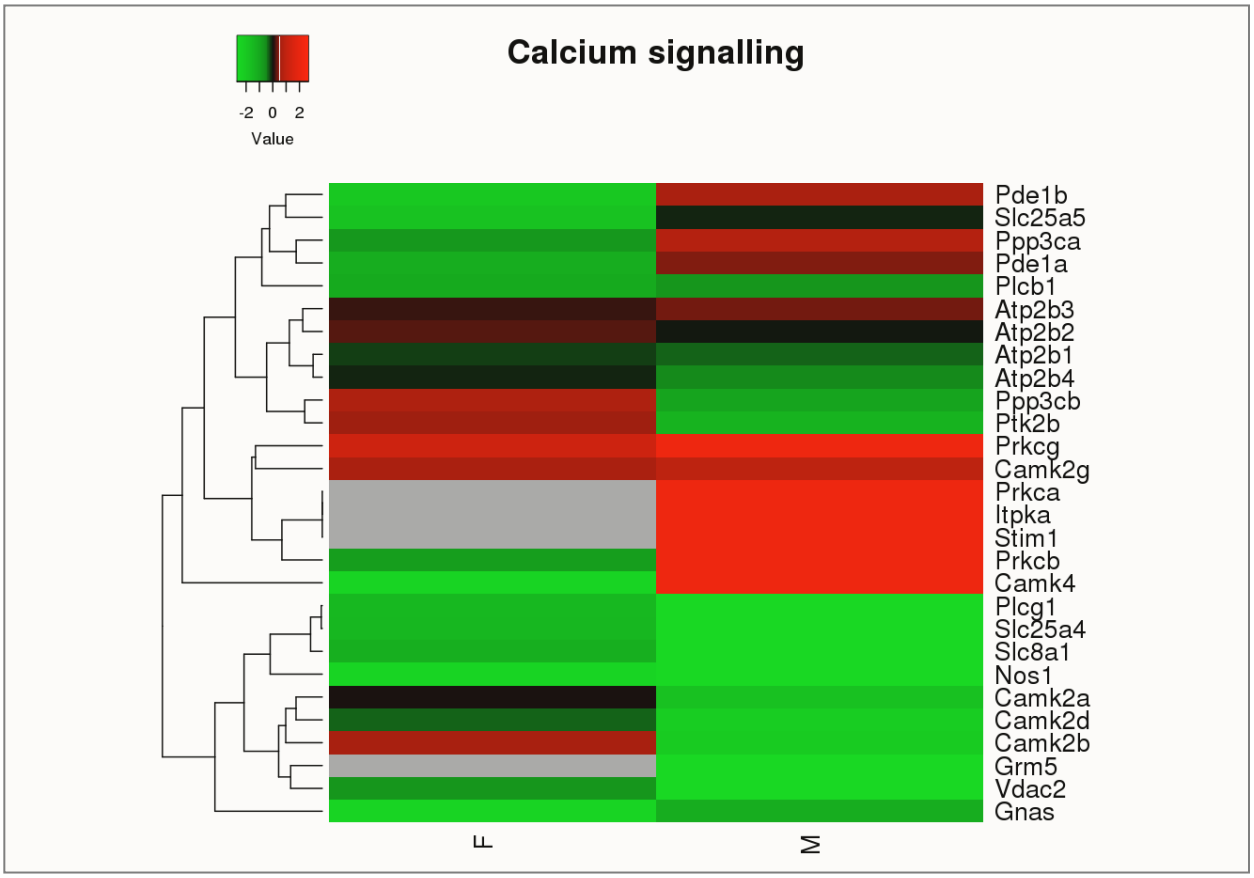


Figure 3.14: Heatmap visualization of identified proteins involved in the "Calcium signalling" KEGG pathway, constructed via log2(Fold change) values, in a comparison of MAM-treated animals with saline for both genders (F:Female & M:Male).

Synaptic vesicle cycle

The majority of proteins grouped under "synaptic vesicle cycle" KEGG pathway appear to be downregulated, in MAM-treated females (Figure 3.15/ no upregulation is observed). Interestingly, some proteins that are involved in vesicular transport (NSF, RAB3A, SYT1, CLTC) are found to be upregulated in MAM males. These findings may indicate increased vesicular trafficking in MAM males, compared to MAM females and saline. In addition, lysosomal ATPases ATP6V1H ATP6V1A, ATP6V1B2 are observed to be upregulated in MAM males. Though, ATP6V0A1 is not regulated and ATP6V0D1 appears to be downregulated in the MAM-treated male PFC.

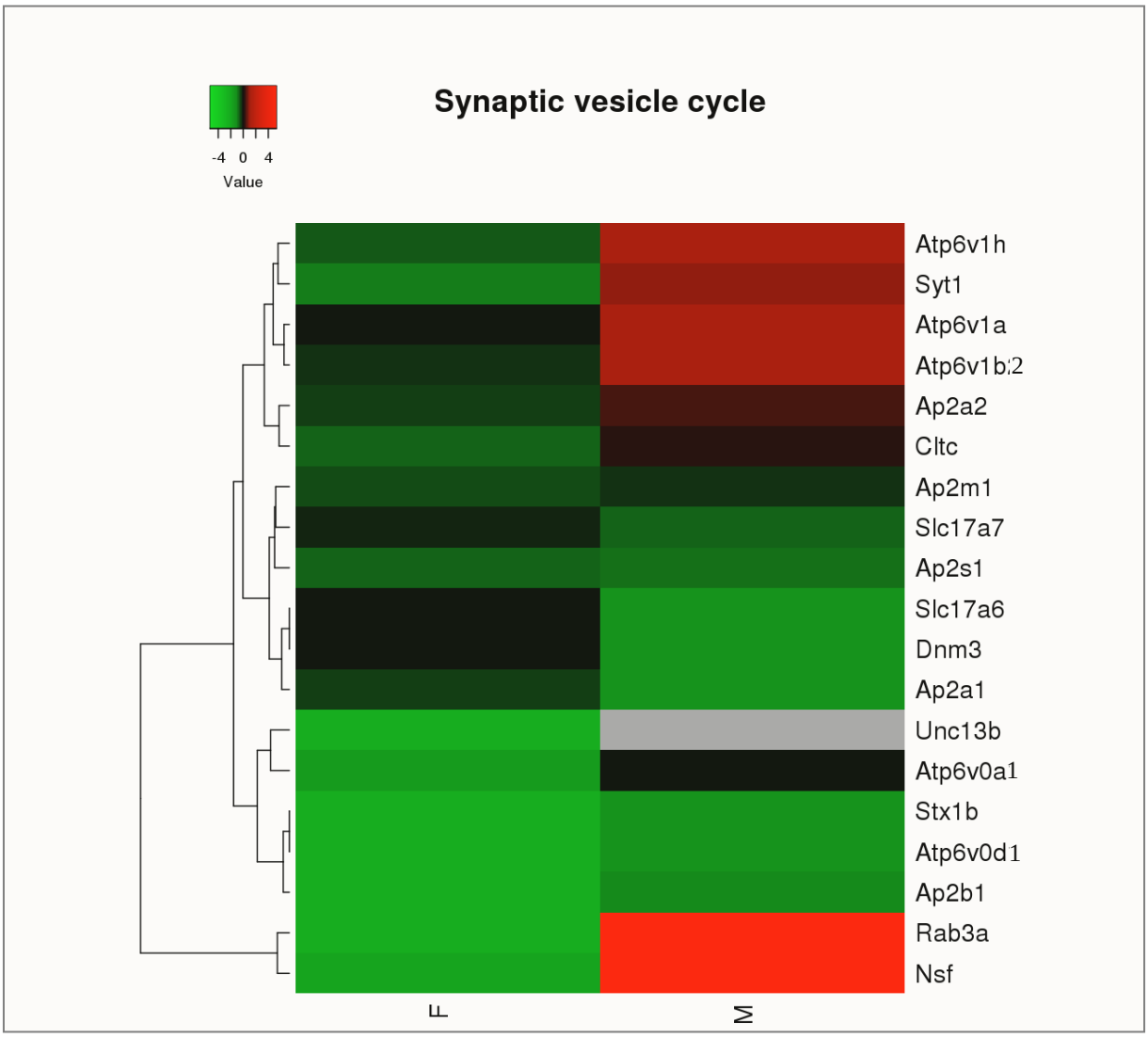


Figure 3.15: Heatmap visualization of identified proteins involved in the "Synaptic vesicle cycle" KEGG pathway, constructed via log2(Fold change) values, in a comparison of MAM-treated animals with saline for both genders (F:Female & M:Male).

Endocytosis

A substantial number of proteins are presented as strongly upregulated in MAM-treated males (Figure 3.16/ 65% of regulated proteins are upregulated in males/41% in females). Multiple molecules involved in intracellular trafficking (SNX1, SNX2, SNX5, SNX6, EEA1, EPN1, EPN2, BIN1) are upregulated in the MAM male PFC, to a higher degree than in the MAM-treated females. These results may point towards increased endocytosis in MAM males, compared to MAM females and saline animals, already suggested by an enrichment in unique proteins participating in endocytosis (section 3).

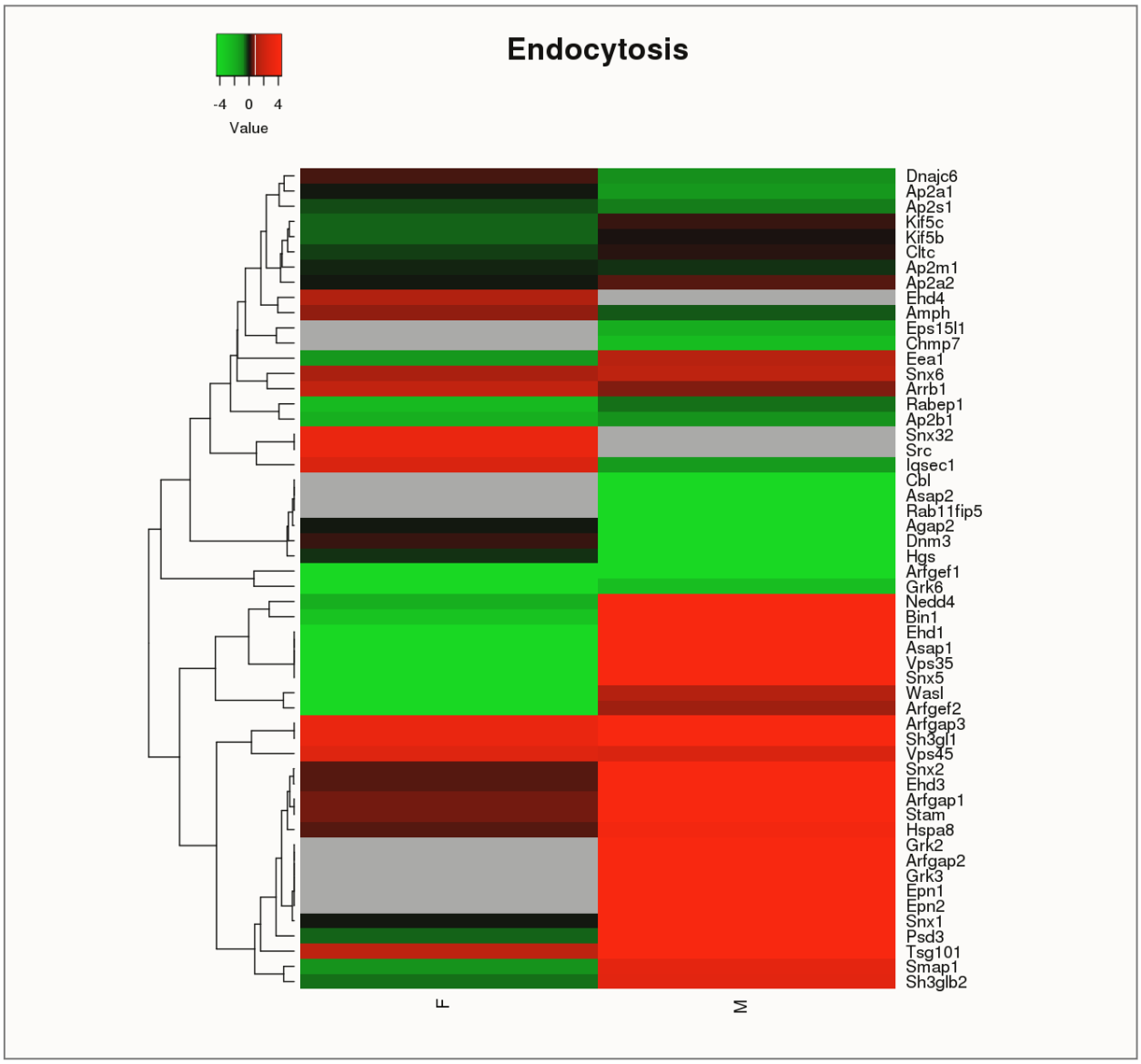
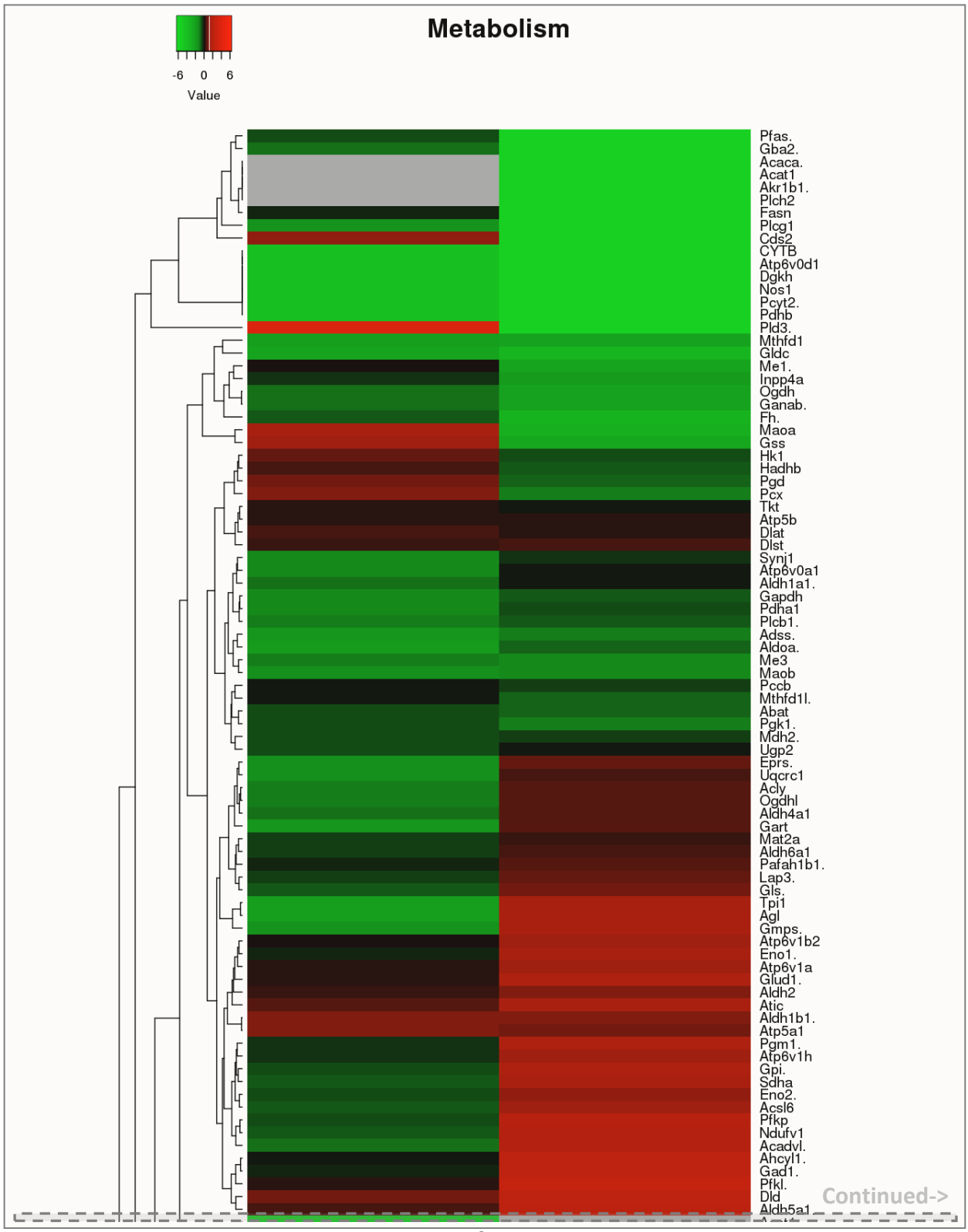


Figure 3.16: Heatmap visualization of identified proteins involved in the "Endocytosis" KEGG pathway, constructed via log2(Fold change) values, in a comparison of MAM-treated animals with saline for both genders (F:Female & M:Male).

Metabolism

A stronger tendency for upregulation in proteins participating in multiple metabolic pathways is evident (Figure 3.17/ 65% of regulated proteins exhibit upregulation in MAM males and 27% in MAM females), an observation that is consistent with an earlier suggestion (section 3: Unique proteins in MAM-treated animals). Given the size and complexity of this KEGG pathway enrichment (the heatmap, below, visualizes the relative abundance of 165 proteins) and the need to identify a potential biological meaning for the observed upregulation in MAM-treated males, further KEGG pathway enrichment analysis was conducted (Table 2 presents the 15 highest ranked KEGG pathways, in terms of percentage coverage). This process revealed that the upregulated proteins in metabolism are involved in multiple metabolic pathways (biosynthesis of antibiotics, carbon metabolism, biosynthesis of amino acids, glycolysis-

gluconeogenesis, valine/leucine and isoleucine degradation, purine metabolism, fatty acid degradation and citrate cycle among other KEGG pathways) and may suggest an overall increase in the metabolic activity of the MAM-treated male PFC (compared to saline and MAM-treated females).



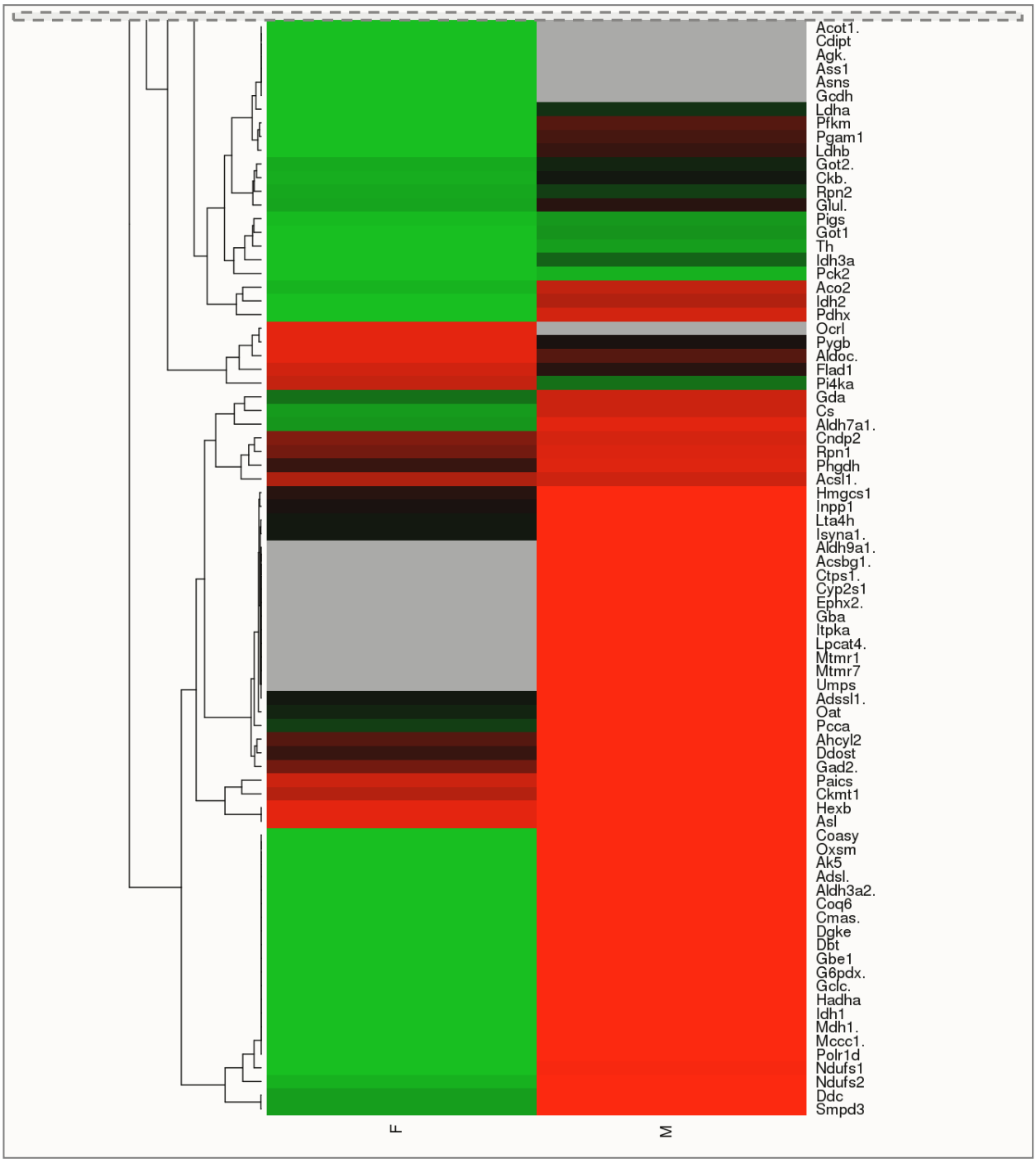


Figure 3.17: Heatmap visualization of identified proteins involved in the "Metabolism" KEGG pathway, constructed via log2(Fold change) values, in a comparison of MAM-treated animals with saline for both genders (F:Female & M:Male).

Table 2: KEGG pathway enrichment analysis on significantly upregulated metabolic proteins (over 2-fold change), in MAM-treated males

KEGG Pathway	Count	Per. %	P-Value	Benjamini Value
Metabolic pathways	79	100	1.60E-62	1.50E-60
Biosynthesis of antibiotics	31	39.2	2.60E-27	1.20E-25
Carbon metabolism	18	22.8	8.6E-16	2.8E-14
Biosynthesis of amino acids	12	15.2	2.4E-10	5.8E-09
Glycolysis / Gluconeogenesis	11	13.9	8.2E-10	1.6E-08
Valine, leucine and isoleucine degradation	9	11.4	6.3E-08	9.9E-07
Purine metabolism	9	11.4	0.00042	0.0021
Fatty acid degradation	8	10.1	5E-07	6.8E-06
Citrate cycle (TCA cycle)	7	8.9	6.4E-07	7.6E-06
beta-Alanine metabolism	7	8.9	7.8E-07	8.2E-06
Alanine, aspartate and glutamate metabolism	7	8.9	1.6E-06	0.000015
Oxidative phosphorylation	7	8.9	0.0027	0.01
Arginine and proline metabolism	6	7.6	0.00012	0.00099
Fatty acid metabolism	6	7.6	0.00015	0.001
Inositol phosphate metabolism	6	7.6	0.00067	0.003

6. Comparison between functional groups

The elementary functional clustering methodology, described in the previous section, that utilized KEGG pathway enrichment analysis, permitted a gross, yet possibly, informative, final comparison between different biological pathways. This minimal procedure, so as to describe the overall tendency of each functional group (significant upregulation/ balance between upregulation and downregulation/significant downregulation), as a whole, was based on percentage change values, between the percentage of upregulation and the percentage of downregulation, over the sum of regulated proteins, for each gender [Percentage change=-(Percentage of downregulation-Percentage of upregulation)/Percentage of upregulation].The results were presented through a heatmap visualization, utilizing the color code of previous figures (green for downregulation/ red for upregulation). Interestingly, the comparison revealed a notable tendency for downregulation (over saline) in almost all examined functional groups in the MAM-treated female PFC (except glutamatergic synapse proteins, where a balance between upregulation and downregulation is observed), which is substantially stronger in proteins of the synaptic vesicle cycle (the majority of the identified molecules is found to be significantly downregulated). The MAM-treated male PFC, however, is characterized by a balance between upregulation/downregulation in proteins of the synaptic vesicle cycle, noticeable downregulation in proteins participating in calcium signaling, dopaminergic synapse and glutamatergic synapse and a tendency for upregulation in molecules involved in endocytosis, GABAergic synapse and metabolism.

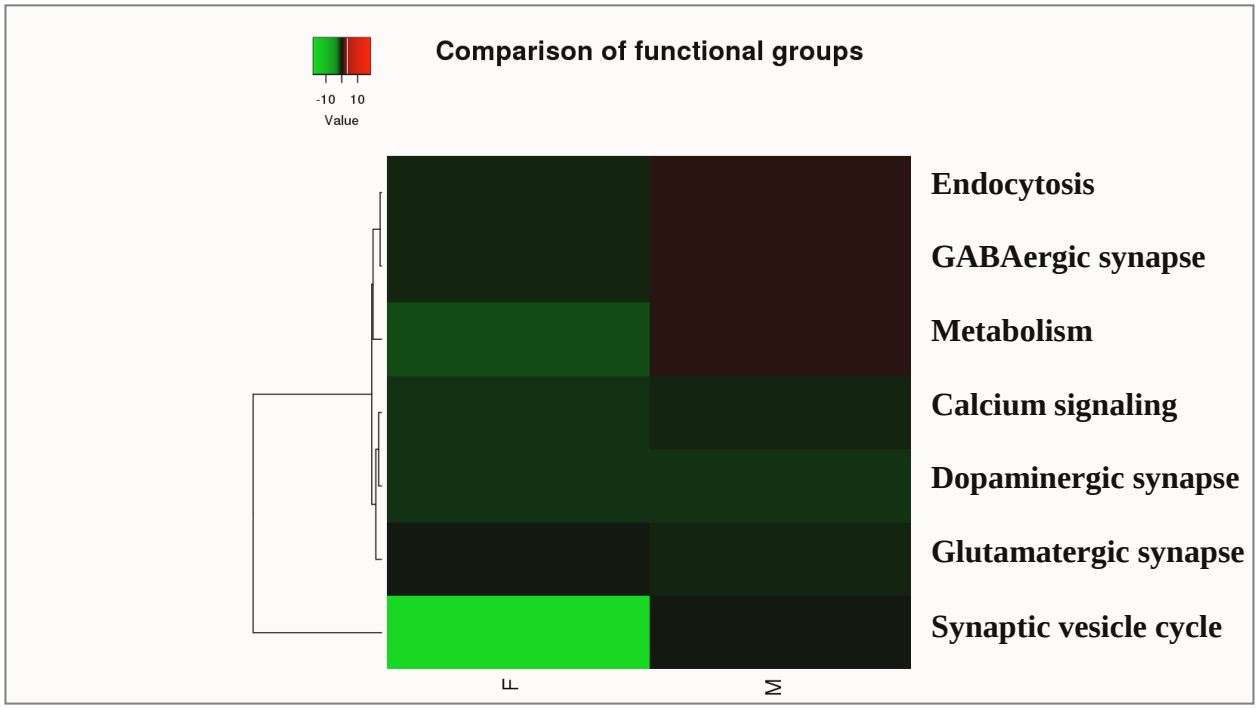


Figure 3.18: Heatmap visualization of a comparison between the examined KEGG pathways, constructed via Percentage change values (based on the Percentage of upregulation and the Percentage of downregulation of each pathway), in a comparison of MAM-treated animals with saline for both genders (F:Female & M:Male).

4. Discussion

The present work comprised a comparative mass spectrometry based proteomic analysis, in an effort to characterize, in molecular terms, "the prenatal MAM" (methylazoxymethanol acetate) mouse model of schizophrenia (induced at embryonic day 16). This early investigation put a primary focus on gender differences, which have been suggested to comprise an important factor in translational neuropsychiatric research[99,100]. Label-free relative protein quantitation, in this regard, enabled the identification of significantly enriched and underrepresented proteins (over saline). A functional component was included to the analytical procedure (by clustering identified proteins under specific enriched KEGG pathway entries), as a means to determine biologically informative pathways.

Overall, the MAM-exposed male PFC is shown to present a greater number of unique proteins, more pronounced quantitative differences and increased "proteomic complexity" compared to the MAM-treated female PFC. Some faint indications for decreased dopaminergic transmission in both genders, impaired calcium and glutamatergic signaling as well as more intense regulation of GABAergic pathways in MAM-treated males, have been highlighted. In addition, multiple results have suggested increased vesicular trafficking and endocytosis in the MAM-treated male PFC (compared to MAM females and saline animals). Furthermore, MAM males exhibited prominent upregulation in numerous metabolic pathways, a finding that suggests increased metabolic activity in the MAM-treated male PFC.

Based on unpublished observations by Dr. Sidiropoulou and colleagues (manuscript in preparation) the MAM-exposed males have exhibited decreased performance in behavioral studies (delayed alternation task in a T-maze) compared to MAM-treated females, a finding that has suggested a pronounced impairment in the male PFC function. This result was further reinforced through recordings of field excitatory postsynaptic potentials (fEPSPs/ layer II of the PFC), at induced long-term potentiation (LTP), via tetanic stimulation, where decreased fEPSP was observed at the MAM-treated male PFC (in contrast to more intense fEPSP recordings for both saline and MAM females), an indication of defective LTP in MAM males. Moreover, the fEPSP peak was found to be reduced in male MAM-treaded mice, at multiple intensities of current stimulation. These results, cooperatively, point towards PFC function deficits in MAM-exposed males.

The proposed increased metabolic activity in MAM-treated males, at a proteomic level, constitutes the most notable statement of this study and may further complement the behavioral and electrophysiological indications for non-canonical PFC function. A significant body of research work has linked hypermetabolism with brain damage in humans and other species[108-110]. Oxidative stress and increased metabolic rates have also been associated with the onset and progression of synaptic pathology in Alzheimer's disease[111]. Additionally, substantial correlations between metabolic stress, neuro-inflammation and cognitive impairments have been established[112]. Indications for increased metabolic rates in the frontal cortex of unmedicated SCZ patients, by a functional neuroimaging study using FDG-PET (positron emission tomography scanning with fluorodeoxyglucose) may, also, validate a case for links between hypermetabolism and PFC functional perturbations[113].

Interestingly, research in another mouse model of psychosis ("acute ketamine") has presented hypermetabolism as a hallmark of hippocampal dysfunction (concurrent atrophy and pathology of parvalbumin-expression interneurons was highlighted)[51]. In addition, proteomic analysis of the "acute PCP" model of SCZ, at the hippocampus, has revealed a marked increase in transketolase, an enzyme that connects the pentose phosphate cycle to glycolysis, while pronounced abnormalities in energy metabolism have also been described in the "chronic PCP"[85,90]. Extensive analysis of the prefrontal cortex of SCZ patients, finally, has provided evidence for increased utilization and depletion of glycogen (upregulation of glycogen phosphorylase and glucose-6-phosphate transporter-1), a finding that implies increased glycose demand and highly altered energy metabolism in SCZ (associations with mitochondrial dysfunction, oxidative stress and possible hypoxia within the PFC have, also been made)[114].

In conclusion, this research effort has featured an attempt to investigate gender differences in the "prenatal MAM" mouse model, a previously unexplored direction. Considerable alterations in the relative abundance of a plethora of identified proteins (compared to saline) was observed in both sexes. A substantial number of the upregulated proteins, in MAM-exposed males, was shown to be involved in metabolic processes, a finding that may correspond to aberrant functioning of the prefrontal cortex and may evidence, an already suggested by other experiments, more intense "schizotypal" phenotype in MAM-treated males, compared to females. These results, though, ought to be further examined, in a more detailed study.

5. Appendix

1. Ingredients of utilized buffers/solutions

Table 1: Lysis Buffer	
HEPES	50 mM (Stock concentration: 0,5 M)
NaCl	150mM (Stock concentration: 1,5 M)
Glycerol	1%
Triton X-100	1%
MgCl2	1,5 mM (Stock concentration: 15 mM)
EGTA	5 mM (Stock concentration: 0,5 mM)
Cocktail of inhibitors	1:1000
*HEPES 0,5 M and NaCl 1,5 M are jointly prepared at one Stock solution . *ddH_2O is added to final volume. *Store Stock solutions at 4$_o$C and Lysis buffer at -20$_o$C.	

Table 2: Sample Buffer 2X	
Tris 2M/pH:6.8	0,625 ml
SDS 10%	4 ml
DTT 1M (or b-mercaptoethanol)	0,4 ml
Glycerol	1 ml
Bromophenol blue	~2 ng (at the least possible quantity)
ddH_2O	added to 10 ml
*Stored at -20$_o$C.	

Table 3: 30% Acrylamide Stock Solution	
Acrylamide	29,2 gr
N_2N-Methylene bis-acrylamide	0,8 gr
ddH_2O	added to 100 ml
*The solution is thoroughly mixed for more than 30 minutes at dark conditions and then filtered by passing through a syringe with a 0,45 filter. Stored at 4$_o$C.	

Table 4 (A): Separating Gel Buffer	
Tris 1,5 M	18,161 gr
SDS	0,4 gr (0,4% w/v)
ddH_2O	added to 100 ml
*The solutions' pH value is adjusted at 8.8 with HCl. Stored at 4$_o$C.	

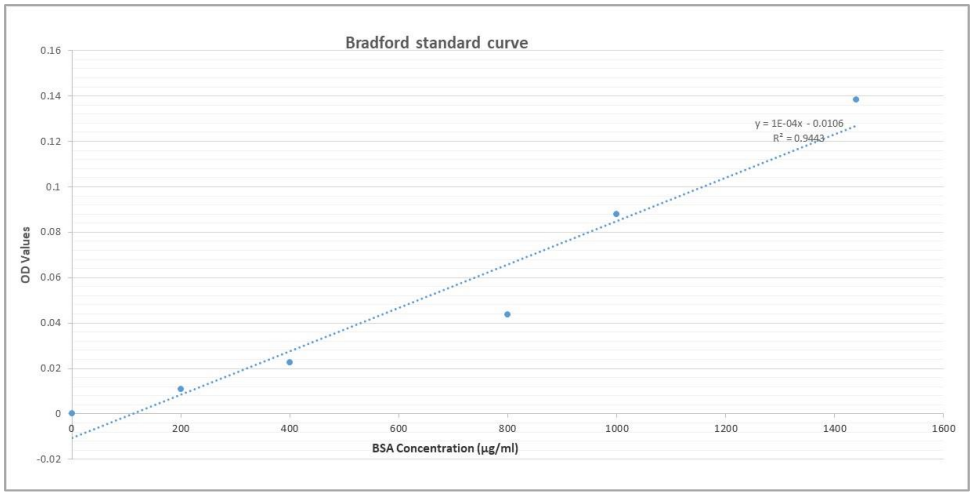
Table 4 (B): Stacking Gel Buffer	
Tris 0,5 M	6,05 gr
SDS	0,4 gr (0,4% w/v)
ddH_2O	added to 100 ml
*The solutions' pH value is adjusted at 6.8 with HCl. Stored at 4$_o$C.	

Table 4 (C): Gel Recipes			
Stacking Gel 4%	**10 ml**	**5 ml**	**3 ml**
ddH_2O	6,1 ml	3,05 ml	1,83 ml
30% Acrylamide	1,33 ml	0,665 ml	0,399 ml
Stacking Buffer	2,5 ml	1,25 ml	0,75 ml
10% SDS	100 µl	50 µl	30 µl
10% APS	100 µl	50 µl	30 µl
TEMED	10 µl	5 µl	3 µl
Separating Gel 10%	**12 ml**	**6 ml**	
ddH_2O	4,9 ml	2,45 ml	
30% Acrylamide	3,94 ml	1,97 ml	
Stacking Buffer	3 ml	1,5 ml	
10% SDS	120 µl	60 µl	
10% APS	60 µl	30 µl	
TEMED	6 µl	3 µl	

Table 5: 10X Running Buffer	
Tris	30,3 gr
Glycine	144,2 gr
SDS	10 gr
* The solutions' pH value is adjusted at 8.3 with HCl. Stored at 4$_o$C.	

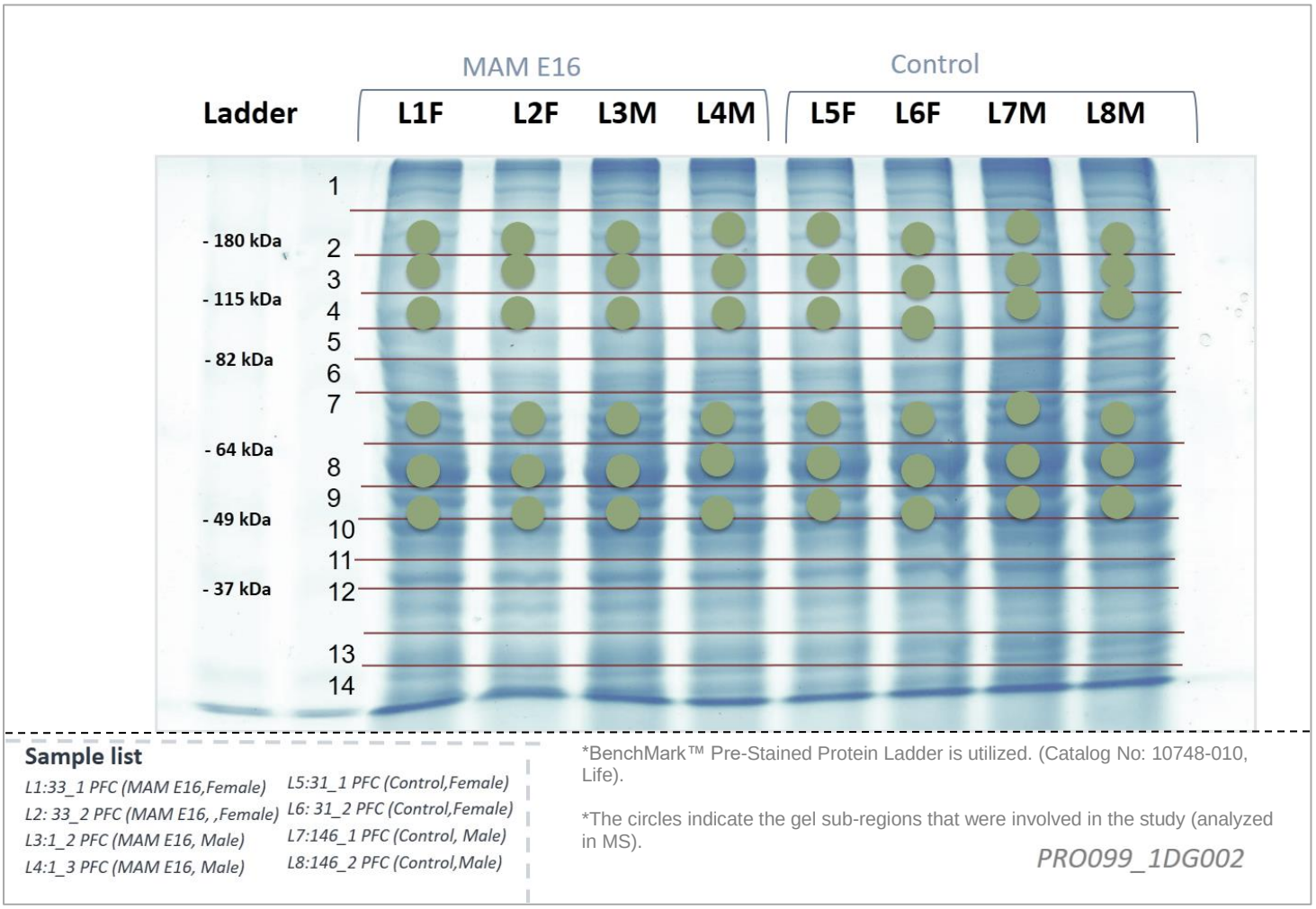
Table 6: 10X Transfer Buffer	
Tris	30,3 gr
Glycine	144,2 gr
* The solutions' pH value is adjusted at 8.3 with HCl. Stored at 4$_o$C.	

2. Bradford measurements



Sample	Volume loaded for 40 µg of protein (in µl)
33_1 PFC (MAM E16,Female)	6,1 µl
33_2 PFC (MAM E16, ,Female)	7,3 µl
1_2 PFC (MAM E16, Male)	6,4 µl
1_3 PFC (MAM E16, Male)	6,0 µl
31_1 PFC (Control,Female)	7,5 µl
31_2 PFC (Control,Female)	5,8 µl
146_1 PFC (Control, Male)	5,8 µl
146_2 PFC (Control,Male)	6,2 µl

3. Coomassie staining/Sample Set-up



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