Differential protein expression of Hippocampal cells associated with Heavy metals (Pb, As, and MeHg) Neurotoxicity: Deepening into the Molecular Mechanism of Neurodegenerative Diseases

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Abstract: Chronic exposure to heavy metals such as Pb, As, and MeHg can be associated with an increased risk of developing neurodegenerative diseases. Our in vitro bioassays results showed the potency of heavy metals in the order of Pb<As<MeHg on hippocampal cells. The main objective of this study was combining in vitro label free proteomics and systems biology approach for elucidating patterns of biological response, discovering underlying mechanisms of Pb, As, and MeHg toxicity in hippocampal cells. The omics data was refined by using different filters and normalization and multilevel analysis tools were employed to explore the data visualization. The functional and pathway visualization was performed by using Gene ontology and Pathvisio tools. Using these all integrated approaches, we identified significant proteins across treatments within the mitochondrial dysfunction, oxidative stress, ubiquitin proteome dysfunction, and mRNA splicing related to neurodegenerative diseases. The systems biology analysis revealed significant alterations in proteins implicated in Parkinson's disease (PD) and Alzheimer's disease (AD). The current proteomics analysis of three metals support the insight into the proteins involved in neurodegeneration and the altered proteins can be useful for metal-specific biomarkers of exposure and its adverse effects.

Key words: Metal exposure; Proteomics; Pathway visualization; Neurodegenerative diseases.

Abbreviation: AD= Alzheimer's disease, APP= Amyloid precursor protein, Aβ =Amyloid-beta, CAM= Calmodulin, ETC= Electron transport chain, , GAD= glutamate decarboxylase, Glu= Glutamate, GO= Gene ontology, LFQ= Label free quantification, LTP= Long term potentiation, MAPK= Mitogen-activated protein kinase, MAO-A= Monoamine oxidase A, NMDA= N-methyl D-aspartate, OD= Optical density, ORA= over-representation analysis, PD= Parkinson's disease, PS= Phosphatidyl serine, PI= Propidium iodide, PKC= protein kinase-C, ROS= Reactive oxygen species ,UPS= Ubiquitin proteasome System

1. Introduction

Many environmental pollutants have been associated with human diseases [1]. Among various pollutants, heavy metals contribute to a great proportion of air, soil and water pollution and cause major health problems to human beings [2-4]. An increased worldwide industrialisation [5] has led to higher levels of pollution by potent neurotoxins such as lead (Pb), arsenic (As), and methyl mercury (MeHg) [6]. Both environmental and occupational exposures to any of the three metals are of significant toxicological concern [7, 8]. Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide spreading in the environment [9, 10]. Heavy metal exposure can occur through contaminated air, food, water, and/or hazardous occupations [11-14]. The toxicity of heavy metals depends on several factors including dosage, route of exposure and chemical species, as well as on the age, gender, genetics, and nutritional status of exposed individuals [15-17]. Human exposure to three heavy metals (Pb, As, and MeHg) can disrupt brain function [18, 19], and increase the risk of diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [20-22].

These three heavy metals (Pb, As, and MeHg) are well known potent toxicants, and humans are exposed to each one of them via different routes [23, 24]. Firstly, the main routes of exposure for Pb are inhalation and ingestion [25, 26]. Inhalation exposure to Pb is a much more efficient route of absorption than ingestion [27, 28]. Pb metal is relatively common in the environment, and its toxicity impacts the children health [29]. Pb exposure in the workplace is responsible for a wide range of adverse effects, mainly on the brain [30]. Secondly, humans are exposed to various forms of As mainly via oral consumption of contaminated water, food or drugs [31, 32]. As metal can also enter the body via inhalation, which is particularly important for certain types of occupational exposure [33-35]. Also As metal is rapidly absorbed, distributed and stored in different body organs such as liver, kidney, and lung. Moreover, it can easily cross the blood brain barrier (BBB) and accumulate in the brain [36, 37]. Reported studies state that As metal has been directly linked to neurodegenerative diseases [38]. These findings raised concern over As induced neurotoxicity in humans [39-41]. However, the underlying molecular mechanisms not clear. Finally, MeHg contamination is possible through consumption of fish and other seafood [42, 43]. In humans, MeHg accumulates in kidneys and neurological tissues [44]. It is well known that ingested MeHg can interact with proteins due to its strong affinity to sulphur (-SH) containing functional groups, and cause organ dysfunction in the central nervous system [45, 46].

In the past few years, many studies have been conducted to understand the mechanisms underlying Pb, As and MeHg toxicity on the hippocampus region of the brain [47-49] Exposure to Pb and MeHg have significant effects on the human brain [50, 51]. Many reported evidence linked As exposure with developmental neurotoxicity [52]. Recently, Karri et al. reported that Pb, As, and MeHg exposure induce damage to the hippocampus region of the brain [53]. However, the risk level depends on exposure intensity and metal nature in the brain [39, 54]. Basha et al. observed that developmental exposure to Pb exhibits latent effects [20], through the epigenetic interaction of Pb with amyloid precursor protein (APP) gene causing neurodegeneration at an older age. As can disrupt the cognitive function of the brain in children [52]. MeHg directly disrupts the mitochondrial function by generating an uncontrolled release of Ca^{+2} [55], resulting in the dysregulation of the mitochondrial electron transport chain (ETC) [56] and causes the cell death. MeHg effect on mitochondria could be a potential cause of neurodegenerative diseases [57].

Most of the reported studies focused on the effects of metals on the brain in a generalized manner [46, 52, 58]. In the past few years, substantial improvements in toxicogenomics knowledge have led to an increase in the application of proteomics and systems biology knowledge to answer these mechanistic biological questions [59, 60]. To assess the biological effects of heavy metals, we are conducting studies engaging a systems toxicology approach that combines evaluation of classical toxicological endpoints with extensive molecular measurements and pathway analysis approaches [61-64]. The main goal of this project was taking advantage of high throughput proteomic technologies and systems biology tools for assessing the neurotoxicity mechanism of Pb, As, and MeHg on the hippocampal cells. We choose the mouse hippocampal HT-22 cell line due to the known sensitivity of chronic Pb, As, and MeHg

exposure and relevant to the disease [65]. Here, a label-free quantitative proteomics approach [66] was used to detect the effects of Pb, As, and MeHg exposure at the protein level by using a non-cytotoxic dose (IC_{10}) of each metal to avoid secondary cytotoxic responses. The differential protein expression patterns involved in the heavy metal toxic response were quantified to deeply reveal the integrative molecular network of protein response to heavy metal stress. The proteomics data were integrated with a systems biology approach [67], in which the analysis tools Pathvisio, GO-elite, and Cytoscape were used to provide a more global view of the molecular] changes elicited by heavy metals in hippocampal cells [68, 69].

2. Materials and Methods

2.1. Chemicals

Lead chloride ($PbCl_2$ [CAS no: 7758-95-4]), Sodium metaarsenite ($NaAsO_2$ [CAS no: 7784-46-5]), Methyl mercury chloride ($MeHgCl_2$ [CAS no: 115-09-3]), Dimethyl sulphoxide (DMSO [D5879]), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT [M5655]), trypsin (TrypLE [Gibco: 12604013]), and proteomics reagents: Urea (GE HealthCare, Life Sciences, CAS Number: 57-13-6), Sodium Dodecyl Sulphate (SDS) (Merck, CAS Number: 151-21-3), Ammonium Hydroxide (Fluka, CAS Number: 1066-33-7), Dithiothreitol (GE HealthCare, Life Sciences, CAS Number: 3483-12-3), Iodoacetamide (GE HealthCare, Life Sciences, CAS Number: 144-48-9), Formic Acid (Merck, CAS Number: 64-18-6), Acetonitrile (HPLC grade) (Fisher Chemical, CAS Number: 75-05-8), and Water (HPLC grade) (Fisher Chemical, CAS Number: 7732-18-5) are analytical grade and purchased from Sigma-Aldrich Química, S.L-Madrid (Spain).

2.2. Cell line and Reagents

The HT-22 cells have been used as a hippocampal neuronal cell model in numerous studies [70]. The HT-22 cells were a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). HT-22 cells were maintained in Dulbecco's modified eagle's medium (DMEM [D6429]) containing 10% fetal bovine serum (FBS Gibco [10500-064]), 100 U/mL penicillin, and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% CO_2 in air at 37^0 C. For all the experiments cells were grown at 70-80% confluence. The cells were cultured in 75 cm^2 cell culture flasks. For experimental purpose, cells were plated at 0.56 x 10^6 cells/mL and grown for 24 hours (hrs.) before the metal treatment. Duplicates wells of cells were treated with 10 exposure levels of Pb, As, and MeHg ranging from 10 to 100 µM, 0.4 to 4.2 µM, and 0.6 to 12 µM, respectively; due to the 8 days exposure-period, medium containing the given concentration was refreshed at 2 days interval in order to maintain the metal exposure along time. Metal stock solutions 100X was prepared in deionized distilled water (for poorly soluble $PbCl_2$ < 0.5% DMSO was added) and sterilized by filtration through 0.2 µm and different concentrations of a working solution for each individual metal were prepared by prior dilution of the stock solution in phosphate buffered saline (pH = 7.4) and then applying 10% working solution on DMEM culture medium.

2.3. Cytotoxicity

The MTT assay was carried out using a modification of the method of Mossman [71]. The HT-22 cells were seeded in 96-well plates. After 24 hrs, when the cells had reached a confluence of 70–80%, they were exposed for 8 days to various concentrations of Pb, As, and MeHg. After the incubation period, the medium was aspirated and MTT working solution of 0.5 mg/mL was added to each well. The absorbance of the solubilized reduced MTT was then measured in a microtiter plate spectrophotometer reader at a wavelength of 570 nm. The measured optical density (OD) values were converted to percent of cell viability (%) with respect to control. The cell viability results were used for calculating the sub-lethal dose (IC_{10}) of each metal for apoptosis and proteomics studies.

2.4. Analysis of apoptosis by annexin V-FITC/ Propidium iodide (PI)

To evaluate the translocation of phosphatidylserine (PS) from inner leaflets to outer leaflets of the plasma membrane, annexin V-FITC apoptosis detection kit (BD Pharmingen, Poland) was utilized. In this kit, annexin V and propidium iodide (PI) were used to distinguish the apoptotic and necrotic cells from the viable cells. According to the manufacture's protocol, the exponentially proliferating cells were exposed to the IC_{10} to IC_{30} of heavy metals in culture plate at a density of 0.56 x 10^6/mL during 8 days; control cells were made without chemical. Cells were harvested by trypsinization, washed twice with ice cold PBS (pH = 7.4). Thereafter, cells were centrifuged at 1200 rpm for 5 min at 4^0C, resuspended in 1mL 1X binding buffer and then 100 µL of the solution was transferred to a 5 mL culture tube, and 5 µL of both annexin-V and PI was added to the samples. After staining, cells were incubated for 15 minutes in the dark place at room temperature. Finally, cells were re-washed with 400 µL 1X binding buffer and analyzed by flow cytometry (Beckman coulter, Germany). Three independent experiments were performed.

2.5. Protein extraction and digestion

For the proteomics study, the exponentially proliferating HT-22 cells were exposed to the selected sub-lethal dose (IC_{10}) of each heavy metal at a density of 0.025 x 10^6/mL during 8 days; control cells were made without chemical exposure. Pb, As, and MeHg treatment (and control culture) protein was extracted from HT-22 cells with 200 µL lysis buffer (8M urea, 0.1% SDS, 50 mM ammonium bicarbonate [ABC]) and quantified using the Micro BCA™ Protein Assay Kit (Thermo Scientific). 30 µg of protein from each sample was digested in a filter assisted sample preparation (FASP) approach. The volume corresponding to 30 µg of protein was adjusted to 270 µL with 8M Urea/50 mM ABC. The reduction was done with 20 mM dithiothreitol (DTT) treatment for 1h 30 min at 32^O C. Samples were then alkylated using 30 mM of iodoacetamide (IAA) and incubated in the dark for 30 min at room temperature. Afterwards, samples were loaded in 10 kDa filters (Amicon) and centrifuged for 30 min, 12,000g at room temperature followed by two washes with 300 µL 1M urea/50 mM ABC by centrifugation (30 min, 12,000g, room temperature) in order to remove interferents and establish optimal pH (7.5-8.5) and urea concentration for proteins to be denatured. The filter remaining material was resuspended in 400 µL 1 M urea/50 mM ABC. Digestion was done in two steps: an initial digestion with 1:30 (w/w) porcine trypsin 0.25 µg/µL (Sequence grade modified trypsin, Promega) for 3 hrs at 32^OC followed by a digestion with 1:50 (w/w) trypsin 0.25 µg/µL for 16h at 32^OC. Peptides were eluted by centrifugation (12,000g for 15 min at room temperature) and the filters were cleaned with 200 µL 50 mM ABC by centrifugation at 12,000g for 15 min at room temperature. Samples were prepared for chromatographic analysis. Briefly, peptides resuspended in 100 µL of 1% formic acid (FA) were charged in the tip columns (previously washed with 70% acetonitrile [ACN] in 0.1 % FA and equilibrated with 0.1% FA) by centrifugation (300 × g for 1 min 30 sec). Columns were washed twice with 100 µL 0.1% FA by centrifugation (300g for 1 min) and then peptides were eluted in 2 x 100 µL of 70% ACN / 0.1% FA by centrifugation (300g for 1 min). The peptides were dried in speed vacuum (Eppendorf) and stored at 20 ^{O}C until LC-MS analysis.

2.6. LC-MS/MS analysis

The peptide mixtures were resuspended (1% FA, 2% ACN) and the volume corresponding to 500 ng of protein was analysed by an LTQ-Orbitrap velos mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were injected into the capillary column (75 µm× 25 cm) in full loop mode and separated by a 5 µm C18 column using a nano-acquity liquid chromatography system (Waters). Peptides were eluted with a linear gradient of 1-35 % buffer B (0.1% FA, 100% ACN) for 150 min, followed by 35-45 % buffer A for 20 min (0.1% A). The mass spectrometer was operated in positive ion mode (source voltage 2000V) and data-dependent manner. The full MS scans were performed in the Orbitrap at the range of 300–1,700 m/z at a resolution of 60,000. For MS/MS scans, the 15 most abundant ions with multiple charge states were selected for collision induced dissociation (CID) fragmentation following one MS full scan.

2.7. Label-free quantitative (LFQ) data analysis using Progenesis QI software

Proteomics quantitative data analysis was done by using Progenesis QI data analysis software v4 (Non-Linear Dynamics, Waters, U.S.). Ion feature matching was achieved by aligning consistent ion m/z and retention times. Peptide abundances were calculated as the area under the MS peak for every matched ion feature. Progenesis label

free quantification (LFQ) was done using non-conflicting unique peptides and protein grouping, and the software normalization algorithm was applied to all proteins. Mascot search results were filtered based on peptide ion score≥ 40 and contaminants were removed and abundances were normalized to all proteins. A total of 428,801 spectra were obtained from the LFQ LC−MS/MS proteomic analysis of samples including a control and test samples. After data filtering to eliminate low-scoring spectra 27,088 peptides were retained and used for protein identification. Total 3,140 proteins were quantified from 27,088 peptide ions. Only the proteins quantified with minimal 2 peptides were included in the further statistical analysis.

2.8. Protein identification

Proteins identification was done using Mascot search engine (v. 2.3.01). All MS/MS spectra passing an arbitrary cut-off in the Progenesis software were included in the list used to perform the search against SwissProt Mouse database using the Mascot search program (Matrix Science, London, UK, www.matrixscience.com). The following criteria were applied: (1). trypsin as an enzyme; (2). allowed two missed cleavages; (3). variable modifications, acetyl (N-terminus, protein) and oxidation (M); Carbamidomethyl (C) fixed modification and (4). Peptide tolerance, ±10 ppm (MS); MS/MS tolerance, ± 0.6 Da.

2.9. Statistical analysis

Label free quantitative data was submitted to T-test method ($p \leq 0.05$) using Microsoft excels in order to find out significant differences among the treatments and control. Data were further curated applying the following filters: minimum 1.2-fold change and p-value< 0.05. Meaboanalyst 3.0 (http://www.metaboanalyst.ca) programme was used for presenting the unsupervised data in the principal component analysis (PCA), volcano plot, and hierarchical clustering analysis.

2.10. Systems biology analysis

In our systems biology analysis three main steps were included for the quantitative functional interpretation of proteomics data. The first step was aimed to identify the statistically significant proteins by using the T-test method. The next step was to identify significantly altered functional Gene Ontology (GO) categories using GO analysis. The altered GO terms and the differentially expressed proteins in these GO terms were visualized in a network to identify the links between the terms. In the final step, the changes in protein expression were visualized in selected pathways related to neurodegenerative diseases.

2.10.1. Functional interpretation of significantly altered genes using GO analysis

GO-Elite (http://www.genmapp.org/go_elite/), a flexible and powerful tool for GO functional analysis [72] was used to find altered biological processes, molecular functions, and cellular components based on changes in proteins expression. GO-Elite performs an over-representation analysis (ORA) on any structured ontology annotations, or biological identifiers (e.g. gene, protein or metabolite) [73]. The program utilizes the structured nature of biological ontologies to report a minimal set of non-overlapping terms called as pruned terms. Pruning is the process of intelligently examining the hierarchical structure of ontology, such as GO, and comparing the ORA scores of enriched terms based on their relationships to each other. Highly related terms highlighted by ORA are considered to be redundant with each other. Hence, pruning is used to select the highest scoring term among a set of related terms, while retaining any distinct related terms. GO-Elite ranks each analyzed pruned GO term according to a Z-score along with a p-value [74]. Significantly altered categories were identified based on the p value <0.05, Z-score > 1.96, and a minimum of 3 proteins changed within each specific GO term. The results were visualized as networks linking the GO terms and differentially expressed proteins in the GO terms using the network analysis tool Cytoscape [75]. In addition, on the GO-protein network in Cytoscape, the log2 fold change (log2FC) between each metal exposure group and control were visualized.

2.10.2. Pathway visualization

Pathvisio [76] is a commonly used pathway editor, visualization, and analysis software for omics based experiments. First, the required curated mus musculus pathway collection was obtained from Wikipathways (http://www.wikipathways.org) [77]. Pathway analysis was performed in Pathvisio version 3.2.4 (http://www.pathvisio.org) to interpret and visualize the molecular changes on a pathway level. In the present study, we visualized the changes in proteins after metal exposure in biological pathways relevant to neurodegeneration processes in which a minimum of 3 proteins was changed. The log2FC and p-value were shown on pathways using the visualization module in Path Visio. A flow chart of experimental label free proteomics and systems biology is shown in the supplementary file 1 (Figure 1.1)

3. Results and Discussion

To characterize the proteomics changes of Pb, As, and MeHg individually on mouse hippocampal HT-22 cells during 8 days of exposure, firstly we performed cell viability studies by using the MTT assay. The cell confluence (80-85%) was stable until 8 days exposure; consequently, we considered the 8 days exposure as chronic for further mechanistic studies. As expected, all three metals showed concentration and time-dependent cytotoxic effect. Results and discussion for Pb, As and MeHg cytotoxicity experiments at chronic conditions were reported by Karri et al [65]. Upon the Pb exposure (10-100µM)) cell death was significantly lower indicating that sensitivity of hippocampal cells towards Pb is relatively low. Previous studies reported that As showed cytotoxicity even at a micromolar concentration [78], and similar results were observed in HT-22 cells treated during 8 days. However, MeHg (IC_{50} =0.6µM) showed high potency in hippocampal cells (HT-22) compared to Pb (IC_{50}= 74.3 µM) and As (IC_{50} =0.8 µM) The obtained MeHg results were consistent with previous studies in cerebellar granule cells [79].

The obtained 8 days exposure MTT results were useful for further mechanistic based assays such as apoptosis to know the chronic risk of heavy metals on HT-22 cells. For that purpose, in a previous study, cells were treated with a range of IC_{10} to IC_{30} concentrations (µM), and results were reported in Karri et al. [65]. This report confirmed that Pb induced apoptosis process was low in HT-22 cells and agreement with reported studies in (Figure 1) [80]. The other two metals, As and MeHg, showed significant effects even at low concentration of exposure [81, 82]. The results of the present study show that non-cytotoxic (IC_{10}) apoptosis mechanism also similar to cell viability in the potency of metal. The selecting sub lethal (non-cytotoxic) concentration effects suggest that for the three metals, the necrotic process was not initiated under mild stress conditions. The MTT and apoptosis results indicate that the toxicity potency of metals on hippocampal cell follows: MeHg>As>Pb. Figure 2 shows the apoptosis effect of Pb, As, and MeHg onHT-22 cells after 8 days of exposure.

The findings from the MTT and apoptosis assay are interesting for extending further to mechanistic experiments to find the underlying molecular mechanisms. The high throughput label free omics analysis could be helpful for understanding the complete overview of metal induced stress in hippocampal cells and also validate the potency of metals in HT-22 cells by analysing the protein expression. Here, we selected the IC_{10} /sub lethal concentration of Pb, As, and MeHg (63.50 µM, 0.09 µM, and 0.42 µM) on HT-22 cells for omics studies.

3.1. Identification and quantification of proteins in HT-22 cells by using the label free proteomic analysis

In order to identify new molecular pathways related to heavy metal neurotoxicity, we have used a proteomics label-free quantitative approach with sub lethal (IC_{10}) concentration in 8 days exposure. The quantitative analysis was performed by Progenesis QI software as explained in the methods section 2.7. In total 3,140 proteins were quantified from 27,088 peptide ions. From them, 2161 proteins were identified with a minimum of 2 peptides. Only the proteins with at least two unique identified peptides were retained for further quantification analysis. The unsupervised PCA was performed by using the normalized proteins (≥2 unique peptide) that were present in all four (n=4) replicates (Figure 2A). The first principal component (PC1: 55.1%) describes the largest variation in the dataset in which the samples spread the most in the variable space. The second component (PC2: 13.2%) describes the next largest variation and is orthogonal to the first component. The third component describes the last largest variation (PC3: 6.3%). Figure 2A highlights clustering or pattern formations in a three-dimensional space which provides a view of the similarities and dissimilarities among the samples. For each individual metal, we extracted

the proteins showing significant changes in abundance when compared to control cells (547, 1249 and 1156 proteins for Pb, As, and MeHg, respectively). The resulting proteins of the three metal treatments were compared in a venn diagram and shown in Figure 2B. We found that 373 proteins commonly altered due to metal exposure. Interestingly, some proteins were exclusively altered due to the exposure to each metal: 70 for Pb, 244 for As and 193 for MeHg. The experimental groups (control, Pb, As and MeHg) were clustered based on the log standard abundance of the unsupervised data with the ward's hierarchical clustering algorithm. The clusters clearly separated according to the expression level in each replicate as shown in Figure 2C, and the spot maps of MeHg are distinguished the most from the other spot maps. Differences were also found between the controls, MeHg and As; Pb and Control were very close in the cluster. We can notice the clear presence of a group of proteins that are systematically upregulated (red) and down regulated (green) in treatment Vs control. Volcano plot presents the protein expression response to Pb, As and MeHg, respectively (Figure 3). The replicates for the four study groups were averaged and ratios for each metal treatment group to control were calculated. To ease visualization of proteome alterations, ratios (or fold change; FC) were log(2) transformed and plotted against –log(10) transformed ANOVA p-values (Figure 4, A-C) The presented pink dots (log(2)FC ≥0.26 or ≤0.26 and p-value≤ 0.05) were considered as differentially expressed among each metal treated and control comparison. A total of 547, 1249 and 1156 proteins were found to respond to Pb, As, and MeHg exposure respectively. Among them, 347, 563, 632 proteins were significantly down regulated, while 200, 686, 524 proteins were significantly upregulated by Pb, As, and MeHg respectively.

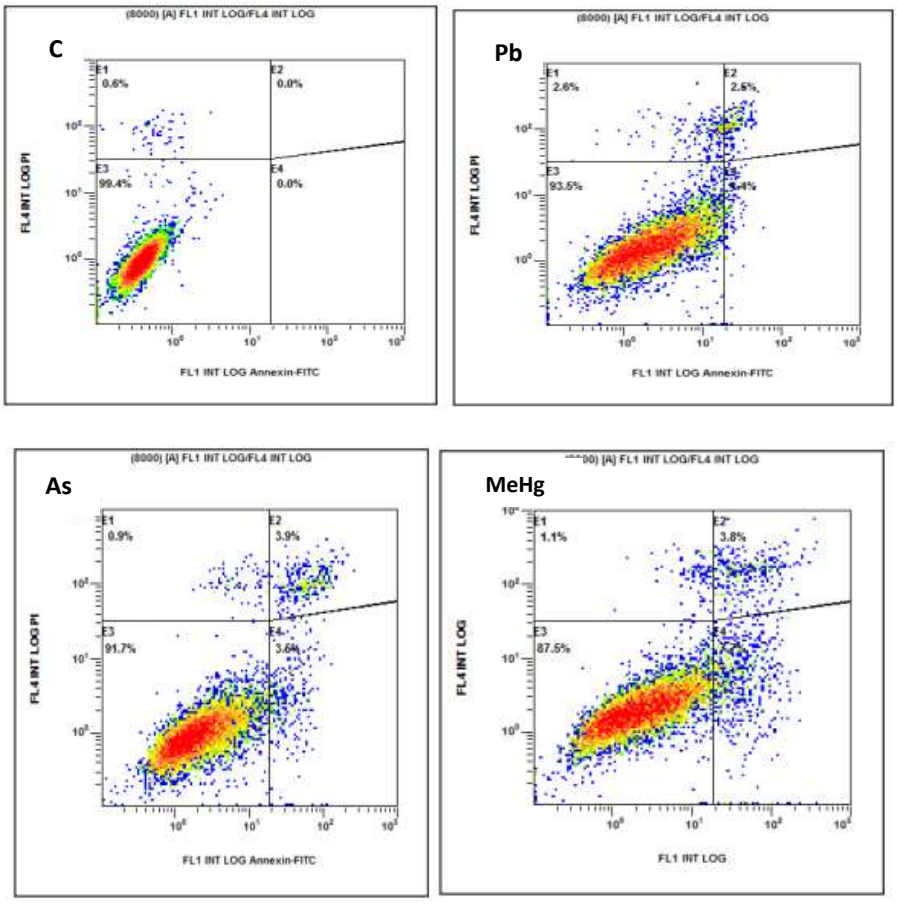

Figure 1: Representative dot plots showing the apoptosis effect of Pb, As, and MeHg to HT-22 cells upon 8 days of exposure at sub lethal experimental concentration (n=3). C= Control, Pb= 63.50 µM, As= 0.42 µM, MeHg= 0.09

µM. Lower left (LL)/ E3 = Live cells (Annexin V−/ PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+/ PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

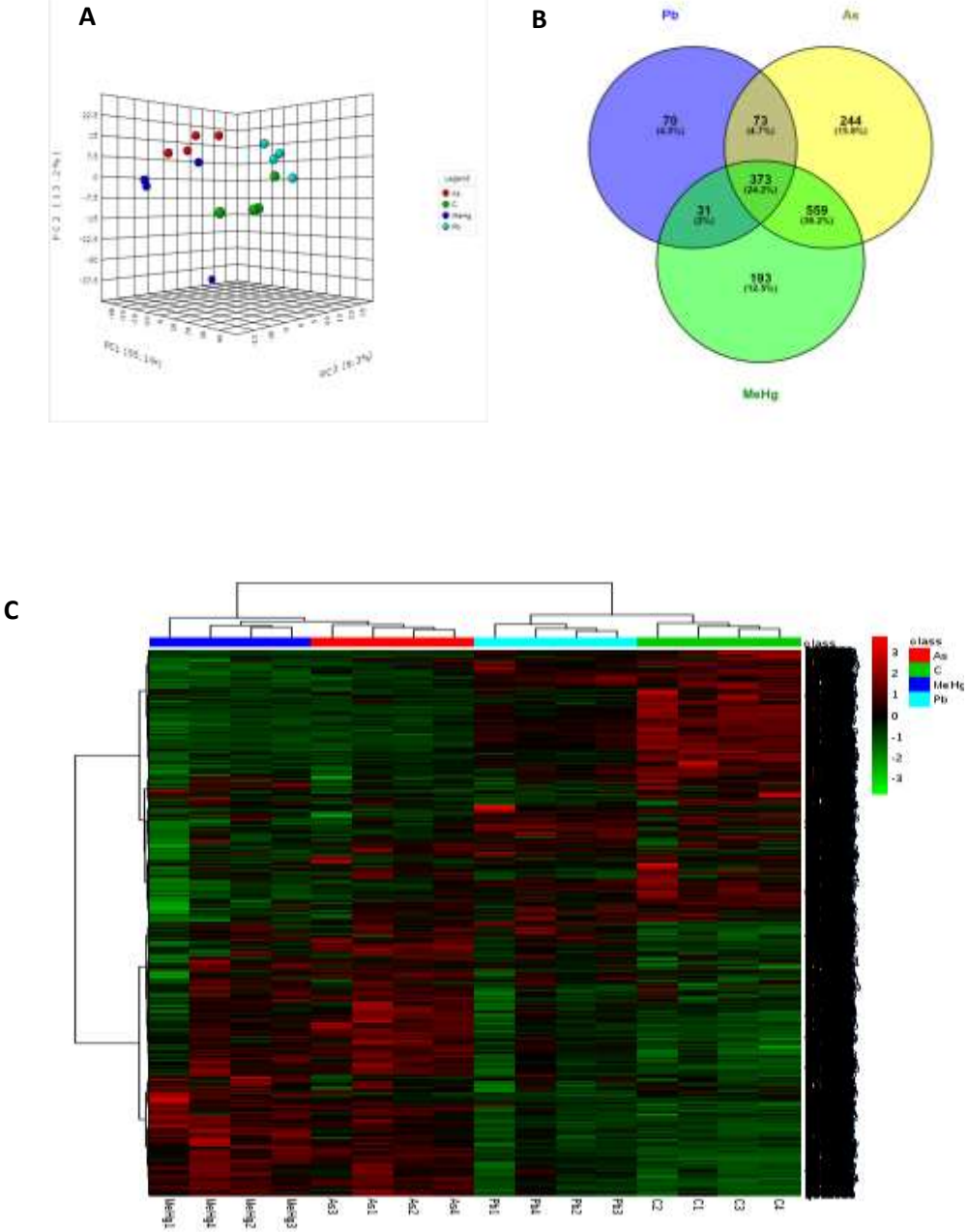

Figure 2: A. Diagram presenting the principle component analysis (PCA) score plot (PC1, PC2, and PC3) of the unsupervised proteomic profiles of HT-22 cell **B.** Venn diagram showing the overlap between significantly changed ($p < 0.05$) proteins in Pb, As, and MeHg metals. **C.** Heat map for hierarchical Euclidean clustering of normalized protein expression of the experimental groups (control, Pb, As, and MeHg).

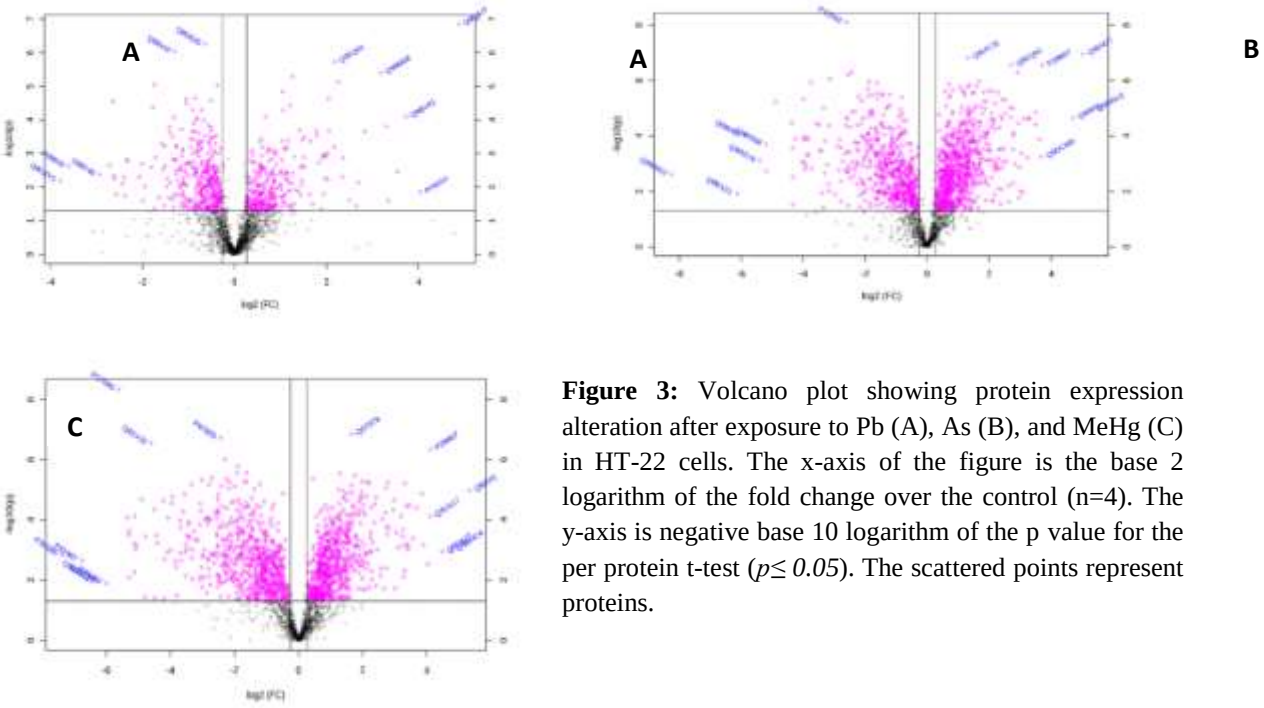

Figure 3: Volcano plot showing protein expression alteration after exposure to Pb (A), As (B), and MeHg (C) in HT-22 cells. The x-axis of the figure is the base 2 logarithm of the fold change over the control (n=4). The y-axis is negative base 10 logarithm of the p value for the per protein t-test ($p \leq 0.05$). The scattered points represent proteins.

3.2. Functional classification and enrichment analysis of protein data by using GO –Elite

The functional interpretation and the pathway analysis of protein expression are always challenging. The application of GO mapping and pathway analysis with omics data to interpret the complex protein expression profiles in the context of the molecular pathways [83]. This study demonstrated that GO analysis by using GO–Elite method for the functional interpretation of proteomics data. Our GO results contain multiple files, in that over-representation analysis (ORA) pruned list are most informative to understand the GO terms and proteins functional relation (supplementary file 1), which includes all summary term statistics and associated gene symbols. The ability to determine in which GO terms differentially expressed proteins are overrepresented provides an ideal model to gain an understanding of what GO classes are altered after metal exposure. The pruned ORA list contains the GO terms; i) are pruned to only include non-redundant information and ii) are significantly altered based on changes in protein expression. The GO-Elite results represent a global picture of biological processes, cellular components, and molecular functions that are significantly altered at the protein level after treatment of Pb, As and MeHg. Relationships between all regulated proteins and pruned terms can also be easily visualized as networks in cytoscape using the pruned sif file. We showed global networks (supplementary file 2) of the all pruned GO terms with protein expression, which gives an overview of all the significantly expressed proteins and which can be helpful to determine the heavy metal's impact on HT-22 cells proteome. For instance, by observing the GO terms and protein expression relation, we found that Pb metal impact was very low while As and MeHg showed a greater impact on HT-22 cells. A major challenge in the GO interpretation of omics data is the functional interpretation and the linking of potentially altered proteins to most relevant disease processes. However, from the overview of the network file, it was difficult to relate the results to specific neurodegeneration processes.

Using the GO hierarchical classification system, we identified enriched processes within the collection of genes identified as significantly altered by Pb, As, and MeHg exposure. Generally, ORA GO terms are crucial to evaluate the significant GO processes with respect to metal exposure. We observed that the obtained GO processes were different for each metal exposure; although few of them were linked to neurodegenerative diseases. Table 1 shows the different GO processes for Pb. As and MeHg related to neurotoxicity in HT-22 cells. Regarding Pb, proteins related to cysteine-type endopeptidase inhibitor activity involved in the regulation of caspase related apoptosis are downregulated after Pb exposure, which indicates that the apoptosis process was initiated by Pb in HT-22 cells.

Some studies suggested the importance of the JAK/STAT pathway for the survival of neurons [84]. This pathway has a pro-apoptotic nature in cerebellar granule neurons [85]. Thus, the Pb exposure in HT-22 cells initiated the cell death through apoptosis. In relation to As metal (Table 1), oxidative stress and transition metal ion homeostasis GO processes are critically acclaimed to metal toxicity. The regulation of oxidative stress is indicated by the upregulation of antioxidant proteins, super oxide dismutase 1 (SOD1), reduced glutathione (GSH) and the neuronal part GO term are indicated by the amyloid precursor protein (APP), and parkin7 (PARK7) protein expression that is critical for the neurodegenerative diseases. Most enriched and relevant to neurotoxicity in MeHg are the GO terms: pyruvate metabolic process, spliceosomal and transition metal ion homeostasis. In AD, the process of β-amyloid (Aβ) misfolding and plaque aggregation is greatly influenced by alterations in the transition metal ion homeostasis [86]. Transition metal ions can influence the APP metabolism [87]. We found in relation to the transition metal ion homeostasis GO term, that APP protein expression is differently altered by MeHg (down regulated) than by As (up regulated), which indicates that heavy metal exposure controls the APP expression but in opposite ways best supporting the underlying mechanism of the AD by As.

Our data demonstrate that GO analysis constitutes a powerful unbiased approach to define the biological pathways altered in mice HT-22 hippocampal cells in response to heavy metals exposure. The GO processes shown in Table 3 for the three metals (Pb, As, and MeHg) suggests that the involved proteins are sensitive indicators of metal induced response in association with neurotoxicity. GO based omics data analysis can provide profoundly important and novel information in toxicity mechanism. However, we are currently facing the difficult challenge of how quantitatively to interpret the differentially expressed proteins. As presented in Table 3, the conventional GO process terms do not provide any quantitative information about current data on metal-induced neurotoxicity. Thus, we integrated the selected pruned GO processes in a Cytoscape framework to evaluate functional relations between the proteins, as illustrated in Figures 5, 6, and 7 for Pb, As, and MeHg, respectively. This analysis can useful to visualize how each protein with its significant change in expression connects to the neurotoxicity GO terms. In particular, we were able to show the effects of the metal on the protein expression profiles in HT-22 cells as presented by Pb, As, and MeHg networks. We quantitatively assessed the absolute magnitude of change in expression levels associated with significant proteins within enriched GO terms at IC_{10}. It was observed that MeHg caused the most prominent changes in protein abundance than other two metals. So that, here we will mainly focus on the MeHg induced damage in HT-22 cells which is critical to evaluate the relationship between the protein expression and AD and PD; the other two metals (Pb and As) are discussed in a comparative manner.

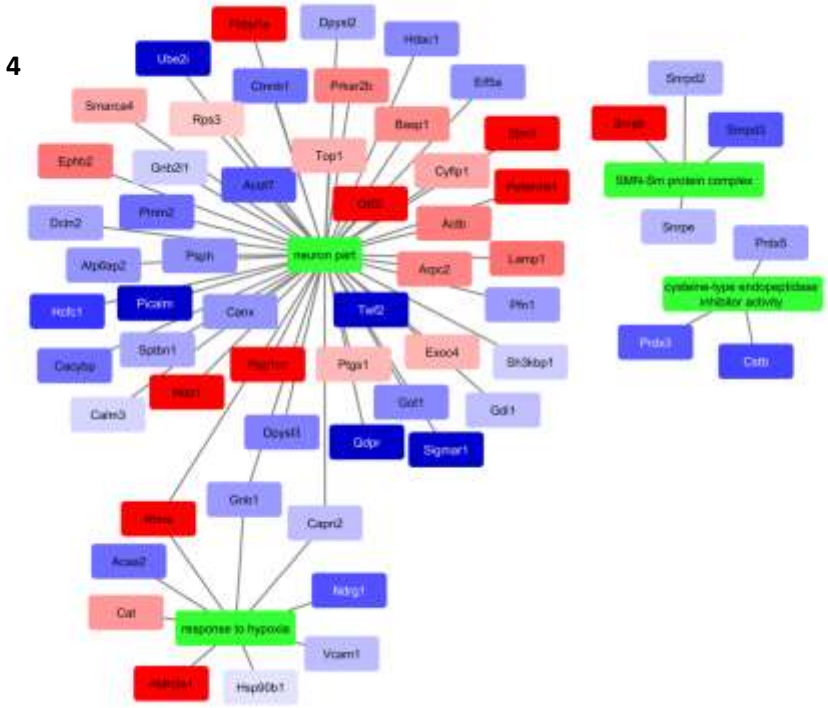

Figure 4: Interaction of differentially expressed proteins and selected GO classes after Pb exposure. The network is based on the selection of the pruned GO terms and was visualized in Cytoscape. The network includes over-represented GO terms and associated differentially expressed proteins. The changes in protein expression are coloured as a gradient going from blue (=downregulated) to red (=upregulated) and GO terms are coloured in green.

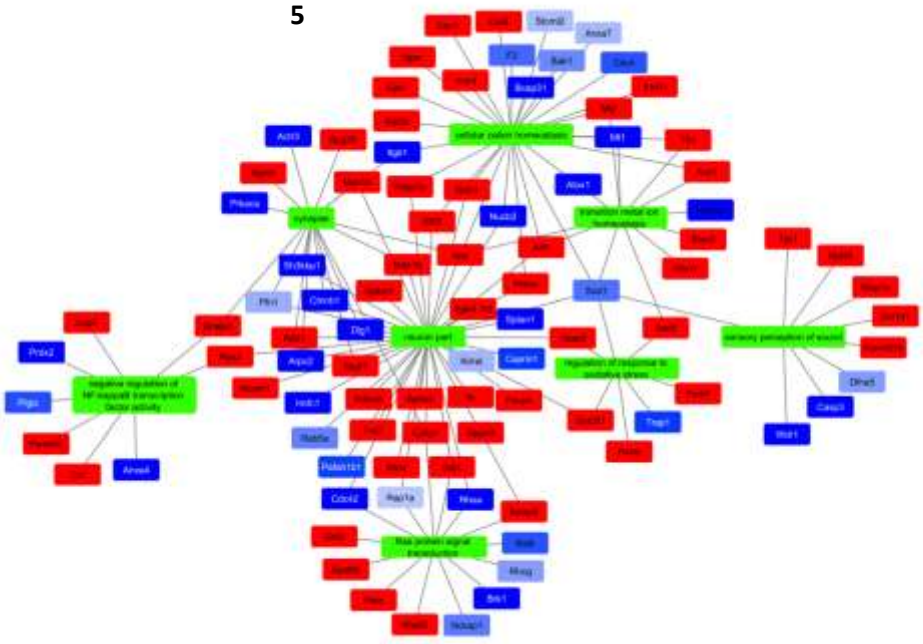

Figure 5: Interaction of differentially expressed proteins and selected GO classes after As exposure. The network is based on the selection of the pruned GO terms and was visualized in Cytoscape. The network includes over-represented GO terms and associated differentially expressed proteins. The changes in protein expression are coloured as a gradient going from blue (=downregulated) to red (=upregulated) and GO terms are coloured in green.

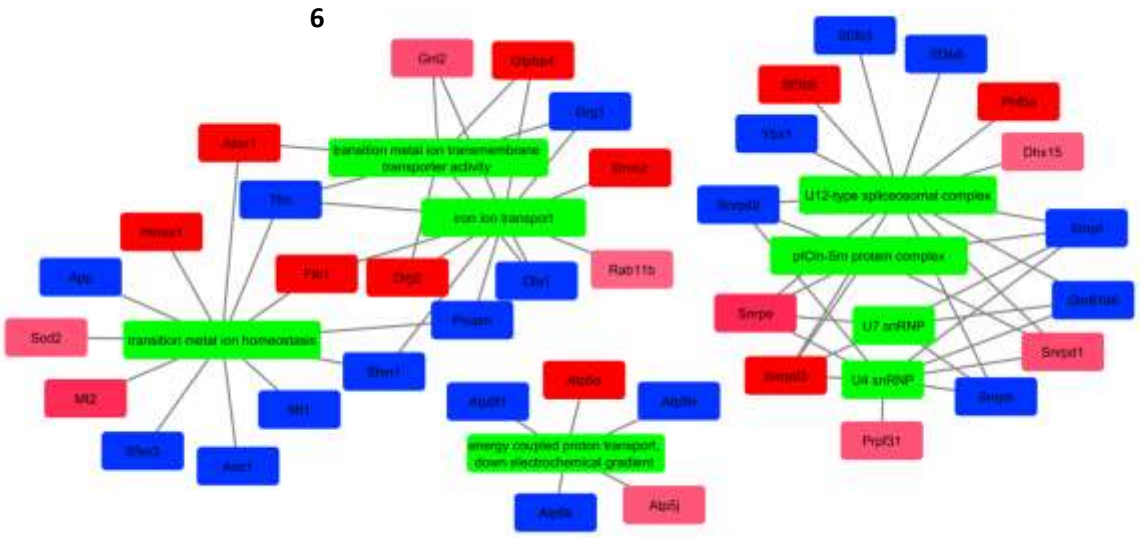

Figure 6: Interaction of differentially expressed proteins and selected GO classes after MeHg exposure. The network is based on the selection of the pruned GO terms and was visualized in Cytoscape. The network includes over-represented GO terms and associated differentially expressed proteins. The changes in protein expression are coloured as a gradient going from blue (=downregulated) to red (=upregulated) and GO terms are coloured in green.

Table 1: Statistically significantly changed GO terms related to neurotoxicity in HT-22 cells for Pb, As and MeHg

Pb						
Ontology-ID	**Ontology Name**	**Ontology Type**	**Changed genes**	**Measured**	**Ontology**	**Z Score**
GO:0043028	cysteine-type endopeptidase regulator activity involved in apoptotic process	molecular function	5	5	36	3.8
GO:0046426	negative regulation of JAK-STAT cascade	biological process	3	3	12	2.9

GO:0045738	negative regulation of DNA repair	biological process	3	4	8	2.2
GO:0034719	SMN-Sm protein complex	cellular component	6	11	17	2.2
GO:0097458	neuron part	cellular component	46	141	927	2.0
As						
Ontology-ID	**Ontology Name**	**Ontology Type**	**Changed**	**Measured**	**Ontology**	**Z Score**
GO:0032088	negative regulation of NF-kappaB transcription factor activity	biological process	8	8	63	2.4
GO:1902882	regulation of response to oxidative stress	biological process	7	7	27	2.2
GO:0007265	Ras protein signal transduction	biological process	19	24	99	2.1
GO:0055076	transition metal ion homeostasis	biological process	12	14	99	2.0
GO:0097458	neuron part	cellular component	95	141	927	2.3
MeHg						
Ontology-ID	**Ontology Name**	**Ontology Type**	**Changed**	**Measured**	**Ontology**	**Z Score**
GO:0006090	pyruvate metabolic process	Biological process	18	21	55	2.9
GO:0050877	neurological system process	biological process	31	43	1863	2.4
GO:0055076	transition metal ion homeostasis	biological process	12	14	99	2.4
GO:0043028	cysteine-type endopeptidase regulator activity	molecular function	5	5	36	2.0

	involved in apoptotic process					
GO:0005687	U4 snRNP	cellular component	8	9	10	2.1
GO:0005683	U7 snRNP	cellular component	5	5	8	2.0
GO:0005689	U12-type spliceosome complex	Cellular component	13	13	25	3.3

3.3. Pathway analysis: visualization of pathways relevant to heavy metal (Pb, As, and MeHg) induced neurodegeneration in HT-22 cells

Pathway analysis was used to further explore the complex interactions in a biological system and to discover the relations between differentially expressed proteins and their relevance to (AD, and PD. In general, toxicological studies related to Pb, As and MeHg have proposed few common predominant mechanisms that relate to neurotoxicity. The obtained results suggest that for AD and PD the pathways: mRNA regulation/splicing, ubiquitin proteasome system (UPS), oxidative stress, electron transport chain (ETC) dysfunction, are the most affected by MeHg and As exposure; however, the Pb metal impact was low. Notable proteins found in the ETC dysfunction, oxidative stress, mRNA splicing process, and UPS degradation also relate to AD and PD. Table 2 shows the pathways that significantly changed proteins relevant to neurodegeneration process in HT-22 cells.

Table 2: Six pathways showed significantly changed proteins relevant to neurodegeneration process in HT-22 cells.

		Pb	As	MeHg
Pathway	**measured (n)**	**positive (r)**	**positive (r)**	**positive (r)**
Electron transport chain	31	12	17	21
mRNA processing	191	51	104	115
Proteasome degradation	40	9	18	7
Alzheimer's disease pathway	17	3	9	20
Parkinson's disease pathway	10	1	7	6
Oxidative stress	12	4	9	7

(*Measured (n) is the number of gene products in the pathways that are measured in the Pb, As and MeHg data sets; positive (r) is the number of differentially expressed proteins in the presented pathway for each metal)

3.3.1. Effect of Pb, As and MeHg on mitochondrial electron transport chain (ETC) dysfunction

The mitochondrion is a critical regulator of neuronal cell death [88]; a failure of one or more of the mitochondrial ETC enzymes generates reactive oxygen species (ROS), and leads to neuronal death [89]. It is a tempting to

speculate that the modification of ETC proteins leads to depletion of ATP production in HT-22 cells. We found protein dysregulation in all complexes of the ETC, suggesting a broad toxicity of metals on ETC of HT-22 cells. As shown in Figure 7, MeHg treatment (IC_{10}) induced significant alteration of proteins in complex I–V. The pattern of protein expression alerted by As in the ETC pathway was similar to that of MeHg. In the present study, cytochrome c oxidase (COX)/complex-IV and F_1 F_0-ATPase activities seemed highly dysregulated by the MeHg and As exposure. MeHg is known to directly disrupt the mitochondrial activity by generating the uncontrolled release of Ca^{+2} from the mitochondria and inhibiting phosphorylation, Yee et al. reported that MeHg inhibits the mitochondrial ETC in a cultured brain cell line [90]. The findings of mitochondrial ETC damage due to MeHg and As metal exposure is an agreement with previously reported results in AD patients from post mortem hippocampus [91]. The reduced activity of F_1 F_0-ATPase (complex-V) could compromise the ATP production and induce the generation of ROS leading to neuronal cell death [92]. The ETC dysfunction after metal exposure in HT-22 cells seems to correlate with the previously reported findings of Valla et al. [93]. Reported studies for deficiency in the activity of the mitochondrial ETC NADH dehydrogenase (complex I) in PD [94], which is in an agreement with ETC dysfunction in the HT-22 cells. Our findings confirm that the metals disrupt the ETC functionin HT-22 cell, and that may relate to energy deficiency, a characteristic feature of both AD and PD [95]. The observed ETC changes raise further questions [96]. Not only direct effects of ETC function, but also other proteins (next section) involved in AD and PD can also impact various mitochondrial functions [97]. There is no evidence that any of the Pb, As, and MeHg metals directly produce only mitochondria based neurodegeneration processes [98], although other pathways present the direct or indirect relation to ETC dysfunction.

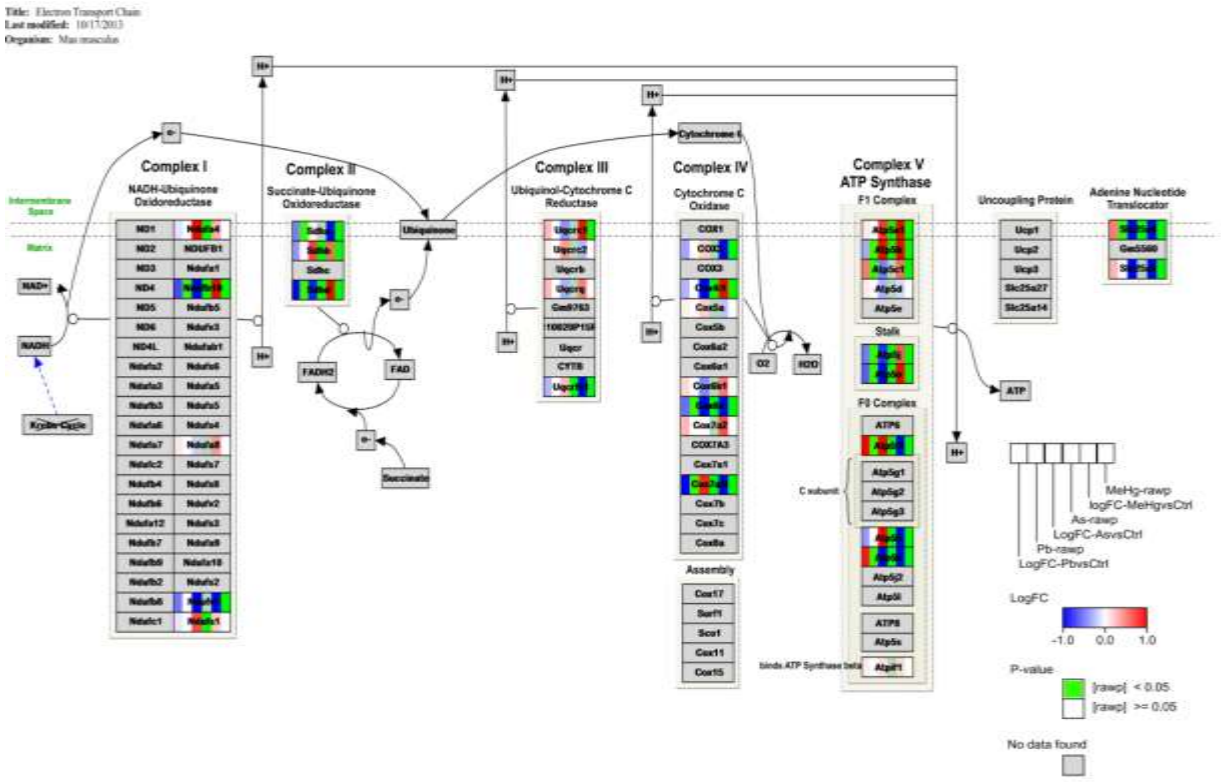

Figure 7: Changes in protein expression in electron transport chain (ETC) pathway in response to Pb, As and MeHg in HT-22 cells. The pathway changes in protein expression are visualized as log2 fold change comparing treatment with control expression. In addition, the significance level of these changes is indicated by *p* values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from

blue (=downregulated) over white (unchanged) to red (=upregulated) and p values < 0.05 are coloured in green and p values ≥ 0.05 are in white. Grey indicates that the selection criteria were not met, but the protein is measured.

3.3.2. Effect of Pb, As, and MeHg on ROS mediated oxidative stress

The present results (Figure 8) indicate that generation of ROS can relate to ETC damage and monoamine oxidase A (MAO-A) elevation in the HT-22 cells. The presented oxidative stress pathway gives an adaptive response in HT-22 cells to maintain redox homeostasis to fight against the heavy metal induced stress. We found that MAO-A protein expression in HT-22 cells in the all three metals, MAO-A participates in the degradation of amines and thereby controls neurotransmitter levels in the brain [99]. In the present study, MAO-A expression changed in a contradictive manner among three metals; As (downregulation) while Pb and MeHg (upregulation), which indicates that Pb and MeHg might be enhancing the monoamine metabolism in HT-22 cells. The obtained MAO-A expression in MeHg is in disagreement with reported MeHg effect in vitro embryo culture experiments [100]. As metal also showed that MAO-A expression was opposite to reported studies, instead of upregulation the effect was downregulated [101]. Pb altered mitogen-activated protein kinase (MAPK) functions leading to the production of ROS causing neuronal cell death [102]. The relationship between AD and increased enzyme activity for MAO has been known for a long time; MAO-A protein activity levels are elevated in the AD [103]. The MAO-A elevated AD brain is subjected to increased oxidative stress resulting from ROS [104]. Thus, the MAO-A activity could be altered by Pb, As and MeHg indirectly through the changes in -SH groups [105]. The results support that MAO-A may be a useful biomarker for Pb, As and MeHg on the hippocampal cells. The MAPKs plays an important role in the cascades of cellular responses evoked by extracellular stimuli such as the heavy metal exposure [106]. Our results demonstrated a significant alteration in MAPKs mediated oxidative stress response (Figure 8). The induction of antioxidant and phase II detoxifying enzymes acts as an important defensive mechanism against heavy metal induced stress in HT-22 cells.

Pb, As and MeHg in HT-22 cell line induced significant effects in MAPKs mediated oxidative stress response such as anti-oxidant proteins, hemeoxygenase1 (HMOX1), NAD (P)-H dehydrogenasequinone1 (NQO1), glutathione disulfide reductase (GSR), superoxidedismutase1 (SOD1), superoxide dismutase2 (SOD2), catalase (CAT), thioredoxin reductase1(TXNRD1), glutamate cysteine ligase catalytic (GCLC) subunit, and metallothionein-1(MT1). All proteins are involved in the activation of antioxidant activity in HT-22 cells supporting the heavy metals stress. The increase in the antioxidant proteins is a compensating mechanism for the decrease in the ATP producing due to ETC dysfunction in HT-22 cells. In contrast to Pb metal; MeHg and As significantly alter some forms of antioxidant and detoxification enzyme which indicates clearly the higher potency of As and MeHg. Also, MeHg and As induce significant upregulation of GSR and SOD2; although CAT protein expression was observed similarly in both Pb and As. The obtained results show that expression/fold change of antioxidant proteins depends on the potency of each metal; MeHg considerably alters the normal pattern of antioxidant elements expression than other two metals. Biochemically MeHg has high affinity to sulphur (-SH) containing molecules, mainly targeting cysteine containing proteins [107].

Oxidative stress has been implicated in the wide variety of biological reactions such as cell death in the central nervous system [108]. ROS induced oxidative stress has been demonstrated to activate members of the MAPKs via phosphorylation [109] and it is also implicated in neurodegenerative disorders [110]. Thus, heavy metal induced ROS can elicit oxidative stress that can be the main cause to trigger various neurodegenerative diseases [111, 112]. ROS is also well known to cause mitochondrial dysfunction and induces cell death in neurons [113]. Previous studies indicated that MeHg disrupts cellular redox homeostasis and the mitochondrial ETC, via excessive generation of ROS [114, 115]. The disruption of ETC function can also correlate with various proteins in oxidative stress such as MT1, SOD, GSH, CAT, HMOX1, and NQ01. Oxidative stress has been shown not only to initiate the onset of disease but also exacerbates specific diseases like AD and PD [116].

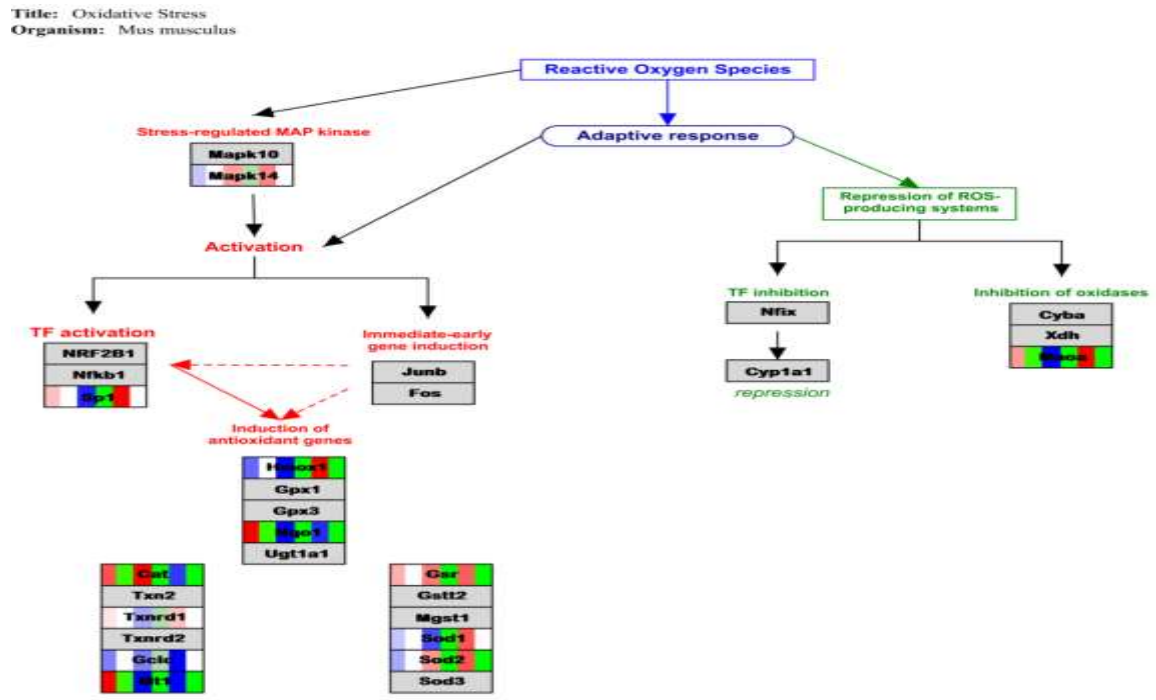

Figure 8: Changes in protein expression in oxidative stress pathway in response to Pb, As and MeHg in HT-22 cells. In the pathway, changes in protein expression are visualized as log2 fold change comparing treatment with control expression. In addition, the significance level of these changes is indicated by *p* values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p* values < 0.05 are coloured in green and *p* values ≥ 0.05 are in white. Grey indicates that the selection criteria were not met, but the protein is measured.

3.3.3. Effect of Pb, As, and MeHg on UPS pathway

In addition to selective damage of ETC and oxidative stress in HT-22 cells, UPS may also contribute to neurodegeneration processes [117]. The UPS is essential for the non-lysosomal degradation and clearance of short-lived, misfolded, mutant, and damaged proteins in eukaryotic cells. Structural and functional deficits in the 26/20S proteasome can lead to AD, and PD [118, 119]. The UPS is a highly conserved cellular pathway that plays an important role in the selective degradation of cellular proteins that are essential for the regulation of a variety of vital cellular functions [120]. Disruption of this system can have significant downstream effects on critical cellular functions. In Figure 9, Pb, As and MeHg metal showed nonspecific proteasome alterations of proteasome subunits of the 20S catalytic core and the 26S (PA700) regulatory complex. The pattern of expression in UPS is metal specific with relevance for neurodegeneration. For instance, ubiquitin carboxyl-terminal esterase L3 (UCHL3) expressions were upregulated in the As exposure, although the other two metals (Pb and MeHg) had no effect. UCHL3 is a deubiquitinating enzyme that is involved in the pathogenesis of both AD and PD [121]. Recently, an increased amount of oxidatively modified UCHL3 in the brains of the AD and PD patients, compared to normal brains was reported [122]. Ubiquitin-like modifier activating enzyme 1 (UBA1) is a key regulator of protein homeostasis and expression of UBA1 is essential for cell survival. Our results suggest that Pb, As, and MeHg metal upregulates the UBA1 expression in HT-22 cells. Moreover, UBA1 canbe sequestered into PD disease associated protein aggregates [123]. Additionally, for MeHg, we observed downregulation of the NEDD4 (ubiquitin E3 ligase/ parkin), which indicates that MeHg has a significant impact on the number of protein targets in HT-22 cells. In contrast, Pb and As metal upregulates the NEDD4 protein in UPS pathway. NEDD4 plays a crucial role in

ubiquitination; this enzyme enhances the cell survivability by decreasing the apoptosis process in HT-22 cells. We found that the expression was downregulated during MeHg exposure, which indicates the UPS dysfunction. The accumulation of parkin specific substrates, as a consequence of the loss of NEDD4 activity, might underlie the damage of dopaminergic neurons [124]. Other proteins in the PD pathway (Figure 12) such as SEPT-5 and ATAXIN-2 have interaction/connection with NEDD4 function and also play a key role in PD (both proteins are upregulated in PD pathway). This upregulation process can decrease the NEDD4 activity of the proteasome after MeHg exposure in the hippocampal cell line. We found the bunch of proteins dysregulation in the proteasome 26 subunit, in which the PSMC5 protein has a critical role in the degradation of unfolded proteins. The expression was downregulated in HT-22 cells after MeHg exposure but upregulated with Pb and As.

Overall, the UPS pathway disruption shows that most of the protein expression was similar for all three metals (except NEDD4 expression). The findings with the UPS pathway clearly show that the potency and molecular mechanism related to PD is different for the three metals. For instance, comparisons of the protein expression level between the three metals by omics data revealed the common targets in the UPS of the HT-22 cells but the way of influence/interaction on the protein is different. Pb, As and MeHg treatment in HT-22 cells induced significant alteration of UPS pathway proteins, including the significant alterations in the proteasome 26S regulatory and 20S catalytic core unit and in the ubiquitin enzyme system (UBA1, NEDD4, and UCHL3), which are highly essential for protein quality control during normal cell function. MeHg effect on E3 ligase enzyme was reported in human cell lines [125, 126]. This report suggests that MeHg induces the cellular accumulation of certain proteins that cause neurodegeneration. Mouse neuronal HT4 cells treated with toxic metals have been reported to induce accumulation of ubiquitination with a decrease in cell viability [127]. This report also shows the critical role of the UPS in the removal of proteins that are oxidatively modified and suggests that accumulation of ubiquitinated proteins in the HT- 22 cells contributes to an overall toxicity. In Figure 9, the comparison of alterations in protein expression between the metals Pb, As, and MeHg revealed both common and unique targets in the UPS. Although the involvement of the UPS pathway might be different among various cell types, results from this omics data strongly support that the disruption of the UPS function is involved in metal-induced neurotoxic effects and denotes the potential protein targets in this pathway. Several studies have identified the critical role of the UPS in the metal induced toxicity [128-132]. It is clear that alterations in UPS function are key factors in the final development of AD and PD; more research is needed to elucidate the exact mechanisms and order of events concerning the role of the UPS in neurodegenerative diseases.

3.3.4. Effect of Pb, As, and MeHg on mRNA splicing pathway

Alternative splicing is one of the most important mechanisms to generate a large number of mRNA and protein isoforms from the low number of human genes [133]. It is a widespread gene regulatory process by which exons of primary transcripts (pre-mRNAs) are spliced into different arrangements to produce structurally distinct mRNA variants [134]. This mechanism of gene product plays a critical role in cell function [135]. It is estimated that more than 75% of genes in the human genome are alternatively spliced in the central nervous system [136]. Dysregulation of alternative splicing has been linked to the number of human diseases including neurodegenerative diseases [137]. The spliceosome is a complex of five small nuclear ribonucleoprotein particles (snRNP: U1, U2, U4, U5, and U6) and numerous protein factors. In Figure 10showed snRNP bind to the pre-mRNA in a sequential manner, following the binding of U4/U6, U5 tri-snRNP, the spliceosome undergoes extensive structural remodelling leading to the release of U1, U2 subunits, and activation of the spliceosome [138-140]. In Figure 10, we addressed the impact of Pb, As and MeHg on splicing and the relationship between neurological disease and the regulation of alternative splicing. Alternative splicing is particularly applicable in the present results, because of the great number of protein changes observed in the mRNA processing. In addition, we identified several components of the U1 small nuclear ribonucleoprotein (U1 snRNP) alterations in HT-22 cells. Our findings demonstrated a unique damage of splicing in HT-22 cells. The functional consequences of these observations can reflect the neurodegeneration processes [141]. We found that U1snrp70K (U1 small nuclear ribonucleoprotein 70 kDa) protein expression was upregulated in As metal exposure; the other two metals have no significant effect. This protein has a critical role in the APP protein metabolism [142]. The alterations of U1snRNP are in agreement with discovered U1 snRNP defect in the AD brain [143]. We found U2 snRNP protein dysregulation with all three metal exposures. A reported study denoted that the

mutation in U2 snRNP genes causes defects in pre-mRNA splicing, leading to neurodegeneration [144]. Our results showed a high number of altered proteins in the U2snRNP unit, and U2 snRNP plays an essential role in the formation of the catalytically active spliceosome by base pairing with both the intron branch point and the U6 snRNA [145]. The last functional tri-protein (U4, U5, and U6) system function was downregulated in HT-22 cells, which indicates the mechanism of splicing is completely altered by heavy metal exposure. Additionally, we found abnormal changes in the serine/arginine rich proteins (SR) Sfrs1, Sfrs2, Sfrs3, Sfr5, and Sfr6; SR proteins are one family of splicing factors involved in the alternative splicing of tau protein. The changes in SR proteins probably influence the regulation of the alternative splicing of the tau gene [146]. In that way, heavy metal induced protein alterations in SR proteins may contribute to the AD by controlling the tau protein expression [147]. The present findings enable mechanistic insight into how aberrant changes in alternative transcript expression occur in the AD and highlight the susceptibility network of splice-regulatory proteins which can potentially link the number of susceptibility pathways [148]. Taken together, these observations imply that alterations in snRNP accumulation and consequently pre-mRNA splicing can contribute to the etiology of multiple neurodegenerative disorders [149]. Alternative splicing is tissue specific and especially important for brain tissue, thus the metal disrupted spliceosome has a big impact in HT-22 cells.

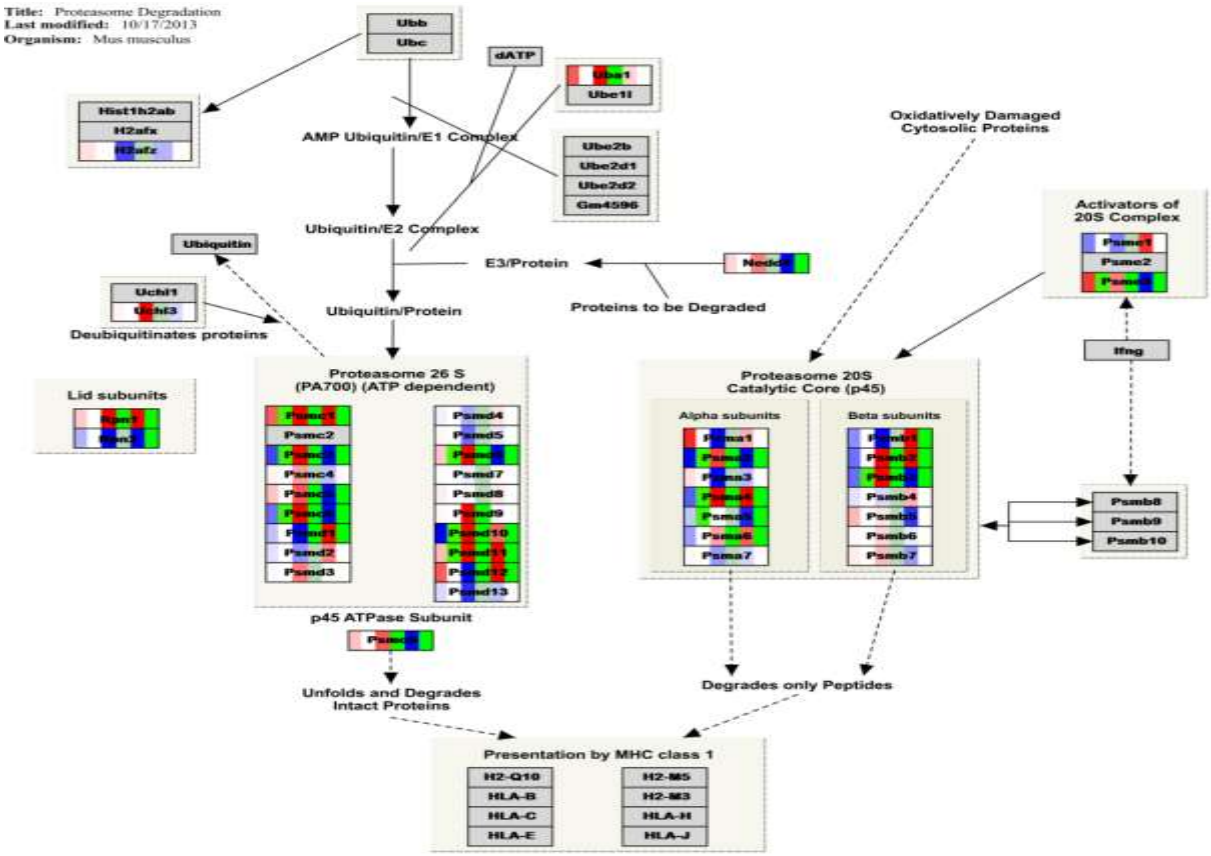

Figure 9: Changes in UPS in response to Pb, As, and MeHg in HT-22 cells. In the pathway, changes in protein expression are visualized as log2 fold change comparing treatment with control expression. In addition, the significance level of these changes is indicated by *p* values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p* values < 0.05 are coloured in green and p values ≥ 0.05 are in white. Grey indicates that the selection criteria were not met, but the protein is measured.

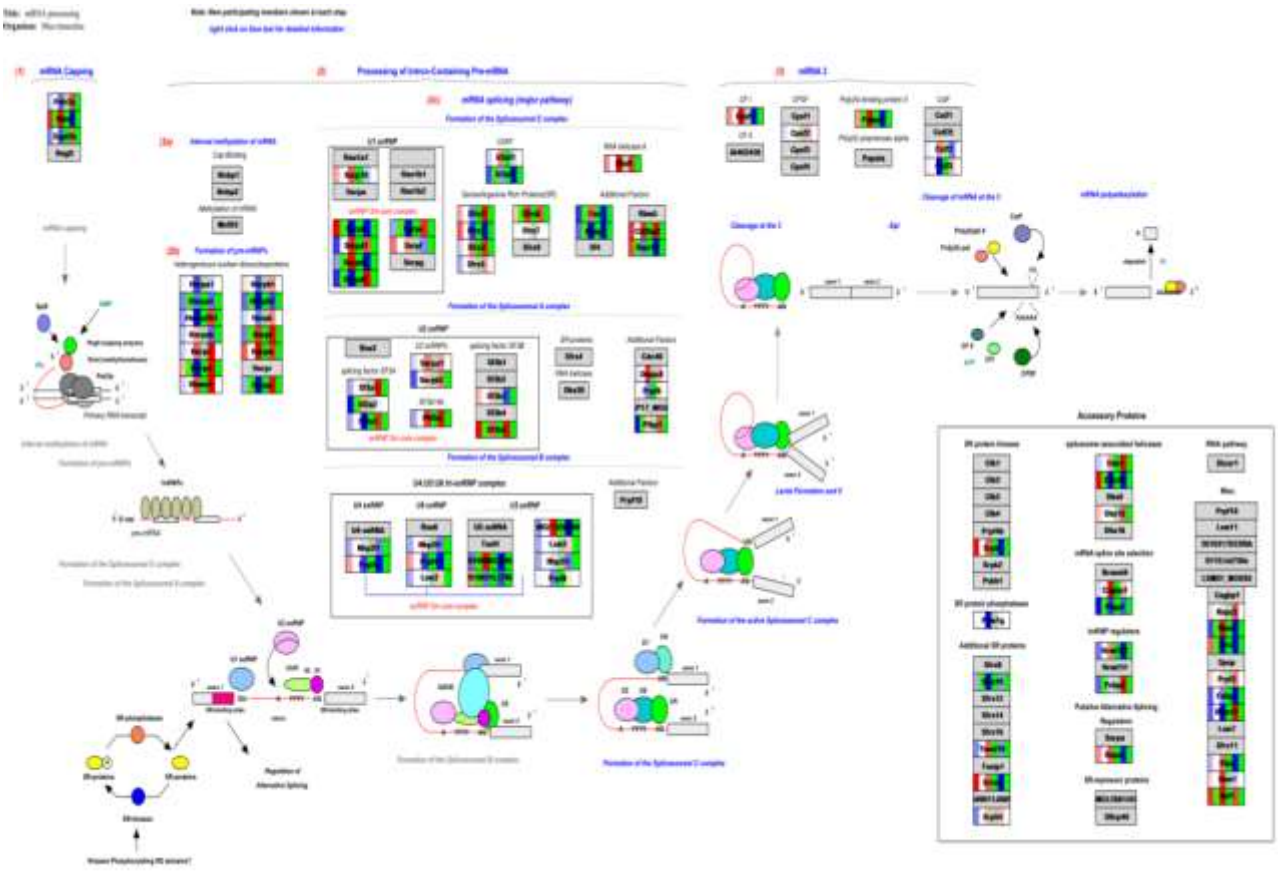

Figure 10: Changes in mRNA splicing in response to Pb, As and MeHg in HT-22 cells. In the pathway, changes in protein expression are visualized as log2 fold change comparing treatment with control expression. In addition, the significance level of these changes is indicated with *p* values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p* values < 0.05 are coloured in green and *p* values ≥ 0.05 are white. Grey indicates that the selection criteria were not met, but the protein is measured.

3.4.5. Effect of Pb, As, and MeHg on Alzheimer Disease (AD) pathway

The AD is a complex, irreversible neurodegenerative disease characterized by the impairment of cognitive function. In the present study, it is conceivable that the observed alterations in the AD specific proteome of HT-22 cells could cause the alterations in the APP. Our findings in Pb and As comply with a previous study that showed an increased hippocampal and cortical APP protein processing causing cognitive dysfunction [150]. Also, we observed significant changes in CAPN1 and CAPN2 (calcium-dependent, and non-lysosomal cysteine proteases). It's known that calpains is implicated in neuronal cell death and long term potentiation (LTP) in the hippocampus [151]. In our study, CAPN2 expression was upregulated by MeHg and As, which clearly suggests the AD relationship with heavy metals exposure. Previously it was reported that CAPN2 can improve survival of hippocampal neurons [152]. The other protein, low-density lipoprotein receptor-related protein1 (LRP1 protein) was upregulated with As and MeHg. LRP1 is implicated in the effective clearance of Aβ from the brain. It indicates that HT-22 cells compensate for the Aβ clearance through the LRP1 protein [153]. NEDD8-activating enzyme E1 regulatory subunit (NAE1/APPBP1) expression was found in As exposure, that binds to the APP protein carboxyl-terminal domain [154], results in neuronal cell death [155]. The AD is also characterized by the activation of apoptotic pathways. However, the hypothesis that apoptosis plays a role in AD neurodegeneration remains controversial. Caspase activation can occur independent of cell death and may be neuroprotective [156, 157]. CASP8 expression was found altered after the As

exposure, which leads to the receptor mediated apoptotic pathway to activate CASP3 within neurons of the AD brain [158]. We found that expression of CASP3 that indicates that HT-22 cell death may be initiated in an apoptosis-dependent manner. Generally, Pb, As and MeHg predominantly induce CASP3 like protease activity in brain cell culture [159-161] The activation of CASP3 is known to an enhanced generation of alternative presenilin cleavage fragments in the AD, but exactly not clear [162]. Here, we found CASP3 expression in relation to tau protein in the AD pathway (Figure 11) which is entirely different from the metal induced apoptosis assumption [163] and thus, may contribute to cellular demise in a different manner [164].We also found that heavy metals induced AD in relation to other pathways. For instance, APP dysregulation can be possibly due to mitochondrial ETC, oxidative stress, transition metal ion homeostasis, and mRNA splicing. These AD related a protein has been previously discussed in other pathways sections 3.3.1, 2, 3, and 4. For instance, the impairment of mitochondrial function has been well known in AD patients [165]. ETC dysfunction (Figure 7) and reduced ATP have an immense impact on brain metabolism [166]. PCR analysis revealed that the downregulation of mitochondrial proteins of complex- I in AD brain, suggesting energy deficits in AD brains [167]. In addition, several in vitro studies of APP and mitochondrial function have reported that APP expression affects the mitochondrial DNA and proteins, leading to impairments of the ETC [168, 169]. With As and MeHg we found that the transition metal ion homeostasis GO term represents a process necessary for the correct functioning of antioxidant systems in the cell [170]. Biometals such as iron (Fe), zinc (Zn) and copper (Cu) play an important role in several neurodegenerative disorders [171]. As and MeHg are such toxic metal which influences the essential metal transportation that relates to Aβ aggregation [172], and also Fe, Pb and Mn and their mixture may raise the risk of PD [173]. The expression of APP in the brain suggests these metals could have a direct or indirect role in neuronal metal homeostasis [174], for instance, Cu deficiency downregulates APP transcription [175]. The association between APP and transition metals such as Fe and Cu lead to the generation of hydrogen peroxide, exacerbating the oxidative damage [176]. Few studies have investigated the link between alternative splicing dysregulation, and AD progression [177]. Evidence of abnormal mitochondrial ETC gene expressions from our studies suggests that APP in AD pathway may affect mitochondrial function, which generates ROS, ultimately leading to oxidative damage [178, 179]. However, the precise mechanistic link between mitochondrial oxidative damage and abnormal APP processing by heavy metals has not been elucidated. Further studies are needed to support the mechanistic link between abnormal mitochondrial gene expression and oxidative damage in AD progression by heavy metal exposure.

3.4.6. Effect of Pb, As, and MeHg on Parkinson Desease (PD) pathway

PD is the second most prevalent progressive neurodegenerative disease [180]. In this study, several proteins are showed differential expression relates to PD. The pathway analysis of metal exposure on HT-22 cells proved particularly valuable for PD because we found significantly changed proteins belonging to the PD pathway. These PD related proteins also appeared in other pathways, for instance, ETC dysfunction, oxidative stress, UPS pathway, and mRNA splicing. A detailed study of the PD pathway (Figure 12) made it plausible that the observed alterations at the protein level in HT-22 cells could be related to PD such as PARK7, CYCS (cytochrome c, somatic), and CASP3 as key regulators of proteins for cell homeostasis. We found that the expression was upregulated by the three metals treatment, which clearly shows that HT-22 cells are under stress. Also, other proteins such as UBE2L3 (Ubiquitin-conjugating enzyme E2 L3), SEPT-5 (septin), and ATAXIN-2 protein expression was altered in the PD pathway. SEPT-5 protein interacts with NEED4 in PD. Indeed, we found that As and MeHg altered UBA1 and NEDD4 (Figure 9) expression, respectively, in the UPS pathway. This SEPT-5 upregulation in MeHg can decrease the activity of NEDD4 resulting in lower ubiquitin proteasome degradation in the hippocampal cell line. In As exposure, the interaction mechanism between NEDD4 and SEPT-5 proteins is entirely opposite to MeHg. NEDD4 has been intimately linked to mitochondrial quality control, which evidence suggests that PARK7, ATP13A2, and FBXO7 may also be involved [181]. In our data, the expression of CYCS and CASP3 were upregulated by the three metals. The loss of mitochondrial membrane potential and translocation of CYCS into the cytosol lead to cell death [182]. In parallel, PARK7 protein is upregulated, which will protect the cells from the apoptosis stress. PARK7 has been thought as an endogenous redox sensor, which prevents the cell from stress-induced apoptosis [183]. Thus,

PARK7 is believed to be protective against oxidative stress in PD brains [184]. PARK7 expression in HT-22 cells was suggested to be related to the oxidative stress and dopaminergic neurodegeneration [185].
The expression pattern of PARK7, CYCS and CASP3 supports that the three metals affect mitochondrial function which is relevant to PD, with the potency in order of Pb<As<MeHg. The pattern of expression of interlinked proteins in the PD and UPS pathways of the MeHg and As metal exposures is somehow different. As metal significantly upregulates the UBA1 and NEDD4 and downregulates SEPT-5 in HT-22 cells. Thus, UBA1 might be sequestered into disease associated protein aggregates, also shown for lewy bodies in As exposure models of PD [186]. For MeHg, we observed downregulation of NEDD4 (Figure 10). NEED4 has a role of the E3 ubiquitin ligase, participating in the proteasome degradation system [187], of which the loss of function allows the formation of these toxic aggregates in neurons. Target proteins of MeHg treatment in HT-22 cells related to PD suggest a direct relation to the UPS pathway. The downregulated NEDD4 proteins during MeHg exposure is critical for inducing the indirect way of PD by affecting the UPS function. The results of our proteomics analysis using hippocampal HT-22 cells strongly suggest that the disruption of UPS function is an important mechanism in metal induced neurotoxicity. Moreover, additional evidence supports not only the important role of NEED4 as an E3 ligase in PD but also this protein can lead to mitochondrial impairment in the brain [188]. Our findings comply with the previous report that NEDD4 protein expression can correlate to alternative splicing defects, which is one of the reasons for PD [189].

The heavy metal induced PD is likely to be multifactorial; ETC dysfunction, oxidative stress, and the impairment of UPS dependent protein degradation have been identified as the main processes associated with PD [190]. Our findings confirm that the metals disrupt the ETC pathway in HT-22 cell and influence the PARK7, and NEDD4 expression [191]. We found that NEED4 expression was downregulated in the MeHg exposure, which correlates with previously reported alternative splicing defects and PD [188](Tan et al., 2005). We also found that the heavy metal induces oxidative stress and mitochondrial dysfunction, which is directly or indirectly related to PD [192, 193]. Our results also support that As and MeHg treatments highly influence the ETC, oxidative stress and UPS pathway which are relevant to the PD pathway [194]. Finally, PD is associated with a number of biochemical abnormalities, in which the UPS and ETC plays a major role. Indeed, inhibition of proteasomal function has been shown to cause oxidative stress and mitochondrial dysfunction [195-199].

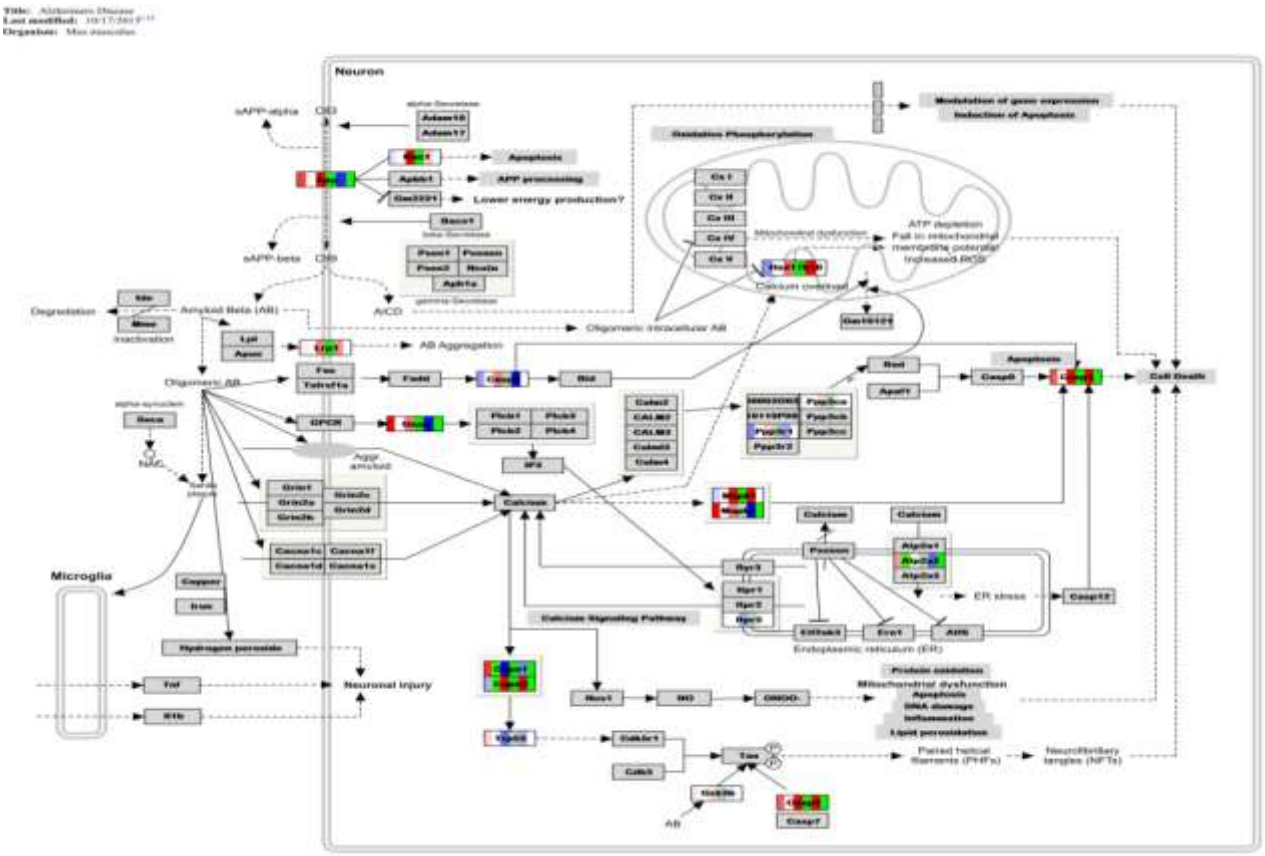

Figure 11: Changes in AD in response to Pb, As, and MeHg in HT-22 cells. In addition, the significance level of these changes is indicated with *p* values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p* values < 0.05 are coloured in green and *p* values ≥ 0.05 are in white. Grey indicates that the selection criteria were not met, but the protein is measured.

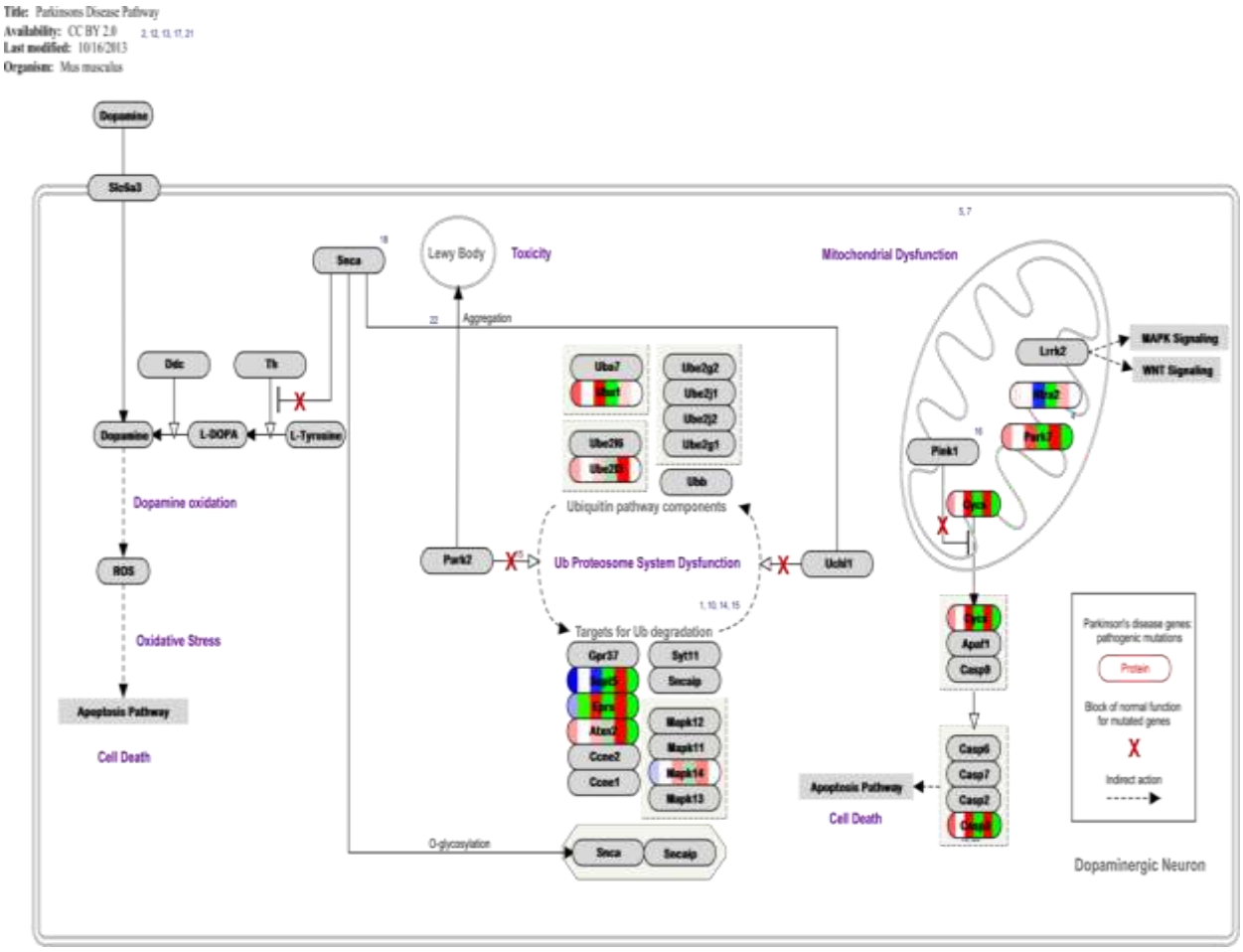

Figure 13: Changes in Parkinson's disease in response to Pb, As, and MeHg in HT-22 cells. In addition, the significance level of these changes is indicated by *p* values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p* values < 0.05 are coloured in green and *p* values ≥ 0.05. Grey indicates that the selection criteria were not met, but the protein is measured.

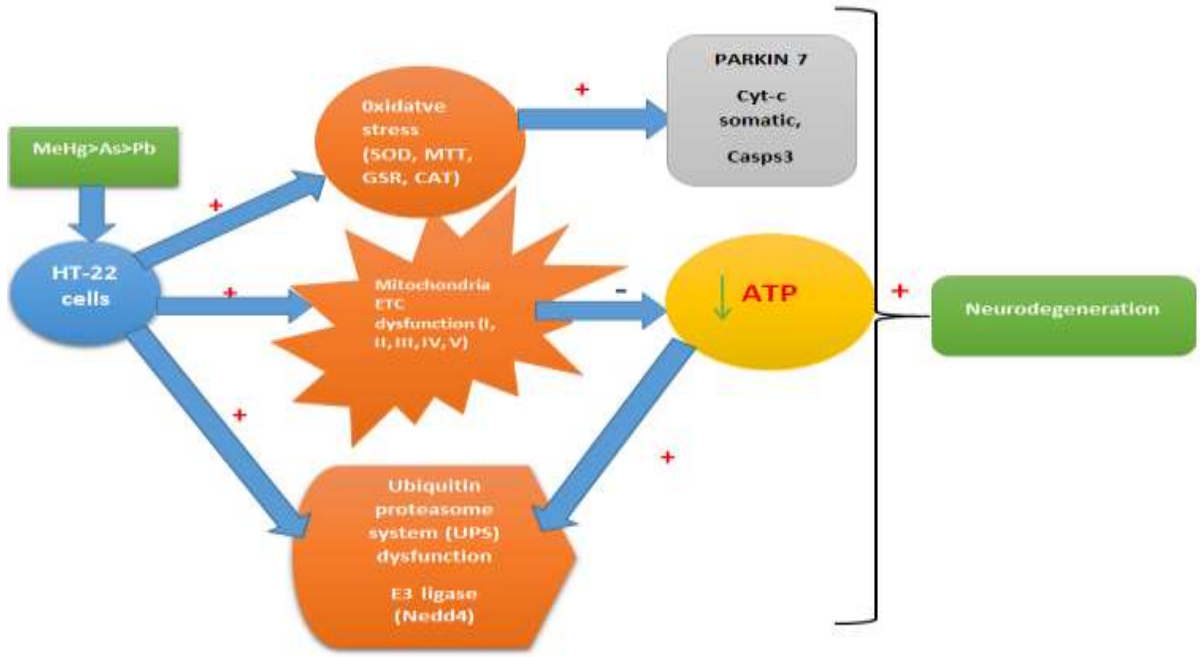

Figure 14: Proposed potential underlying neurotoxicity mechanisms of Pb, As, and MeHg. Metals exposure cause proteomic alterations in hippocampal cells including the proteins related to energy metabolism, oxidative stress, UPS, PD, AD (+ sign enhance the damage, - sign ATP depletion)

4. Conclusions and Future Directions

Chronic exposure to the toxic metals Pb, As, and MeHg has been associated with the neurological degeneration. Nowadays, knowledge of neurodegenerative diseases has advanced rapidly, and the field holds great promise for improving the understanding and the eventual treatment of disease. To date, this study is the first report of comparative heavy metals neurotoxicity assessment by using the omics and systems biology tools. For the proteomics analyses, we selected sub lethal toxic concentrations (IC_{10}) of each metal on HT-22 cells on 8 days exposure as a chronic. Omics based analysis concentration of testing chemical has had a critical role for the success of this study. If the chemical concentration is too high, the secondary effects could mask the primary response. Thus, preliminary cytotoxicity, genotoxicity, and apoptosis observations were used to select the defined concentration. The functional interpretation of protein expressions is always challenging. In this study, we employed both GO analysis and Pathvisio approaches for systems biology analysis. Using these integrated tools, we identified significant protein expression changes across treatments related to AD, PD pathways. Our results confirmed that GO and Pathvisio analysis is a powerful approach to generate an unbiased view of the functional protein alterations by heavy metals. The application of such quantitative interpretation of toxicogenomics data is likely to become increasingly useful for evaluating the mechanistic similarity of novel chemicals.

The current study describes toxicity profile in the form of protein expression that is observed in response to toxic metals exposure. These changes may provide further insight into mechanisms underlying the development of metal-induced diseases. These proteins are also candidate biomarkers of metal exposure that could potentially be used diagnostically in molecular and epidemiologic studies. The findings from our results have greatly expanded understanding of the role of mitochondria in the pathogenesis of neurodegenerative diseases. Mitochondrial ETC dysfunction, oxidative stress, UPS dysfunction and mRNA splicing changes occur early in all major neurodegenerative diseases, and there is strong evidence that this dysfunction has a causal role in disease pathogenesis. Greater improvement of these pathways will be required to understand fully the pathogenesis of

neurodegenerative disorders. Both Pathvisio and GO interpretation integrated systems biology supports that the potency of heavy metals is as follow: Pb<As<MeHg. In summary, the comparison of protein expression among Pb, As and MeHg revealed both common and unique protein targets that relate to neurodegenerative diseases. The involvement of mRNA splicing and the UPS pathway are new findings. The results of our proteomics analysis strongly suggest that the disruption of mRNA splicing, UPS, ETC and oxidative stress plays a crucial role in metal induced neurodegenerative diseases. Obviously, the utility of toxicogenomics has promising advantages on assistance and refinement of the risk assessment process, particularly the dose response analysis.

Future work will focus on using these data to explore basic neurodegeneration mechanisms of metals and the toxicity of their mixtures to generate new hypotheses. Although relatively less toxic doses (IC_{10}) were used in the current analysis, a wide dose range study including an environment relevant dose is needed to further examine the association of metal exposure and its neurodegeneration. These heavy metal response patterns may shed new light on the mechanisms of toxic metal induced neurodegeneration, and may also be useful for the development of molecular biomarkers of exposure in mechanistic studies and risk assessment

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References

1. Centers for Disease Control and Prevention (CDC), Fourth National Report on Human Exposure to Environmental Chemicals, (2014) 1–108. Available online: https:// www.cdc.gov/exposurereport/.
2. R.I.S. Alves, C.F. Sampaio, M. Nadal, M. Schuhmacher, J.L. Domingo, S.I. Segura munoz, Metal concentrations in surface water and sediments from Pardo River, Brazil: Human health risks, Environ. Res. 133 (2014) 149–155.
3. M.D. Angelica, Y. Fong, Environmental Toxicity and Poor Cognitive Outcomes in Children and Adults Jianghong, October. 141 (2014) 520–529.
4. M. Nadal, M. Schuhmacher, J.L. Domingo, Metal pollution of soils and vegetation in an area with petrochemical industry, Sci. Total Environ. 321 (2004) 59–69.
5. E. Fonfria, M.T. Vilaro, Z. Babot, E. Rodriguez-Farre, C. Sunol, Mercury compounds disrupt neuronal glutamate transport in cultured mouse cerebellar granule cells, J. Neurosci. Res. 79 (2005) 545–553.
6. A.P. Neal, Mechanisms of Heavy Metal Neurotoxicity: Lead and Manganese, J. Drug Metab. Toxicol. 06 (2015).
7. D. Caserta, A. Graziano, G. Lo Monte, G. Bordi, M. Moscarini, Heavy metals and placental fetal-maternal barrier: A mini-review on the major concerns, Eur. Rev. Med. Pharmacol. Sci. 17 (2013) 2198–2206.
8. X.D. Yu, C.H. Yan, X.M. Shen, Y. Tian, L.L. Cao, X.G. Yu, L. Zhao, J.X. Liu, Prenatal exposure to multiple toxic heavy metals and neonatal neurobehavioral development in Shanghai, China, Neurotoxicol. Teratol. 33 (2011) 437–443.
9. S. Chowdhury, M.A.J. Mazumder, O. Al-Attas, T. Husain, Heavy metals in drinking water: Occurrences, implications, and future needs in developing countries, Sci. Total Environ. 569–570 (2016) 476–488.
10. G. Tóth, T. Hermann, M.R. Da Silva, L. Montanarella, Heavy metals in agricultural soils of the European Union with implications for food safety, Environ. Int. 88 (2016) 299–309.
11. E. Ha, N. Basu, S. Bose-O'Rielly, J. Dorea, Current progress on understanding the impact of mercury on human health, Environ. Res. 152 (2017) 419–433.
12. T. Li, Y. Wan, Y. Ben, S. Fan, J. Hu, Relative importance of different exposure routes of heavy metals for humans living near a municipal solid waste incinerator, Environ. Pollut. 226 (2017) 385–393.

13. Z. Tang, M. Chai, J. Cheng, J. Jin, Y. Yang, Z. Nie, Q. Huang, Y. Li, Contamination and health risks of heavy metals in street dust from a coal-mining city in eastern China, Ecotoxicol. Environ. Saf. 138 (2017) 83–91.
14. Y. Xu, J.C. Khoury, H. Sucharew, K. Dietrich, K. Yolton, Low-level gestational exposure to mercury and maternal fish consumption: Associations with neurobehavior in early infancy, Neurotoxicol. Teratol. 54 (2016) 61–67.
15. S. Bolan, A. Kunhikrishnan, B. Seshadri, G. Choppala, R. Naidu, N.S. Bolan, Y.S. Ok, M. Zhang, C.G. Li, F. Li, B. Noller, M.B. Kirkham, Sources, distribution, bioavailability, toxicity, and risk assessment of heavy metal(loid)s in complementary medicines, Environ. Int. 108 (2017) 103–118.
16. E. Deshommes, R.C. Andrews, G. Gagnon, T. McCluskey, B. McIlwain, E. Doré, S. Nour, M. Prévost, Evaluation of exposure to lead from drinking water in large buildings, Water Res. 99 (2016) 46–55.
17. K. Khan, Y. Lu, H. Khan, M. Ishtiaq, S. Khan, M. Waqas, L. Wei, T. Wang, Heavy metals in agricultural soils and crops and their health risks in Swat District, northern Pakistan, Food Chem. Toxicol. 58 (2013).
18. M. Farina, D.S. Avila, J.B.T. Da Rocha, M. Aschner, Metals, oxidative stress and neurodegeneration: A focus on iron, manganese and mercury, Neurochem. Int. 62 (2013) 575–594.
19. E.B. Montgomery, Heavy metals and the etiology of Parkinson ’s disease and other movement disorders, Toxicology. 97 (1995) 3–9.
20. M.R. Basha, M. Murali, H.K. Siddiqi, K. Ghosal, O.K. Siddiqi, H. a Lashuel, Y.-W. Ge, D.K. Lahiri, N.H. Zawia, Lead (Pb) exposure and its effect on APP proteolysis and Abeta aggregation., FASEB J. 19 (2005) 2083–4.
21. A.B. Cholanians, A. V. Phan, E.J. Ditzel, T.D. Camenisch, S.S. Lau, T.J. Monks, Arsenic induces accumulation of α-synuclein: Implications for synucleinopathies and neurodegeneration, Toxicol. Sci. 153 (2016) 271–281.
22. C. Prakash, M. Soni, V. Kumar, Biochemical and Molecular Alterations Following Arsenic-Induced Oxidative Stress and Mitochondrial Dysfunction in Rat Brain, Biol. Trace Elem. Res. 167 (2015) 121–129.
23. M.C. Mauriello, C. Sbordone, P. Montuori, R. Alfano, M. Triassi, I. Iavicoli, M. Manno, Biomonitoring of toxic metals in incinerator workers: A systematic review, Toxicol. Lett. 272 (2017) 8–28.
24. R. Persoons, D. Arnoux, T. Monssu, O. Culié, G. Roche, B. Duffaud, D. Chalaye, A. Maitre, Determinants of occupational exposure to metals by gas metal arc welding and risk management measures: A biomonitoring study, Toxicol. Lett. 231 (2014) 135–141.
25. D.W. Anderson, W. Mettil, J.S. Schneider, Effects of low level lead exposure on associative learning and memory in the rat: Influences of sex and developmental timing of exposure, Toxicol. Lett. 246 (2016) 57–64.
26. C. Li, M. Xu, S. Wang, X. Yang, S. Zhou, J. Zhang, Q. Liu, Y. Sun, Lead exposure suppressed ALAD transcription by increasing methylation level of the promoter CpG islands, Toxicol. Lett. 203 (2011) 48–53.
27. Chaumont, M. Nickmilder, X. Dumont, T. Lundh, S. Skerfving, A. Bernard, Associations between proteins and heavy metals in urine at low environmental exposures: Evidence of reverse causality, Toxicol. Lett. 210 (2012) 345–352.
28. Y. Li, Y. Chen, H. Jiao, F. Zhou, G. Du, C. Feng, J. Yan, Z. Liu, X. Xiao, G. Fan, [Effect of lead exposure on brain iron in aged rats based on the MRI]., Wei Sheng Yan Jiu. 42 (2013) 33–38.
29. S.A.M.A.S. Eqani, R. Khalid, N. Bostan, Z. Saqib, J. Mohmand, M. Rehan, N. Ali, I.A. Katsoyiannis, H. Shen, Human lead (Pb) exposure via dust from different land use settings of Pakistan: A case study from two urban mountainous cities, Chemosphere. 155 (2016) 259–265.
30. M. Mari, M. Nadal, M. Schuhmacher, J.L. Domingo, Exposure to heavy metals and PCDD/Fs by the population living in the vicinity of a hazardous waste landfill in Catalonia, Spain: Health risk assessment, Environ. Int. 35 (2009) 1034–1039.
31. J.C. Ng, J. Wang, A. Shraim, A global health problem caused by arsenic from natural sources, Chemosphere. 52 (2003) 1353–1359.
32. Sarkar, B. Paul, The global menace of arsenic and its conventional remediation - A critical review, Chemosphere. 158 (2016) 37–49.
33. S. V. Jadhav, E. Bringas, G.D. Yadav, V.K. Rathod, I. Ortiz, K. V. Marathe, Arsenic and fluoride contaminated groundwaters: A review of current technologies for contaminants removal, J. Environ. Manage. 162 (2015) 306–325.

34. P. Mandal, An insight of environmental contamination of arsenic on animal health, Emerg. Contam. 3 (2017) 17–22.
35. R. Singh, S. Singh, P. Parihar, V.P. Singh, S.M. Prasad, Arsenic contamination, consequences and remediation techniques: A review, Ecotoxicol. Environ. Saf. 112 (2015) 247–270.
36. C.H. Tseng, Y.K. Huang, Y.L. Huang, C.J. Chung, M.H. Yang, C.J. Chen, Y.M. Hsueh, Arsenic exposure, urinary arsenic speciation, and peripheral vascular disease in blackfoot disease-hyperendemic villages in Taiwan, Toxicol. Appl. Pharmacol. 206 (2005) 299–308.
37. C.H. Wang, C.K. Hsiao, C.L. Chen, L.I. Hsu, H.Y. Chiou, S.Y. Chen, Y.M. Hsueh, M.M. Wu, C.J. Chen, A review of the epidemiologic literature on the role of environmental arsenic exposure and cardiovascular diseases, Toxicol. Appl. Pharmacol. 222 (2007) 315–326.
38. V.M. Rodríguez, M.E. Jiménez-Capdeville, M. Giordano, The effects of arsenic exposure on the nervous system, Toxicol. Lett. 145 (2003) 1–18.
39. J. Luo, Z. Qiu, W. Shu, Y. Zhang, L. Zhang, J. Chen, Effects of arsenic exposure from drinking water on spatial memory, ultra-structures and NMDAR gene expression of hippocampus in rats., Toxicol. Lett. 184 (2009) 121–125.
40. V.M. Rodriguez, L. Carrizales, M.S. Mendoza, O.R. Fajardo, M. Giordano, Effects of sodium arsenite exposure on development and behavior in the rat., Neurotoxicol. Teratol. 24 (2002) 743–750.
41. J. hua Luo, Z. qun Qiu, W. qun Shu, Y. yan Zhang, L. Zhang, J. an Chen, Effects of arsenic exposure from drinking water on spatial memory, ultra-structures and NMDAR gene expression of hippocampus in rats, Toxicol. Lett. 184 (2009) 121–125.
42. P.W. Davidson, J.J. Strain, G.J. Myers, S.W. Thurston, M.P. Bonham, C.F. Shamlaye, A. Stokes-Riner, J.M.W. Wallace, P.J. Robson, E.M. Duffy, L.A. Georger, J. Sloane-Reeves, E. Cernichiari, R.L. Canfield, C. Cox, L.S. Huang, J. Janciuras, T.W. Clarkson, Neurodevelopmental effects of maternal nutritional status and exposure to methylmercury from eating fish during pregnancy, Neurotoxicology. 29 (2008) 767–775.
43. K. Murata, P. Weihe, S. Araki, E. Budtz-JØrgensen, P. Grandjean, Evoked Potentials in Faroese Children Prenatally Exposed to Methylmercury, Neurotoxicol. Teratol. 21 (1999) 471–472.
44. S. Morais, F. Garcia, Heavy Metals and Human Health, (2010). Available online: cdn.intechopen.com.
45. S. Ceccatelli, E. Daré, M. Moors, Methylmercury-induced neurotoxicity and apoptosis, Chem. Biol. Interact. 188 (2010) 301–308.
46. Johansson, A.F. Castoldi, N. Onishchenko, L. Manzo, M. Vahter, S. Ceccatelli, Neurobehavioural and molecular changes induced by methylmercury exposure during development., Neurotox. Res. 11 (2007) 241–260.
47. K. Von Stackelberg, E. Guzy, T. Chu, B.C. Henn, Exposure to Mixtures of Metals and Neurodevelopmental Outcomes : A Multidisciplinary Review Using an Adverse Outcome Pathway Framework, Risk Anal. (2015).
48. W. Zheng, M. Aschner, J.F. Ghersi-Egea, Brain barrier systems: A new frontier in metal neurotoxicological research, Toxicol. Appl. Pharmacol. 192 (2003) 1–11.
49. R.O. Wright, A. Baccarelli, Metals and neurotoxicology., J. Nutr. 137 (2007) 2809–2813.
50. M. Aschner, T. Syversen, D.O. Souza, J.B.T. Rocha, M. Farina, Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity., Braz. J. Med. Biol. Res. 40 (2007) 285–291.
51. T. Sanders, Y. Liu, V. Buchner, P.B. Tchounwou, Neurotoxic effects and biomarkers of lead exposure: a review., Rev. Environ. Health. 24 (2009) 15–45.
52. C.R. Tyler, A.M. Allan, The Effects of Arsenic Exposure on Neurological and Cognitive Dysfunction in Human and Rodent Studies: A Review., Curr. Environ. Heal. Reports. 1 (2014) 132–147.
53. V. Karri, M. Schuhmacher, V. Kumar, Heavy metals (Pb, Cd, As and MeHg) as risk factors for cognitive dysfunction: A general review of metal mixture mechanism in brain, Environ. Toxicol. Pharmacol. 48 (2016) 203–213.
54. K. Krüger, H. Straub, A. V Hirner, J. Hippler, N. Binding, U. Muβhoff, Effects of monomethylarsonic and monomethylarsonous acid on evoked synaptic potentials in hippocampal slices of adult and young rats, Toxicol. Appl. Pharmacol. 236 (2009) 115–123.
55. Atchison, F. Hare, Mechansims of methyl mercury induced neurotoxicity. 8(1994) 622–629, Available online: http://www.fasebj.org/.

56. M. Farina, J.B.T. Rocha, M. Aschner, Mechanisms of methylmercury-induced neurotoxicity: evidence from experimental studies, Life Sci. 89 (2011) 555–563.
57. P.W. Ho, J.W. Ho, H.-F. Liu, D.H. So, Z.H. Tse, K.-H. Chan, D.B. Ramsden, S.-L. Ho, Mitochondrial neuronal uncoupling proteins: A target for potential disease-modification in Parkinson's disease, Transl. Neurodegener. 1 (2012) 3–12.
58. S. Sadiq, Z. Ghazala, A. Chowdhury, D. Büsselberg, Metal toxicity at the synapse: Presynaptic, postsynaptic, and long-term effects, J. Toxicol. 2012 (2012). .
59. S. Cuello, S. Ramos, Y. Madrid, J.L. Luque-Garcia, C. Camara, Differential protein expression of hepatic cells associated with MeHg exposure: Deepening into the molecular mechanisms of toxicity, Anal. Bioanal. Chem. 404 (2012) 315–324.
60. D.M.R. Han, M.R. Choi, K.H. Jung, H.T. Lee, J.H. Park, T. Ohn, Y.G. Chai, Proteomic analysis of the copper ion-induced stress response in a human embryonic carcinoma cell line., Int. J. Toxicol. 31 (2012) 397–406.
61. E. A. Soares, E.G. Werth, L.J. Madronero, J.A. Ventura, S.P. Rodrigues, L.M. Hicks, P.M.B. Fernandes, Label-free quantitative proteomic analysis of pre-flowering PMeV-infected Carica papaya L., J. Proteomics. 151 (2016).
62. Elamin, B. Titz, S. Dijon, C. Merg, M. Geertz, T. Schneider, F. Martin, W.K. Schlage, S. Frentzel, F. Talamo, B. Phillips, E. Veljkovic, N. V Ivanov, P. Vanscheeuwijck, M.C. Peitsch, J. Hoeng, Quantitative proteomics analysis using 2D-PAGE to investigate the effects of cigarette smoke and aerosol of a prototypic modified risk\ tobacco product on the lung proteome in C57BL/6 mice, J. Proteomics. 145 (2016) 237–245.
63. X. Liang, A. Feswick, D. Simmons, C.J. Martyniuk, Environmental toxicology and omics: A question of sex, J. Proteomics. 172 (2018) 152–164.
64. G. Xu, Z. Li, L. Wang, F. Chen, Z. Chi, M. Gu, S. li, D. Wu, J. Miao, Y. Zhang, L. Hao, Y. Fan, Label-free quantitative proteomics reveals differentially expressed proteins in high risk childhood acute lymphoblastic leukemia, J. Proteomics. 150 (2017) 1–8.
65. V. Karri, V. Kumar, D. Ramos, E. Oliveira, M. Schuhmacher, Comparative In Vitro Toxicity Evaluation of Heavy Metals (Lead, Cadmium, Arsenic, and Methylmercury) on HT-22 Hippocampal Cell Line, Biol. Trace Elem. Res. 184 (2018) 226–239.
66. S. Correa, C. Panis, R. Binato, A.C. Herrera, L. Pizzatti, E. Abdelhay, Identifying potential markers in Breast Cancer subtypes using plasma label-free proteomics, J. Proteomics. 151 (2016) 33–42.
67. N.P. Manes, A. Nita-Lazar, Application of targeted mass spectrometry in bottom-up proteomics for systems biology research, J. Proteomics. (2018).
68. S.A. Ali, G. Kaur, J.K. Kaushik, D. Malakar, A.K. Mohanty, S. Kumar, Examination of pathways involved in leukemia inhibitory factor (LIF)-induced cell growth arrest using label-free proteomics approach, J. Proteomics. 168 (2017) 37–52.
69. Titz, A. Elamin, F. Martin, T. Schneider, S. Dijon, N. V. Ivanov, J. Hoeng, M.C. Peitsch, Proteomics for systems toxicology, Comput. Struct. Biotechnol. J. 11 (2014) 73–90.
70. K. Niska, M.J. Santos-Martinez, M.W. Radomski, I. Inkielewicz-Stepniak, CuO nanoparticles induce apoptosis by impairing the antioxidant defense and detoxification systems in the mouse hippocampal HT22 cell line: Protective effect of crocetin, Toxicol. Vitr. 29 (2015) 663–671.
71. T. Mossmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65 (1983) 55–63.
72. A.C. Zambon, S. Gaj, I. Ho, K. Hanspers, K. Vranizan, C.T. Evelo, B.R. Conklin, A.R. Pico, N. Salomonis, GO-Elite: A flexible solution for pathway and ontology over-representation, Bioinformatics. 28 (2012) 2209–2210.
73. D.W. Huang, B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists, Nucleic Acids Res. 37 (2009) 1–13.
74. J. Ren, N. Williams, L. Clementi, S. Krishnan, W.W. Li, Opal web services for biomedical applications, Nucleic Acids Res. 38 (2010) 724–731.
75. Gang Su, H.J Morris, B. Demchak, and G.D. Bader, BIOLOGICAL NETWORK EXPLORATION WITH CYTOSCAPE 3. Curr Protoc Bioinformatics. 49 (2014), 1841-1850.

76. M. Kutmon, M.P. van Iersel, A. Bohler, T. Kelder, N. Nunes, A.R. Pico, C.T. Evelo, PathVisio 3: An Extendable Pathway Analysis Toolbox, PLoS Comput. Biol. 11 (2015) 1–13.
77. M. Kutmon, A. Riutta, N. Nunes, K. Hanspers, E.L. Willighagen, A. Bohler, J. Mélius, A. Waagmeester, S.R. Sinha, R. Miller, S.L. Coort, E. Cirillo, B. Smeets, C.T. Evelo, A.R. Pico, WikiPathways: capturing the full diversity of pathway knowledge, Nucleic Acids Res. 44 (2016) D488–D494.
78. Y. Hu, L. Su, E.T. Snow, Arsenic toxicity is enzyme specific and its affects on ligation are not caused by the direct inhibition of DNA repair enzymes, Mutat. Res. - DNA Repair. 408 (1998) 203–218.
79. M.E. Crespo-López, A. Lima de Sá, A.M. Herculano, R. Rodríguez Burbano, J.L. Martins do Nascimento, Methylmercury genotoxicity: A novel effect in human cell lines of the central nervous system, Environ. Int. 33 (2007) 141–146.
80. L. He, A.T. Poblenz, C.J. Medrano, D.A. Fox, Lead and calcium produce rod photoreceptor cell apoptosis by opening the mitochondrial permeability transition pore, J. Biol. Chem. 275 (2000) 12175–12184.
81. W.P. Roos, B. Kaina, DNA damage-induced cell death by apoptosis, Trends Mol. Med. 12 (2006) 440–450.
82. L.T. Ndountse, H.M. Chan, Methylmercury increases N-methyl-d-aspartate receptors on human SH-SY 5Y neuroblastoma cells leading to neurotoxicity, Toxicology. 249 (2008) 251–255.
83. Camon, The Gene Ontology Annotation (GOA) Database: sharing knowledge in Uniprot with Gene Ontology, Nucleic Acids Res. 32 (2004) 262–266.
84. Yadav, A. Kalita, S. Dhillon, K. Banerjee, JAK/STAT3 pathway is involved in survival of neurons in response to insulin-like growth factor and negatively regulated by suppressor of cytokine signaling-3, J. Biol. Chem. 280 (2005) 31830–31840.
85. F.A. Loucks, S.S. Le, A.K. Zimmermann, K.R. Ryan, H. Barth, K. Aktories, D.A. Linseman, Rho family GTPase inhibition reveals opposing effects of mitogen-activated protein kinase kinase/extracellular signal-regulated kinase and Janus kinase/signal transducer and activator of transcription signaling cascades on neuronal survival, J. Neurochem. 97 (2006) 957–967.
86. H. Kozlowski, A. Janicka-Klos, J. Brasun, E. Gaggelli, D. Valensin, G. Valensin, Copper, iron, and zinc ions homeostasis and their role in neurodegenerative disorders (metal uptake, transport, distribution and regulation), Coord. Chem. Rev. 253 (2009) 2665–2685.
87. S.A. Bellingham, D.K. Lahiri, B. Maloney, S. La Fontaine, G. Multhaup, J. Camakaris, Copper Depletion Down-regulates Expression of the Alzheimer's Disease Amyloid-β Precursor Protein Gene, J. Biol. Chem. 279 (2004) 20378–20386.
88. R. Castellani, K. Hirai, G. Aliev, K.L. Drew, A. Nunomura, A. Takeda, A.D. Cash, M.E. Obrenovich, G. Perry, M.A. Smith, Role of mitochondrial dysfunction in Alzheimer's disease, J. Neurosci. Res. 70 (2002) 357–360.
89. A.R. Esteves, J. Lu, M. Rodova, I. Onyango, E. Lezi, R. Dubinsky, K.E. Lyons, R. Pahwa, J.M. Burns, S.M. Cardoso, R.H. Swerdlow, Mitochondrial respiration and respiration-associated proteins in cell lines created through Parkinson's subject mitochondrial transfer, J. Neurochem. 113 (2010) 674–682.
90. S. Yee, B.H. Choi, 1996. Oxidative stress in neurotoxic effects of methylmercury poisoning. Neurotoxicology 17 (1996), 17–26.
91. Bosetti, F. Brizzi, S. Barogi, M. Mancuso, G. Siciliano, E. a Tendi, L. Murri, S.I. Rapoport, G. Solaini, Cytochrome c oxidase and mitochondrial F 1 F 0 -ATPase (ATP synthase) activities in platelets and brain from patients with Alzheimer' s disease, Neurobiol. Aging. 23 (2002) 371–376.
92. M. Orth, A. H. V Schapira, Mitochondrial involvement in Parkinson's disease, Neurochem. Internatinational. 40 (2002) 533–541.
93. J. Valla, J. D. Berndt, F. Gonzalez-Lima, Energy hypometabolism in posterior cingulate cortex of Alzheimer's patients: superficial laminar cytochrome oxidase associated with disease duration. J. Neurosci. 21 (2001) 4923–4930.
94. E Lezi and Russell H. Swerdlow, Advances in Mitochondrial Medicine, 942 (2012) 269–286. Available online: http://www.springer.com/gp/book/9789400728684.

95. M.F. Beal, E. Brouillet, B.G. Jenkins, R.J. Ferrante, N.W. Kowall, J.M. Miller, E. Storey, R. Srivastava, B.R. Rosen, B.T. Hyman, Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid., J. Neurosci. 13 (1993) 4181–4192.
96. M.T. Lin, M.F. Beal, Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, Nature. 443 (2006) 787–795.
97. X. Wang, B. Su, H. Lee, X. Li, G. Perry, M.A. Smith, Impaired Balance of Mitochondria Fission and Fusion in Alzheimer Disease, J. Neurosci. Res. 29 (2010) 9090–9103.
98. K. Nakamura, V.M. Nemani, F. Azarbal, G. Skibinski, J.M. Levy, K. Egami, L. Munishkina, J. Zhang, B. Gardner, J. Wakabayashi, H. Sesaki, Y. Cheng, S. Finkbeiner, R.L. Nussbaum, E. Masliah, R.H. Edwards, Direct Membrane Association Drives Mitochondrial Fission by the Parkinson Disease-associated Protein α-Synuclein, J. Biol. Chem. 286 (2011) 20710–20726.
99. S.S. Jossan, P.G. Gillberg, C.G. Gottfries, I. Karlsson, L. Oreland, Monoamine oxidase B in brains from patients with Alzheimer's disease: A biochemical and autoradiographical study, Neuroscience. 45 (1991) 1–12.
100. P. Beyrouty, C.J. Stamler, J.N. Liu, K.M. Loua, S. Kubow, H.M. Chan, Effects of prenatal methylmercury exposure on brain monoamine oxidase activity and neurobehaviour of rats, Neurotoxicol. Teratol. 28 (2006) 251–259.
101. R.S. Yadav, R.K. Shukla, M.L. Sankhwar, D.K. Patel, R.W. Ansari, A.B. Pant, F. Islam, V.K. Khanna, Neuroprotective effect of curcumin in arsenic-induced neurotoxicity in rats., Neurotoxicology. 31 (2010) 533–539.
102. J.C. Yin, M. Del Vecchio, H. Zhou, T. Tully, CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in Drosophila., Cell. 81 (1995) 107–115.
103. L. Emisson, P. Saetre, J. Balciuniene, A. Castensson, N. Cairns, E.E. Jazin, Increased monoamine oxidase messenger RNA expression levels in frontal cortex of Alzheimer ' s disease patients, 326 (2002) 56–60.
104. D.A. Butterfield, S.M. Yatin, S. Varadarajan, T. Koppal, Amyloid β-peptide-associated free radical oxidative stress, neurotoxicity, and Alzheimer's disease, Methods Enzymol. 309 (1999) 746–768.
105. M. Aschner, J.L. Aschner, Mercury neurotoxicity: mechanisms of blood-brain barrier transport., Neurosci. Biobehav. Rev. 14 (1990) 169–176.
106. L. Chang, M. Karin, Mammalian MAP kinase signalling cascades, Nature. 410 (2001) 37–40.
107. T. Suzuki, S. Shishido, N. Ishihara, Different behaviour of inorganic and organic mercury in renal excretion with reference to effects of D-penicillamine., Br. J. Ind. Med. 33 (1976) 88–91.
108. S. Cuello, L. Goya, Y. Madrid, S. Campuzano, M. Pedrero, L. Bravo, C. Cámara, S. Ramos, Molecular mechanisms of methylmercury-induced cell death in human HepG2 cells, Food Chem. Toxicol. 48 (2010) 1405–1411.
109. N. El-Najjar, M. Chatila, H. Moukadem, H. Vuorela, M. Ocker, M. Gandesiri, R. Schneider-Stock, H. Gali-Muhtasib, Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling, Apoptosis. 15 (2010) 183–195.
110. E.K. Kim, E.-J. Choi, Pathological roles of MAPK signaling pathways in human diseases, Biochim. Biophys. Acta - Mol. Basis Dis. 1802 (2010) 396–405.
111. A.I. Bush, Metals and neuroscience, Curr. Opin. Chem. Biol. 4 (2000) 184–191.
112. S.V.S. Rana, Metals and apoptosis: Recent developments, J. Trace Elem. Med. Biol. 22 (2008) 262–284.
113. T.H. Lu, C.H. Chen, M.J. Lee, T.J. Ho, Y.M. Leung, D.Z. Hung, C.C. Yen, T.Y. He, Y.W. Chen, Methylmercury chloride induces alveolar type II epithelial cell damage through an oxidative stress-related mitochondrial cell death pathway, Toxicol. Lett. 194 (2010) 70–78.
114. Shanker, J.L. Aschner, T. Syversen, M. Aschner, Free radical formation in cerebral cortical astrocytes in culture induced by methylmercury, Mol. Brain Res. 128 (2004) 48–57.
115. Z. Yin, D. Milatovic, J.L. Aschner, T. Syversen, J.B.T. Rocha, D.O. Souza, M. Sidoryk, J. Albrecht, M. Aschner, Methylmercury induces oxidative injury, alterations in permeability and glutamine transport in cultured astrocytes, Brain Res. 1131 (2007) 1–10.

116. T.H. Lu, S.Y. Hsieh, C.C. Yen, H.C. Wu, K.L. Chen, D.Z. Hung, C.H. Chen, C.C. Wu, Y.C. Su, Y.W. Chen, S.H. Liu, C.F. Huang, Involvement of oxidative stress-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic signals in methylmercury-induced neuronal cell injury, Toxicol. Lett. 204 (2011) 71–80.
117. N.P. Dantuma, L.C. Bott, The ubiquitin-proteasome system in neurodegenerative diseases: precipitating factor, yet part of the solution, Front. Mol. Neurosci. 7 (2014) 1–18.
118. K.S.P. McNaught, C.W. Olanow, B. Halliwell, O. Isacson, P. Jenner, Failure of the ubiquitin–proteasome system in Parkinson's disease, Nat. Rev. Neurosci. 2 (2001) 589–594.
119. K.S.P. McNaught, C.W. Olanow, Schapira, Jenner, Isacson, Hunot, Tatton, Beal, Proteolytic stress: A unifying concept for the etiopathogenesis of Parkinson's disease, Ann. Neurol. 53 (2003) 73–86.
120. C.A.P. Joazeiro, T. Hunter, Ubiquitination--More Than Two to Tango Ubiquitination--More Than Two to Tango, Science. 289 (2000) 2061-2062.
121. R. Setsuie, K. Wada, The functions of UCH-L1 and its relation to neurodegenerative diseases, Neurochem. Int. 51 (2007) 105–111.
122. A, Castegna, M. Aksenov, V. Thongboonkerd, J.B. Klein, W.M. Pierce, R. Booze, W.R. Markesbery, D.A. Butterfield, Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71., J. Neurochem. 82 (2002) 1524–32.
123. E.J.N. Groen, T.H. Gillingwater, UBA1: At the Crossroads of Ubiquitin Homeostasis and Neurodegeneration, Trends Mol. Med. 21 (2015) 622–632.
124. T.M. Dawson, L. Valina, Molecular Pathways of Neurodegeneration in Parkinson's disease, 302 (2003) 819–823.
125. T. Furuchi, G.-W. Hwang, A. Naganuma, Overexpression of the ubiquitin-conjugating enzyme Cdc34 confers resistance to methylmercury in Saccharomyces cerevisiae., Mol. Pharmacol. 61 (2002) 738–741.
126. G.W. Hwang, T. Furuchi, A. Naganuma, A ubiquitin-proteasome system is responsible for the protection of yeast and human cells against methylmercury, FASEB J. 16 (2002) 709–711.
127. M.E. Figueiredo-Pereira, G. Cohen, The ubiquitin/proteasome pathway: friend or foe in zinc-, cadmium-, and H2O2-induced neuronal oxidative stress, Mol. Biol. Rep. 26 (1999) 65–69.
128. Y. Di, M.J. Tamás, Regulation of the arsenic-responsive transcription factor Yap8p involves the ubiquitin-proteasome pathway, J. Cell Sci. 120 (2007) 256–264.
129. M.E. Figueiredo-PEreira, S. Yakushin, G. Cohen, Disruption of the intracellular sulfhydryl homeostasis by cadmium-induced apoptosis in human promonocytic cells., J. Biol. Chem. 273 (1998) 12703–12709.
130. Stanhill, C.M. Haynes, Y. Zhang, G. Min, M.C. Steele, J. Kalinina, E. Martinez, C.M. Pickart, X.P. Kong, D. Ron, An Arsenite-Inducible 19S Regulatory Particle-Associated Protein Adapts Proteasomes to Proteotoxicity, Mol. Cell. 23 (2006) 875–885.
131. D.S. Kirkpatrick, K. V. Dale, J.M. Catania, A.J. Gandolfi, Low-level arsenite causes accumulation of ubiquitinated proteins in rabbit renal cortical slices and HEK293 cells, Toxicol. Appl. Pharmacol. 186 (2003) 101–109.
132. Stewart, E. Killeen, R. Naquin, S. Alam, J. Alam, Degradation of Transcription Factor Nrf2 via the Ubiquitin-Proteasome Pathway and Stabilization by Cadmium, J. Biol. Chem. 278 (2003) 2396–2402.
133. Daguenet, G. Dujardin, J. Valcárcel, The pathogenicity of splicing defects: mechanistic insights into pre-mRNA processing inform novel therapeutic approaches, EMBO Rep. 16 (2015) 1640-1655.
134. B.R. Graveley, Alternative splicing: Increasing diversity in the proteomic world, Trends Genet. 17 (2001) 100–107.
135. O. Kelemen, P. Convertini, Z. Zhang, Y. Wen, M. Shen, M. Falaleeva, S. Stamm, Function of alternative splicing, Gene. 514 (2013) 1–30.
136. J.M. Johnson, Genome-Wide Survey of Human Alternative Pre-mRNA Splicing with Exon Junction Microarrays, Science. 302 (2003) 2141–2144.
137. N.A. Faustino, T. a Cooper, N. Andre, Pre-mRNA splicing and human disease, Genes Dev. 17 (2003) 419–437.

138. C.M. Hales, E.B. Dammer, I. Diner, H. Yi, N.T. Seyfried, M. Gearing, J.D. Glass, T.J. Montine, A.I. Levey, J.J. Lah, Aggregates of Small Nuclear Ribonucleic Acids (snRNAs) in Alzheimer's Disease, Brain Pathol. 24 (2014) 344–351.
139. J. E. Love, E.J. Hayden, T.T. Rohn Alternative Splicing in Alzheimer's Disease, J. Park. Dis. Alzheimer's Dis. 2 (2015) 2–7.
140. V. La Cognata, V. D'Agata, F. Cavalcanti, S. Cavallaro, Splicing: is there an alternative contribution to Parkinson's disease? Neurogenetics. 16 (2015) 245–263.
141. R.H. Fu, S.-P. Liu, S.-J. Huang, H.-J. Chen, P.-R. Chen, Y.-H. Lin, Y.-C. Ho, W.-L. Chang, C.-H. Tsai, W.-C. Shyu, S.-Z. Lin, Aberrant alternative splicing events in Parkinson's disease, Cell Transplant. 22 (2013) 653–661.
142. N. L. Inguglia, J. Walters, P. Picone, M. Di Carlo, A Shotgun Proteomics Approach Reveals a New Toxic Role for Alzheimer's Disease Aβ Peptide: Spliceosome Impairment, J. Proteome Res. 16 (2017) 1526–1541.
143. B. Bai, C.M. Hales, P. Chen, Y. Gozal, E. B. Dammer, J.J Fritz, et al. U1 small nuclear ribonucleoprotein complex and RNA splicing alterations in Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 110 (2013), 16562–16567.
144. Y. Jia, J.C. Mu, S.L. Ackerman, Mutation of a U2 snRNA Gene Causes Global Disruption of Alternative Splicing and Neurodegeneration, Cell. 148 (2012) 296–308.
145. Y. Lee, D.C. Rio, S. Biology, C. Biology, Mechanisms and Regulation of Alternative Pre-mRNA Splicing, Annu.Rev. Biochem. (2015) 291–323.
146. W. Qian, F. Liu, Regulation of alternative splicing of tau exon 10, Neurosci. Bull. 30 (2014) 367–377.
147. T. Geuens, D. Bouhy, V. Timmerman, The hnRNP family: insights into their role in health and disease, Hum. Genet. 135 (2016) 851–867.
148. J. Wong, Altered expression of RNA splicing proteins in Alzheimer's disease patients: evidence from two microarray studies, Dement Geriatr Cogn Dis Extra. 3 (2013) 74–85.
149. B.K. Dredge, A.D. Polydorides, R.B. Darnell, The splice of life: Alternative splicing and neurological disease, Nat. Rev. Neurosci. 2 (2001) 43–50.
150. Ashok, N.K. Rai, S. Tripathi, S. Bandyopadhyay, Exposure to As, Cd, and Pb-Mixture Induces A , Amyloidogenic APP Processing and Cognitive Impairments via Oxidative Stress-Dependent Neuroinflammation in Young Rats, Toxicol. Sci. 143 (2015) 64–80.
151. M. Grammer, S. Kuchay, A. Chishti, M. Baudry, Lack of phenotype for LTP and fear conditioning learning in calpain 1 knock-out mice, Neurobiol. Learn. Mem. 84 (2005) 222–227.
152. M.B. Bevers, E. Lawrence, M. Maronski, N. Starr, M. Amesquita, R.W. Neumar, Knockdown of m-calpain increases survival of primary hippocampal neurons following NMDA excitotoxicity, J. Neurochem. 108 (2009) 1237–1250.
153. B.Z. R. Deane, R.D Bell, A Sagare, Clearance of amyloid-β peptide across the blood-brain barrier: Implication for therapies in Alzheimer's disease, CNS Neurol Disord Drug Targets. 8 (2009) 16–30.
154. Y. Chen, D.L. McPhie, J. Hirschberg, R.L. Neve, The amyloid precursor protein-binding protein APP-BP1 drives the cell cycle through the S-M checkpoint and causes apoptosis in neurons, J. Biol. Chem. 275 (2000) 8929–8935.
155. Y. Chen, R. Neve, H. Zheng, W. Griffin, S. Barger, R. Mrak, Cycle on Wheels: Is APP Key to the AppBp1 Pathway? Austin Alzheimers Parkinsons Dis 27 (2015), 320–331.
156. McLaughlin, K.A. Hartnett, J.A. Erhardt, J.J. Legos, R.F. White, F.C. Barone, E. Aizenman, Caspase 3 activation is essential for neuroprotection in preconditioning, Proc Natl Acad Sci U S A. 100 (2003) 715–720.
157. M.P. Mattson, J.N. Keller, J.G. Begley, Evidence for Synaptic Apoptosis, Exp. Neurol. 153 (1998) 35–48.
158. T.T. Rohn, E. Head, W.H. Nesse, C.W. Cotman, D.H. Cribbs, Activation of Caspase-8 in the Alzheimer's Disease Brain, Neurobiol. Dis. 8 (2001) 1006–1016.
159. T. Nishioku, N. Takai, K. Miyamoto, K. Murao, C. Hara, K. Yamamoto, H. Nakanishi, Involvement of caspase 3-like protease in methylmercury-induced apoptosis of primary cultured rat cerebral microglia, Brain Res. 871 (2000) 160–164.

160. Y. Akao, Y. Nakagawa, K. Akiyama, Arsenic trioxide induces apoptosis in neuroblastoma cell lines through the activation of caspase 3 in vitro, FEBS Lett. 455 (1999) 59–62.
161. J. Xu, L.D. Ji, L.H. Xu, Lead-induced apoptosis in PC 12 cells: Involvement of p53, Bcl-2 family and caspase-3, Toxicol. Lett. 166 (2006) 160–167.
162. T. Kim, Alternative Cleavage of Alzheimer-Associated Presenilins During Apoptosis by a Caspase-3 Family Protease, Science 277 (1997) 373–376.
163. C.W. Chung, Y.M. Hong, S. Song, H.N. Woo, Y.H. Choi, T. Rohn, Y.K. Jung, Atypical role of proximal caspase-8 in truncated Tau-induced neurite regression and neuronal cell death, Neurobiol. Dis. 14 (2003) 557–566.
164. R.A. Rissman, W.W. Poon, M. Blurton-Jones, S. Oddo, R. Torp, M.P. Vitek, F.M. LaFerla, T.T. Rohn, C.W. Cotman, Caspase-cleavage of tau is an early event in Alzheimer disease tangle pathology, J. Clin. Invest. 114 (2004) 121–130.
165. K. Hirai, G. Aliev, A. Nunomura, H. Fujioka, R.L. Russell, C.S. Atwood, A.B. Johnson, Y. Kress, H. V Vinters, M. Tabaton, S. Shimohama, A.D. Cash, S.L. Siedlak, P.L.R. Harris, P.K. Jones, R.B. Petersen, G. Perry, M.A. Smith, Mitochondrial Abnormalities in Alzheimer' s Disease, 21 (2001) 3017–3023.
166. I.G. Onyango, J. Dennis, S.M. Khan, Mitochondrial Dysfunction in Alzheimer's Disease and the Rationale for Bioenergetics Based Therapies, Aging Dis. 7 (2016) 201-214.
167. M. Manczak, B.S. Park, Y. Jung, P.H. Reddy, Differential expression of oxidative phosphorylation genes in patients with Alzheimer's disease: implications for early mitochondrial dysfunction and oxidative damage, Neuromolecular Med. 5 (2004) 147–162.
168. P.H. Reddy, Amyloid beta, mitochondrial structural and functional dynamics in Alzheimer's disease, Exp Neurol. 218 (2009) 1–15.
169. M. Manczak, T.S. Anekonda, E. Henson, B.S. Park, J. Quinn, P.H. Reddy, Mitochondria are a direct site of Aβ accumulation in Alzheimer's disease neurons: Implications for free radical generation and oxidative damage in disease progression, Hum. Mol. Genet. 15 (2006) 1437–1449.
170. Liu, W. Huang, R.D. Moir, C.R. Vanderburg, B. Lai, Z. Peng, R.E. Tanzi, J.T. Rogers, X. Huang, Metal exposure and Alzheimer's pathogenesis, J. Struct. Biol. 155 (2006) 45–51.
171. A.R. White, R. Reyes, J.F. Mercer, J. Camakaris, H. Zheng, A.I. Bush, G. Multhaup, K. Beyreuther, C.L. Masters, R. Cappai, Copper levels are increased in the cerebral cortex and liver of APP and APLP knockout mice, Brain Res. 2 (1999) 439–444.
172. C.S. Atwood, R.D. Moir, R.C. Scarpa, N.E. Michael, D.M. Romano, a Mariana, R.E. Tanzi, I. Ashley, X. Huang, N.M.E. Bacarra, M. a Hartshorn, A.I. Bush, Dramatic aggregation of alzheimer A(by Cu (II) is induced by conditions representing physiological acidosis. J Biol Chem. 273(1998), 12817–12826.
173. B.C.L. Lai, S.A. Marion, K. Teschke, J.K.C. Tsui, Occupational and environmental risk factors for Parkinson's disease, Parkinsonism Relat. Disord. 8 (2002) 297–309.
174. Ding, K.M. Chen, H.W. Ling, F. Sun, X. Li, T. Wan, W.M. Chai, H. Zhang, Y. Zhan, Y.J. Guan, Correlation of iron in the hippocampus with MMSE in patients with Alzheimer's disease, J. Magn. Reson. Imaging. 29 (2009) 793–798.
175. C.S. Atwood, R.C. Scarpa, X. Huang, R.D. Moir, W.D. Jones, D.P. Fairlie, R.E. Tanzi, A.I. Bush, Characterization of Copper Interactions with Alzheimer Amyloid β Peptides, J. Neurochem. 75 (2008) 1219–1233.
176. X. Huang, C.S. Atwood, M.A. Hartshorn, G. Multhaup, L.E. Goldstein, R.C. Scarpa, M.P. Cuajungco, D.N. Gray, J. Lim, R.D. Moir, R.E. Tanzi, A.I. Bush, The A β Peptide of Alzheimer ' s Disease Directly Produces Hydrogen Peroxide through Metal Ion Reduction Accelerated Publications The A Peptide of Alzheimer ' s Disease Directly Produces Hydrogen Peroxide through Metal Ion Reduction, 38 (1999). 7609–7616
177. J. Wong, Altered Expression of RNA Splicing Proteins in Alzheimer's disease Patients: Evidence from Two Microarray Studies, Dement. Geriatr. Cogn. Dis. Extra. 3 (2013) 74–85.
178. C. Caspersen et al, Mitochondrial Aβ: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease, FASEB J. 19 (2005) 2040–2041.
179. G.E. Gibson, K.F.R. Sheu, J.P. Blass, Abnormalities of mitochondrial enzymes in Alzheimer disease, J. Neural Transm. 105 (1998) 855–870.

180. D.J. Moore, V.L. Dawson, T.M. Dawson, Role for the Ubiquitin-Proteasome System in Parkinson's Disease and Other Neurodegenerative Brain Amyloidoses, NeuroMolecular Med. 4 (2003) 95–108.
181. J. Blackinton, M. Lakshminarasimhan, K.J. Thomas, R. Ahmad, E. Greggio, A.S. Raza, M.R. Cookson, M.A. Wilson, Formation of a stabilized cysteine sulfinic acid is critical for the mitochondrial function of the parkinsonism protein DJ-1, J. Biol. Chem. 284 (2009) 6476–6485.
182. R. Tofighi, C. Johansson, M. Goldoni, W.N.W. Ibrahim, V. Gogvadze, A. Mutti, S. Ceccatelli, Hippocampal neurons exposed to the environmental contaminants methylmercury and polychlorinated biphenyls undergo cell death via parallel activation of calpains and lysosomal proteases, Neurotox. Res. 19 (2011) 183–194.
183. K.G. Shadrach, M.E. Rayborn, J.G. Hollyfield, V.L. Bonilha, DJ-1-Dependent Regulation of Oxidative Stress in the Retinal Pigment Epithelium (RPE), PLoS One. 8 (2013) e67983.
184. J.C. Fitzgerald, H. Plun-Favreau, Emerging pathways in genetic Parkinson's disease: Autosomal-recessive genes in Parkinson's disease - A common pathway?, FEBS J. 275 (2008) 5758–5766.
185. R. Requejo-Aguilar, I. Lopez-Fabuel, D. Jimenez-Blasco, E. Fernandez, A. Almeida, DJ1 represses glycolysis and cell proliferation by transcriptionally upregulating pink1, 36 (2016) 1011–1014.
186. J. N. Groen, T. H. Gillingwater, UBA1: At the Crossroads of Ubiquitin Homeostasis and Neurodegeneration. Trends Mol. Med. 21 (2015.), 622–632.
187. K. Tanaka, H. Shimura, N. Hattori, S. Kubo, Y. Mizuno, S. Asakawa, S. Minoshima, N. Shimizu, K. Iwai, T. Chiba, T. Suzuki, No Title, Nat. Genet. 25 (2000) 302–305.
188. J. Park, S.B. Lee, S. Lee, Y. Kim, S. Song, S. Kim, E. Bae, J. Kim, M. Shong, J.-M. Kim, J. Chung, Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin, Nature. 441 (2006) 1157–1161.
189. E.K. Tan, H. Shen, J.M.M. Tan, K.L. Lim, S. Fook-Chong, W.P. Hu, M.C. Paterson, V.R. Chandran, K. Yew, C. Tan, Y. Yuen, R. Pavanni, M.C. Wong, K. Puvan, Y. Zhao, Differential expression of splice variant and wild-type parkin in sporadic Parkinson's disease, Neurogenetics. 6 (2005) 179–184.
190. K. Kalinderi, S. Bostantjopoulou, L. Fidani, The genetic background of Parkinson's disease: current progress and future prospects, Acta Neurol. Scand. 134 (2016) 314–326.
191. L. Scott, V.L. Dawson, T.M. Dawson, Trumping neurodegeneration: Targeting common pathways regulated by autosomal recessive Parkinson's disease genes, Exp. Neurol. 298 (2017) 191–201.
192. D.J. Moore, V.L. Dawson, T.M. Dawson, Role for the ubiquitin-proteasome system in Parkinson's disease and other neurodegenerative brain amyloidoses. Neuromolecular Med. 4 (2003), 95–108.
193. M.F. Beal, E. Brouillet, B.G. Jenkins, R.J. Ferrante, N.W. Kowall, J.M. Miller, E. Storey, R. Srivastava, B.R. Rosen, B.T. Hyman, Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J. Neurosci. 13 (1993), 4181–4192.
194. C. van der Merwe, Z. Jalali Sefid Dashti, A. Christoffels, B. Loos, S. Bardien, Evidence for a common biological pathway linking three Parkinson's disease-causing genes: Parkin, PINK1 and DJ-1, Eur. J. Neurosci. 41 (2015) 1113–1125.
195. G.U. Hoglinger, G. Carrard, P.P. Michel, F. Medja, A. Lombès, M. Ruberg, B. Friguet, E.C. Hirsch, Dysfunction of mitochondrial complex I and the proteasome: interactions between two biochemical deficits in a cellular model of Parkinson's disease, J. Neurochem. 86 (2003) 1297–1307.
196. N. Jha, M.J. Kumar, R. Boonplueang, J.K. Andersen, Glutathione decreases in dopaminergic PC12 cells interfere with the ubiquitin protein degradation pathway: relevance for Parkinson's disease?, J. Neurochem. 80 (2002) 555–561.
197. J.N. Keller, E. Dimayuga, Q. Chen, J. Thorpe, J. Gee, Q. Ding, Autophagy, proteasomes, lipofuscin, and oxidative stress in the aging brain, Int. J. Biochem. Cell Biol. 36 (2004) 2376–2391.
198. H.-J. Lee, S.Y. Shin, C. Choi, Y.H. Lee, S.-J. Lee, Formation and Removal of α-Synuclein Aggregates in Cells Exposed to Mitochondrial Inhibitors, J. Biol. Chem. 277 (2002) 5411–5417.
199. Shang, A. Taylor, Ubiquitin-proteasome pathway and cellular responses to oxidative stress, Free Radic. Biol. Med. 51 (2011) 5–16.