Quantitative Microproteomics Based Characterization of the Central and Peripheral Nervous System of a Mouse Model of Krabbe Disease

Davide Pellegrini$^{1,2}$, Ambra del Grosso$^{1,3}$, Lucia Angella$^3$, Nadia Giordano$^4$, Marialaura Dilillo$^2$, Ilaria Tonazzini$^3$, Matteo Caleo$^4$, Marco Cecchini$^{1,3}$, Liam A. McDonnell$^{2,5}$$^*$

$^1$ NEST, Scuola Normale Superiore, Pisa 56127, Italy.

$^2$ Fondazione Pisana per la Scienza ONLUS, 56107 San Giuliano Terme, Pisa, Italy

$^3$ NEST, Istituto Nanoscienze-CNR, Pisa, Italy

$^4$ Neuroscience Institute-CNR, Pisa, Italy

$^5$ Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands

* Corresponding author:

Liam A. McDonnell, Tel. +39 050 8753547; l.a.mcdonnell@outlook.com

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## Abbreviations

| Abbr. | Definition |
|-------|------------|
| AGC: | Automatic Gain Control |
| BH: | Benjamini-Hochburg |
| CNS: | Central Nervous System |
| FDR: | False Discovery Rate |
| GAGs | Glycosaminoglycans |
| Gal-cer: | Galactosylceramide |
| GO: | Gene Ontology |
| HCD: | Higher-energy collisional dissociation |
| HSCT: | Hematopoietic stem cell transplantation |
| IAM: | Iodoacetamide |
| KD: | Krabbe disease |
| LCD | Lysosomal cell death |
| LCM: | Laser Capture Microdissection |
| LMP | Lysosome membrane permeabilization |
| LSD: | Lysosomal Storage Disorder |
| NCE: | Normalized collision energy |
| ORA: | Overrepresentation Enrichment Analysis |
| PCA: | Principal Component Analysis |
| PNS: | Peripheral Nervous System |
| Abbreviation | Definition |
|--------------|------------|
| PSMs         | Peptide Spectral Matches |
| PSY          | Psychosine |
| ROIs         | Region of Interest |
| ROS          | Reactive oxygen species |
| RPS          | Reversed phase S |
| SP3          | Single-Pot Solid-Phase-enhanced Sample Preparation |
| SPS          | Synchronous precursor selection |
| TFE          | Trifluoroethanol |
| TMT          | Tandem Mass Tag |
| TWI          | Twitcher |
| WT           | Wild-type |
Abstract

Krabbe disease is a rare, childhood lysosomal storage disorder caused by a deficiency of galactosylceramide beta-galactosidase (GALC). The major effect of GALC deficiency is the accumulation of psychosine in the nervous system and widespread degeneration of oligodendrocytes and Schwann cells, causing rapid demyelination. The molecular mechanisms of Krabbe disease are not yet fully elucidated and a definite cure is still missing.

Here we report the first in-depth characterization of the proteome of the Twitcher mouse, a spontaneous mouse model of Krabbe disease, to investigate the proteome changes in the Central and Peripheral Nervous System. We applied a TMT-based workflow to compare the proteomes of the corpus callosum, motor cortex and sciatic nerves of littermate homozygous Twitcher and wild-type mice. More than 400 protein groups exhibited differences in expression and included proteins involved in pathways that can be linked to Krabbe disease, such as inflammatory and defense response, lysosomal proteins accumulation, demyelination, reduced nervous system development and cell adhesion. These findings provide new insights on the molecular mechanisms of Krabbe disease, representing a starting point for future functional experiments to study the molecular pathogenesis of Krabbe disease. Data are available via ProteomeXchange with identifier PXD010594.
Krabbe disease (KD), also known as globoid cell leukodystrophy, is a rare autosomal recessive sphingolipidosis and is one of a larger group of lysosomal storage disorders (LSDs). KD is a neurodegenerative disorder and occurs due to mutations in the β-galactocerebrosidase gene (galc). These mutations lead to a reduced, or loss of, activity of the encoded enzyme (β-galactocerebrosidase, GALC), leading to a disruption of myelin turnover in the central nervous system (CNS) and peripheral nervous system (PNS) [1][2].

The incidence of KD in the US is 1:100,000 and in 95% of cases onset occurs within the first 6 months of life (infantile KD) [3][4]. Patients develop progressive blindness, ataxia and psychomotor regression, with death typically occurring within 2 years [5][6]. Late onset KD (late infantile, late juvenile and adult form) is characterized by milder progression and severity [7][3]. Currently there are neither long-term therapies nor a definitive cure for KD. Hematopoietic stem cell transplantation (HSCT) is the only treatment available and has been shown to slow the course of the disease in pre-symptomatic infantile patients [8]. Additional treatment strategies, including gene therapy, substrate reduction therapy, chemical chaperones and enzyme replacement therapy, are currently under investigation in animal models of KD [9][10][11][12][13][14]. The Twitcher mouse is the most common animal model of human KD, as it shows similar clinical and histopathological features [15].

The “psychosine hypothesis” is the established explanation for KD pathogenesis [16]. Defective GALC leads to impaired degradation of the two glycolipids: galactosylceramide (Gal-cer), the primary substrate of GALC, and psychosine (PSY) [17]. Whereas Gal-cer is also degraded by GM1 ganglioside β-galactosidase [18], there is not another degradation pathway for PSY. PSY is
a cytotoxic sphingolipid that is produced by galactosylation of sphingosine by ceramide galactosyltransferase (CGT or UGT8). CGT is mainly expressed in myelinating cells and so in KD psychosine accumulates in oligodendrocytes and Schwann cells and is believed to be the main cause of demyelination [19][20]. The pathophysiological effect is not limited to oligodendrocytes and Schwann cells death, but includes also other cell types of the nervous system because the consequent accumulation of myelin debris triggers an inflammatory response with astrocytosis and microgliosis [8][17][21]. Furthermore it has been shown that PSY accumulates in membrane microdomains affecting lipid rafts and signaling pathways [22][23][24].

Metabolic profiling of the Twitcher mouse has identified metabolic pathways influenced by KD, revealing decreased levels of long chain fatty acids, increased levels of short chain fatty acids and alteration of several metabolites involved in mitochondrial fuel selection, energy production, inflammation, neurotransmitter metabolism and osmotic regulation [25][26][27][28]. However, the proteome changes associated with KD and the pathogenic mechanisms are still not well understood.

Mass-spectrometry based proteomics has become an indispensable tool for molecular biology and clinical research because of its ability to identify and quantify thousands of proteins [29][30][31]. When combined with laser capture microdissection (LCM), MS-based proteomics may be used to investigate disease-associated changes in the proteome of specific tissue regions or cell populations. Such specificity is essential because different anatomical regions in the brain have distinct and diverse functions, and may behave differently under pathological conditions [32]. Strict anatomical/cellular specification usually yields micrograms or submicrograms
amounts of proteins [33][34], and thus ultrasensitive microproteomics protocols are required to analyze these small sample amounts while maintaining high proteome coverage [35][36][37]. MS-based proteomics has been used to study several LSDs, including Niemann-Pick type C disease [38][39][40], Gaucher disease [41][42] and Fabry disease [43][44]. Mass spectrometry has been extensively used to quantify psychosine in several tissues and cells used to study KD, including the Twitcher mouse brain [22], spinal cord [45], serum [46], newborn dried blood spots from infants [47] and a cell model of KD derived from the Twitcher mouse [48]. However, to date an in-depth proteome characterization of the central and peripheral nervous system during KD progression is lacking. Here we report the first in-depth characterization of the central and peripheral nervous system of the Twitcher mouse. 10-plex Tandem Mass Tags (TMT) based experiments were used to compare the proteomes of the corpus callosum, motor cortex and sciatic nerves of littermate homozygous Twitcher and wild-type mice.
Experimental procedures

*Experimental design and statistical rationale*

We investigated the proteome changes associated with Krabbe disease by comparing the proteomes extracted from the corpus callosum, motor cortex and sciatic nerves of littermate homozygous Twitcher and homozygous wild-type mice. We performed a TMT 10-plex experiment for each tissue region using independent biological replicates (n = 5) for each mouse type. The five TWI and five WT samples within each TMT 10-plex set were randomized using the Random.org list randomizer (www.random.org), and the expression levels of the confidently identified proteins compared using a two-sided Student’s $t$-test with a permutation-based FDR cutoff (250 randomizations, FDR 0.01, S0 1).

*Materials*

Trypsin/LysC mix Mass Spec grade was purchased from Promega (Madison, WI). Tandem Mass Tags (TMT 10-plex) kits and microBCA protein assay kit were purchased from Thermo Fisher Scientific (Rockford, IL). Polyethylene naphthalate (PEN) membrane slides were purchased from Carl Zeiss (Carl Zeiss Microsystems GmbH, Göttingen, Germany). All other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO).

*Mice*

Twitcher (strain B6.CE-Galctwi/J; Jackson Labs, Bar Harbor, Maine, USA) animals were bred at the Center for Experimental Biomedicine of CNR in Pisa, which is authorized for the use of animals for scientific purposes by the Ministry of Health (Authorization No. 114/2003-A of...
16/9/2003). Animals were maintained under standard housing conditions and used according to the protocols and ethical guidelines of the Italian (DLGS 26/2014; Permit Number: PT5.15, July 2015) and European Union (2010/63/EU) laws. Experiments were conducted in parallel on Twitcher-wild type mice (WT) and littermate Twitcher-mutant homozygous mice (TWI), while Twitcher-mutant heterozygous mice (Het) for the TWI colony maintenance. Genomic DNA was extracted from the clipped tails of mice by Proteinase K digestion and subsequent genomic DNA extraction (EUROGOLD Tissue-DNA Mini kit, Euroclone) as previously described [49][50]. The genetic status of each mouse was determined from the genome analysis of the Twitcher mutation (Supplementary Figure S1), following the method reported by Sakai et al. [51].

Five WT (3 male and 2 female) and five TWI (4 male and 1 female) mice were sacrificed by cervical dislocation, dissected, and the brain extracted [52] and snap frozen in liquid nitrogen for 15 s. Sciatic nerves were dissected by making a 5 mm vertical incision along the thigh [53]. The muscles were split until the entire length of the sciatic nerve in the thigh region was exposed. The nerves were then gently lifted using forceps and removed by cutting at the proximal and distal ends. Brains and sciatic nerves were stored at -80°C until analysis.

*Laser capture microdissection*

Consecutive coronal brain sections, 10µm thick, were cut at 0.74-0.98 mm from Bregma using a cryostat (CM1950, Leica Microsystems Srl, Milan, Italy) and thaw mounted onto PEN membrane slides (previously conditioned in UV light for 30 minutes). After a light hematoxylin staining, small regions of 2.5 - 3.3mm² were isolated from the corpus callosum and the motor cortex using a PALM microbeam laser capture microdissection system (Carl Zeiss MicroImaging, Munich, Germany). Microdissection was performed using a x40 ocular lens and a
355nm laser for cutting the tissue and catapulting the isolated regions of tissue into adhesive cap tubes (Carl Zeiss). Samples were stored at -80°C until analysis.

**Protein extraction and digestion**

The isolated brain samples were dissolved in 20µL of lysis buffer consisting of 50% trifluoroethanol (TFE), 0.5% sodium dodecyl sulfate (SDS), 2.5mM ethylenediaminetetraacetic acid (EDTA), 2.5mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.5 and protease inhibitor (cOmplete™, Mini, EDTA-free Inhibitor Cocktail). The sciatic nerves were homogenized in 100µL of the same lysis buffer. Proteins were extracted by sonication at 4°C using a Bioruptor Pico (Diagenode, Seraing, Belgium - 10 cycles of 30s ON and 30s OFF). Protein quantification was performed on a 1µL aliquot of each sample using a modified microBCA assay [35]. Each anatomical region was compared using an identical amount of extracted protein, and which corresponded to the maximum amount that could be obtained from all ten animals, namely 1.5, 2.5 and 3µg for the corpus callosum, motor cortex and sciatic nerves, respectively. Protein digestion was performed using a modified SP3 protocol [35][36]. The protein extracts were mixed with 20µL of lysis buffer and 2µL of paramagnetic beads (100mg/mL solution of 50% Speedbeads A (GE45152105050250, Sigma) and 50% Speedbeads B (GE65152105050250, Sigma)) in 0.2mL PCR tubes (Sarstedt). Briefly, proteins were denatured at 95°C for 5 min. Reduction (DTT 200mM), alkylation (IAM 400mM), protein purification and overnight trypsin/Lys-C digestion (1:25 enzyme/protein) steps were performed in the same tube.
**TMT labeling**

The protein expression levels in the tissue samples from all ten animals were compared using 10-plex TMT isobaric labeling. After digestion the samples were vortexed and sonicated for 5min with a Bioruptor Pico (5 cycles of 30s ON and 30s OFF). In-solution TMT labeling was performed using a 1:20 peptide/TMT proportion. Three TMT 10-plex reagents (0.8mg) were dissolved in 26, 16, and 13µL of ACN and used for the labeling of the peptides obtained from the corpus callosum, motor cortex and sciatic nerves, respectively (note the TMT solutions were made up using different volumes in order to ensure 2µL of the TMT solutions provided the correct excess for each tissue extract). The TMT label was added in two equal steps of 1µL, each followed by an incubation period of 30min. TMT labeling was quenched by adding 2µL of 4% hydroxylamine and incubating for 15min. After TMT labeling, the samples were transferred to 0.5mL LoBind tubes (Eppendorf) for peptide purification. Samples were rinsed with 100% ACN to promote peptide binding on the carboxylate coated beads. Peptides were washed twice with 70% ethanol and once with 100% ACN. The purified peptides were eluted from the beads with a 2% DMSO aqueous solution. The samples from the five WT and five TWI mice were each labeled with a different, randomized TMT label; the labeled peptides from the ten mice were then combined in a 1:1:1:1:1:1:1:1:1:1 ratio. An aliquot corresponding to 1.5µg of total protein content was collected from each TMT set, diluted 1:1 with 10% formic acid and injected into a nanoLC system for the evaluation of the TMT labeling reaction efficiency. The remaining samples were dried down with a speedvac (Eppendorf) and stored at -20°C.
**Automated high-pH fractionation**

High-pH fractionation was performed using an AssayMap Bravo robot (Agilent Technologies) using the fractionation protocol V1.0 [35]. Peptide fractionation was performed using all of the sample remaining after TMT labeling, approximately 15, 25 and 30 µg for the corpus callosum, motor cortex and sciatic nerve extracts, respectively. Briefly, the dried samples were resuspended in 10µL of 2% DMSO, mixed with 100µL of 10mM NH₄OH (pH 10) and loaded on to reversed-phase (RP-S) cartridges. Peptides were isocratically eluted with 35µL plugs of 12, 18, 24, 30, 36 and 80% ACN in 10mM NH₄OH (pH 10). The six fractions and the sample flow-through were transferred to 0.5mL LoBind tubes (Eppendorf), dried down with a speedvac and stored at -20°C.

**LC-MS3 analysis**

Peptides were resuspended in 10% formic acid and injected into an Easy-nLC1000 (Thermo Scientific) coupled to an Orbitrap Fusion (Thermo Scientific). Peptides were first trapped using a nanoviper trap column (2cm x 75µm, C18, 3µm, 100A; Thermo Scientific) and then separated using an EASY-Spray™ analytical column (ES803: 50cm x 75µm, C18, 2µm, 100A; Thermo Scientific) using a flow rate of 300nl/min and a 140min gradient. Peptides were loaded at 800 bar followed by a non-linear gradient: 0-1min, 8%B; t=105min, 25%B; t=120min, 35%B; t=130min, 90%B; t=140min, 90%B. Buffer A consisted of 0.1% FA and Buffer B of 99.9% ACN and 0.1% FA.

The Orbitrap Fusion was operated in a data dependent top-speed method using a 2 second maximum cycle time and multi-notch synchronous precursor selection (SPS) for MS3 based
TMT quantification. The survey scan was performed in the Orbitrap (m/z 375-1500, 120k resolution, AGC target 2e5, 50 ms maximum injection time). Monoisotopic precursor selection and a dynamic exclusion of 70s were adopted. Ions with charge states from 2+ to 7+ and intensity greater than 5e3 were selected for CID fragmentation (35% NCE) using an isolation window of 1.6 m/z.

MS2 spectra were recorded in the linear ion trap with a rapid scan rate, 5e3 AGC target and 125ms maximum injection time. Following fragmentation, multinotch (synchronous) precursor selection was performed to select the 8 most abundant fragment ions for HCD-MS3 (50% NCE). MS3 scanning was performed in the Orbitrap at a resolution of 60K, with an AGC target of 1e5 and 150ms maximum injection time.

Data analysis

Raw data files were analyzed using Proteome Discoverer 2.1 (Thermo Scientific). For each tissue type the raw files from the 7 fractions were merged and searched with the SEQUEST HT [54] search engine against the Mus Musculus Swiss-Prot protein database (July 2016, 16,808 entries) supplemented with a common contaminant database (246 entries). Searches were performed using the TMT reagents (+229.163 Da, lysine and N-termini) and carbamidomethyl (+57.021 Da, cysteine) as static modifications, methionine oxidation (+15.995 Da) as a dynamic modification, 20 ppm precursor mass tolerance, 0.6 Da fragment mass tolerance, and 20 ppm reporter ion tolerance. The search was performed using fully tryptic peptides with a minimum length of 6 amino acids and up to 2 missed cleavages. The results were then filtered for a 1% false discovery rate (FDR) using the Percolator algorithm and additionally filtered for a minimum Xcorr score of 1.8. At least one unique peptide was required for definitive protein
identification. Protein intensities were calculated based on the label intensities of the 3 most abundant peptides with a co-isolation threshold of 50% taking into account unique peptides. Search results were exported as txt files and processed with Perseus 1.5 [55]. For each TMT experiment the protein intensities were log2 transformed and subject to a median normalization. Figure S3 shows the boxplots of the log2 transformed protein intensities before and after normalization for all the datasets. Data were filtered such that each protein was quantified in at least four TWI and four WT mice. Principal Component Analysis was performed on the normalized and filtered datasets. Significantly different protein levels between TWI and WT mice for the three TMT experiments were calculated using a two-sided Student’s t-test using a permutation-based FDR cutoff (250 randomizations, FDR 0.01, S0 1). Proteins were considered as differentially regulated if their adjusted p-value corresponded to an FDR lower or equal to 0.01 and their fold change (expressed as log2 ratio) was <-1 or >+1. Gene ontology was performed with WebGestalt [56] using the Overrepresentation Enrichment Analysis (ORA) method. For each TMT experiment the input protein list included the proteins significantly up or down regulated in the TWI mice. The reference protein list included all proteins identified in the TMT experiment. A Benjamini-Hochburg (BH) method for multiple test adjustment was used and the FDR was set at 0.05.

**TMT labeling efficiency**

TMT labeling efficiency and over labeling rate were evaluated using 1.5μg aliquots of each TMT set. TMT labeling efficiency was evaluated by setting the TMT modification (+229.163 Da) at N-termini and lysine residues as dynamic and determining the percentage of labeled PSMs. TMT overlabeling was evaluated by setting the TMT modification at N-termini and lysine residues as
static, and TMT modification at serine, threonine, histidine and tyrosine as dynamic, and then determining the percentage of TMT-labeled serine, threonine, histidine and tyrosine.

**Western blot analysis**

Western blot analysis was performed on the sciatic nerve extracts to validate the LC-MS/MS results of seven proteins that play important roles in the molecular mechanisms of Krabbe disease. Proteins were extracted from the sciatic nerves and quantified identically as for the LC-MS/MS analysis (see *Protein extraction and digestion* section above). A WT mice pooled sample and a TWI mice pooled sample were prepared by combining the sciatic nerve extracts of three WT and three TWI mice, respectively. The western blot analysis was performed in technical triplicate on the two pooled samples. The samples were boiled in Laemli buffer containing b-mercaptoethanol (5% final concentration) for 5min and centrifuged at room temperature. The supernatants were used for gel electrophoresis (SDS-PAGE). Samples (25µg) were resolved by SDS-PAGE using Gel Criterion XT-Precasted polyacrylamide gel 4–12% Bis-Tris (Bio-Rad, Hercules, CA) and subsequently transferred to nitrocellulose membranes [57]. Immunodetection was performed for ATG16L1 (Abcam, Cambridge, UK; catalog No. ab188642), VAMP8 (Abcam; catalog No. ab76021), UGT8 (Abcam; catalog No. ab170351), CTSB (Abcam; catalog No. ab58802), SQSTM1 (Abcam; catalog No. ab56416), HEXB (Santa Cruz Biotechnology, Dallas, Texas (USA); catalog No. sc-376781), LAMP1 (Santa Cruz Biotechnology, Dallas, Texas (USA); catalog No. sc-20011), and α-tubulin (Sigma Aldrich; catalog No. T6074). On the following day, the blots were incubated with the corresponding peroxidase-linked secondary antibodies and after incubation the membranes were developed with
Clarity enhanced chemiluminescent substrates (Bio-Rad). The chemiluminescent signal was acquired with an ImageQUANT LAS400 scanner (GE Healthcare Life Sciences, Uppsala, Sweden), and the density of immunoreactive bands quantified in ImageJ. The results were normalized to α-tubulin.

**Data availability**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [58] partner repository with the data set identifier PXD010594.

Reviewer access via Pride -- [https://www.ebi.ac.uk/pride/archive/login](https://www.ebi.ac.uk/pride/archive/login)

Login: reviewer86461@ebi.ac.uk

Password: 40IWTMpZ
Results

We applied a quantitative microproteomics workflow for the characterization of the changes in the proteome of the central and peripheral nervous system of the Twitcher mouse, the most widely used animal model of Krabbe disease. For the characterization of the central nervous system we focused the analyses on the corpus callosum and the motor cortex. The corpus callosum represents one of the main regions of the brain showing extensive demyelination in Krabbe patients [59][60][61][62] and in the Twitcher mouse [63]. The motor cortex was selected because in humans and rodents KD leads to muscle weakness, spasticity and paralysis [2]. In order to investigate the effects of KD on the peripheral nervous system we chose the sciatic nerves as they show marked demyelination, decreased number of axons and axonopathy in the Twitcher mouse [49][64][65]. Figure 1 shows an overview of the experimental approach. 10-plex TMT experiments were used to compare the proteomes of the corpus callosum, motor cortex, and sciatic nerve of five TWI and five WT littermate mice.

Tissue sampling and protein extraction

Laser capture microdissection (LCM) was used to isolate and excise small regions of interest (ROIs) from the corpus callosum and the motor cortex of the mouse brains. The brain morphology was found to be different between WT and TWI mice (Figure S2). The corpus callosum of TWI mice exhibited a greater cell density and reduced white matter compared to WT mice. This morphological feature, due to demyelination of the corpus callosum, is consistent with the known histopathology of the Twitcher mouse brain [63]. The motor cortex did not show any gross morphological difference between TWI and WT mice.
For each mouse the small, localized regions of the corpus callosum and motor cortex were isolated from sequential tissue sections to ensure approximately 2µg of protein was available from each region of each mouse. The corpus callosum ROI was isolated from 4-8 brain sections per animal (total area of 2.6-3.6 mm²) and the motor cortex ROI was isolated from 5-10 sections (total area of 2.5-4.2 mm²). The protein amount extracted from each sample was estimated using a modified microBCA assay performed on a 1µL aliquot [35]. This assay allowed us to quantify the protein content of each sample using only 5% of its total volume. We extracted 1.5-2.4µg of proteins from the corpus callosum samples and 2.5-4.2µg from the motor cortex samples. Table S1 summarizes the number of tissue sections, ROI areas and protein amounts extracted from the corpus callosum and motor cortex.

**Proteome profiling**

The quantitative microproteomics workflow combined SP3 protein digestion [35][36], in-solution TMT labeling and high-pH fractionation followed by LC-MS3. The corpus callosum and motor cortex analyses used the maximum protein amount available from all mice for each microdissected region (1.5µg for the corpus callosum and 2.5µg for the motor cortex). The analysis of the sciatic nerve was performed on 3µg aliquots of the tissue extracts. We assessed TMT labeling efficiency on the three datasets in terms of the percentage of PSMs with the TMT modification at lysine and N-termini residues (Figure 2A). The labeling efficiency was greater than 96% for all the datasets for both lysine and N-terminal residues. We also assessed the over labeling rate (TMT labeling of serine, threonine, histidine and tyrosine), Figure 2B shows a bar graph indicating the percentage of PSMs with a TMT modification at serine,
threonine, histidine and tyrosine residues. The total over labeling rate was less than 5% for all datasets.

The MS3 analyses of the high-pH fractionated TMT-labeled samples resulted in the identification of 3699, 4394 and 3388 protein groups from the corpus callosum, motor cortex and sciatic nerve isolates, respectively. Figure 2C summarizes the number of identified protein groups, peptides PSMs and MS/MS spectra. For the determination of group-wise relative protein quantification the datasets were filtered such that each protein needed to be quantified in at least four TWI and four WT mice. This filtering reduced the number of protein groups to 2607, 3579 and 2350 for the corpus callosum, motor cortex and sciatic nerve, respectively. A histogram of the mass errors of the identified peptides can be found in Supplementary Figure S2, which demonstrates that >95% of all peptide identifications were within 2 ppm, and that >99.9% were within 5 ppm.

Proteome changes in the CNS and PNS of the Twitcher mouse

Principal Component Analysis (PCA) was performed on the filtered, quantitative data matrices to summarize differences in protein expression between TWI and WT mice. The PCA score plot revealed that one mouse, originally considered a TWI mouse, fell into the cluster of WT mice for each tissue region (Figure S4). The incorrect assignment of this mouse was further indicated by a GALC enzymatic activity assay performed on the sciatic nerve extract, which showed a GALC activity comparable to that of WT mice. The initial genotyping of the littermate mice is shown in Figure S1A, which clearly indicates the homozygous TWI and WT mice selected for the experiment. These results indicated a labeling error with one of the TWI mice selected for the experiment; a subsequent genotyping of the suspect TWI-annotated mouse confirmed its status.
as a WT mouse (Figure S1). Thus, this mouse was considered as a WT in the subsequent statistical analysis.

In the PCA score plots of the corpus callosum and the sciatic nerve datasets (Figure S4A, C) the same WT mouse was well separated by PC1 (thus maximum variance in the data) from both the TWI and WT clusters. We repeated the TMT experiment on another aliquot of the sciatic nerve extracts, from the same cohort of mice, and the same mouse remained an outlier and was thus excluded from subsequent statistical analysis. Once the outlier was removed the TWI and WT mice were well separated as two distinct clusters, separated by PC1, for all tissue regions (Figure 3A-C). The percentage of variation explained by PC1 was 42.3%, 18.5%, and 68% for the corpus callosum, motor cortex, and sciatic nerve datasets, respectively.

Significantly different protein levels between TWI and WT mice for the three datasets were calculated using a two-sided Student’s $t$-test with FDR correction for multiple testing. Figure 3D-F show the volcano plots for the datasets from the three tissue regions.

We found 75, 14 and 387 protein groups differentially expressed in the corpus callosum, motor cortex and sciatic nerves of TWI mice, respectively. Specifically, in the corpus callosum 63 protein groups were significantly up-regulated and 12 down-regulated in TWI mice. In the motor cortex 9 protein groups were significantly up-regulated and 5 down-regulated in TWI mice. The sciatic nerve dataset exhibited the largest difference in protein expression, with 16% of the total number of quantified protein groups being differentially expressed (244 protein groups significantly up-regulated in TWI mice and 143 down-regulated).

Table 1 summarizes the number of protein groups identified, quantified and significantly different between TWI and WT mice for the three datasets. The total number of deregulated protein groups was 75, 14 and 386 for the corpus callosum, motor cortex and sciatic nerve
datasets, respectively. Table S2 summarizes the adjusted p-value and fold change of all the quantified protein groups in the three datasets.

**Gene Ontology Enrichment Analysis**

Gene Ontology (GO) analysis was performed to understand which GO terms are represented in the differentially expressed proteins. We performed an Overrepresentation Enrichment Analysis (ORA) using WebGestalt on the corpus callosum and sciatic nerve datasets (the small number of differentially expressed proteins in the motor cortex dataset was insufficient for the analysis). Figure 4 shows the enriched GO terms for both datasets, in which the bubble graphs show the number of differentially expressed proteins contributing to the terms and the FDR value. Most of the enriched biological processes in the TWI mice are related to inflammatory and defense response. In the sciatic nerve dataset we also found down regulation of processes consistent with axon demyelination (reduced axon development, reduced neuron ensheathment, and reduced peripheral nervous system development) as well as reduced microtubule cytoskeleton organization. KEGG pathways upregulated in the TWI mice included those related to inflammatory response (e.g. antigen processing and presentation, leukocyte transendothelial migration, and complement pathway) and to phagocytosis and lysosomes. The glycosaminoglycan degradation pathway, a subclass of the lysosome pathway, was also found to be up-regulated in the TWI mice. Table S3 summarizes the list of protein groups that were linked to the enriched biological processes and pathways.

A cellular component ontology analysis was performed to investigate the subcellular localization of the deregulated proteins because Golgi apparatus, endosomes and lysosomes have previously been demonstrated to play critical roles in LSD’s [40][66][67]. Figure 5 demonstrates that a
significant number of the differentially regulated proteins in TWI mice were linked to these organelles.

**Validation by western blot analysis**

A set of the differentially expressed proteins, including proteins related to lysosomes, autophagy and psychosine synthesis, were selected for validation by western blot analysis. These validation experiments were performed using sciatic nerve extracts. Figure 6 shows the western blots of the seven selected proteins. The relative expression of all the proteins selected for validation by western blot analysis was in agreement with the LC-MS/MS data. ATG16L1 and UGT8 were down-regulated in the TWI mice. ATG16L1 belongs to the autophagy-related proteins and plays a crucial role in the autophagy pathway as part of a complex with autophagy proteins ATG5 and ATG12 [68]. Down-regulation of ATG16L1 may indicate a reduced efficiency of autophagosome assembly. UGT8 (or CTG) is the enzyme that catalyzes the synthesis of psychosine[69]. The down-regulation of UGT8 in the TWI mice may indicate a homeostatic response of the cell to the accumulation of psychosine. LAMP1, HEXB, CTSB and VAMP8 are lysosomal proteins that were up-regulated in the TWI mice. Deregulation of the lysosomal pathway is a general hallmark of all LSD’s, including KD [66][70]. SQSTM1 (also known as ubiquitin binding protein p62) is an autophagy substrate and a marker used to study autophagic flux [71]. SQSTM1 was detected at higher levels in the TWI mice.
Discussion

This work represents the first mass spectrometry-based in-depth characterization of the Twitcher mouse proteome to study changes associated with KD. 10-plex TMT experiments were used to compare the proteomes of the corpus callosum, motor cortex and sciatic nerves of five TWI and five WT control mice. LCM was combined with a microproteomics approach [35] to focus the analysis on localized anatomical regions from individual animals; high pH fractionation [72] and multinotch MS3 was used to ensure high proteome coverage and higher relative quantitation precision [73][74], and together enabled the relative quantitation of 3000 to 4000 protein groups from the microdissected samples. Statistical analysis highlighted 75, 14 and 386 protein groups differentially expressed between TWI and WT mice in the corpus callosum, motor cortex and sciatic nerves, respectively. The expression levels of several proteins were further validated by western blot analysis of the sciatic nerve extracts. The roles of the differentially expressed proteins in the central and peripheral nervous system of the Twitcher mouse are discussed below.

Proteome changes in the Peripheral Nervous System of the Twitcher mouse

The analysis of the sciatic nerve extracts showed an activation of inflammatory response in the TWI mouse. Gene ontology analysis, Figure 4, revealed an up-regulation of several biological processes and pathways related to immune response, cytokines production, antigen processing and presentation pathway, complement and phagosome pathways, indicating a marked inflammation of the peripheral nervous system. Seventeen proteins belonging to the complement pathway were up-regulated in the TWI mouse (A2M, C1QA, C1QB, C3, C8A, C8B, CFB, F2, FGA, FGB, FGG, ITGAM, ITGB2, KNG1,
PLG, SERPINF2, VTN). CD55, an inhibitor of complement activation, was also found to be significantly down-regulated in the TWI mouse, indicating an activation of the complement pathway. Moreover, the up-regulation of several macrophage markers (e.g. CSF1R, MPEG1, MSR1) suggests macrophage infiltration of the sciatic nerves. Complement activation has been shown to be involved in the initiation and/or progression of inflammation by attracting macrophages, stimulation of phagocytosis and tissue injury in diseases of both CNS and PNS, including Alzheimer’s [75] and Gaucher disease [76]. The observed up-regulation of 18 proteins involved in phagocytosis (e.g. CORO1A, CALR and FCGR1) may be an effect of complement activation.

Neuroinflammation is a well-known feature of KD that was believed to be a consequence of demyelination [77]. Recent studies showed a neuroimmune activation in murine models of KD several weeks before symptoms onset, suggesting that neuroinflammation precedes demyelination [78]. The finding that complement proteins are up-regulated in TWI mice may be of interest since complement activation has been shown to be involved in progressive demyelination of the PNS in Guillain-Barré syndrome [79] and Miller Fisher syndrome [80], and to induce tissue inflammation in Gaucher disease [76]. In addition, 39 proteins associated with the PNS, axon development and neuron ensheathment (e.g. MAG, MPZ, MPZL1, JAM3, NEFL, NCAM2, STXBP1) were found to be down-regulated in the TWI mouse, indicating neuron damage and extensive demyelination.

Our data show a marked neuroinflammation, demyelination and complement up-regulation at the terminal stage of the disease (the mice were sacrificed at postnatal day 30 and the mean lifespan of the TWI mouse is 35-40 days). Future work will focus on younger TWI mice in order to address the role of complement activation in the pathogenesis of KD.
The lysosome pathway was found to be up-regulated in the TWI mouse (Figure 4). Defective lysosomal function is a general hallmark of LSD’s and leads to an accumulation of nondegraded macromolecules and metabolites [66]. We found 29 lysosomal proteins up-regulated in the TWI mouse (Figure 5), including lysosome membrane proteins (e.g. LAMP1 and VAMP8), hydrolases (e.g. CTSB, CTSD) and vacuolar H+-ATPases (ATP6V1C1). A subset of lysosomal proteins associated with glycosaminoglycan (GAG) degradation were also up-regulated in the TWI mice, specifically four hydrolases (GUSB, HEXA, HEXB and GNS) that are involved in the degradation of hyaluronan, heparan sulfate, chondroitin sulfate and keratan sulfate [81]. GAGs are important constituents of the cell membrane and the extracellular matrix and play important roles not only in cell adhesion but also in inflammation, neurodevelopment and neuropathology [82]. GAGs have been shown to be involved in leukocyte transmigration at inflammatory sites and in the modulation of chemokines [83]. Accumulation and impaired degradation of specific GAGs in mucopolysaccharidosis disorder leads to cell death and a chronic inflammatory response resulting in neurodegeneration [84]. Our data indicate altered GAG degradation in TWI mice, which has not been reported previously. Further studies are needed to elucidate the potential role of GAG dysregulation in KD pathogenesis and/or progression.

The up-regulation of hydrolases suggests inefficient lysosomal degradation, while the up-regulation of lysosomal membrane proteins (e.g. LAMP1) may indicate a larger number of lysosomes in the TWI mouse [85]. An accumulation of lysosomes was already observed in induced neurons derived from an adult onset KD patient [86]. The accumulation of lysosomes and lysosomal proteins may indicate a disruption of the autophagy pathway, which has previously been shown in several LSD’s [87][67]. Autophagy was shown to be active in a cell
line model of KD after psychosine administration, and the treatment with Lithium (an autophagy stimulator) improved cell viability [88]. Here ATG16L1 was found to be down-regulated in the TWI mouse. ATG16L1 belongs to the autophagy related protein family and forms part of a large protein complex that is necessary for autophagy [89]. Specifically, ATG16L1 forms a complex with ATG12 and ATG5 that localizes to phagophores and pre-phagophore structures [90] and is essential for the proper elongation of the nascent autophagosome and for the lipidation of LC3 [91][92]. Down-regulation of ATG16L1 may lead to a reduced rate of autophagosome assembly, and thus a reduced rate of autophagy [93]. A deregulation of the autophagic flux in TWI mice also explains the observed up-regulation of p62. The ubiquitin-binding protein p62 can bind to LC3 on the autophagosome membrane and be degraded by autophagy or target other proteins for degradation[94]. Accumulation of p62 and formation of p62 aggregates have been observed to occur upon autophagy inhibition and in several LSDs characterized by a disruption of the autophagy pathway, including mucopolysaccharidoses, mucolipidoses, Niemann-Pick C1, Pompe, Gaucher and Fabry diseases [87]. Thus, the accumulation of p62 is consistent with a reduced autophagic activity due to down-regulation of ATG16L1. Lysosomal protein accumulation could also be due to decreased autophagosome-lysosome fusion. A significant down-regulation of 28 proteins associated with microtubule cytoskeleton organization (e.g. MAP6, MAP7D2, CHMP3, CLASP2) were observed in TWI mice (Figure 4C), MAP6 and MAPK8IP3 have already been shown to be involved in the active transport of lysosomes [95][96]. The two kinesin motor proteins (KIF5C and KIF21A), which play an active role in the anterograde transport of lysosomes [97], were also found to be down regulated in TWI mice. Moreover we observed a down-regulation of CHMP3, which is a protein that forms part of the
ESCRT-III complex whose inactivation has been shown to induce autophagosome accumulation and autophagy disruption [98].

An accumulation of lysosomal proteins may also cause a destabilization of the lysosomal membrane that leads to lysosome membrane permeabilization (LMP) [99]. LMP have been shown to play a crucial role in lysosomal cell death (LCD) because of the release of lysosomal proteins in the cytosol. Cathepsins are believed to be the main mediators of LCD, even if they can also trigger LCD [100]. Among the lysosomal proteins that were found to be up-regulated in TWI mice there were 4 cathepsins: CTSB, CTSD, CTSS, CTSZ. In particular, CTSB and CTSD are involved not only in the triggering of LMP but also in cell death since they remain active at neutral pH, and so they remain active in the cytosol [101]. LMP may also be induced by reactive oxygen species (ROS) and apolipoproteins [99][102]. ROS production in Krabbe disease has been linked to psychosine accumulation, which activates secretory phospholipase A2 in oligodendrocytes MO3.13 [103]. Here we found an up-regulation in TWI mice of PLAA (an activator of phospholipase A2) and 4 apolipoproteins (APOA1, APOB, APOD, APOE), supporting the hypothesis of LMP in the TWI mouse. APOE is a high density lipoprotein produced mostly by astrocytes in the CNS and secreted by macrophages at the site of injury in the PNS [104][105]. Accumulation of APOE has been shown to delay the regeneration of sciatic nerves, contributing to degeneration of the nervous system [105]. Moreover, APOE seems to impair autophagy in astrocytes leading to a reduced capacity of Aβ plaque clearance in the CNS of Alzheimer’s disease patients [106]. Apolipoproteins have not previously been reported to accumulate in the TWI mouse and future studies will be needed to establish their role in KD.

Among the proteins down-regulated in the TWI mouse we also found UGT8, which is the enzyme that catalyzes the synthesis of psychosine. This result is in agreement with previous
Studies that reported a down-regulation of UGT8 in the brain and spinal cord of the TWI mouse at a late stage of the disease [107][108][69]. The down-regulation of UGT8 in TWI mice is thought to be a homeostatic response of the cell to psychosine accumulation.

**Proteome changes in the Central Nervous System of the Twitcher mouse**

We characterized the Central Nervous System of the TWI mouse by analyzing the corpus callosum and motor cortex regions.

The corpus callosum dataset contained a larger number of deregulated proteins than the motor cortex, consistent with the known mechanisms of KD in which the accumulation of psychosine occurs mainly in myelinating cells (such as oligodendrocytes and Schwann cells), impairing remyelination and damaging the brain’s white matter [2].

The main biological processes up-regulated in the CNS of the TWI mouse are related to inflammatory and immune response and leukocyte infiltration (Figure 4A-B), as we found in the PNS. We found an up-regulation of the Complement and JAK/STAT pathways in both the corpus callosum and motor cortex, indicating neuroinflammation in the TWI mouse brain. The JAK/STAT pathway is essential for both innate and adaptive immunity and its aberrant activation has previously been reported in the neuroinflammatory diseases Multiple Sclerosis and Parkinson’s Disease [109]. The use of JAK1 and JAK2 inhibitors in several murine models of Multiple Sclerosis has been shown to suppress clinical symptoms, reduce demyelination and suppress the production of pro inflammatory cytokines and chemokines [110]. The up-regulation of both STAT1 and STAT3 in the CNS of the TWI mouse suggests that the JAK/STAT pathway may be a therapeutic target for KD treatment.
We also found an up-regulation in the TWI mouse of GFAP and VIM, which are markers of reactive astrocytes and microglia activation [111][112]. GFAP and VIM up-regulation in the TWI mouse have been previously shown to occur before demyelination and in concomitance with macrophage infiltration, suggesting that CNS astrogliosis and inflammation may precede demyelination [113][114].

Several lysosomal proteins were found up-regulated in the corpus callosum of the TWI mouse (Figure 5A-B), including HEXB, CTSB, CTSD and LAMP2. These results are in agreement with that found for the PNS of the TWI mouse, indicating that the accumulation of nondegraded material and the related biological alterations (see section “Proteome changes in the Peripheral Nervous System of the Twitcher mouse”) also occurs in the CNS of the TWI mouse.

Four proteins involved in membrane raft organization (ANXA2, CAV1, DOCK2 and PTPRC) were found to be up-regulated in the corpus callosum of the TWI mouse. Disruption of lipid raft domains have been shown to occur in sphingolipid storage disorders and KD as a consequence of undigested lipids and psychosine accumulation [22][115]. Lipid rafts are involved in several signaling pathways and an alteration in raft composition can lead to altered membrane fluidity, and deregulation of cell signaling, influencing survival signals such as PKC, Akt and ERK [23][24].

Among the proteins down-regulated in the motor cortex of the TWI mouse we found UGT8, and which was also found to be down regulated in the sciatic nerve (see section “Proteome changes in the Peripheral Nervous System of the Twitcher mouse”). This result is in agreement with previous studies, which reported a down-regulation of UGT8 at late stages of KD in both CNS and PNS [107].
Conclusions

This work represents the first in-depth characterization of the Twitcher mouse proteome to study Krabbe disease. We applied a quantitative microproteomic workflow to detect changes associated with Krabbe disease in the Central and Peripheral Nervous System of the Twitcher mouse. The proteomes extracted from the corpus callosum, motor cortex and sciatic nerves of five homozygous Twitcher and five wild-type mice were compared at postnatal day 30. More than 3300 protein groups were identified for each dataset. Statistical analysis revealed a total number of deregulated protein groups of 75, 14 and 386 for the corpus callosum, motor cortex and sciatic nerve datasets, respectively.

Most of the enriched biological processes and pathways in the TWI mice were related to neuroinflammation, immune response, accumulation of lysosomal proteins, demyelination, membrane raft organization and reduced peripheral nervous system development. These results on the proteome changes in the Twitcher mouse help provide new insights into the molecular mechanisms of Krabbe disease. Future experiments will focus on the proteomic characterization of younger mice (pre-symptomatic and at symptoms onset) in order to understand how these deregulated pathways are linked to the pathogenesis of Krabbe disease and specifically, which of these trigger damage to the CNS and PNS and can act targets for new therapies.
Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Summary of the number of protein groups identified, quantified, up-regulated and
down-regulated in TWI mice for the corpus callosum, motor cortex and sciatic nerves datasets.

| # Protein groups         | Corpus callosum | Motor cortex | Sciatic nerves |
|-------------------------|-----------------|--------------|---------------|
| Identified              | 3699            | 4394         | 3388          |
| Quantified              | 2607            | 3579         | 2350          |
| Up-regulated in TWI     | 63              | 9            | 243           |
| Down-regulated in TWI   | 12              | 5            | 143           |
Figure legends

Figure 1. Ultrasensitive microproteomic workflow for the characterization of CNS and PNS of the Twitcher mouse. The brains and sciatic nerves were collected from five TWI and five WT mice at 30 days of age. Laser capture microdissection was used to isolate ROIs from the corpus callosum and motor cortex. Proteins were extracted from the corpus callosum, motor cortex and sciatic nerves, quantified with microBCA assay and digested with the SP3 protocol. Peptides from each dataset were pooled and labeled with TMT 10-plex reagents. Labeled peptides were purified and fractionated on a RPS cartridge. MS3 spectra were acquired on an Orbitrap Fusion mass spectrometer and proteins were identified with Proteome Discoverer 2.1. Data and statistical analysis were performed with Perseus and WebGestalt softwares. The proteomic data were validated by western blot analysis on the sciatic nerves extracts.

Figure 2. (A) TMT labeling efficiency expressed as the percentage of PSMs with the TMT modification at N-termini and lysine residues. (B) Over-labeling rate expressed as the percentage of PSMS with the TMT modification at serine, histidine, threonine and tyrosine residues. (C) Protein identification metrics: number of identified protein groups, peptides, PSMs and acquired MS/MS spectra. Grey, violet and white bars refer to the corpus callosum, motor cortex and sciatic nerves datasets.

Figure 3. Principal Component Analysis performed on the filtered protein groups expression values for the corpus callosum (A), motor cortex (B), and sciatic nerves (C) datasets. Blue circles indicate TWI mice, orange circles indicate WT mice. Volcano plots for the corpus callosum (D),
motor cortex (E), and sciatic nerves (F) datasets. Grey circles indicate non significant protein
groups (log2(fold change) < 1 and >-1, FDR > 0.01). Blue circles indicate protein groups
significantly up-regulated in the TWI mice (log2(fold change) >1, FDR < 0.01). Orange circles
indicate protein groups significantly down-regulated in the TWI mice (log2(fold change) < -1,
FDR < 0.01).

Figure 4. Gene ontology Overrepresentation Enrichment Analysis (ORA) performed on the
protein groups that are significantly up- or down-regulated between TWI and WT mice for the
corpus callosum and sciatic nerves datasets. (A) Biological processes enriched in the corpus
callosum dataset, (B) KEGG pathways enriched in the corpus callosum dataset, (C) biological
processes enriched in the sciatic nerves dataset, (D) KEGG pathways enriched in the sciatic
nerves dataset. The number in the bubble indicates the number of protein groups in the
experimental dataset that matched with the corresponding GO term. Blue and orange bubbles
indicate biological processes/pathways enriched in the protein groups that are significantly up-
and down-regulated in TWI mice, respectively.

Figure 5. GO - Cellular component analysis. List of deregulated protein groups that are localized
on Golgi apparatus, endosomes and lysosomes for the corpus callosum (A), motor cortex (B),
and sciatic nerves (C) datasets. Blue and orange indicate protein groups that are significantly up-
and down-regulated in TWI mice, respectively.
Figure 6. Western blots showing the expression levels of SQSTM1, VAMP8, ATG16L1, LAMP-1, HEXB, CTSB and UGT8 in the sciatic nerve extracts of WT mice compared to TWI mice. Results were normalized to $\alpha$-tubulin. The WT mice pooled sample and the TWI mice pooled sample were prepared by combining the sciatic nerves extracts of three WT and three TWI mice, respectively. The western blot analysis was performed in technical triplicate on the pooled samples. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; Student’s $t$-test. Blue and orange bars indicate TWI and WT mice, respectively. Error bars indicate standard deviation.
Figure 1

Experimental design

Wild-type (WT)  
Twitcher (TWI)

p30

n=5

n=5

Brain and sciatic nerves

LCM

WT  
TWI

Motor cortex  
Corpus callosum

Sample preparation

Droplet-based MicroBCA

1μL sample

SP3 digestion

Denaturation  
Reduction  
Alkylation  
Protein clean-up  
Digestion  
TMT labeling  
Peptide clean-up

TMT labeling  
High-pH fractionation

EASY-nLC 1000 Orbitrap Fusion

nLC-MS3 analysis

Proteome

Discoverer 2.1  
Perseus 1.5  
WebGestalt

Data analysis
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6