isas Leibniz-Institut für Analytische Wissenschaften

Leibniz Association

Final Report
Leibniz Competition

Title: Post-translation modifications of the synaptic scaffold controlling age-induced memory impairment (SAW-2019-ISAS-4-SyMetAge)

Project number: K169/2018

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Leibniz Institute in charge:

Leibniz-Institut für Analytische Wissenschaften – ISAS – e.V.

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Project leader: Prof. Dr. Albert Sickmann

Executive Summary

Please briefly summarize the most important points of the individual chapters of the interim report here (max. 4500 characters). The executive summary may be similar to the performance review report, but unlike the latter, it will be published.

The collaborative project SyMetAge between FU Berlin (Berlin), LIN (Magdeburg) and ISAS (Dortmund) was aimed to establish causal relations between lifetime-associated post-translational modifications (PTMs) and memory impairment (MI). Our central hypothesis was that an age-associated change in PTMs provokes alterations in structural and functional plasticity/dynamics of synapses and is thereby a major driver of cognitive decline. This interplay study of PTMs in synaptic proteins in aging with respect to the exchange and alteration of function has not been studied systematically before. Hence, this project is the first in establishing molecular fingerprints and essential nodal/entry points to enable successful therapeutic interventions. Sigrist (FU Berlin) and Kreutz labs (LIN Magdeburg) provided the respective biological samples whereas Sickmann lab (ISAS Dortmund) performed liquid chromatography mass spectrometry-based (LC-MS) proteomics analyses.

In *Drosophila melanogaster* (DM; fruit fly), Sigrist lab continued working on a specific and distinct PTM known as hypusination, which is unique to eukaryotic translation initiation factor 5A (eIF5A). We previously showed that this modification enzymatically mediated by spermidine (Spd) that converts lysine into hypusine; plays a vital role in cognitive decline upon aging (Schroeder S et al and Liang Y et al., 2021). For this, fruit fly mutants such as *CG8005/+* (heterozygous for the deoxyhypusine synthase) and *K51R/+* (hypusination site point mutation in eIF5A in heterozygosity) were generated that specifically undermine the hypusination process. Our findings suggest changes in distinct regulatory mechanisms due to different mutations and age, with Spd exhibiting a complex influence on mitochondrial and cytoplasmic activities. Moreover, we investigated the impact of low-protein (2% yeast) and high-protein (12% yeast) diet, ± Spd supplementation in an age-dependent manner (5, 10, 15 and 30 days). Results indicate that dietary restriction enhances mitochondrial respiration e.g., 'respiratory chain complex assembly' and 'electron transport chain' at 2% rearing, while downregulating cytoplasmic translation activities such as 'translational elongation' and 'translation factors'. Spd supplementation in a 2% background enhances mitochondrial gene expression but reduces deacetylation and apoptosis and inhibits translation in a 12% background. Furthermore, we studied the role of the ELKS-family scaffold protein Bruchpilot (BRP)-driven plasticity in the context of sleep homeostasis as well as in learning and memory (Huang S et al., 2020 and 2022). Overall, analysis from site, peptide to the cumulative phosphorylation levels of protein found antagonistic changes, with low BRP promoting phosphorylation and high BRP promoting dephosphorylation at the synapse. These results together suggest a negative correlation between BRP or probably sleep and global synaptic phosphorylation levels.

In mouse, Kreutz lab mainly targeted neddylation, a PTM that adds a ubiquitin-like protein NEDD8 to substrate proteins; in the aging brain that has the potential to rejuvenate activity-dependent gene expression and synaptic function. We could enrich and characterize Nedd8 synaptic proteins with the use of the so-called “CUBAN” (Cullin-Binding domain Associated with NEDD8) domain of KHNYN protein (627-678 aa) that could exclusively recognize neddylated proteins. Cortex and hippocampi of either C57BL/6J wildtype male mice were collected of 3 different ages i.e., 3, 12, and 24 months, which represent young adults, middle-aged, and old mice and synaptosomes were prepared for LC-MS. Around 200 proteins pulled down by the CUBAN domain that, based on SynGO annotations, are present either at pre- or postsynaptic sites. A few target proteins were selected from the interactome based on functional relevance in memory and their role in synaptic plasticity e.g., Bassoon and Piccolo, very large scaffold proteins of the cytomatrix of the active zone (CAZ) were also found neddylated.

In summary, our data enables to move a step closer to understand underlying mechanisms promoting synapse function and consequently cognitive aging and most importantly opens new avenues for therapeutic interventions.

Contents

1. Achievement of objectives and milestones

Please explain briefly the implementation of the most important objectives and milestones of your project compared to the planning presented in the application. If applicable, also explain any objectives/milestones that have not been achieved, or that have been achieved only in part. Please also explain the most important points of the final financial statement and the final financial plan compared to the original financial planning.

As stated in the project application, we divided our project´s main objectives into three work packages between working groups (a) Sickmann lab (ISAS, Dortmund), (b) Sigrist lab (FU Berlin), (c) Kreutz lab (LIN, Magdeburg). Sigrist (FU Berlin) and Kreutz labs (LIN Magdeburg) provided the respective biological samples whereas Sickmann lab (ISAS Dortmund) performed liquid chromatography mass spectrometry-based (LC-MS) proteomics analyses.

a. Mass-spectrometry-based quantitative characterization of PTM dynamics and crosstalk during brain aging.

At ISAS, we primarily focused on the optimization and development of sample preparation protocols that best suited to the downstream LC-MS based proteomics analyses. One such example is implementation of the in house developed semi-automated positive-pressure filter-aided sample preparation (FASP) in 96-Well Format (PF96) TECAN (Loroch et al., 2022). Similarly, for enrichment of phosphopeptides, we adapted the immobilized metal affinity chromatography (IMAC) coupled to the Bravo platform (Agilent). These two modules enabled us to simultaneously process up to 60 samples in one day with high sample preparation reproducibility. The serial isolation of ubiquitinated and acetylated peptides was established during the project and applied to planned samples. Overall, Sickmann lab achieved the work package related milestones.

b. Fast and efficient analysis of PTMs causally associated with cognitive aging in *Drosophila melanogaster* (DM).

Sigrist lab further investigated the molecular mechanisms of protection from cognitive decay in aging DM by dietary Spd supplementation. We concluded that Spd supplementation boosts mitochondrial abundance and protects mitochondrial functionality in aging DM brains via hypusination. We also performed a set of experiments to isolate synaptic and non-synaptic compartments in DM to enrich the synaptic proteins. For a quantitative characterization of dynamically regulated synaptic proteins upon different gene copy number of Bruchpilot (BRP), we combined biochemical synaptosome-isolation with proteomics analysis (ISAS). Per se, the technical procedures including retrieving enough synaptosome from flies to allow for highly sensitive detection became very well established between all partners involved. We found a negative correlation between BRP or probably sleep and global synaptic phosphorylation levels and planned to further validate these findings using other short-sleep mutants. Unfortunately, due to technical issues this set of experiment has to be repeated. We currently test the biological relevance for a spectrum of identified PTM sites by introducing point mutations into the DM genome using CRISPR/Cas9 gene editing. Preliminary results here are encouraging: the potential sleep-related PTM site mutation in Spinophilin induced specific sleep and plasticity phenotypes. Further studies on sleep-related PTM changes are expected to be completed and published within two years. The project has deepened the understanding of the PTM modulation in memory and sleep, particularly in relation to aging. Overall, the aims of the WP2 were fulfilled, resulting in three publications with two more currently in preparation.

c. Acetylation, ubiquitylation, phosphorylation, and neddylation at the crossroads of healthy and high-risk aging in *Mus musculus* (mouse).

Kreutz lab developed a highly efficient strategy that allows for purification of neddylated proteins from synaptic junctions in the male C57BL/6J wildtype and high-risk aging (24 months) mice. Our strategy is based on the highly specific binding of the CUBAN domain. Moreover, ISAS performed proteomic analysis of synaptosomes purified from mouse model in age-dependent manner (3, 12 and 24 months), respectively. We also established suitable *in vitro* and *in vivo* models to examine the early processes and interactions occurring at the prodromal phase of Alzheimer's disease using a novel transgenic model of high cerebral amyloid levels

as a predisposing environment. During the funded studies, we found that neddylation and subsequent activation of cullin-RING ligase complexes induce synaptic insulin resistance by ubiquitylation and degradation of the insulin- receptor substrate IRS1 that organizes synaptic insulin signaling. Accordingly, inhibition of neddylation preserves synaptic insulin signaling and rescues memory deficits in mice with a high amyloid load, which were fed with a high-fat/high-calorie chow diet (western diet) (Confettura et al., 2021). Therefore, it is pertinent to explore the new substrate of neddylation especially from synapses in context to synaptic plasticity and role of neddylation with respect to age.

2. Activities and obstacles

Please outline the work and activities of the project partners during the funding period. Please also explain any obstacles or failures you may have encountered that led to delays in the schedule, to deviations from the original proposal or work plan or to goals not being met. Explain any advances in the research area that have become known during the project's implementation and their impact on the project. Briefly comment on the necessity and appropriateness of the work performed.

In the context of the hypothesis if an age-associated change in PTMs provokes alterations in structural and functional plasticity/dynamics of synapses and is thereby a major driver of cognitive decline, several sets of samples (wildtype and mutants) from both model organisms i.e., *Drosophila melanogaster* and *Mus musculus* were prepared by the Sigrist and Kreutz labs, respectively that were further processed, and analyzed using LC-MS technologies by the Sickmann lab (see Appendix) and subsequently evaluated.

Unfortunately, there were also some difficulties during the project, such as the SARS-CoV2 pandemic. Due to the pandemic, the laboratories and facilities were temporarily closed in 2020, and subsequent restrictions meant that the laboratories could only be used under strict hygiene regulations and for limited periods of time. This all led to a significant delay in the project.

Furthermore, technical issues also arose during the project. We planned to validate our findings in sleep and synaptic hypophosphorylation using a short-sleep mutant, named *sleepless*. Unfortunately, the initial PCA analysis of the proteomic profile suggested potential sample preparation issue in this experiment. Additionally, the protein *sleepless* itself was detected in the null *sleepless* mutants and the groups of *sleepless* mutants in 3×BRP background, which indicates a loss of the credibility of this data. Therefore, we plan to re-perform this set of experiment with new fresh prepared samples.

3. Results and successes

Please present the key results and successes in research (publications, completed theses and dissertations, acquisition of third-party funds, scientific events, etc.) and transfer (consultancy, technology transfer, public relations work). What activities are planned for further exploitation of the project results? Please use the Excel template to register all basic information for this and briefly explain this basic information here.

A. Scientific presentations
    a. Poster: SFB1315 Symposium, 2019 in Rheinsberg (Brandenburg); *Dietary Spd protects mitochondrial function and brain aging via hypusination*.
    b. Talk: Stephan Sigrist, Gordon Research Conference (GRC), 2019 in Waterville Valley, New Hampshire, United States; *Polyamines, Synaptic Plasticity, Autophagy in the aging fruit fly brain.*
    c. Poster: DGMS, 2020 in Münster; *Dietary Spd protects mitochondrial function and brain aging via hypusination*.
    d. Talk: Chengji Piao, EMBO Workshop, Cell biology of the nervous system, 2023 in Heraklion, Greece; *Active zone plasticity optimizes brain aging.*
    e. Poster: Ärztegesellschaft Heilfasten & Ernährung e.V., 2023 in Berlin; *Spd Exerts Neuroprotection from Age-promoting High-protein Diet.*

f. Poster: 72nd ASMS Conference, 2024 in Houston, Texas, United States; *eIF5A hypusination, boosted by dietary Spd, protects from premature brain aging and mitochondrial dysfunction.*

B. Publications
   a. Liang Y et al., *eIF5A hypusination, boosted by dietary Spd, protects from premature brain aging and mitochondrial dysfunction.* Cell Rep. 2021 Apr. 13;35(2):108941 doi: 10.1016/j.celrep.2021.108941.
   b. Confettura et al., *Neddylation-dependent protein degradation is a nexus between synaptic insulin resistance, neuroinflammation and Alzheimer's disease.* Transl. Neurodegener. 2022 11(1):2.doi: 10.1186/s40035-021-00277-8.
   c. Huang S. et al., A brain-wide form of presynaptic active zone plasticity orchestrates resilience to brain aging in *Drosophila*. PLoS Biol. 20(12), e3001730 doi: 10.1371/journal.pbio.3001730
   d. Huang S. & Sigrist, S.J. Presynaptic and postsynaptic long-term plasticity in sleep homeostasis. Current Opinion in Neurobiology 69, 1-10 doi: 10.1016/j.conb.2020.11.010
   e. Furthermore, two publications are currently in preparation.

C. Theses and Dissertations
   a. Liang, Y. (2019). Spermidine versus dietary restriction in the context of brain aging: a role for hypusination and mitochondria. Dissertation, Department of Biology, Chemistry and Pharmacy (Freie Universität Berlin)
   b. Sulaimen, S (2021). Quantitative proteomic and phosphoproteomic analysis of the molecular signatures of sleep need in *Drosophila melanogaster*. Bachelor Thesis, Department of Mathematics and Computer Science (Freie Universität Berlin)
   c. Piao, C. (2022). Proteomic and phospho-proteomic analysis of sleep need uncovers an autoregulatory loop between GABAergic signaling and synaptic plasticity. Dissertation, Department of Biology, Chemistry and Pharmacy (Freie Universität Berlin)
   d. Doumene, M (2022). Impact of spermidine and eIF5A hypusination on dietary restriction effects. Master Thesis, Department of Biology, Chemistry and Pharmacy (Freie Universität Berlin)
   e. Cuboni, E. (2023). Analysis of neddylation in the context of synaptic function and high-risk ageing. Dissertation, Fakultät für Naturwissenschaften (Otto-von-Guericke-Universität Magdeburg)

D. Acquisition of third-party funds
   a. DFG Research Grant - *Project number* 445178831: The role of hypusination-depenedent translation for brain aging. (Stephan Sigrist)

4. Equal opportunities

Please describe briefly your initiatives and measures to ensure equal opportunities concerning gender and internationalisation, especially in staff development and recruitment. Please also describe any measures taken to promote the careers of young researchers. Please use the tables in the Excel template to register all basic information for this and briefly explain this basic information here.

We tried to assure equal opportunities concerning gender (9 males and 9 females currently working in the project) and internationalization (7 nationalities) in staff development and recruitment. ISAS includes equality as a universal guiding principle in all tasks and decisions supports the principle of equal opportunity for all employees, try to improve the compatibility of work and family life and particularly encouraged women to apply. Sigrist lab implemented the general policy for equal-career management of FU Berlin, which has a long successful history

of promoting diversity and reducing inequality and was recognized in 2018 as one of only ten German universities with the title “Outstanding Equal Opportunity!”. FU Berlin also aims to enhance conditions for young scientists through established and evolving programs in qualification, mentoring, counseling, gender equality, diversity, research-oriented teaching, and internationalization. LIN aims to promote the careers of young researchers by establishing an internal doctoral programm and has an annual audit on the topic of work and family. Furthermore, they concentrate on the topic mental health (cooperation ZI Mannheim) and organize an equality female career day.

5. Structures and collaboration

Please describe the structure of collaborations during the funding period, including any obstacles and challenges. Where relevant, describe changes to the governance of your project and/or the addition of new partners or collaborative relationships. If new collaborative relationships were established with institutional partners during the funding period, please briefly describe the scientific benefit for your project. If the changes resulted in additional agreements or changes to the existing collaboration agreement, please attach copies to this report. Please use the tables in the Excel template to register all basic information for this and briefly explain this basic information here.

The structure of existing collaborations according to the original applications in the period under review is as follows:

a. Leibniz-Institut für Analytische Wissenschaften - ISAS - e.V., Dortmund; project leader: Prof. Albert Sickmann. Quantitative strategies (labelled and label-free) for the analysis of proteins and their posttranslational modifications. Nano-separation techniques combined with high-resolution mass spectrometry platforms and computing cluster. The relevant biological material was provided by the Sigrist and Kreutz labs (see below).
b. Freie Universität Berlin, Department of Biology, Chemistry, Pharmacy, Institute of Biology, Sigrist Group - Genetics, Berlin; project leader: Prof. Stephan Sigrist. Analysis of synapse organization and its plasticity. Study of a generic synaptic mechanisms and age-induced memory impairment. Investigation of relationship between polyamine (Spd) feeding and extended life-spam and improvement of cognitive processes. Model organism: *Drosophila melanogaster*
c. Leibniz Institute for Neurobiology, Magdeburg; project leader: Dr. Michael R. Kreutz. Molecular dynamics of the post synapse and the investigation of protein transport from synapse-to-nucleus. Model organism: *Mus musculus*.

During the course of this project, regular interactions (in-person, virtual via video conference platforms, and telephone) took place between the partners involved to discuss about the experimental strategies, data, results and scientific contributions.

Moreover, new collaboration with another Leibniz institute (FMP, Berlin) as well as German and Austrian universities (University of Graz), and other non-university institutions like the Institute for Mathematics and Computer Sciences (Zuse Institute Berlin), German Center for Neurodegenerative Diseases (DZNE, Magdeburg) and Institute of Pharmacology, Charité – Universitätsmedizin Berlin have been established to support the structure.

6. Quality assurance

Please describe briefly your measures for quality assurance, in particular in terms of complying with good scientific practice and making your research results available (open access). Please also state whether or not animal testing has been conducted. If this is the case, please give a short description of your measures ensuring animal welfare.

Data upload, sharing and exchange between the collaboration partners were done using the internal (ISAS) Pure research data repository and cloud server. Supervision of the PhD students, Postdocs and technicians was done by the respective project leaders. Furthermore,

to make our research results available, we will publish our findings in open access journals. To ensure quality, raw data of all publications will be deposited in public repositories. Animal testing (*Mus musculus*) has been conducted at the Leibniz Institute for Neurobiology, Magdeburg. All experiments were approved by the Animal Care and Ethics Authority of the State of Saxony-Anhalt (Landesverwaltungsamt Sachsen-Anhalt, Referat Verbraucherschutz, Veterinärangelegenheiten) and conducted in accordance with ethical animal research standards defined by the German Law and the EU Directive (2010/63/EU) on the protection of animals used for scientific purposes. All fly strains were reared under standard laboratory conditions, as previously reported (Gupta et al., 2013), unless specially mentioned (25 °C, around 70 % humidity with constant 12:12 h light/dark cycle).

7. Additional in-kind resources

Please estimate the value of in-kind resources generated within the project at your institute, the participating Leibniz institutes and/or the university partners. Please differentiate between personnel cost (for scientific and non-scientific staff) and consumables.
For funding programmes with co-funding (i.e. Leibniz Programme for Women Professors, Leibniz Junior Research Groups, Leibniz ScienceCampi, Leibniz Research Alliances), please also indicate the amount of co-funding by the Leibniz institution(s) and (if applicable) the cooperation partners.

For the successful realization of the project, all participants have been financially supporting the project with different measures:

1. FU Berlin and LIN provided scientific and technical support including three postdocs (two postdoc were paid by internal funds and one postdoc from FU Berlin was supported by DFG Research Grant - the role of hypusination-depenedent translation for brain aging; one from LIN), three PhD students who were not paid by the SAW fund (one PhD student (LIN) was partially paid by GRK2413 and one PhD student from FU Berlin was financed by DFG Collaborative Research Centre SFB1315 - Mechanisms and disturbances in memory consolidation: From synapses to systems) and three technicians of the research group NPlast at LIN.
2. FU Berlin, LIN and ISAS provided consumables for sample preparation equipment, antibodies, kits and costs for animals which were mostly covered by internal funds.
3. ISAS provided the infrastructure i.e., LC-MS instrumentation and technical services.

8. Outlook

Please describe the most important future research questions related to the work performed.

In work package one (Sickmann lab) we developed a tailormade protocol for serial enrichment of different posttranslational modification. This protocol is currently applied to our research topics regarding cardiovascular research. The results may deliver new insights in biochemical processes after cardiac infection.

The results of the second work package (Sigrist lab) already form the basis to deeper functionally analyze the interplay between mitochondria, synapses, and intrinsic neuronal excitability in functional brain aging. We are particularly excited about the results of the phosphoproteomics analysis, which allows us the limit the set of kinases/phosphatases involved and seek for drugs and natural metabolites to promote healthy brain aging in conjunction with Spd, our lead compound.

Overall, the project enables us to move a step closer to understand underlying mechanisms promoting synapse function and consequently cognitive aging and most importantly opens new avenues for therapeutic interventions.

9. Appendix

List of experiments performed using fruit fly and mouse models.

Model organism (Lab)	Sample description	Sample#	Proteomics (Sickmann lab)	Remark
Drosophila melanogaster (Sigrist)	*w*^{1118}(wildtype) vs *CG8005/+* synthase mutant Age: 5 days 200 brains/sample	8	Label free (DIA, DDA) and labeling (TMT) + pH 8.0 fractionation LC-MS/MS	Validation/test experiment
Drosophila melanogaster (Sigrist)	*w*^{1118}(wildtype) vs *CG8005/+* Age: 5d, 15d, 30d Diet: ± Spd 18-20 flies pooled	48	Label free DDA LC-MS/MS	Role of Spd in mutant and age-dependent whole fruit fly
Drosophila melanogaster (Sigrist)	Brain synaptosomes (5 fractions S1, S2, P2, $P2_S$ and $P2_M$) *w*^{1118}(wildtype, 5-day old)	5	Labeling (TMT) + pH 8.0 fractionation LC-MS/MS	Validation/test experiment for BRP enriched fraction
Drosophila melanogaster (Sigrist)	Brain synaptosomes (3 fractions S1, S2, P2) 1×BRP: *brp*^{c04298}*/+* 2×BRP: *w*^{1118} 3×BRP: *brp* P[acman]*/+* 4×BRP: *brp* P[acman] 5-day old	36	Enrichment of phosphopeptides using IMAC Bravo platform Label free global and phosphoproteome analyses using DDA LC-MS/MS	Comparison of global and phosphoproteome between different *brp* gene copy numbers in P2, S1 or S2 fractions
Drosophila melanogaster (Sigrist)	Single *w*^{1118}(wildtype) fruit fly Age: 10d Diet: ± Spd and 2% or 12% yeast	20	Label free global proteome analysis using DDA LC-MS/MS	Validation/test experiment to check if protein from single fly is sufficient for further analysis
Drosophila melanogaster (Sigrist)	*w*^{1118}(wildtype) and *CG8005/+* Age: 5d, 15d, 30d Diet: 2% yeast vs. 12% yeast	60	Semi-automated TECAN-FASP Label free global proteome analysis using DDA LC-MS/MS	Role of Spd on the whole single fruit fly
Drosophila melanogaster (Sigrist)	*w*^{1118}(wildtype) and *CG8005/+* Age: 5d, 15d, 30d Diet: ± Spd	60	Semi-automated TECAN-FASP Label free global proteome analysis using DDA LC-MS/MS	Role of Spd on the whole single fruit fly
Drosophila melanogaster (Sigrist)	*w*^{1118}(wildtype) and *K51R/+* Age: 5d, 15d, 30d Diet: ± Spd	60	Semi-automated TECAN-FASP Label free global proteome analysis using DDA LC-MS/MS	Role of Spd on the whole single fruit fly
Drosophila melanogaster (Sigrist)	*w*^{1118}(wildtype) Age: 10d Diet: ± Spd and 2% or 12% yeast	60	Semi-automated TECAN-FASP Label free global proteome analysis using DDA LC-MS/MS	Validation experiment
Drosophila melanogaster (Sigrist)	*w*^{1118}(wildtype) Age: 15d Diet: ± Spd and 2% or 12% yeast	20	Label free global proteome analysis using DDA LC-MS/MS	Validation experiment
Drosophila melanogaster (Sigrist)	*w*^{1118}(wildtype) Age: 15d Diet: ± Spd and 2% or 12% yeast 10 flies pooled/condition	20	Label free global proteome analysis using DDA LC-MS/MS	Validation experiment. Comparison between single and pooled flies

Drosophila melanogaster (Sigrist)	Brain synaptosomes (3 fractions S1, S2, P2) 2×BRP: w^{1118} 3×BRP: *brp* P[acman]/+, *sleepless* mutant: sss^{P1} *sleepless* mutant in 3×BRP background: sss^{P1}; *brp* P[acman]/+ 5-day old	48	Labeling (TMT) + pH 8.0 fractionation Enrichment of phosphopeptides using TiO_2-MOAC Label free global and phosphoproteome analyses using DDA LC-MS/MS	Comparison of global and phosphoproteome between different *brp* gene copy number within P2, S1 or S2 fractions and *sleepless* mutants
Mus musculus C57BL/6J; wildtype (Kreutz)	Brain synaptosomes subjected to CUBAN domain pulldown protocol	10	In gel digestion and DDA LC-MS/MS	Validation/test experiment for neddylation analysis
Mus musculus C57BL/6J; wildtype (Kreutz)	Brain synaptosomes subjected to CUBAN domain pulldown protocol	40	In gel digestion and DDA LC-MS/MS	Validation/test experiment for neddylation analysis
Mus musculus C57BL/6J; wildtype (Kreutz)	Synaptosomes from mouse brain cortex	2	PTMScan HS Ubiquitin/SUMO Kit (Cell Signalling) PTMScan Acetyl-Lysine Kit (Cell Signalling) DDA LC-MS/MS S-trap midi (1 mg of protein) for trypsin digestion	Testing preparation and PTM enrichment protocols
Mus musculus C57BL/6J; wildtype (Kreutz)	P2 fraction of mouse brain synaptosomes. CUBAN domain pulldown IP protocol	2	Organic solvent-based protein precipitation followed by in solution trypsin digestion. DDA LC-MS/MS	To verify if 1D SDS page is needed to identify enriched neddylated proteins
Mus musculus C57BL/6J; wildtype (Kreutz)	P2 synaptosome fraction from WT mouse	6	S-trap mini protocol for eluates digestion DDA LC-MS/MS	Cuban domain pulldown without running SDS-PAGE
Mus musculus C57BL/6J; wildtype (Kreutz)	P2 fraction of mouse brain synaptosomes. CUBAN domain pulldown IP protocol	8	S-trap mini digest for N samples (n=3) with GST controls (C samples, n=3)	Testing preparation and PTM enrichment protocols
Mus musculus C57BL/6J; wildtype (Kreutz)	P2 fraction of mouse brain synaptosomes. CUBAN domain pulldown protocol. Flowthrough for other PTMs enrichment WT (control) Age: 3, 12, 24 months	15	S-trap mini/midi digest. Serial PTMs enrichment (ubi, acetyl and phospho) Label free global and PTMs analyses using DDA LC-MS/MS	To study/compare neddylation and other PTMs simultaneously between 3 different age group mice