Identification of PPT1 substrates to study the role of palmitoylation in synaptic function

Erica Gorenberg[1], Vicky Chou[2], Gregory Wirak[2], TuKiet Lam[3] and Sreeganga S. Chandra[2]
Interdepartmental Neuroscience Program[1], Departments of Neuroscience and Neurology[2],
Yale/NIDA Neuroproteomics Center[3], Yale University, New Haven, CT 06511

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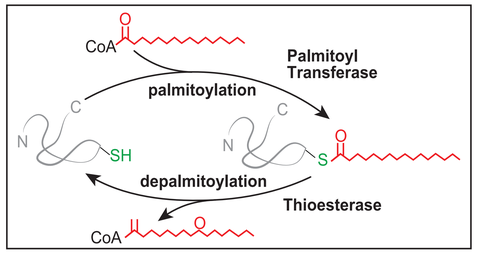
Abstract

Background: The 3D structure and function of proteins is influenced by post-translational modifications. Palmitoylation, one such modification, is the covalent attachment of a 16-carbon fatty acid chain to cysteine residues. Palmitoylation increases hydrophobicity, therefore facilitating protein association with membranes. Unlike other lipid modifications in which the protein is permanently modified, protein palmitoylation is dynamic, and palmitate groups are added and removed as the cell requires. Palmitate groups are attached to proteins by protein acyl transferases, and removed by protein thioesterases, such as palmitoyl protein thioesterase 1 (PPT1), which breaks the thioester link between the palmitate and the protein. In neurons, PPT1 is enriched at synapses, and its dysfunction leads to aberrant increases in palmitoylation of synaptic proteins such as SNAP-25, cysteine string protein α and synaptobrevin 2. Mutations in the PPT1 gene (CLN1) lead to autosomal recessive infantile neuronal ceroid lipofuscinosis (INCL), a progressive neurodegenerative disease. CLN1 mutations lead to a loss of the depalmitoylation activity of PPT1 and result in synaptic trafficking deficits. However, the repertoire of substrates of PPT1 are unknown. **Aim**: To identify synaptic PPT1 substrates. **Method**: We purified palmitoylated proteins from wild type and PPT1 knockout (KO) whole brains and synaptosomes and compared the palmitomes using Label Free Quantification-Mass Spectroscopy. **Results**: We identified putative PPT1 substrates and validated select substrates using orthologous methods. We also mapped and characterized the synaptic pathways most affected by PPT1 KO. **Conclusion**: Our results reveal the critical roles PPT1 and dynamic palmitoylation play in synapse function, and identify targets of protein depalmitoylation deficits in CLN1.

Palmitoyl Protein Thioesterase 1 (PPT1)

Palmitoylation is a dynamic post-translational modification that entails the covalent attachment of a 16-carbon fatty acid chain typically to cysteine residues. Palmitate groups are added by palmitoyl transferrases such as the DHHC proteins and are removed by thioesterases such as PPT1, which cleave the thioester linkage. Palmitoylation allows proteins to associate with membranes.

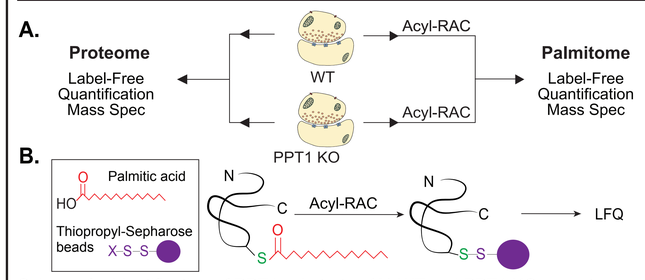


Neuronal ceroid lipofuscinoses

Neuronal ceroid lipofuscinoses are a family of hereditary lysosomal storage disorders characterized by the accumulation of the autofluorescent pigment lipofuscin in the lysosomes. These lysosomal accumulations also contain highly lipidated peptides.

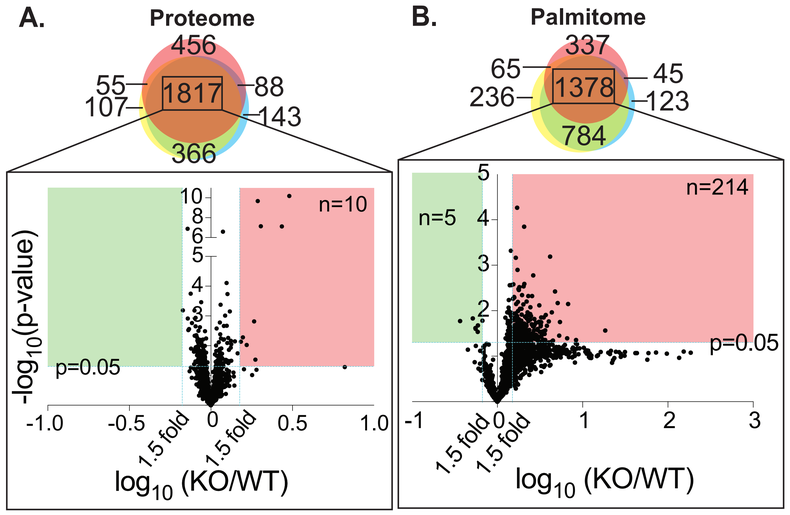
Loss-of function mutations in PPT1 cause infantile NCL, an autosomal recessive form with rapid progression. The average lifespan of INCL patients is 9-11 years.

Isolation of the synaptic palmitome



Scheme for identification of PPT1 substrates (**A**) WT and PPT1 KO synaptosomes were assessed to determine baseline protein levels (proteome) and palmitoylated protein levels (palmitome) following Acyl Resin-Assisted Capture (Acyl-RAC). (**B**) Schematic of Acyl-RAC. Levels of isolated proteins can be quantified using LFQ to assess palmitome changes in PPT1 KO vs. WT.

PPT1 KO results in changes to the synaptic palmitome



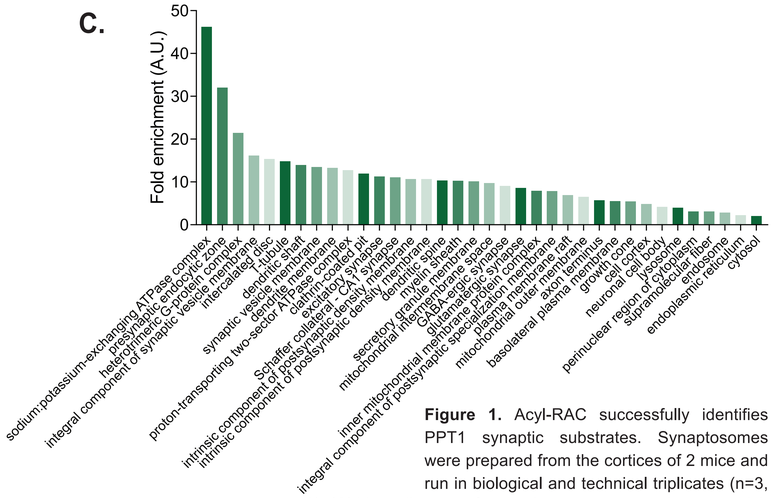
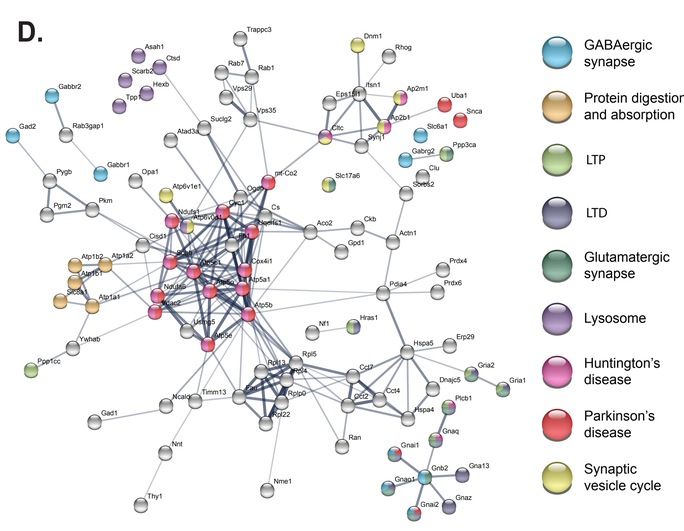
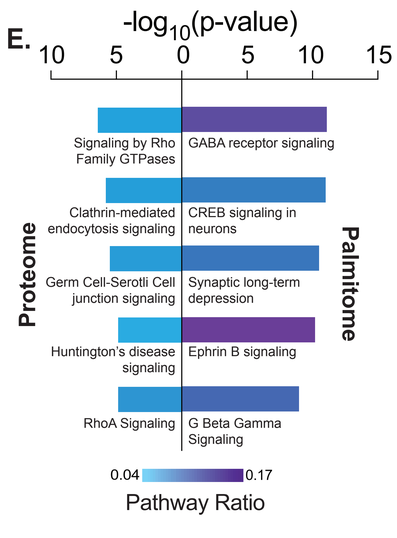


Figure 1. Acyl-RAC successfully identifies PPT1 synaptic substrates. Synaptosomes were prepared from the cortices of 2 mice and run in biological and technical triplicates (n=3, 3). (**A**) Venn diagram of synaptic proteome experiments, which contain 1817 common proteins.Volcano plot of common proteins depicts that only 10 proteins exhibit significantly increased expression in KO (**Red**; p<0.05, fold change>1.5) . These data are the average of common hits. (**B**) Venn diagram of synaptic proteome experiments, which contain 1378 common proteins. Synaptic palmitome exhibits significant decreases (**Green**) in palmitoylation of 5 proteins and significant increases (**Red**) in palmitoylation of 214 proteins. Proteins with significantly increased palmitoylation in 3 experiments were considered putative PPT1 substrates. (**C**) Gene ontology analysis of 214 putative PPT1 substrates identifies cellular components with significantly enriched representation in putative PPT1 substrates (p<0.05). (**D**) STRING analysis of 214 putative PPT1 substrates identifies pathways and disease processes that these proteins are associated with. Notably, lysosomal dysfunciton is associated with NCL, and palmitoylation defects are observed in Huntington's disease brains. (**E**) Ingenuity pathway analysis of proteome and palmitome data identify pathways most significantly impacted by PPT1 KO. P-value indicates the significance between identified proteins and pathways. Ratio represents the change in that pathway's regulation with **Blue** indicating less change and **Purple** representing greater change.





Palmitoylation changes occur independently of protein expression

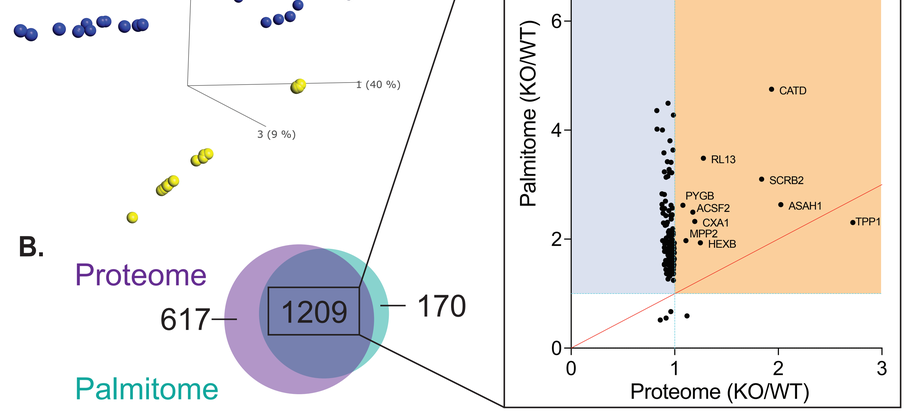


Figure 2. Comparison of proteome to palmitome identifies substrates to be validated first. (**A**) Principal component analysis of proteome and palmitome mass spectrometry data shows that principal componet 2 segregates protein levels by proteome and palmitome. Percentage represents the variance in the data explained by each principal component. Data are not clustered by genotype. (**B**) Venn diagram of proteins for proteome and palmitome experiments, which contain 1209 protein present in all replicates (n=3 biological, n=3 technical replicates for each proteome and palmitome. (**C**) Direct comparison of protein expression and palmitoylation level for common proteins (n=3 proteome, n=3 palmitome). **Light blue** indicates the subset of proteins (n=109) with increased palmitoylation and decreased or unchanged expression in KO/WT, suggesting that they are PPT1 substrates. **Orange** indicates the subset of proteins (n=11) with significant increases in both protein expression and palmitoylation in KO/WT, suggesting that these are PPT1 substrates whose degradation may be regulated by palmitoylation status. The line in **Red** indicates a 1:1 expression to palmitoylation ratio.

Future Directions

We plan to validate hits from Acyl-RAC analysis by incubation of synaptosomes with purified PPT1 followed by Acyl-RAC to assess whether PPT1 itself is able to depalmitoylate putative substrates. As palmitoylation is an activity-dependent modification, we will also stimulate neurons prior to Acyl RAC to identify substrates whose depalmitoylation by PPT1 is activity dependent. We also plan to screen PPT1 substrates for consensus sequences that may be recognized by PPT1. Finally, we would like to assess how loss of PPT1 function results in neurodegenerative disease by studying NCL patient neurons and assessing substrate palmitoylation.

Acknowledgements

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