Investigating the Roles of CLN3/6/8 In Vesicular Transport

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Abstract

A longstanding question in Batten research is whether the selective neuronal vulnerability is driven by neuron-specific roles for the causative proteins. Cellular localization of NCL proteins has been well defined separating NCLs into three broad categories including lysosomal, soluble, and transmembrane NCLs. However, the function of many of these proteins remains largely undercharacterized. To address this problem, CLN3, CLN6, and CLN8 BioID constructs were expressed in N2A cells, labeled candidate interactors were analyzed by mass spectrometry, and a list of shared interacting protein partners was compiled. Physical interactions (direct or indirect) were confirmed by coimmunoprecipitation and colocalization was confirmed with immunocytochemistry. Interestingly, interactomes for all three proteins were enriched for SNARE and tether proteins important for trafficking and binding of vesicles and neurotransmitter release. Early findings suggest that CLN3, CLN6, and CLN8 may serve an important role in the regulation of trafficking, as synaptic depletion of these SNAREs and tethers was observed across all three forms. Additional functional experiments confirm aberrant synaptic SNARE complex formation. Furthermore, neurotransmitter recycling and glutamate release assays were investigated, presenting additional evidence of functional roles of CLN3, CLN6, and CLN8 in synaptic processes. Collectively, these experiments identify novel neuron-specific roles of CLN3, CLN6 and CLN8, and may uncover potential therapeutic targets.

Cln3, *Cln6*, and *Cln8* transcript localize to a similar subset of cells in P21 mouse somatosensory cortex

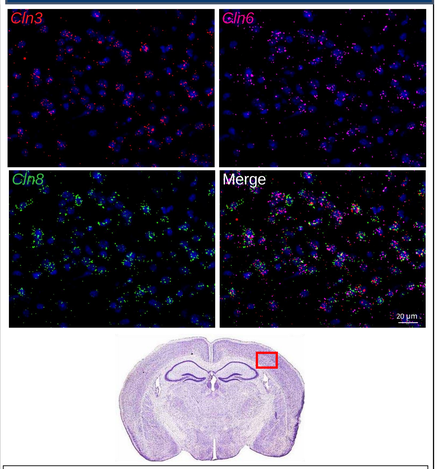


Figure 1. RNAscope transcript localization indicates co-occurrence of CLN3, CLN6, and CLN8 in P21 mouse somatosensory cortex

Shared CLN3/6/8 BioID identified interactors reveals enrichment of SNARE and tether proteins

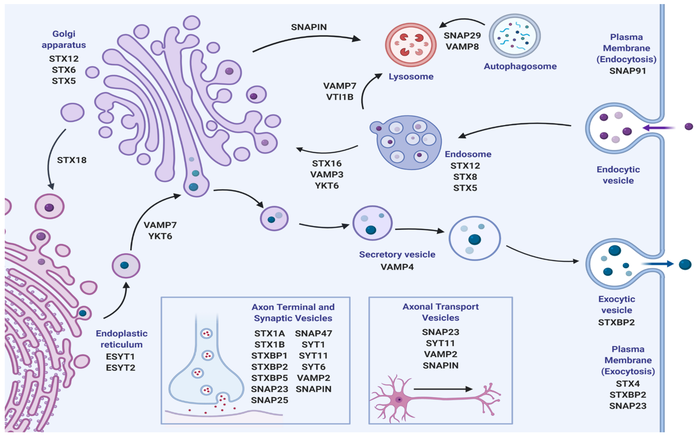


Figure 3. Subcellular localization of SNAREs and tethers identified in BioID screen

Analysis of CLN3/6/8 mutant brain fractions reveals significant differences in synaptic and cytosolic target protein expression

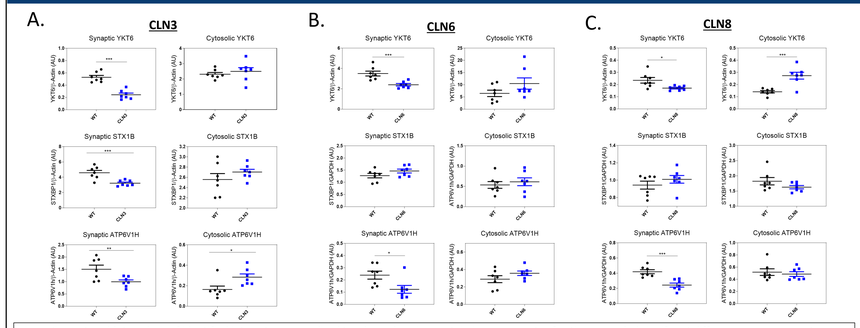
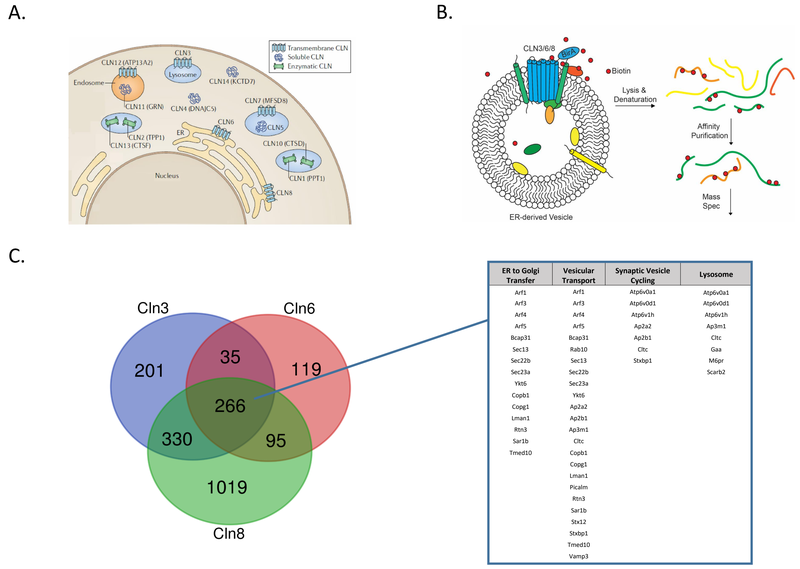
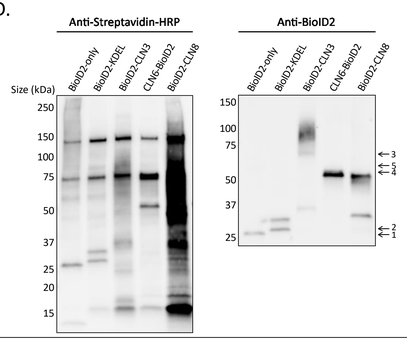


Figure 6. Western blot analysis of identified interactors normalized to a housekeeping protein in mouse cortex fractions revealed significant differences among genotypes in the synaptic and cytosolic fractions.
CLN3, CLN6 and CLN8 synaptic and cytosolic fractions were probed with anti-YKT6, anti-STX1B, and anti-ATP6V1H. (A). CLN3 analyses revealed synaptic depletion of YKT6 and ATP6V1H, and cytosolic depletion of STX1B. Also noted was cytosolic accumulation of ATP6V1H. (B). Analysis of CLN6 fractions revealed a similar effect; both YKT6 and ATP6V1H were depleted in synaptic fractions of CLN6, however no cytosolic accumulation was observed (C). CLN8 brain fractions revealed synaptic depletion of YKT6 and ATP6V1H, while significant accumulation of cytosolic YKT6 was noted. One-tailed t-test (synaptic),*p<0.05, **p<0.01, ***p<0.001, n=7, mean +/- SEM. Two-tailed t-test (cytosolic), *p<0.05, n=7, mean ± SEM.

Interacting protein partners of CLN3/6/8 identified by BioID



ER to Golgi Transfer	Vesicular Transport	Synaptic Vesicle Cycling	Lysosome
Arf1	Arf1	Atp6v0a1	Atp6v0a1
Arf3	Arf3	Atp6v0d1	Atp6v0d1
Arf4	Arf4	Atp6v1h	Atp6v1h
Arf5	Arf5	Ap2a2	Ap3m1
Bcap31	Bcap31	Ap2b1	Cltc
Sec13	Rab10	Cltc	Gaa
Sec22b	Sec13	Stxbp1	M6pr
Sec23a	Sec22b		Scarb2
Ykt6	Sec23a		
Copb1	Ykt6		
Copg1	Ap2a2		
Lman1	Ap2b1		
Rtn3	Ap3m1		
Sar1b	Cltc		
Tmed10	Copb1		
	Copg1		
	Lman1		
	Picalm		
	Rtn3		
	Sar1b		
	Stx12		
	Stxbp1		
	Tmed10		
	Vamp3		



E.

Supplementary Table 1. Coimmunoprecipitation analysis of BioID-identified protein interactions

Target	Stable interaction with CLN3	Stable interaction with CLN6	Stable interaction with CLN8
ATP6V0A1	—	Yes	—
ATP6V0D1	Yes	Yes	—
ATP6V1H	—	Yes	Yes
MUNC18	Yes	Yes	Yes
STX1B	Yes	Yes	Yes
STX7	Yes	Yes	Yes
TBC1D15	Yes	Not tested	Not tested
TBC1D5	Yes	Not tested	Not tested
VTI1B	Yes	Yes	Yes
YKT6	Yes	Yes	—

Figure 2. Shared BioID identified interacting partners of CLN3/6/8 were verified by Co-Immunoprecipitation
(A). Cellular localization of NCL proteins demonstrating lysosome (CLN3), endoplasmic reticulum (CLN6/CLN8), and endosome (CLN12), and may share similar roles in vesicle trafficking (B). A biotin ligase was fused to the protein of interest (CLN3/6/8), and expressed in N2A cells, resulting in the bioitinylation of proximal proteins. N2A cells were lysed, affinity purified, and analyzed by mass spectrometry. (C). Functional analysis of mass spectrometry data identified shared CLN3, CLN6 and CLN8 protein partners important for ER to Golgi transfer, vesicular transport, synaptic vesicle cycling, and lysosomal function. (D). Western blot analysis of BioID lysates demonstrates successful expression of BioID constructs and resulting biotinylation of proximal proteins. Expected band sizes denoted as follows: BioID-only (1) - 26kDa, BioID2-KDEL (2) - 28kDa, BioID2-CLN3 (3) - 68kDa, CLN6-BioID2 (4) - 52kDa, BioID2-CLN8 (5) - 59kDa. (E). Shared BioID-identified interactors of CLN3, CLN6 and CLN8 validated by coimmunoprecipitation. "Yes" denotes stable interaction confirmed while "-" denotes interactions that were not stable under the conditions used for coimmunoprecipitations.

Coimmunoprecipitation confirms CLN3, CLN6, CLN8 candidate interactors identified in BioID screen

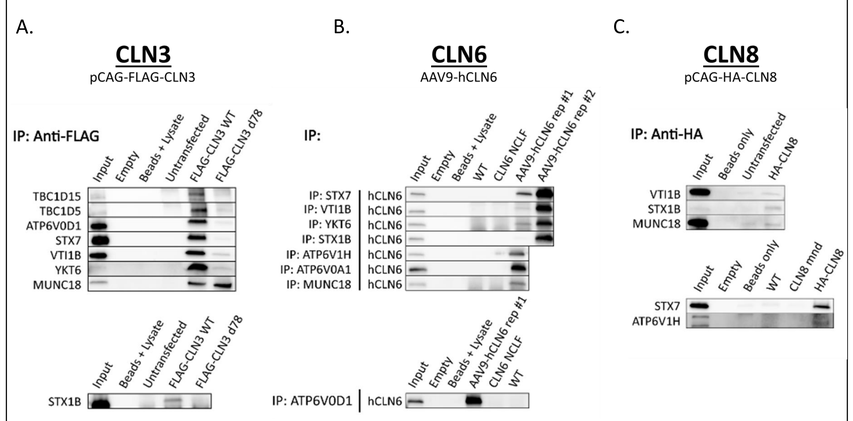


Figure 4. BioID-identified interacting partners of CLN3/6/8 were validated by Coimmunoprecipitation
(A) Western blot analyses confirm stable CLN3 interaction with TBC1D15, TBC1D5, ATP6V0D1, STX7, VTI1B, YKT6, MUNC18, and STX1B, (B) stable hCLN6 interaction with STX7, VTI1B, YKT6, STX1B, ATP6V1H, ATP6V0A1, MUNC18, and ATP6V0D1, (C) and stable CLN8 interaction with VTI1B, STX1B, MUNC18, STX7, and ATP6V1H. Input ~ 3% total protein loaded on IPs. Due to the lack of commercially available antibodies, tagged CLN3 and CLN8 expression plasmids were used for immunoprecipitation experiments.

Synaptic and cytosolic brain fractions were successfully isolated from P30 mouse cortices

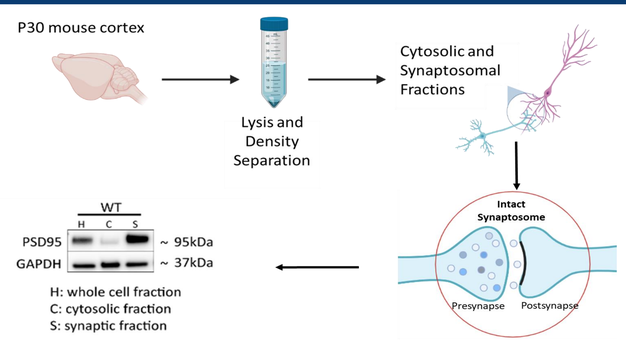
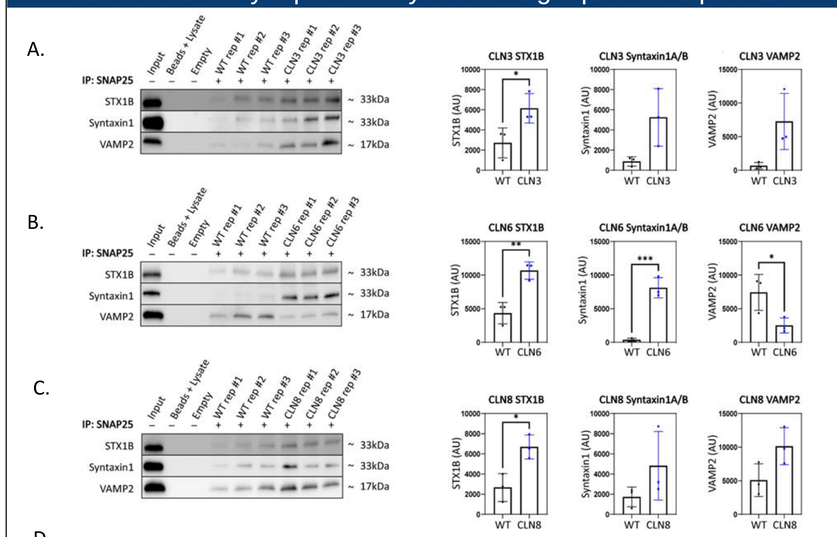


Figure 5. Fresh P30 mouse brain cortices were fractionated following a density separation protocol, yielding a whole cell fraction, cytosolic fraction, and synaptic fraction. Brain fractionation validated by western blot demonstrates significant enrichment of Postsynaptic density protein 95 (PSD-95) in the synaptic fraction coupled with cytosolic depletion.

Analysis of CLN3/6/8 mutant brain fractions reveals significant differences in synaptic and cytosolic target protein expression



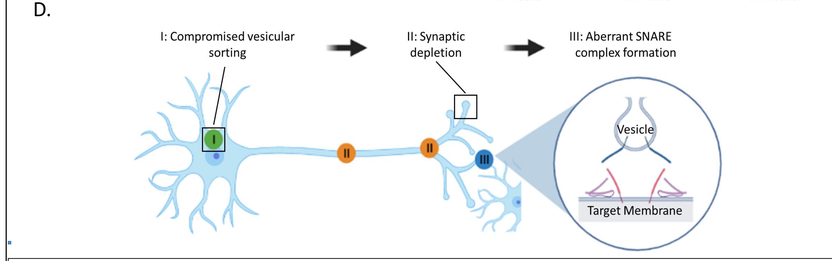


Figure 7: SNAP25 coimmunoprecipitations demonstrate synaptic SNARE dysfunction in *Cln3*$^{\Delta 7/8}$, *Cln6*$^{ncl/}$, and *Cln8*^{mnd} mice.
To analyze core SNARE complex formation, P30 mutant brain lysate was immunoprecipitated with anti-SNAP25 antibody and probed for antibodies directed against STX1B, STX1 (recognizes A and B isoforms), and VAMP. (A) Analyses reveal a significant increase in SNAP25-bound STX1B in CLN3, (B) a significant increase in SNAP25 bound STX1B and STX1, and a significant decrease in VAMP2 in Cln6 mutants, (C) and increased binding of SNAP25 and STX1B in CLN8 mutants. Input ~ 3% total protein loaded on IPs. (D) The data presented here suggests a model wherein SNARE complex dysfunction is a downstream consequence of (I) comprised vesicular sorting leading to (II) synaptic depletion of targets. (III) In turn, protein imbalance at the synapse results in dysfunctional SNARE complexes formation or release. Figure created in BioRender.com. Two-tailed t-test, *p<0.05, **p<0.01, ***p<0.001, n=3, mean +/- SEM.

Summary

- In Vitro BioID reveals a substantial number of interactors shared by CLN3/6/8, specifically SNARE and tether proteins.
- Co-immunoprecipitation confirmed stable interaction between CLN3/6/8 and BioID-identified interactors
- Analyses reveal synaptic depletion and cytosolic accumulation of stable CLN3/6/8 protein interactors
- SNAP25 coimmunoprecipitations demonstrate synaptic SNARE dysfunction in *Cln3*$^{\Delta 7/8}$, *Cln6*$^{ncl/}$, and *Cln8*^{mnd} mice.
- These finding suggest that CLN3/6/8 share neuron-specific functions in the regulation of vesicular sorting or trafficking, resulting in synaptic depletion of protein partners, and aberrant formation of complexes in which they participate.
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