Intracellular Trafficking and Synaptic Function of APL-1 in 
Caenorhabditis elegans

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Abstract

Background: Alzheimer’s disease (AD) is a neurodegenerative disorder primarily characterized by the deposition of β-amyloid plaques in the brain. Plaques are composed of the amyloid-β peptide derived from cleavage of the amyloid precursor protein (APP). Mutations in APP lead to the development of Familial Alzheimer’s Disease (FAD), however, the normal function of this protein has proven elusive. The organism Caenorhabditis elegans is an attractive model as the amyloid precursor-like protein (APL-1) is the single ortholog of APP, and loss of apl-1 leads to a severe molting defect and early larval lethality.

Methodology/Principal Findings: We report here that lethality and molting can be rescued by full length APL-1, C-terminal mutations as well as a C-terminal truncation, suggesting that the extracellular region of the protein is essential for viability. RNAi knock-down of apl-1 followed by drug testing on the acetylcholinesterase inhibitor aldicarb showed that loss of apl-1 leads to aldicarb hypersensitivity, indicating a defect in synaptic function. The aldicarb hypersensitivity can be rescued by full length APL-1 in a dose dependent fashion. At the cellular level, kinesins UNC-104/KIF-1A and UNC-116/kinesin-1 are positive regulators of APL-1 expression in the neurons. Knock-down of the small GTPase rab-5 also leads to a dramatic decrease in the amount of apl-1 expression in neurons, suggesting that trafficking from the plasma membrane to the early endosome is important for apl-1 function. Loss of function of a different small GTPase, UNC-108, on the contrary, leads to the retention of APL-1 in the cell body.

Conclusions/Significance: Our results reveal novel insights into the intracellular trafficking of APL-1 and we report a functional role for APL-1 in synaptic transmission.

Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by the deposition of β-amyloid plaques, loss of cholinergic neurons and accumulation of neurofibrillary tangles within the brain. Plaques are primarily composed of the amyloid-β peptide derived from cleavage of the amyloid precursor protein (APP). Despite the discovery of dominant mutations in APP that lead to the development of Familial Alzheimer’s Disease, the normal functional role of this protein within the neuron is still unclear. Past studies have implicated APP in cell adhesion, synaptogenesis, cell migration, signaling, apoptosis, axonal transport as well as development of the neuromuscular junction suggesting that APP is not restricted to a single function [1] for review.

APP is a type I trans-membrane protein that is conserved from C. elegans to humans. APP knock-out mice are viable and fertile and have mild defects in locomotor activity, forelimb grip strength, behavior and long term potentiation (LTP) [2,3,4,5]. The subtlety of these phenotypes is thought to be due to functional redundancy with the two other members of the APP family, APLP1 and APLP2 as loss of APLP2 along with one of the other two APP homologs results in early postnatal lethality in mice [6,7]. Because of the redundancy of these homologs, using the mammalian system to study the function of APP has proven challenging.

The C. elegans model offers a simplification of the mammalian system in that APL-1 is the only APP ortholog in the nematode and a null mutation leads to early larval lethality [8]. APL-1 is structurally similar to its mammalian counterpart and shares three major regions of homology: the N-terminal E1 and E2 domains and the highly conserved intracellular C-terminal domain [9]. APL-1 does not contain the amyloid-β sequence, similar to the functionally redundant mammalian APP homologs APLP1 and APLP2 [10,11,12].

In this study, we use C. elegans to investigate the normal functional role of APL-1. We report that APL-1 is necessary for viability, molting and regulation of neurotransmission. Full length rescue of the synaptic transmission defect is dose dependent, while the N-terminus of APL-1 is sufficient to rescue the molting and lethality phenotypes. At the cellular level, proper localization and protein levels of APL-1 throughout the neuron are dependent on the kinesin transporters UNC-104/KIF1A and UNC-116/kinesin-
1 as well as the small GTPase RAB-5 and UNC-108/Rab2, indicating their likely role in APL-1 vesicle transport and endocytosis.

Materials and Methods

C. elegans Strains

Strains were cultivated at 20°C as described previously [13]. The strains used in this study include the wild-type Bristol N2 strain, ap1-1(tm385)/lon-2(e678), lon-2(e678), dpy-1(mu62), unc-32(e189), unc-49(e372), rrf-3(pk1426), unc-104(e1265), unc-116(e2310), unc-108(n2363). Crosses were performed using standard genetics methods and final strains identified by phenotype and PCR.

RNAi

RNAi was performed by feeding as previously described [14]. RNAi clones were isolated from the Ahringer RNAi library (Gene Service) by streaking clones onto plates containing 10 μg/ml tetracycline and 100 μg/ml ampicillin. Before use, all RNAi clones isolated from the library were validated by sequencing. Cultures were grown overnight in LB containing 100 μg/ml ampicillin and used to seed NGM plates containing 1 mM IPTG and 50 μg/ml ampicillin.

Development and Brood Size Assays

Ten to twelve young adult worms were placed on individual RNAi plates or NGM plates and allowed to lay eggs overnight. The eggs were collected onto new plates and counted. After 48 hours the worms were scored for their developmental stage. For brood size assays, ten L4 worms were placed on individual NGM plates. Every 24 hours the number of the eggs the worms produced were counted and the mothers transferred to new plates until no more eggs were produced. Brood size assays were analyzed by one-way ANOVA with Bonferroni post-hoc test.

Movement Assays

Body bends per minute were obtained by placing late L4 worms individually on NGM plates and screening 24 hrs later. Body bends were counted over a 3 min time period and then divided to calculate the average number of body bends in one minute. One body bend is completed when the point behind the pharynx reaches the opposite apex of the sinuous curve. Measurements were statistically analyzed by one-way ANOVA with Bonferroni post-hoc test.

Plasmid Construction

All constructs were generated, unless otherwise described, by amplifying target sequences with Phusion High-Fidelity DNA Polymerase (Finnzymes) using primers with overhanging restriction sites. The PCR products were digested with their respective restriction enzymes and ligated to the destination vector backbone. For ap1-1 expression studies and rescue experiments, worm ap1-1 genomic coding DNA was amplified from cosmid C42D8 (Sanger Institute) including 4.4 kb of sequence upstream of the start codon. This fragment was ligated into L3781 (Addgene plasmid 1590; Fire Lab C. elegans Vector Kit, 1999 plate) in frame with the GFP sequence at the C-terminal end of ap1-1 to generate pAPl-1::ap1-1::gfp. The C-terminal truncation construct pAPl-1::ap1-1::T658E::gfp was generated by amplifying the coding region of ap1-1 including the sequence for the last 36 amino acids. This PCR product was cloned into L3781 followed by cloning the ap1-1 promoter upstream. Constructs containing pAPl-1::ap1-1::rab-5::mCherry, pAPl-1::ap1-1::T658E::gfp, and pAPl-1::ap1-1::T658A::gfp were generated from the original pAPl-1::ap1-1::gfp full length construct using site-directed mutagenesis to introduce the individual mutations. For human rescue studies, human APP cDNA containing an mRFP fusion was amplified from N1-APP-RFP and cloned into the L3781 expression vector that had been cut with XmaI and NheI to remove the coding region of GFP. The ap1-1 promoter was amplified from C42D8 then added to the APP::RFP clone to generate pAPl-1::APP::RFP. APLP1 and APLP2 cDNA sequences were amplified from clones 3865417 and 2820109 (Open Biosystems) respectively and also cloned into the L3781 vector with the GFP tag on the 3' end of the gene followed by the insertion of the ap1-1 promoter.

Plasmids for colocalization purposes were made by amplifying mCherry from the vector pCFJ90 (Addgene plasmid 19327; [15]) and cloning the sequence upstream of the start codon of either rab-5 or unc-108 cDNA. For rab-5, the mCherry sequence was cloned into pBZ103 containing phps162/2::zab-5 followed by cloning the ap1-1 promoter to generate the final construct containing pAPl-1::mCherry::zab-5. The unc-108 construct was developed by inserting the amplified mCherry sequence into pAOLO174 containing punc-108::unc-108(Q65L). The Q65L mutation was removed using site-directed mutagenesis to generate the WT sequence. pBZ103 and pAOLO174 were generous donations from the Zhou lab at Baylor College of Medicine.

All constructs were completely sequenced to verify accuracy of promoters and coding regions. All primers used for cloning are referenced in File S1.

Transgenic Strains

Transgenic strains were generated through microinjection of DNA constructs into the worm gonad as previously described [16]. For expression studies, pAPl-1::ap1-1::gfp (40 ng/μl) was co-injected with the marker construct pRF4 (75 ng/μl), which contains rol-6(sa1006). The rescue constructs described above were individually injected into the gonads of the ap1-1(tm385)/lon-2(e678) strain. For human rescue studies, human APP cDNA was co-injected into the gonads of the ap1-1(tm385)/lon-2(e678) strain. F1 progeny containing the array were individually cloned onto new plates and the F2 progeny of the lines were analyzed to determine if rescue of the L1 lethality occurred by absence of both long worms, indicating loss of the balancer, and arrested L1 progeny. Presence of the tm385 deletion was tested by PCR. The full length ap1-1 10 ng/μl expressing strain was isolated by UV irradiation, outcrossed 3X with the lon-2(e678) strain to maintain the tm385 deletion then crossed to the rfy-3(pk1426) background for RNAi studies. For colocalization experiments, constructs were co-injected into N2 worms. Rescue strain construct concentrations are listed in Table 1 with each being co-injected with the pmpro-3::GFP marker (L4816; Fire Lab C. elegans Vector Kit, 1999 plate) (20 ng/μl) and pCR2.1 added to a final injection concentration of 100 ng/μl. For colocalization experiments the following injection concentrations were used: pAPl-1::ap1-1::gfp (20 ng/μl), pAPl-1::mCherry::zab-5 (20 ng/μl), punc-108::mCherry::unc-108 (5 ng/μl), pRF4 (75 ng/μl). Strains created in this study are listed in File S1. Representative strains are listed where multiple strains were generated at the same concentration.

Aldicarb Assay

L4 hermaphrodites were placed on control (L4440) or ap1-1 RNAi plates and allowed to mature and lay eggs overnight. Young adults from the F1 progeny were placed onto NGM plates containing 1 mM aldicarb (PS734, Sigma-Aldrich) in the presence of food then scored semi-blind for response to a harsh touch every 10 minutes for 2 hours. Animals unable to respond to the touch were scored as paralyzed. Adult RNAi aldicarb experiments were performed 48 hrs after young adult animals were placed on the control (L4440) or ap1-1 RNAi plates. All rescue and mutant strains
were tested as young adults. Strains were compared statistically by one-way ANOVA with Bonferroni post-hoc test.

Protein Extraction and Western Blotting

Worms were synchronized by bleaching as previously described [17] and three 10 cm plates containing L4 stage worms were harvested. Samples were washed with TE, pelleted by centrifugation then placed at -80°C for at least 15 min. The pellet was thawed and all liquid replaced with 50 μl RIPA buffer containing protease inhibitors (Roche). Samples were sonicated twice for 10 seconds against GFP 1:5000 (ab290; Abcam) or membrane and then the membrane was blocked for 2 hrs in 5% polyacrylamide gel. Samples were transferred onto nitrocellulose control and compared using the Student’s t-test. Colocalization experiments were performed by taking images on an Olympus IX50 inverted microscope. Images underwent deconvolution using MetaMorph software and then overlayed using ImageJ. Colocalization quantification was performed as previously described [18] using intensity correlation analysis with the following modifications. The neuronal cell body was set as the region of interest and then the intensity correlation quotient (ICQ) was quantified using ImageJ as described by the McMaster Biophotonics Facility. In brief, an ICQ of 0 indicates random staining between the two fluorescent images while 0<ICQ<0.5 indicates colocalization and ICQ≥0.5 occurs during segregated staining. For further description of the analysis refer to Li et al. (2004) [18]. ICQ values of the neurons were statistically analyzed using the one-sample t-test to compare the mean ICQ values against a random staining value of 0.

RNA Extraction and qRT-PCR

Worms were harvested at the L4 stage (~6,000 per sample) by rinsing worms off the plate using TE. Samples underwent a freeze/thaw cycle 4X between liquid nitrogen and a 37°C waterbath followed RNA isolation using the Qiagen RNeasy Lipid Tissue kit method with the addition of the RNase free DNase steps (Qiagen). cDNA was generated by reverse transcription using the Superscript III First Strand kit (Invitrogen) with the input of equal concentrations (1000 ng/μl) of RNA as measured by NanoDrop (NanoDrop Technologies). PCR primers to test apl-1 expression were designed using Primer Express 2.0 (Applied Biosystems) (apl-1 Fwd ACTCACACGTGTCAGACCGTACCA, apl-1 Rev GTGCGGGACCTTGAGAGCCTT) and ama-1 qPCR r1, AACTCGACATGAGCCTGCA, using the ABIprism 7000 and data collected using the 7000 System SDS Software (Applied Biosystems). Primer efficiencies were originally validated using the standard curve method with all subsequent experiments using a 1:50 dilution of cDNA and results analyzed using the comparative Ct method. Bars represent the mean of a triplicate containing a single biological sample with error bars calculated from the sample and endogenous control standard deviations (STD = \sqrt{(Sample\ STD)^2+Sample\ Keeping\ Gene\ STD)^2}).

Results

Characterization of apl-1 Expression

To determine the expression pattern of apl-1, transgenic worms were generated expressing an apl-1::gfp fusion protein driven by the endogenous apl-1 promoter. APL-1::GFP fluorescence is detected in the cell bodies and processes of nerve ring interneurons (Figure 1A,c) and the ventral cord (Figure 1A,g). apl-1::gfp is also expressed in

| Constructs* | Concentration | Rescue/Total Strains |
|-------------|---------------|---------------------|
| papl-1::apl-1::gfp | 20 ng/μl | 4/4 |
| papl-1::apl-1 | 20 ng/μl | 3/3 |
| papl-1::apl-1 | 5 ng/μl | 3/3 |
| papl-1::apl-1::IC::gfp | 20 ng/μl | 0/3 |
| papl-1::apl-1::T658A::gfp | 20 ng/μl | 3/3 |
| papl-1::apl-1::T658E::gfp | 20 ng/μl | 3/3 |
| papl-1::apl-1::DYENPTY::gfp | 20 ng/μl | 10 ng/μl | 4/4 |

*Constructs were used to inject apl-1(tm385)/lon-2(e678) and the F2 progeny were examined for the generation of a rescue strain. **Endogenous apl-1 promoter. doi:10.1371/journal.pone.0012790.t001

Fluorescence Microscopy and Quantification

Imaging was performed by placing live animals anesthetized with 20 mM sodium azide on a 2% agarose pad. Images were obtained using a Zeiss AxioScope 2 Plus upright microscope equipped with an AxioCam MRm camera and Axiovision 4.1 software. Pictures were acquired with 100X or 63X lens. In the case of fluorescence quantification, head neurons of 10–20 worms per genotype were imaged with identical exposure times. Each neuronal cell body was imaged at its widest diameter in the plane of focus. Fluorescence was quantified using ImageJ. Control pictures were taken on the same day to account for differences in bulb intensity. Genotypes were compared using the Student’s t-test.
socket cells and amphids present in the head. Strong expression is seen in junctional cells such as the pharyngeal intestinal valve (Figure 1 A,e), which tethers the pharynx to the intestine, and the uterine seam junction in adults (Figure 1 A,h), which provides the structural connection between the epidermis and the uterus. APL-1 can be weakly detected in many epidermal epithelia including hyp7 (Figure 1 A,f), the hypodermal syncitium surrounding the worm, as well as vulval cells, rectal valve cells, pharyngeal arcade cells, and tail hypodermis. Expression is prominent in the excretory cell (Figure 1 A,h), a long H-shaped cell implicated in fluid balance. APL-1 was notably absent from body wall muscle and intestine. These expression patterns indicate that apl-1 is active in cells with high levels of structural components such as synapses, junctional epithelial cells and cells with apical basal polarity.

apl-1 Loss of Function and Genetic Rescue

Similar to the molting defect caused by apl-1 null mutations, knock-down of apl-1 using RNAi on the RNAi sensitive nrf-3(pk1426) strain led to defective molting starting at the L3/L4 molt and continuing in the L4/YA molt (Figure 1B). A variety of molting phenotypes were seen which ranged from loose cuticle around the head and tail (7.5%, n = 173), internal pinching of the worm body at or just posterior to the head (4.6%), degradation around the mouth area (9.8%) or a cuticle plug around the mouth (38.1%) (Figure S1A, B). All of the worms had a very transparent appearance that, when examined at higher magnification, appeared as empty spaces in the worm spanning the length of the body. apl-1 knock-down also led to delayed development, as most of the population after two days was in the L4 stage while the majority of the control worms had completed development to adulthood (Figure S1C). Furthermore, we observed that worms on apl-1 RNAi exhibited sluggish movement, failing to move normally even when touched.

The apl-1(tm385) allele is a deletion that removes 646 base pairs including exon 3, which deletes 42 amino acids leading to a frame shift of the downstream sequence, resulting in a premature stop.
codon (Figure S2A). Worms homozygous for the apl-1(tm385) deletion are L1 lethal and exhibit internal vacuolization, degradation and loose cuticle phenotypes (Figure 1C) similar to previously reported null mutations [8], indicating that the tm385 lesion is also null. We attempted to rescue the lethality of this mutant using constructs containing either full length apl-1, mutations within the highly conserved C-terminal domain, a C-terminal truncation of apl-1, or human APP, APLP1 or APLP2 (summarized in Table 1). All constructs were driven by the apl-1 promoter and fused to a C-terminal GFP. Rescue constructs included a deletion of the highly conserved YENPTY motif which is known to bind to many different adaptor proteins or mutations of the conserved Thr668 residue (Thr658 in APL-1) which is known to bind to many different adaptor proteins or mutations of the conserved Thr658 residue (Thr658 in APL-1) which is a known to bind to many different adaptor proteins or mutations of the conserved Thr668 residue (Thr658 in APL-1) which is a known to bind to many different adaptor proteins or mutations of the conserved Thr668 residue (Thr658 in APL-1) which is a known to bind to many different adaptor proteins or mutations of the conserved Thr668 residue (Thr658 in APL-1) which is a

To test whether the C-terminus of apl-1 is required for the regulation of neurotransmission, we tested the aldicarb sensitivity of the ΔIC and the ΔYENPTY rescue strains and found that the C-terminal mutants exhibited similar aldicarb hypersensitivity as compared to full length APL-1 expressed at comparable levels (Figure S4). These data provide indirect support against a potent role of the highly conserved C-terminal domain in APL-1 mediated synaptic transmission.

UNC-104/KIF1A, UNC-116/Kinesin 1 and RAB-5 Positively Regulate APL-1 Expression

While the trafficking of APP has been extensively studied in neurons, the movement of APL-1 through the cell is still unknown. APP is normally trafficked in a kinesin-dependent anterograde fashion from the cell body to the nerve terminal [26]. Due to APL-1’s strong expression in neurons, we decided to test whether APL-1 can be transported by two of the major kinesins involved in anterograde transport of synaptic proteins. The first kinesin we investigated is the worm homolog of KIF1A, UNC-104. UNC-104/KIF1A is responsible for transporting synaptic vesicles and dense core vesicles to sites of synaptic transmission [27,28,29]. In order to test if APL-1 transport depends on this neuronal kinesin, we crossed the APL-1::GFP transgenic rescue strain to the hypomorphic mutant unc-104(e1263). Interestingly, rather than observing an accumulation of the GFP fluorescence in the cell body, which traditionally results from the reduction of UNC-104 mediated vesicle transport [27], we found a dramatic decrease in the fluorescence from apl-1::gfp on the unc-104(e1263) background, as measured by fluorescence intensity in a set of three head interneurons that consistently expressed apl-1 (Figure 4A, B).
ally, APL-1 fluorescence was absent from the processes of the neurons, quantified from a specific dorsal process that is consistently visible on the N2 background (Figure 4C). Western blotting from L4 worms also detects a drop in APL-1 protein expression on the unc-104(e1265) background (Figure 4D, E). qRT-PCR comparing apl-1 expression between N2 and unc-104(e1265) backgrounds showed no differences, suggesting that the loss of APL-1::GFP is occurring at the protein level (Figure 4F).

Figure 3. Rescue of aldicarb hypersensitivity by apl-1 is dose dependent. (A) qRT-PCR showing representative rescue strain expression of apl-1 at different injection concentrations. Error bars represent the STD. (B) Aldicarb hypersensitivity is not rescued by apl-1 full length at a lower expression level. Strains expressing half the level of the original full length strain can no longer rescue the aldicarb hypersensitivity, but rather show a dosage dependent effect. Two rescue strains per genotype were used to rule out variability from over-expression of the rescue array (representative strains shown). Control: unc-49 - GABA receptor subunit, aldicarb hypersensitive. (C) Quantification of the rescue strain aldicarb experiment at 90 minutes. (*) P<0.05) Error bars represent the s.e.m. unless otherwise noted.

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Using a similar approach, we tested the ability of UNC-116/kinesin-1 to transport APL-1 by crossing the APL-1::GFP transgenic strain to the hypomorphic unc-116(e2310) background. Kinesin-1 has been found to play a prominent role in the transport of APP along the axon [26]. Again we saw a reduction in the amount of APL-1::GFP expression on the unc-116 mutant background, although to a lesser extent as that on the unc-104 background (Figure S3). However, there was a complete loss of APL-1 expression along the dorsal axon in every nematode analyzed on the unc-116 background. These results suggest that APL-1 localization is dependent on both kinesins.

In addition to being transported by kinesins, APP has previously been shown to localize to the early endosome by its strong colocalization with Rab5 positive compartments in preparations of nerve terminals from rat forebrain and PC12 cells [30,31]. As a small GTPase, Rab5 regulates endosomal trafficking of vesicles from the plasma membrane to the early endosome [32]. To determine whether APL-1 was also present in RAB-5 compartments in the worm we generated strains co-expressing APL-1::GFP and mCherry::RAB-5. We found that these proteins colocalized within a subset of puncta (Figure 5A). This was reconfirmed by observing consistently positive intensity correlation quotient (ICQ) values in the different neurons analyzed (Figure S3B).

To determine whether loss of rab-5 had an effect on the localization of APL-1, we used RNAi to knock-down rab-5 expression in an integrated APL-1::GFP expression strain on the RNAi sensitive rfi-3(pk1426) background. Loss of rab-5 led to a dramatic decrease in the amount of APL-1::GFP in neurons as well as a complete loss of APL-1 in the dorsal process (Figure S3C, D). By contrast, knock-down of two other small GTPases, rab-7 or rab-10, did not affect APL-1 expression, suggesting that RAB-5 compartments specifically are important for the localization of APL-1 (Figure 5E).

unc-108 Mutations Lead to Altered Intracellular Localization of APL-1

UNC-108 is a small GTPase expressed in neurons and engulfing cells that localizes to the Golgi and early endosome [33,34]. UNC-108 has been found to be involved in the maturation of dense core vesicles (DCVs), a distinct vesicular population containing peptide hormones and neuropeptides [34,35,36]. APL-1 likely undergoes fast axonal transport in a vesicular population, therefore we wanted to investigate if UNC-108 is required for the packaging of APL-1 into vesicles destined for anterograde transport.

To study this possibility we crossed the hypomorphic mutant unc-108(n3263) to the APL-1::GFP expressing strain. We found that one of the head inter-neurons appeared to have a back-up of protein in the cell body (Figure 6A, B). This aggregation of protein in a distinct compartment was also seen in ventral cord neurons. We then performed colocalization experiments by generating strains expressing APL-1::GFP and mCherry::UNC-108. Similar to the colocalization with RAB-5, APL-1 is found in overlapping puncta with UNC-108 in neurons, demonstrating APL-1 localization to the same compartment (Figure 6C). The ICQ values of the different neuronal populations were consistently positive, showing that the proteins colocalize together at a similar frequency as with RAB-5 (Figure 6D). These data suggest that UNC-108 is required for the localization of APL-1 and ultimately its transport.

Discussion

N-terminus of APL-1 Is Required for Worm Survival and Molting

We have shown that loss of apl-1 contributes to defects in at least two systems, one of which is molting. The L1 lethality seen in the apl-1(tm385) strain is likely due, at least in part, to the molting defect, which is recapitulated by apl-1 RNAi. Worms with apl-1...
APL-1 Function in C. elegans

knocked-down share the loose cuticle and internal vacuolization phenotypes of *apl-1(tm385)* L1s but exhibit these phenotypes later in development. The clear appearance of these worms may be due to inappropriate release of proteases involved in the melting process, loss of proper adhesion between tissues, and/or loss of fat stores due to starvation from the loose cuticle blocking food intake. Whether the sluggish movement defect seen in adult *apl-1* knock-down worms is due to the melting defect, starvation or a neurotransmission defect remains to be seen. The phenotypes shown in this RNAi study are more severe than those described previously [37], possibly due to our use of an RNAi sensitive strain. Whether the sluggish movement defect seen with *apl-1* RNAi is indicative of a failure to undergo ecdysis, or shedding of the old exoskeleton [38]. Another single-pass transmembrane protein possessing a similar loose cuticle melting defect is the LDL receptor-related protein (LRP-1), which is the *C. elegans* ortholog of LRP-2/megalin and likely functions in cholesterol uptake and homeostasis [38,39,40]. A null mutation in LRP-1, like *apl-1*, leads to arrest and lethality although at later larval stages [40]. These similarities suggest that *lip-1* and *apl-1* may operate in the same or similar pathways to control the molting process. Since we found that *apl-1* does not require its C-terminal domain for rescue of the molting defect and soluble, secreted APP has been shown to bind to an LRP homolog [41], an attractive hypothesis would be that the N-terminal domain of *apl-1* is shed and released at regulated periods followed by binding to LRP-1 to mediate proper ecdysis at each of the four molts.

Our rescue results and previous studies support the notion that only the N-terminus of *apl-1* is required to rescue the lethality seen in the *apl-1* null strain [8]. In mice, expression of APP that has been truncated either at the N-terminus or had the last 15 amino acids removed could ameliorate APP knock-out phenotypes such as reduced body and brain weight, defective LTP and spatial learning, and loss of grip strength [42]. These findings combined support an important function of the N-terminus of APP in the mammalian system as well as in *C. elegans*.

While the behavior defects in the *Drosophila* APPL null can be rescued by expression of human APP [43], we were unable to rescue the *apl-1* null lethality in *C. elegans* by expressing any of the human homologs of APP. Several notable differences between the fly and the worm homologs of APP has been shown to bind to an LRP homolog [41], an attractive hypothesis would be that the N-terminal domain of *apl-1* is shed and released at regulated periods followed by binding to LRP-1 to mediate proper ecdysis at each of the four molts.

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While the behavior defects in the *Drosophila* APPL null can be rescued by expression of human APP [43], we were unable to rescue the *apl-1* null lethality in *C. elegans* by expressing any of the human homologs of APP. Several notable differences between the fly and the worm homologs of APP can account for the differences in cross species rescue. Unlike *apl-1*, expression of the fly homolog is confined to neurons and loss of APPL does not affect Drosophila viability or fertility [43,44]. Furthermore, different domains in APPL-1 and APPL are required for their respective functions. The entire APPL protein is required for its proper function in the fly, whereas in *C. elegans* the *apl-1* N-terminus is the critical domain needed to rescue the lethal *apl-1* null mutant. APPL over-expression induced synaptic bouton formation could be prevented by deletion of the C-terminal domain or the N-terminal E1 and E2 domains, showing that the holoprotein is needed to mediate this function [45]. That APP with a C-terminal truncation expressed in APPL deficient fly lines could no longer induce axonal arborization seen when expressing full length APP further highlights the importance of the C-terminus for proper function of the protein in flies [46]. Since APPL-1 function requires the N-terminus, it is not surprising that the *C. elegans* system cannot use APP with its minimally conserved N-
terminal domains to rescue the severe \textit{apl-1} loss-of-function phenotypes.

Loss of \textit{apl-1} Leads to Neurotransmission Defects

\textit{C. elegans} and mice share many homologs that are involved in synaptic structure and function. Therefore we predicted that \textit{C. elegans} would be an excellent model to study the importance of APL-1 in neurotransmission. In mice, \textit{APP/APLP2} null animals have enhanced nerve sprouting, reduced numbers of synaptic vesicles, defects in neurotransmitter release as well as a large number of defective synapses [24]. Similar to the mammalian system, we reveal here that loss of \textit{apl-1} expression leads to defective neurotransmission. We did not observe any overt defect in general neuronal structure in \textit{apl-1(tm385)} lethal L1s, therefore defects in the development of the neuronal network are not likely to contribute to the phenotype. Interestingly, we would predict that the defect on aldicarb would be resistance rather than hypersensitivity if the worms lacking APL-1 also have a reduction in synaptic vesicle number and decreased number of functional synapses. These differences may be due to the fact that the mammalian system uses purely cholinergic connections at the neuromuscular junction while worm movement is modulated by both GABAergic and cholinergic synapses. Aldicarb cannot distinguish between cholinergic or GABAergic defects, nor can we rule out contributions from dense core vesicles, which also modulate neurotransmission [47,48,49]. The hypersensitivity we see during \textit{apl-1} knock-down may be due to improper regulation of this pathway due to varying levels of interaction with G_{\alpha}. The fact that loss of \textit{apl-1} also leads to enhanced pharyngeal pumping [52] supports a regulatory role through the EGL-30 pathway as pharyngeal pumping is one of the many functions modulated by this G-protein [53].

We have found that the regulation of neurotransmission by APL-1 does not appear to be related to its regulation of molting. A molting defect was not seen in any of the rescue strains, or purposely avoided by performing \textit{apl-1} RNAi knock-down in adults, whereas the aldicarb hypersensitivity was present in these worms. This dual regulation could not be dissected by removing the C-terminal domain or YENPTY motif, since the full length APL-1 rescue of lethality at a lower expression level still could not rescue the aldicarb hypersensitivity. Together, these data support a model in which the function of APL-1 in molting is independent of its function in neurotransmission.
Regulation of APL-1 Localization and Transport

We have found that localization of APL-1 in neurons is regulated through the action of the kinesins UNC-104/KIF1A and UNC-116/kinesin-1 as well as the small GTPases RAB-5 and UNC-108/Rab2 (Figure 7). In mice, APP undergoes fast axonal transport to the nerve terminal through the action of the kinesin-1 transporter [26]. However, in worms, APL-1 localization is dependent on both UNC-104/KIF1A and UNC-116/Kinesin-1. Rather than causing a back-up of protein, loss of either of these kinesins led to a general loss of APL-1::GFP. This indicates that the protein is being broken down rather than being allowed to accumulate in the cell body. Neither of these hypomorphic mutants have a molting defect, possibly through compensation of APL-1 trafficking by the other transporter, or the decrease in function of the kinesin is not severe enough to prevent APL-1 from operating in the molting pathway.

After being exposed to the cell surface, APP is rapidly internalized and sorted to the early endosome by the action of the small GTPase Rab5 [31]. Like APP, we have found that APL-1 movement through the endosomal pathway utilizes RAB-5 as loss of RAB-5 reduces the level of APL-1 within the neuron. With the loss of RAB-5 through RNAi, we speculate that APL-1 trafficking by the other transporter, or the decrease in function of the kinesin is not severe enough to prevent APL-1 from operating in the molting pathway.

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Unc-108/Rab2 is known for its role in COPI-mediated retrograde transport between the Golgi and ER [54]. However, in C. elegans, loss of unc-108 does not affect COPI transport, but rather leads to an accumulation of early endosomal compartments [55]. UNC-108 is also involved in the maturation of dense core vesicles by preventing loss of cargo to specific endosomal compartments [34,36]. We suspect that APL-1 accumulation in unc-108(n3263) mutants may be due to incorrect sorting of APL-1 into the proper vesicular population upstream of anterograde transport. It is still unknown whether APL-1 is present in dense core vesicles, although this subset of vesicles is primarily transported by UNC-104 [56], which also appears to be the transporter most involved in proper localization and expression levels of APL-1 (Figure 4). Since the localization of APL-1 is dependent upon the presence of functional UNC-108, and the two proteins colocalize, it is possible that APL-1 may play a role either within the DCVs, or actively operating with UNC-108 in the maturation of DCVs. This may be another plausible explanation for the ability of APL-1 to regulate synaptic transmission as dense core vesicle cargos have been found capable of modulating cholinergic signaling [49].

In summary, our results show that APL-1 regulates neurotransmission independently of its function in the molting process. APL-1 moves through the neuron in a similar fashion to APP, with the distinction that two kinesins are needed for anterograde transport and to maintain proper expression levels of APL-1. Like APP, this transport is followed by endocytosis through the action of RAB-5. The ability of UNC-108 to alter the localization of APL-1 points to a novel process by which APL-1 is regulated in the cell. Overall, we predict that transport of APL-1 within the neuron enables APL-1 to properly perform its multiple functions by introducing the protein to molecules that can cleave and regulate release of the critically important N-terminal portion of the protein. This has implications for the biology of APP and its homologs where the N-termini of these proteins may also act as ligands to stimulate downstream pathways that modulate neurotransmission.

Supporting Information

File S1 Supplementary tables. Found at: doi:10.1371/journal.pone.0012790.s001 (0.07 MB DOC)

Figure S1 Molting defect seen during apl-1 RNAi. (A) Representative pictures of the various molting defects seen in RNAi sensitive strain nrf-3(pk1426) L4s during apl-1 knock-down. Arrowheads point to regions described by the inlaid text. (Scale...
bar, 20 μm). (B) Percentages of the different molting defects seen in worms on apl-1 RNAi after 48 hours from egg stage (n = 173). (C) Percentage of different progeny stages 48 hours from egg stage on both control (L4440) and apl-1 RNAi.

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**Figure S2** Rescue strains of apl-1 (tm385) have slower development. (A) Map of apl-1 (tm385) deletion and premature stop codon. (B) Percentage of different larval stages after 48 hours of growth. The N2 strain and heterozygous apl-1 (tm385)/lon-2(e678) are shown as negative controls. (N2, n = 697; apl-1/lon-2, n = 186; N2 Ex(apl-1::gfp) - 20 ng/μl, n = 528). Missing worms were those unaccounted for after 48 hours from the original total of eggs placed on the plate. These are likely in large proportion part of the L1 lethal population that are no longer visible. (C) No movement placed on the plate. These are likely in large proportion part of the unaccounted for after 48 hours from the original total of eggs.

References

1. Zheng H, Koo EH (2006) The amyloid precursor protein: beyond amyloid. Mol Neurodegener 1: 5.
2. Zheng H, Jiang M, Trumbauer ME, Srinath Singhji DJ, Hopkins R, et al. (1995) beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81: 325–331.
3. Dawson GR, Seabrook GR, Zheng H, Smith DW, Graham S, et al. (1999) Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. Neuroscience 90: 1–13.
4. Phinney AL, Calhoun ME, Lipp HP, Zheng H, et al. (1999) No hippocampal neuron or synaptic bouton loss in learning-impaired aged beta-amyloid precursor protein-null mice. Neuroscience 90: 1207–1216.
5. Ananth LG, Johnson PA, Fetter RD, Tully T, Goodman RS, et al. (2003) Mechanisms contributing to the deficits in hippocampal synaptic plasticity in mice lacking amyloid precursor protein. Neuropharmacology 38: 349–359.
6. von Koch CS, Zheng H, Chen H, Trumbauer M, Thinakaran G, et al. (1997) Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice. Neurobiol Aging 18: 661–669.
7. Heber S, Herms J, Gajic V, Hainfellner J, Aguzzi A, et al. (2000) Mice with combined gene knockouts reveal essential and partially redundant functions of amyloid precursor protein family members. J Neurosci 20: 7951–7963.
8. Horsten A, Liebertal J, Fadia S, Malini Rs, Ha L, et al. (2007) APL-1, a Caenorhabditis elegans protein related to the human beta-amyloid precursor protein, is essential for viability. Proc Natl Acad Sci U S A 104: 1971–1976.
9. Duangle J, Li C (1993) apl-1, a Caenorhabditis elegans gene encoding a protein related to the human beta-amyloid precursor protein. Proc Natl Acad Sci U S A 90: 12043–12049.
10. Shutt HH, Thinakaran G, Von Koch C, Lo AG, Tanzi RE, et al. (1994) Expression of a ubiquitins, cross-reactive homologue of the mouse beta-amyloid precursor protein (APP). J Biol Chem 269: 2637–2644.
11. Wasco W, Bopp K, Magendanz M, Gusella JF, Tanzi RE, et al. (1992) Characterization of a mouse brain cDNA that encodes a protein related to the Alzheimer disease-associated amyloid beta protein precursor. Proc Natl Acad Sci U S A 89: 10758–10762.
12. Wasco W, Gurubhagavatula S, Paradiso MD, Romano DM, Gusella JF, et al. (1993) Isolation and characterization of APLP2 encoding a homologue of the Alzheimer’s-associated amyloid beta protein precursor. Nat Genet 3: 95–100.
13. Bocuyk BS (1974) The genetics of Caenorhabditis elegans. Genetics 77: 71–94.
14. Kamastr S, Martinez-Campos M, Zipperlen P, Fraser AG, Ahringer J (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. Genome Biol 2: RESEARCH0002.

**Figure S4** C-terminal mutation rescue strains cannot rescue the aldicarb hypersensitivity. (A) Western blot showing protein expression levels from the different rescue strains. (B) Rescue strains with either a C-terminal truncation or deletion of the YENPTY domain could not rescue the aldicarb hypersensitivity. (C) Quantification of the aldicarb experiment at the 90 min time point. (*, P<0.05; **, P<0.01) Error bars represent the s.e.m.

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**Figure S5** Loss of kinesin-1 function also leads to a reduction in APL-1::GFP fluorescence. (A) Representative pictures of head APL-1::GFP strain were normal fluorescence (n = 10 per strain). (*, P<0.05) Error bars represent the s.e.m.

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Author Contributions

Conceived and designed the experiments: MW AA HZ. Performed the experiments: MW AA HZ. Contributed reagents/materials/analysis tools: MW AA HZ. Wrote the paper: MW AA HZ.
30. Marquez-Sterling NR, Lo AC, Sisodia SS, Koo EH (1997) Trafficking of cell-surface beta-amyloid precursor protein: evidence that a sorting intermediate participates in synaptic vesicle recycling. J Neurosci 17: 140–151.
31. Ikin AF, Annaert WG, Takei K, De Camilli P, Jahn R, et al. (1996) Alzheimer amyloid protein precursor is localized in nerve terminal preparations to Rab5-containing vesicular organelles distinct from those implicated in the synaptic vesicle pathway. J Biol Chem 271: 31783–31786.
32. Bucci C, Parton RG, Mather H, Stenzenberg H, Simons K, et al. (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell 70: 715–728.
33. Mangahas PM, Yu X, Miller KG, Zhou Z (2008) The small GTPase Rab2 functions in the removal of apoptotic cells in Caenorhabditis elegans. J Cell Biol 180: 357–373.
34. Sumakovic M, Hegermann J, Luo L, Husson SJ, Schwarze K, et al. (2009) UNC-108/RAB-2 and its effector RIC-19 are involved in dense core vesicle maturation in Caenorhabditis elegans. J Cell Sci 122: 987–994.
35. Leyssen M, Ayaz D, Hebert SB, Reeve S, De Strooper B, et al. (2005) Amyloid precursor protein promotes post-developmental neurite arborization in the Drosophila embryo. J Neurosci 24: 2944–2953.
36. Edwards SL, Charlie NK, Richmond JE, Hegermann J, Eimer S, et al. (2009) Impaired dense core vesicle maturation in Caenorhabditis elegans mutants lacking Rab2. J Cell Biol 186: 881–895.
37. Niwa R, Zhou F, Li C, Slack FJ (2008) The expression of the Alzheimer's amyloid precursor protein-like gene is regulated by developmental timing microRNAs and their targets in Caenorhabditis elegans. Dev Biol 315: 418–425.
38. Randles AR, Russell S, Rulek G (2005) Functional genomic analysis of C. elegans molt. PLoS Biol 3: e312.
39. Grigorenko AP, Moliaka YK, Soto MC, Mello CC, Rogaev EI (2004) The Caenorhabditis elegans IMPAS gene, imp-2, is essential for development and is functionally distinct from related presenilins. Proc Natl Acad Sci U S A 101: 14953–14960.
40. Yoshie J, Tuck S, Greenwald I, Han M (1999) Ap330/megalin-related protein is required in the major epidermis of Caenorhabditis elegans for completion of molting. Development 126: 597–606.
41. Kounnas MZ, Moir RD, Rebeck GW, Bush AI, Argraves WS, et al. (1995) LDL-receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. Cell 82: 331–340.
42. Ring S, Weyer SW, Kilian SB, Waldron E, Pietrzik CU, et al. (2007) The secreted beta-amyloid precursor protein ectodomain APPs alpha is sufficient to rescue the anatomical, behavioral, and electrophysiological abnormalities of APP-deficient mice. J Neurosci 27: 7817–7826.