

μ 2 adaptin facilitates but is not essential for synaptic vesicle recycling in *Caenorhabditis elegans*

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Synaptic vesicles must be recycled to sustain neurotransmission, in large part via clathrin-mediated endocytosis. Clathrin is recruited to endocytic sites on the plasma membrane by the AP2 adaptor complex. The medium subunit (μ 2) of AP2 binds to cargo proteins and phosphatidylinositol-4,5-bisphosphate on the cell surface. Here, we characterize the *apm-2* gene (also called *dpy-23*), which encodes the only μ 2 subunit in the

nematode *Caenorhabditis elegans*. APM-2 is highly expressed in the nervous system and is localized to synapses; yet specific loss of APM-2 in neurons does not affect locomotion. In *apm-2* mutants, clathrin is mislocalized at synapses, and synaptic vesicle numbers and evoked responses are reduced to 60 and 65%, respectively. Collectively, these data suggest AP2 μ 2 facilitates but is not essential for synaptic vesicle recycling.

Introduction

After synaptic vesicle fusion, vesicle proteins are retrieved from the plasma membrane and recycled into new synaptic vesicles to sustain neuronal transmission. Recycling is thought to be initiated by the recruitment of clathrin to patches of membrane containing synaptic vesicle proteins. The reformed vesicle with a geodesic coat is budded into the cytoplasm. This model is supported by extensive associative and functional evidence. In electron micrographs of the frog neuromuscular junction, invaginating vesicles at presynaptic terminals are enveloped by a coat (Heuser and Reese, 1973). Purification of vesicles from rat brain indicate that clathrin is associated with synaptic vesicle proteins (Maycox et al., 1992). Genetic disruption of clathrin-associated endocytic proteins such as AP180, synaptojanin, dynamin, and endophilin leads to a depletion of synaptic vesicles (De Camilli et al., 1995; Nonet et al., 1999; Harris et al., 2000; Verstreken et al., 2002, 2003; Schuske et al., 2003; Newton et al., 2006). Finally, specific disruption of clathrin interactions with the adaptor protein AP180 disrupts synaptic vesicle recycling (Augustine et al., 2006; Granseth et al., 2006). These data suggest that clathrin-mediated endocytosis is the main mechanism used by synapses to recycle vesicles after exocytosis.

Clathrin is linked to cargo and membranes by the clathrin adaptor complex (Keen, 1987). Four different adaptor complexes

have been identified in mammals: AP1, AP2, AP3, and AP4 (Keen, 1987; Simpson et al., 1997; Dell'Angelica et al., 1999). These adaptor protein complexes localize to different membranes in the cell and coordinate cargo selection and vesicle biogenesis (Lewin and Mellman, 1998; Robinson and Bonifacino, 2001; Robinson, 2004). AP2 is the adaptor complex functioning during endocytosis at the plasma membrane (Mahaffey et al., 1990; Traub, 2003). There are four different subunits in the AP2 complex: α (large), β 2 (large), μ 2 (medium), and σ 2 (small; Matsui and Kirchhausen, 1990), and each subunit serves a specific function. In particular, the μ 2 subunit recruits cargo proteins containing the tyrosine-based Yxx ϕ motif (Owen and Evans, 1998) and mediates in part the association of the AP2 complex to membranes (Gaidarov and Keen, 1999; Rohde et al., 2002; Honing et al., 2005).

Here, we characterize mutants that lack μ 2 adaptin, encoded by the *apm-2* gene (also called *dpy-23*), in the nematode *Caenorhabditis elegans*. We demonstrate that μ 2 is partially required for synaptic localization of clathrin and for the stability of the AP2 complex. However, synaptic vesicles are still recycled in the absence of μ 2. Our data suggest that despite previous predictions, μ 2 is not absolutely required for synaptic vesicle endocytosis. Moreover, the decrease in synaptic vesicle number

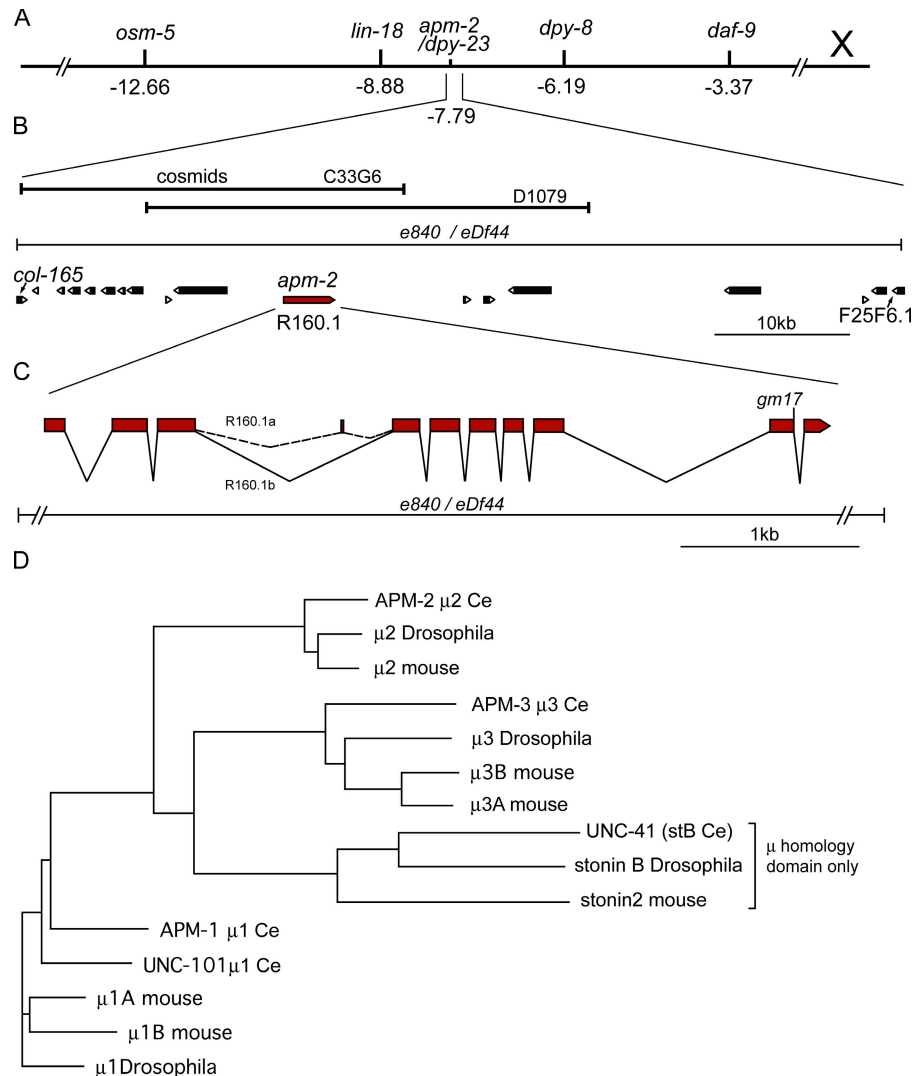
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Abbreviations used in this paper: CHC, clathrin heavy chain; ePSC, evoked postsynaptic current; mPSC, miniature postsynaptic current; ORF, open reading frame.

The online version of this article contains supplemental material.

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Figure 1. ***apm-2* cloning.** (A) Genetic map position of *apm-2* on chromosome X. (B) Either of two overlapping cosmids, C33G6 and D1079, rescue *apm-2(gm17)*. Below, the mutation *e840* (also called *eDf44*) is a deletion of 100 kb that removes 18 ORFs from *col-165* to F25F6.1. (C) Genomic structure of *apm-2* gene. The *e840* allele deletes the entire ORF; *gm17* is a G to A transition at the donor site of the last intron. The splice form R160.1a (Lee et al., 1994) includes an exon encoding six amino acids. This splice form is not essential because it is not required for rescue of *apm-2* mutant phenotypes (see Fig. 6 B) and is also not conserved in other species. (D) Phylogenetic tree of μ subunits from mouse, *Drosophila*, and *C. elegans*. For stonin B, only the μ homology domains were used in the alignment. See Materials and methods for accession numbers.



does not cause a locomotion defect in μ 2 knockout mutants, so a smaller reserve pool might be adequate for *C. elegans* under normal condition.

Results

dpy-23/apm-2 encodes μ 2 adaptin in *C. elegans*

Two mutant alleles for the locus *dpy-23* (*e840* and *gm17*) have been identified. Both mutants have a variable dumpy (Dpy) phenotype in which animals vary from almost wild-type length to approximately half the size (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200806088/DC1>). The dumpy phenotype is likely caused by defects in cuticle morphology. Specific defects in the cuticle are observed in the head and along the body. About 5% of the animals have “jowls” or protrusions on either side of the head (Fig. S1 B). The cuticular ridges along the body, called alae, are distorted and have multiple breaks along their length (Fig. S1 C). In addition, mutant worms are slightly uncoordinated (Unc) and have a strong egg-laying defect suggesting a role for *dpy-23* in the nervous system.

The *dpy-23* mutant phenotype was mapped to the interval between -7.91 to -7.55 on chromosome X (Fig. 1 A). The gene encoding μ 2 adaptin, called *apm-2*, maps to this interval, and RNA interference to this gene gave rise to a variable dumpy phenotype suggesting *dpy-23* is likely to encode μ 2 (Grant and Hirsh, 1999). We cloned the *dpy-23* gene and demonstrated that the mutated gene is *apm-2*. Two overlapping cosmids, D1079 and C33G6, in the region rescued the *dpy-23* mutant phenotype. A 12-kb genomic PCR fragment (5 kb upstream, 5 kb coding sequence of μ 2 adaptin, and 2 kb downstream) could fully rescue the dumpy, uncoordinated, and egg-laying defects of *dpy-23(e840)* and *dpy-23(gm17)* (Fig. S1 A). Interestingly, overexpression of *dpy-23* in a wild-type background causes the same phenotypes as *dpy-23* loss-of-function mutations (Fig. S1 A). Because *dpy-23* encodes μ 2 we will refer to the gene by its alternative name, *apm-2* (adaptor protein medium subunit 2), throughout the remainder of the manuscript.

apm-2 encodes the only μ 2 subunit in *C. elegans* (Fig. 1 D). It is somewhat surprising that disruption of the single μ 2 subunit in the worm gives rise to a viable animal. To determine whether μ 2 adaptin is completely disrupted in the mutants, the lesions were identified. Genomic Southern analysis showed that *apm-2(e840)*

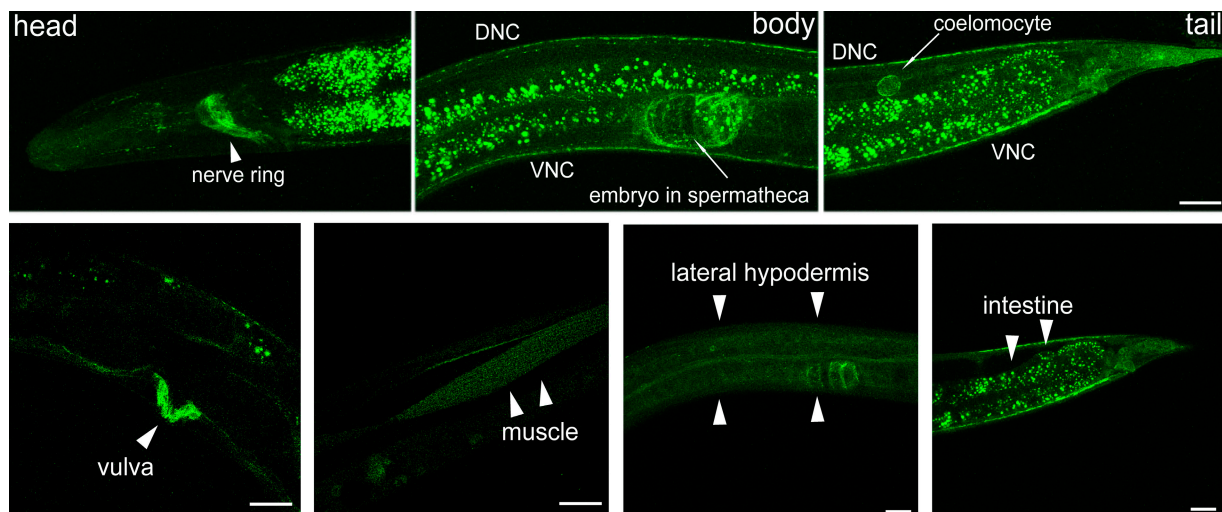


Figure 2. APM-2 is expressed ubiquitously. The expression pattern of translational fusion protein APM-2::GFP (Fig. S4, pMG4, available at <http://www.jcb.org/cgi/content/full/jcb.200806088/DC1>) in young adult hermaphrodites. Worms are oriented anterior left and dorsal up. The image of the muscle is from the transcriptional fusion of GFP driven by the *apm-2* promoter (Fig. S4, pMG3) in a young adult hermaphrodite. Muscle expression was not observed using GFP-tagged μ 2, perhaps because of faint expression or synaptic localization, which overlaps strong APM-2 expression in the presynaptic terminals. The vulva and muscle expression figures are single-slice confocal images. The rest of the images are z-stack projections through the whole worm or the tissues of interest. The contrast for the image of the vulva was increased to show the outline of the worm. Bars, 20 μ m. DNC, dorsal nerve cord; VNC, ventral nerve cord.

contains a deletion of ~ 100 kb that includes the entire coding sequence of μ 2 adaptin. To identify the endpoints of the deletion, individual open reading frames (ORFs) were PCR amplified from *apm-2(e840)* mutant DNA and it was found that the deletion removes 17 additional ORFs from *col-165* to F25F6.1 (Fig. 1 B). *apm-2(gm17)* was found to contain a G to A point mutation in the splice donor site of the last intron (Fig. 1 C). Failure to splice at this intron would introduce a stop codon 27 nt downstream of the splice junction. If translated, 9 amino acids encoded by the intron would replace the 40 amino acids at the C terminus comprising β strands 15, 16, and 17. β 16 has been shown to be critical for binding cargo containing the YXX ϕ motif (Ohno et al., 1995; Owen and Evans, 1998). Although *apm-2(gm17)* appears to have a slightly dominant phenotype, the recessive phenotypes of *apm-2(e840)* and *apm-2(gm17)* are virtually identical, suggesting that *gm17* fully disrupts μ 2 function.

μ 2 is thought to be a critical component of the AP2 complex and should therefore be present in all tissues. To determine where μ 2 is expressed, a construct fusing GFP to the APM-2 protein was expressed under the control of the endogenous *apm-2* promoter (Fig. S4, pMG4, available at <http://www.jcb.org/cgi/content/full/jcb.200806088/DC1>). Because the N terminus of μ 2 adaptin is involved in assembly of the AP2 complex (Aguilar et al., 1997; Collins et al., 2002), GFP was fused to the C terminus of the APM-2 protein. The APM-2::GFP fusion protein fully rescues *apm-2* mutant phenotypes, suggesting the tagged protein is functional and is expressed in tissues that require μ 2 function. Fluorescence is observed in the nervous system, coelomocyte, spermatheca, and vulva (Fig. 2). In addition, weaker expression is observed in the intestine and the hypodermis. Although fluorescence is not detected in body muscles of animals expressing the tagged protein, muscle expression is observed in animals expressing a transcriptional reporter (Fig. 2

and Fig. S4, pMG3). Thus, *apm-2* is expressed in all tissues examined, which confirms and extends a previous paper claiming that *apm-2* is expressed in neurons and some hypodermal cells (Shim and Lee, 2000).

μ 2 is not essential for synaptic vesicle recycling in *C. elegans*

AP2 is thought to recruit synaptic vesicle proteins and clathrin to the endocytic zone. If μ 2 is required for synaptic vesicle recycling, then several predictions can be made. First, μ 2 should be localized to synapses. Second, μ 2 should contribute to clathrin localization at the synapse. Third, the focus of the uncoordinated phenotype should be the nervous system. Fourth, *apm-2* mutants will have a depletion of synaptic vesicles as assayed by electron microscopy. Fifth, *apm-2* mutants will have impaired synaptic transmission as assayed by electrophysiology because of an inability to recycle vesicles.

Because *apm-2* is expressed in virtually all tissues, it is not possible to assay synaptic localization with the rescuing GFP construct, the fluorescence signal is simply too high. To look at a small subset of neurons, the *apm-2* cDNA (R160.1b) was placed under the control of a GABA neuron-specific promoter and GFP was fused at the C terminus. APM-2::GFP is localized at synapses and colocalizes with the synaptic vesicle protein synaptobrevin/VAMP (Fig. 3 A). In addition, APM-2::GFP colocalizes with C-terminal RFP-tagged clathrin heavy chain (CHC) at the synapse (Fig. 3 B). Interestingly, synaptic localization of APM-2::GFP is not dependent on AP180 (*unc-11*), synaptotagmin (*unc-26*), synaptobrevin (*snt-1*), or stonin (*unc-41*) (Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200806088/DC1>). These data indicate that μ 2 associates with synaptic varicosities as predicted, but this localization is independent of other endocytosis proteins.

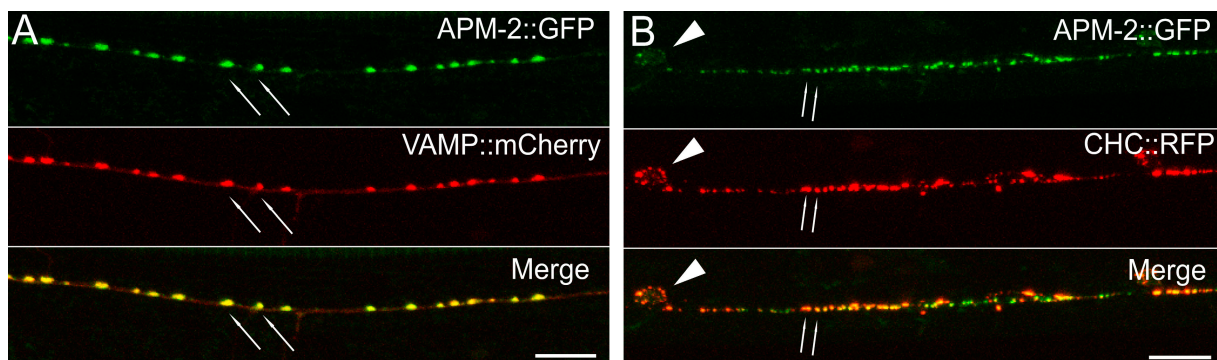


Figure 3. **APM-2 colocalizes with synaptic proteins and clathrin.** Young adult hermaphrodites were used for imaging. (A) APM-2 is localized to synapses. (top) GFP-tagged APM-2 in the GABA neuron processes in the dorsal nerve cord. (middle) mCherry-tagged synaptobrevin in the GABA neuron processes in the dorsal nerve cord. Synaptobrevin is localized to synaptic regions. The fluorescent puncta corresponds to synaptic varicosities along the dorsal muscles (arrows). (bottom) Merged image demonstrates that APM-2::GFP colocalizes with synaptobrevin at synapses. (B) APM-2 is colocalized with CHC. (top) GFP-tagged APM-2 in the GABA neuron processes in the ventral nerve cord. (middle) RFP-tagged CHC-1 in the GABA neuron processes in the ventral nerve cord. CHC is localized to both synaptic regions (arrows) and GABA neuron cell bodies (arrowhead). (bottom) Merged image demonstrates that APM-2::GFP colocalizes with CHC at synapses. Images are confocal z-stack projections through the worm nerve cord. Bars, 10 μ m.

Clathrin interacts with AP2 via the appendage domain of the β 2 subunit (Dell'Angelica et al., 1998). Thus, if *apm-2* mutations disrupt AP2 function, then it is possible that clathrin localization at the synapse should be altered. We analyzed the distribution of N-terminal GFP-tagged clathrin (GFP::CHC) in the dorsal and ventral nerve cords of GABA neurons in *apm-2* mutants. In the dorsal cord of *apm-2* mutants clathrin is diffuse compared with the wild type (Fig. 4; percentage of animals scored with diffuse clathrin in the dorsal cord: in the wild type, 31%, $n = 29$; in *dpy-23(e840)*, 80%, $n = 15$, $P < 0.01$; in *dpy-23(gm17)*, 70%, $n = 30$, $P < 0.01$). However, in the ventral nerve cord clathrin distribution is punctate, similar to wild-type animals (Fig. S2 B). It is possible clathrin localization near cell bodies in the ventral nerve cord is caused by AP1 function at the Golgi apparatus. Clathrin is still localized at dorsal synapses in 20–30% of the mutant animals; perhaps by other clathrin-binding proteins such as AP180, epsin, or amphiphysin. Thus, μ 2 contributes to, but is not essential for, clathrin synaptic localization.

Defects in synaptic vesicle endocytosis cause the mislocalization of synaptic vesicle proteins. For example, in the absence of the endocytosis proteins, such as AP180 (Nonet

et al., 1999), synaptojanin, and endophilin (Schuske et al., 2003), synaptic vesicle proteins are diffuse along the axon instead of clustering at synaptic varicosities. In contrast, the vesicle proteins synaptobrevin, synaptogyrin, and synaptotagmin are localized properly in the dorsal and lateral nerve cords of *apm-2* mutants (Fig. 4). These results suggest that μ 2, unlike other endocytosis proteins, is not required to maintain vesicle proteins at the synapse.

Mutants with defects in synaptic vesicle endocytosis exhibit reduced synaptic transmission and are uncoordinated (Nonet et al., 1999; Harris et al., 2000; Schuske et al., 2003). *apm-2* mutants are also uncoordinated but the uncoordinated phenotype arises from defects in the hypodermis rather than the nervous system. When APM-2::GFP is expressed under the control of a ubiquitous promoter the tagged μ 2 protein rescues all *apm-2(e840)* mutant phenotypes (Fig. 5, A and B). When μ 2 protein is expressed under a pan-neuronal promoter, the *apm-2* mutants are still dumpy, uncoordinated, and egg-laying defective, effectively looking the same as the original *apm-2(e840)* mutants. In contrast, when μ 2 protein is expressed under a hypodermal promoter, the *apm-2(e840)* transgenic animals are

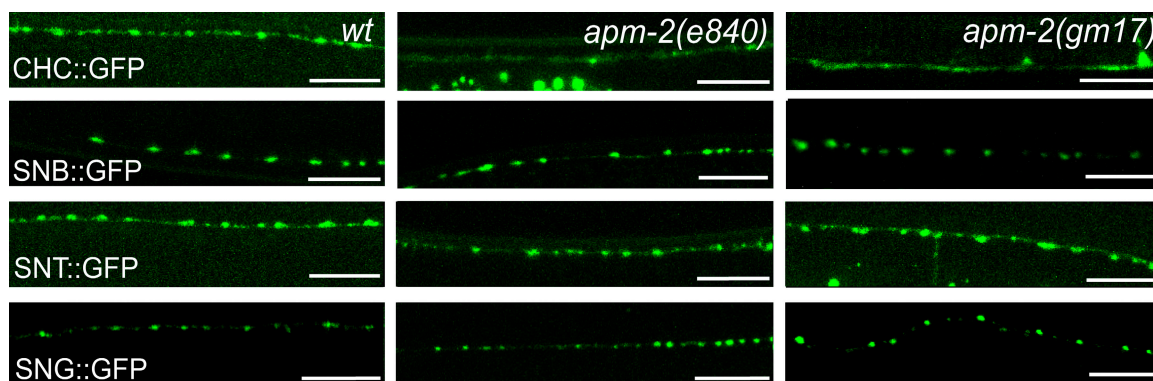


Figure 4. **Clathrin but not synaptic vesicle proteins are mislocalized in *apm-2* mutants.** For CHC, synaptobrevin (SNB), and synaptotagmin (SNT), GFP-tagged proteins were expressed in the GABA neurons and imaged in the dorsal nerve cord. Presynaptic varicosities of neuromuscular junctions along the dorsal nerve cord of an adult hermaphrodite are visible as fluorescent puncta. For synaptogyrin (SNG), GFP-tagged protein is expressed in all neurons under its own promoter and imaged in the lateral cord. Images are confocal z-stack projections through the worm nerve cord. Bars, 10 μ m.

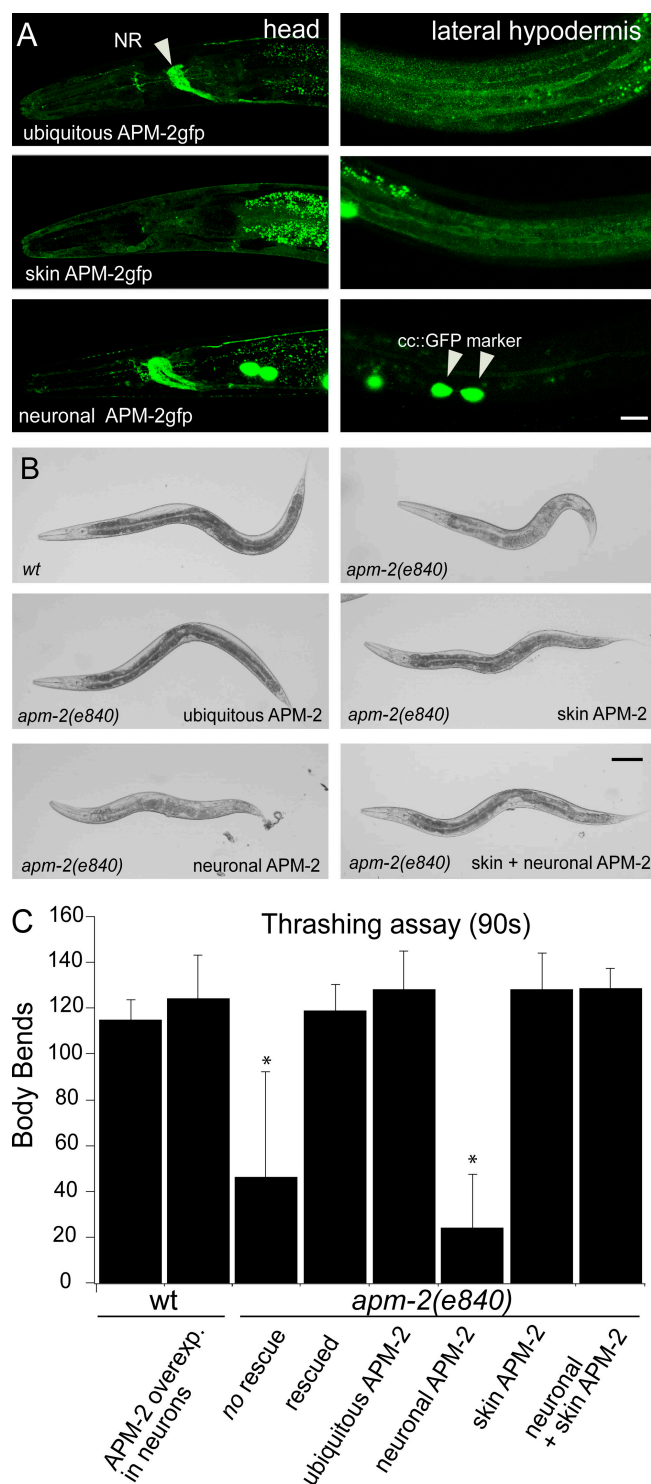


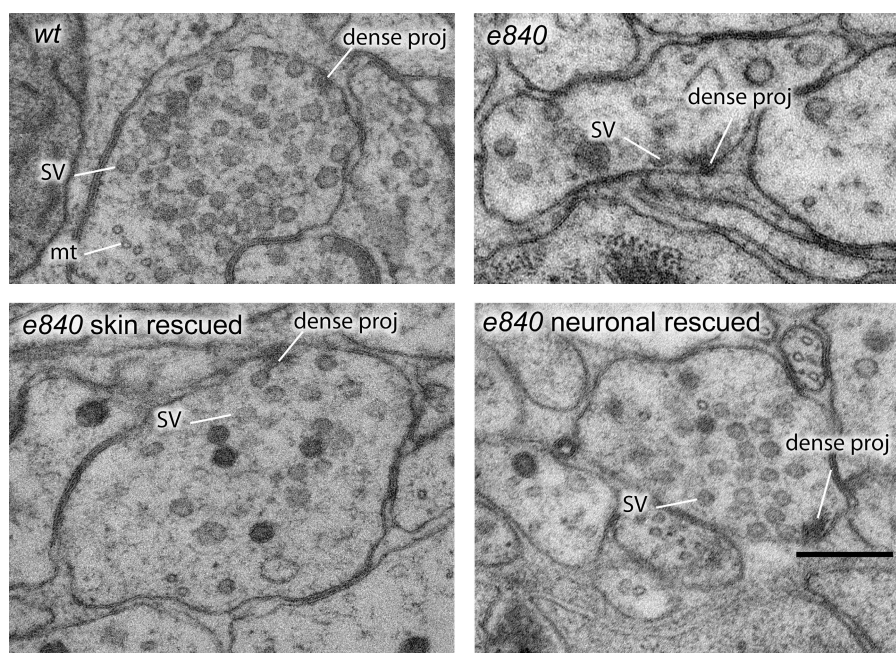
Figure 5. *apm-2(e840)* tissue-specific rescue. (A) APM-2::GFP expression pattern under different promoters. Ubiquitous expression is driven by the *dpy-30* promoter, hypodermal expression (skin) is driven by the *pdi-2* promoter, and neuronal expression is driven by the *rab-3* promoter (Fig S4, pMG10, pMG8, and pMG9, respectively; available at <http://www.jcb.org/cgi/content/full/jcb.200806088/DC1>). Worms are oriented anterior left and dorsal up. Images are confocal z-stack projections through the whole worm or the tissue of interest. All worms were imaged under identical conditions; the contrast for skin APM-2gfp panel was increased to show skin-specific expression. NR, nerve ring. Bar, 20 μ m. (B) Expression of APM-2 in the skin rescues the dumpy phenotype. See Materials and methods for full genotypes. The injection concentration of *apm-2::GFP* DNA is at 1 ng/ μ l in all genotypes. Bar, 100 μ m. (C) Thrashing assay.

not dumpy (Fig. 5 B, skin APM-2); moreover, the jerky uncoordinated phenotype is also rescued. Previous studies have demonstrated that the μ 2 functions in the skin rescue developmental defects in the nervous system (Pan et al., 2008); our data suggest that these nonautonomous defects might extend to development or function of motor neurons as well. However, skin-rescued animals are egg-laying defective and the body bends are increased in amplitude, suggesting neuronal function is somewhat altered. To quantify locomotion in the mosaic strains, animals were placed in a drop of liquid and body bends were counted for 90 s for each strain (Fig. 5 C). Thrashing rates of the hypodermal rescued strains are the same as in the wild type; whereas neuronal expression of μ 2 does not rescue thrashing. The reduced thrashing is not due to a dominant-negative effect because overexpression of μ 2 in the neurons does not impair locomotion in the wild type. Collectively, these data suggest the uncoordinated phenotype of *apm-2* mutants is almost exclusively caused by hypodermal defects rather than defects in the nervous system.

To directly visualize synaptic vesicles, we characterized *apm-2* mutant synapses using electron microscopy. In AP180 mutants the diameter of synaptic vesicles is increased, implicating a role for this adaptin in the control of the diameter of the reforming vesicles (Zhang et al., 1998; Nonet et al., 1999). In *apm-2(e840)* mutants, however, the diameters of synaptic vesicles are the same as in the wild type (Fig. 6 and Fig. 7 A), suggesting μ 2 adaptin, unlike AP180, is not required for regulating the size of synaptic vesicles. In *apm-2(e840)* mutants, the number of remaining vesicles are 58% in acetylcholine neurons and 64% in GABA neurons compared with the wild type (Fig. 6 and Fig. 7 B). Similar vesicle reductions relative to the wild type were also observed in *apm-2(gm17)* (unpublished data). Vesicle number is rescued in *apm-2(e840)* animals containing the neuron-specific APM-2::GFP construct but not in animals that contain the hypodermal specific APM-2::GFP, indicating that the defect is caused by a loss of neuronal APM-2 function (Fig. 6 and Fig. 7 B). The decrease in synaptic vesicle number in *apm-2* mutants suggests μ 2 has a significant role in synaptic vesicle recycling. Other mutants lacking endocytosis proteins such as synaptojanin and endophilin have only 38 and 30% the normal number of vesicles, respectively (Harris et al., 2000; Schuske et al., 2003). Thus the endocytosis defect in the absence of μ 2 is less severe than other endocytosis mutants. Interestingly, the reduced vesicle pool in *apm-2* mutants is able to sustain neuronal transmission because animals lacking μ 2 in the nervous system are not uncoordinated. Consistent with this observation, the number of docked vesicles in *apm-2* mutants is only slightly decreased in GABA neurons and is almost normal in acetylcholine neurons (Fig. 7 C).

Expression of APM-2 in the skin rescues the locomotory phenotype. Worms were placed in buffer and body bends were counted for 90 s. Overexpression of APM-2::GFP in the nervous system (EG4017) does not create a dominant-negative phenotype (*, $P < 0.01$). Expression of APM-2 under its own promoter (EG1616), a ubiquitous promoter (EG4015), or a skin promoter (EG4029 and EG4030) rescues thrashing. Expression of APM-2 in neurons alone does not rescue thrashing (EG4213). The data are presented as mean \pm SD.

Figure 6. ***apm-2(e840)* neuromuscular junction ultrastructure.** Representative images of neuromuscular junctions in the ventral nerve cord from wild-type, EG3622 *dpy-23(e840)*, EG4029 *dpy-23(e840) Ex[Ppdi-2::APM-2::GFP]*, and EG4213 *dpy-23(e840) Ex[Prab-3::APM-2::GFP]* adult hermaphrodites. *apm-2(e840)* shows reduced numbers of synaptic vesicles. Vesicle number is restored in the neuronal-rescued animals but not in the skin-rescued animals. Bar, 200 nm. SV, synaptic vesicle; mt, microtubule; dense proj, dense projection.



To assay neurotransmitter release, animals were tested for sensitivity to the acetylcholinesterase inhibitor aldicarb (Nguyen et al., 1995). *apm-2* mutants are slightly hypersensitive to aldicarb (Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200806088/DC1>), and the neuronally rescued worms exhibit an identical hypersensitivity to the *apm-2* strain. Expression of *apm-2* in the hypodermis rescued the hypersensitivity to a wild-type level (Fig. S3 B), suggesting that the hypersensitivity could be caused by defects in the cuticle. To directly measure synaptic transmission, we recorded currents at neuromuscular junctions. In the *apm-2* mutants, the miniature frequency and the evoked amplitude are reduced to 60 and 82% of the wild-type levels, respectively (Fig. 8, skin rescue). These values correlate fairly well with the 55 and 69% levels of synaptic vesicles observed at GABA and acetylcholine synapses (Fig. 7 B, skin rescue). Full rescue of synaptic transmission is only observed when *apm-2* is simultaneously expressed in both the hypodermis and neurons (Fig. 8). Collectively, our data demonstrate that $\mu 2$ has a detectable role in synaptic vesicle recycling, although this role is not visible in locomotion assays.

Knocking out $\mu 2$ is not equal to knocking down AP2

Residual function from the AP2 complex could still remain in the absence of the medium subunit. There are two lines of evidence: genetic and biochemical. First, α adaptin knockdowns are more severe than $\mu 2$ adaptin knockdowns in the yolk uptake assay (Grant and Hirsh, 1999). RNA interference against clathrin, α adaptin, or $\beta 2$ adaptin each abolished uptake of a GFP-tagged vitellogenin (YP170-GFP) into oocytes. In contrast, RNA interference against either $\mu 2$ or $\sigma 2$ adaptin did not disrupt uptake, suggesting that not all of the subunits of the AP2 complex are required for this process. Because RNA interference is not always fully penetrant we used our deletion allele to assay $\mu 2$ function in yolk uptake. Approximately 120 gonads of each genotype were assayed for the presence of YP170-GFP

fluorescence in oocytes. Over 98% of the gonads in both *apm-2(e840)* and *apm-2(gm17)* mutants were scored as positive for yolk protein uptake (Fig. 9 A). However, the number of oocytes within each gonad containing YP170-GFP was reduced in both alleles of *apm-2* compared with the wild type (wild type: one oocyte, 7%; two or more, 93%; *apm-2(e840)*: one oocyte, 42%; two or more oocytes, 58%; *apm-2(gm17)*: one oocyte, 36%; two or more oocytes, 64%; Fig. 9 B). In all genotypes yolk accumulation is greatest in the oldest oocyte, which is found adjacent to the spermatheca. The defect is not as severe as that seen when the α or $\beta 2$ subunits of the complex are depleted by RNA interference (Grant and Hirsh, 1999), suggesting that $\mu 2$ adaptin subunits are less important than the α adaptin or $\beta 2$ adaptin subunits in this process.

Second, some α adaptin remains in a complex with $\sigma 2$ adaptin in the $\mu 2$ adaptin mutants. Previous results suggested that each subunit is required for function and stability of the AP2 complex; e.g., RNA interference of the $\mu 2$ subunit in HeLaM cells greatly reduced the expression level of the α subunit (Motley et al., 2003). In addition, expression of any single AP2 subunit in bacteria produces an insoluble protein; only simultaneous expression of all subunits produces a soluble protein complex (Collins et al., 2002). To determine if the AP2 complex is stable in the absence of the $\mu 2$ subunit, we performed quantitative Western blot analysis of α adaptin. α adaptin is reduced to 60% in *apm-2(e840)* and to 12% in *apm-2(gm17)* (Fig. 9 C). Oddly, the reduction in α adaptin is more severe in the truncated allele of $\mu 2$ rather than in the null mutant; it is possible that incorporation of truncated $\mu 2$ leads to a destabilization of the whole complex. Consistent with this result, *apm-2(e840)/+* heterozygous animals are wild type, whereas *apm-2(gm17)/+* animals are slightly uncoordinated and have an egg-laying defect. The reduction in α adaptin levels in both alleles indicates that $\mu 2$ is required to stabilize the AP2 complex; however, some residual α adaptin remains in the absence of $\mu 2$.

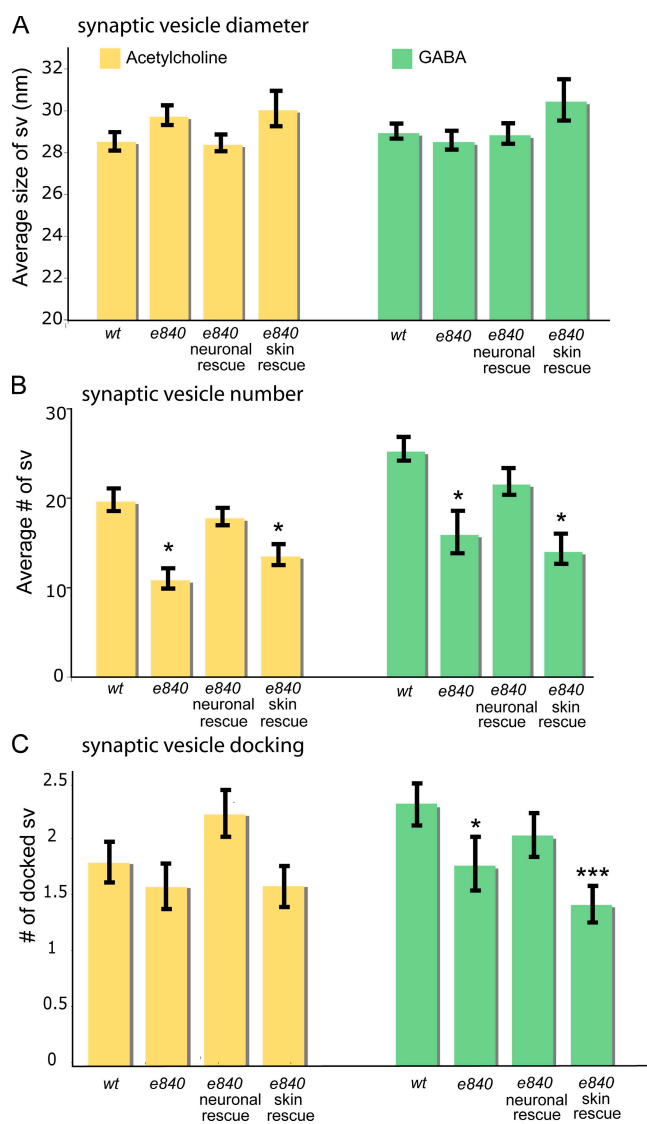


Figure 7. Synaptic vesicle numbers are reduced in *apm-2(e840)* mutants. The ventral nerve cord was reconstructed from serial electron micrographs and distribution of synaptic vesicles at neuromuscular junctions was measured from two young adult hermaphrodites for each genotype. (A) Vesicle diameters are identical in wild-type, *apm-2(e840)*, and *apm-2(e840)* tissue-specific rescued animals. Mean size of synaptic vesicles per profile containing a dense projection in nanometers \pm SEM is as follows: wild-type acetylcholine, 28.52 ± 0.38 , $n = 734$ vesicles; *apm-2(e840)* acetylcholine, 29.71 ± 0.47 , $n = 377$ vesicles; neuronal-rescued *apm-2(e840)* acetylcholine, 28.38 ± 0.40 , $n = 744$ vesicles; skin-rescued *apm-2(e840)* acetylcholine, 28.38 ± 0.85 , $n = 675$ vesicles; wild-type GABA, 28.70 ± 0.35 , $n = 904$ vesicles; *apm-2(e840)* GABA, 28.27 ± 0.44 , $n = 474$ vesicles; neuronal-rescued *apm-2(e840)* GABA, 28.59 ± 0.48 , $n = 1073$ vesicles; skin-rescued *apm-2(e840)* GABA, 30.15 ± 0.96 , $n = 700$ vesicles. (B) The number of synaptic vesicles is reduced in neurons lacking APM-2. Mean number of synaptic vesicles per profile containing a dense projection \pm SEM: wild-type acetylcholine, 19.63 ± 1.28 , $n = 38$ synapses; *apm-2(e840)* acetylcholine, 10.83 ± 1.13 , $n = 35$ synapses; neuronal-rescued *apm-2(e840)* acetylcholine, 17.74 ± 0.97 , $n = 42$ synapses; skin-rescued *apm-2(e840)* acetylcholine, 13.5 ± 1.18 , $n = 50$ synapses; wild-type GABA, 25.11 ± 1.17 , $n = 36$ synapses; *apm-2(e840)* GABA, 15.83 ± 2.23 , $n = 30$ synapses; neuronal-rescued *apm-2(e840)* GABA, 21.46 ± 1.34 , $n = 50$ synapses; skin-rescued *apm-2(e840)* GABA, 13.92 ± 1.51 , $n = 50$ synapses. (C) The number of docked synaptic vesicles is slightly reduced in neurons lacking APM-2. Mean number of docked synaptic vesicles per profile containing a dense projection \pm SEM: wild-type acetylcholine, 1.82 ± 0.13 ; *apm-2(e840)* acetylcholine, 1.60 ± 0.19 ; neuronal-rescued *apm-2(e840)* acetylcholine, 2.24 ± 0.20 ; skin-rescued

The residual α adaptin remains bound to $\sigma 2$ adaptin. α adaptin tagged with GFP was immunoprecipitated from the wild type and *apm-2* mutants. β adaptin coimmunoprecipitated from the wild-type animals but not from $\mu 2$ mutant animals (Fig. S5 A, available at <http://www.jcb.org/cgi/content/full/jcb.200806088/DC1>). In contrast, $\sigma 2$ adaptin (HA tagged) coimmunoprecipitated from both the wild-type and $\mu 2$ mutant animals (Fig. S5, B and C). Moreover, residual α adaptin can still be localized in the absence of $\mu 2$. Coelomocytes are scavenger cells in *C. elegans* with high levels of endocytosis. N-Terminal tagged α adaptin is localized properly to the plasma membrane of mutant coelomocytes (Fig. 9 D). Similarly, tagged α adaptin is localized to synapses in *apm-2* mutants. These data suggest that the protein is folded and transported correctly in mutant cells. Thus, it is possible that AP2 is at least partially functional in *apm-2* mutants.

Discussion

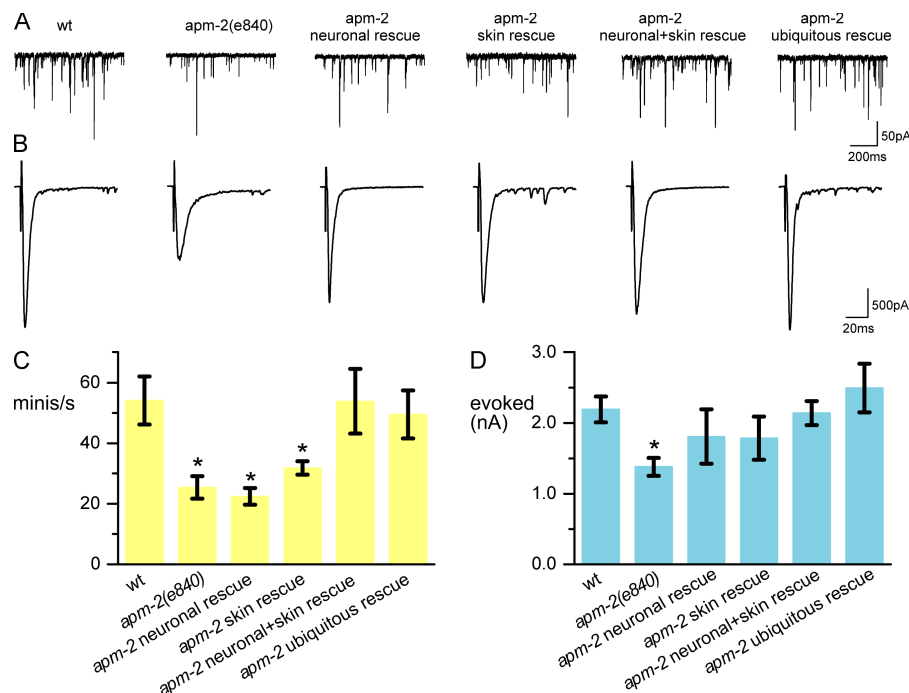
In this study, we characterized the only AP2 $\mu 2$ adaptin subunit in *C. elegans* and its function in synaptic vesicle endocytosis. $\mu 2$ adaptin is encoded by the gene *dpy-23* (*apm-2*). It is expressed ubiquitously in adult worms and is highly expressed in the nervous system. Absence of $\mu 2$ impairs but does not eliminate synaptic vesicle endocytosis. Animals lacking $\mu 2$ have $\sim 60\%$ of the normal number of vesicles at synaptic varicosities, and synaptic vesicle proteins are properly localized at the synapse. This phenotype is much less severe than worm mutants lacking other recycling proteins such as AP180 (*unc-11*), synaptotagmin (*unc-26*), and endophilin (*unc-57*) (Nonet et al., 1999; Harris et al., 2000; Schuske et al., 2003). For example, the number of synaptic vesicles in synaptotagmin and endophilin mutants is reduced to $\sim 35\%$, the normal number of synaptic vesicles found at neuromuscular junctions.

The conclusion that $\mu 2$ is not essential for synaptic vesicle recycling leads to several considerations. (a) Do other proteins recruit cargo? (b) Can other medium subunits stabilize AP2? (c) Do other proteins recruit clathrin?

The specific role of $\mu 2$ is in cargo recruitment, in particular, its interactions with synaptotagmin were thought to be essential for synaptic vesicle biogenesis (Zhang et al., 1994; Jorgensen et al., 1995; Haucke et al., 2000). However, our data indicate that $\mu 2$ is not required to recruit proteins to synaptic vesicles. The essential synaptic vesicle proteins are synaptobrevin, synaptotagmin, and the neurotransmitter transporters. Other ancillary proteins have been identified that recruit these synaptic vesicle proteins to sites of endocytosis. AP180 is required to recruit synaptobrevin to synaptic vesicles (Zhang et al., 1998; Nonet et al., 1999; Bao et al., 2005). Stonin, which is distantly related to $\mu 2$, is required for synaptotagmin recycling (Fergestad and Broadie, 2001; Martina et al., 2001; Walther et al., 2004). The vesicular GABA transporter is recruited by a

apm-2(e840) acetylcholine, 1.60 ± 0.16 ; wild-type GABA, 2.36 ± 0.17 ; *apm-2(e840)* GABA, 1.8 ± 0.22 ; neuronal-rescued *apm-2(e840)* GABA, 2.06 ± 0.18 ; skin-rescued *apm-2(e840)* GABA, 1.44 ± 0.14 . The number of synapses is the same as in B. *, $P < 0.05$; ***, $P < 0.001$.

Figure 8. Electrophysiological analysis at neuromuscular junctions of wild-type, *apm-2(e840)*, and various *apm-2(e840)* tissue-specific rescued worms. (A) sample traces of mPSC recorded from wild-type, *apm-2(e840)*, neuronal-rescued *apm-2(e840)*, skin-rescued *apm-2(e840)*, neuronal- and skin-rescued *apm-2(e840)*, and ubiquitously rescued *apm-2(e840)* worms. (B) sample traces of ePSC recorded from the aforementioned animals. (C) Summary of mPSC frequencies (Hz \pm SEM): wild type, 54.1 ± 8.0 , $n = 8$; *apm-2(e840)*, 25.4 ± 3.7 , $n = 9$; neuronal-rescued *apm-2(e840)*, 22.4 ± 2.7 , $n = 8$; skin-rescued *apm-2(e840)*, 31.9 ± 2.2 , $n = 12$; neuronal- and skin-rescued *apm-2(e840)*, 53.9 ± 10.7 , $n = 8$; ubiquitously rescued *apm-2(e840)*, 49.5 ± 7.9 , $n = 8$. (D) Summary of ePSC amplitudes (nA \pm SEM): wild type, 2.19 ± 0.18 , $n = 8$; *apm-2(e840)*, 1.38 ± 0.13 , $n = 7$; neuronal-rescued *apm-2(e840)*, 1.81 ± 0.39 , $n = 7$; skin-rescued *apm-2(e840)*, 1.79 ± 0.31 , $n = 8$; neuronal- and skin-rescued *apm-2(e840)*, 2.14 ± 0.17 , $n = 6$; ubiquitously rescued *apm-2(e840)*, 2.50 ± 0.34 , $n = 7$. *, $P < 0.05$, compared with wild-type; unpaired t test.



LAMP-related protein called UNC-46 (Schuske et al., 2007). Because of these defects in cargo recruitment, all of these mutants are severely uncoordinated in worms. In contrast, mutants lacking $\mu 2$ in the nervous system are not uncoordinated and evoked responses are at 82% of the levels observed in the wild type, indicating that synaptic transmission is largely intact. Thus, if $\mu 2$ recruits cargo to recycling vesicles it is unlikely to be a component essential for neurotransmission.

The medium subunit $\mu 2$ is also known to stabilize the AP2 complex (Motley et al., 2003). One could imagine that medium subunits from the AP1 or AP3 complexes (there is no AP4 in *C. elegans*) could substitute for $\mu 2$ and provide AP2 complex function. However, adaptins from different complexes do not appear to be redundant in other organisms. For example, in yeast, overexpression of $\sigma 2$ cannot substitute for the loss of $\sigma 1$ (Phan et al., 1994). Similarly, we found that other medium subunits cannot substitute for $\mu 2$ in *C. elegans*. Mutants lacking $\mu 1$ (*unc-101*) are severely uncoordinated and exhibit defects in anterograde transport of olfactory receptors to olfactory cilia (Dwyer et al., 2001). Overexpression of $\mu 2$ cannot rescue the severely uncoordinated phenotype of the $\mu 1$ mutant in a thrashing assay (unpublished data). Moreover, *apm-2 unc-101* ($\mu 1 \mu 2$) double mutants exhibit an additive dumpy and uncoordinated phenotype rather than a synthetic phenotype, suggesting that these proteins are not acting redundantly. Mutants lacking $\mu 3$ (*apm-2(tm920)*) are outwardly wild type but slightly aldicarb resistant. Again, $\mu 2$ and $\mu 3$ mutations do not show synthetic interactions: *apm-2 apm-3* double mutants (with $\mu 2$ rescued in skin) exhibit similar aldicarb sensitivity as the $\mu 3$ mutant alone. In addition, our data show that AP2 is destabilized in the absence of $\mu 2$, suggesting $\mu 2$ is the only medium subunit used by AP2 in *C. elegans*.

Our data are consistent with a study in other systems suggesting that clathrin can be recruited by alternative adap-

tors (Traub, 2003). In particular, AP180 is required for normal synaptic vesicle endocytosis and it is likely that AP180 could recruit clathrin and form vesicles in the absence of AP2 (Zhang et al., 1998; Nonet et al., 1999). Our results for $\mu 2$ seem to conflict with previous studies that suggest that the AP2 subunit α adaptin is essential for synaptic vesicle recycling in *Drosophila melanogaster*. A weak mutation in the *Drosophila* α adaptin subunit *D- α Ada¹* leads to slowly moving larvae, which die as pupae (Gonzalez-Gaitan and Jackle, 1997). The neurons of these animals are not efficient in taking up FM1-43 dye at boutons upon stimulation. The null mutants die before hatching and the electron microscopy data suggest that these animals are depleted of synaptic vesicles. However, in *C. elegans*, the behavioral defects of *apm-2*-null mutants are less pronounced. The hypodermal rescued mutants have almost normal movement and the evoked current upon stimulation is close to the wild type. Thus the neurotransmission defect in $\mu 2$ mutants in worms is less severe than that of α adaptin mutants in flies. Although it is possible that residual AP2 function accounts for vesicle recycling in $\mu 2$ mutants, it is also possible that AP2 is not essential for synaptic vesicle recycling at *C. elegans* neuromuscular junctions.

Materials and methods

Mapping and mutation analysis

The mutation *e840* was isolated in an x-ray mutagenesis by S. Brenner (Agency for Science, Technology, and Research, Biopolis, Singapore). The mutation *gm17* was isolated in an EMS screen for egg-laying defective animals. *gm17* was mapped between two polymorphisms on the X chromosome, *gmP¹* and *pgP2*, and was successfully rescued by two overlapping cosmids, D1079 and C33G6. The sequence of *gm17* was determined by DNA sequencing. The molecular nature of *e840* was determined by genomic southern analysis and the break points of *e840* deletion were further characterized by PCR against *apm-2* neighboring ORFs. Because *e840* is a multigene deletion, an alternative name, *eDf44*, has been given by J. Hodgkin (University of Oxford, Oxford, England).

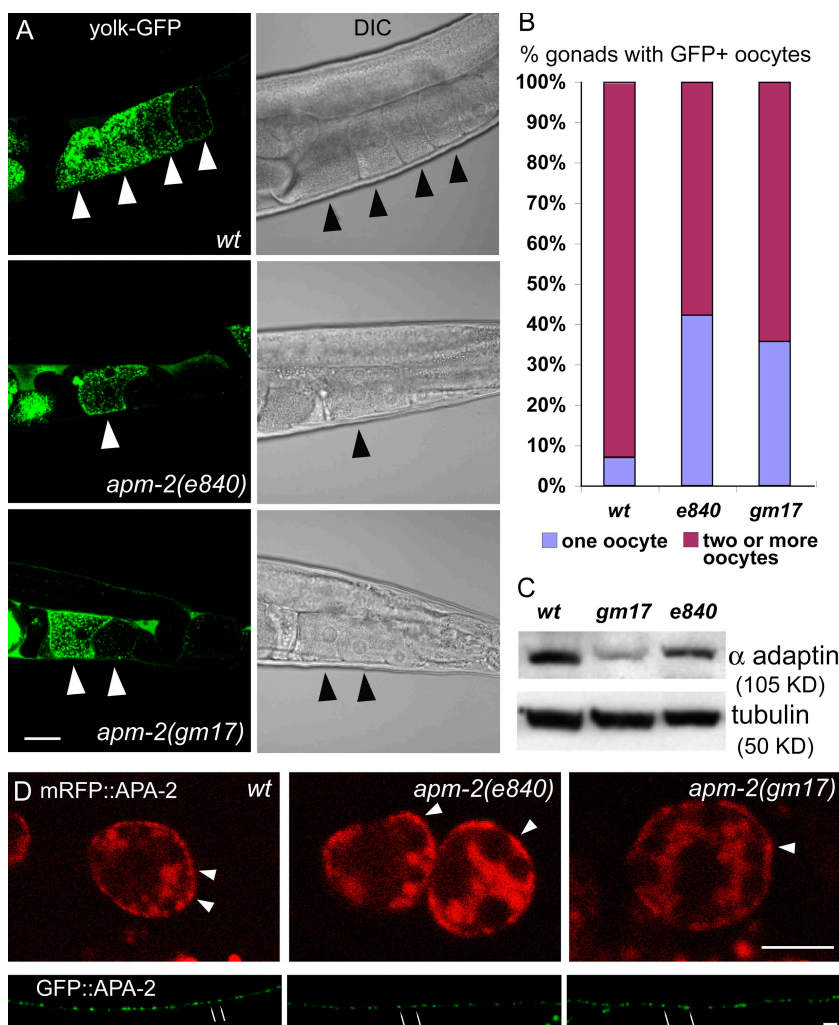


Figure 9. Yolk protein endocytosis in *apm-2* mutants.

(A) Oocytes from adult worms expressing yolk protein YP170::GFP. Arrowheads indicate the GFP-positive oocytes. Figures are single-slice confocal images. Bar, 20 μ m. (B) A bar graph showing the percentage of gonads with different numbers of GFP-positive oocytes. (C) A Western blot of the wild-type and *apm-2* mutant worm lysates probed with an antibody against α adaptin (APA-2). 200 μ g of protein was loaded in each lane. (D) APA-2 localization in scavenger cell, coelomocytes, and GABA synapses. Images were taken from adult worms. Arrowheads indicate the RFP-positive plasma membrane of coelomocytes. Arrows indicate the GFP-positive synapses. Coelomocyte figures are single-slice confocal images. GABA synapse images are z-stack projections. Bars, 5 μ m.

C. elegans strains

The wild strain is Bristol N2. The reference strains for *e840* and *gm17* were outcrossed twice before phenotypic analysis. The outcrossed strains are EG2988 *apm-2(gm17)*X and EG3622 *apm-2(e840)*X.

The strains used in the synaptic vesicle protein distribution assays were EG4052 *lin-15(n765ts)*X; *oxEx761[Punc-47:APM-2(cDNA)::GFP Punc-47::SNB::mCherry lin-15(+)]* and EG4051 *lin-15(n765ts)*X; *oxEx759[Punc-47:APM-2(cDNA)::GFP Punc-47::CHC-1::RFP lin-15(+)]*.

The strains used in the clathrin distribution assays were MT8247 *lin-15(n765ts)* *nls52[Punc-25::SNB::GFP lin-15(+)]*X, EG3733 *apm-2(e840)* *nls52[Punc-25::SNB::GFP lin-15(+)]*X, EG3229 *apm-2(gm17)* *nls52[Punc-25::SNB::GFP lin-15(+)]*X, NM1233 *jsls219[SNG-1::GFP rol-6(su1006)]*III, EG3563 *jsls219[SNG-1::GFP rol-6(su1006)]*III; *apm-2(e840)*X, EG3736 *jsls219[SNG-1::GFP rol-6(su1006)]*III; *apm-2(gm17)*X, EG3855 *lin-15(n765ts)* *oxls224[Punc-47::GFP::SNT-1 lin-15(+)]*X, EG3889 *apm-2(e840)* *oxls224[Punc-47::GFP::SNT-1 lin-15(+)]*X, and EG3891 *apm-2(gm17)* *oxls224[Punc-47::GFP::SNT-1 lin-15(+)]*X.

The strains used in the clathrin distribution assays were EG3381 *oxls164[Punc-47::GFP::CHC-1 lin-15(+)]*IV; *lin-15(n765)*X, EG3735 *oxls164[Punc-47::GFP::CHC-1 lin-15(+)]*IV; *apm-2(e840)*X and EG3564 *oxls164[Punc-47::GFP::CHC-1 lin-15(+)]*IV; *apm-2(gm17)*X.

The strains used in APM-2 distribution assays were EG4055 *snt-1(n2665)*II; *oxEx763[Punc-47::APM-2(cDNA)::GFP Pmyo-2::GFP]*, EG4091 *unc-41(n268)*IV; *oxEx767[Punc-47::APM-2(cDNA)::GFP Pmyo-2::GFP]*, EG4103 *unc-11(e47)*I; *oxEx767[Punc-47::APM-2(cDNA)::GFP Pmyo-2::GFP]*, and EG4089 *unc-26(s1710)*IV; *oxEx767[Punc-47::APM-2(cDNA)::GFP Pmyo-2::GFP]*.

The strains used in APM-2 tissue-specific rescue assay were EG4017 *lin-15(n765)*X; *oxEx747[Prab-3::APM-2(cDNA)::GFP (pMG9) lin-15(+)]*, EG1616 *apm-2(e840)*X; *oxEx730[APM-2::GFP (pMG4) Punc-122::GFP]*, EG4213 *apm-2(e840)*X; *oxEx789[Prab-3::APM-2(cDNA)::GFP (pMG9)*

Punc-122::GFP], EG4015 *apm-2(e840)*X; *oxEx745[Pdp-30::APM-2(cDNA)::GFP (pMG10) Punc-122::GFP]*, EG4029 *apm-2(e840)*X; *oxEx753[Pdp-2::APM-2(cDNA)::GFP (pMG8) Punc-122::GFP]*, EG4030 *apm-2(e840)*X; *oxEx745[Pdp-2::APM-2(cDNA)::GFP (pMG8) Prab-3::APM-2(cDNA)::GFP (pMG9) Punc-122::GFP]*, and EG4093 *apm-2(e840)*X; *oxEx773[Pdp-2::APM-2(cDNA)::GFP (pMG8) Pmyo-2::GFP]*. *Punc-122* is the coelomocyte promoter.

The strains used in yolk uptake assay were DH1033 *sqt-1(sc103)*II; *bls1[vit-2::GFP rol-6(su1006)]*X, EG4062 *apm-2(e840)* *bls1[vit-2::GFP rol-6(su1006)]*X, EG4059 *apm-2(gm17)* *bls1[vit-2::GFP rol-6(su1006)]*X.

The strain used in α adaptin coelomocyte plasma membrane localization assay were RT490 *unc-119(ed3)* III; *pwl177[Punc-122:mRFP::apa-2; cb-unc-119(+)]*, EG4188 *apm-2(e840)* X; *pwl177[Punc-122:mRFP::apa-2; cb-unc-119(+)]*, EG4189 *apm-2(gm17)* X; *pwl177[Punc-122:mRFP::apa-2; cb-unc-119(+)]*. They were provided by B.D. Grant (Rutgers University, Piscataway, NJ).

The strains used in α adaptin GABA synaptic localization assay were GK275 *unc-119(ed3)* III; *dkls160[Punc-25::GFP::apa-2,unc-119(+)]*, EG4203 *apm-2(e840)* X; *dkls160[Punc-25::GFP::apa-2,unc-119(+)]*, and EG4204 *apm-2(gm17)* X; *dkls160[Punc-25::GFP::apa-2,unc-119(+)]*. They were provided by K. Sato (Gunma University, Gunma, Japan).

The strains used in AP2 assembly immunoprecipitation were EG5264 *oxEx1275[Papa-2::apa-2::GFP Punc-122::GFP]*, EG5265 *apm-2(e840)*X, *oxEx1275[Papa-2::apa-2::GFP Punc-122::GFP]*, EG5266 *oxEx1276[Papa-2::apa-2::GFP Paps-2::aps-2::HA Punc-122::GFP]*, and EG5267 *apm-2(e840)*X *oxEx1276[Papa-2::apa-2::GFP Paps-2::aps-2::HA Punc-122::GFP]*.

Phylogenetic analysis

The phylogenetic tree of μ adaptin was made by ClustalX (1.83.1 Mac) and Treeview X. The protein accession numbers are as follows: μ 3A mouse (Q9JKC8); μ 3B mouse (Q8R2R9); μ 3 *Drosophila* (NP_788873); APM-3

(NP_508184); stB *Drosophila* (Q24212); APT-10 (stB Ce; NP_505566); stB mouse (NP_780576); μ 2 *Drosophila* (NP_732744); μ 2 mouse (NP_033809); APM-2 (NP_001024865); APM-1 (NP_491572); UNC-101 (NP_001040675); μ 1A mouse (AAF61814); μ 1B mouse (AF067146); μ 1 *Drosophila* (NP_649906).

GFP constructs

pMG1, *apm-2* genomic region. A 12-kb *apm-2* genomic PCR fragment was amplified from cosmid C33G6, including 5 kb upstream of the start codon, 5 kb of coding sequence, and 2 kb downstream of the stop codon, and was cloned between BamHI and PstI restriction sites in pGEM-3zf(+) (Promega). Primers used were 5'-ATTAGGATCCAGGTGGTGGTGAAGA-3' and 5'-AGATCTGCAGTCGGCTAACGGCTAATTCGGCTAA-3'.

pMG2, *apm-2* transcriptional GFP. The construct comprises the *apm-2* promoter only driving GFP. This construct does not show expression in neurons, suggesting that a neuronal enhancer is contained within an intron.

pMG3, *apm-2* transcriptional GFP. GFP with the *unc-54* 3'UTR was PCR amplified from the plasmid pPD95.77 (provided by A. Fire, Carnegie Institution of Washington, Baltimore, MD). The primers used were 5'-GGC-TGAAATCACTCAACGATGG-3' and 5'-TACAGTCGACTACGGCCG-CTAGTAGGAAACAGT-3'. This fragment contains Sall restriction sites at both ends and was inserted into the Sall site of pMG1, which is in the second exon of *apm-2*.

pMG4, *apm-2* translational GFP. *apm-2* 10-kb genomic PCR fragment, including 5 kb upstream of the start codon and 5 kb of coding sequence without the stop codon, was amplified from pMG1 and cloned between the BamHI and PstI restriction sites in pGEM-3zf(+). Primers used were 5'-ATTAGGATCCAGGTGGTGGTGAAGA-3' and 5'-ACAGCTG-CAGGCATCTGGTTTCATACAGTCCCGA-3'. GFP with the *unc-54* 3'UTR was cloned from the plasmid pPD95.77 with a PstI site at one end and a HindIII site at the other end and was then inserted after the *apm-2* coding sequence to make a GFP fusion protein. Primers used were 5'-ACATCTG-CAGTTGGCCAAAGGACCCAAAGGTATG-3' and 5'-ACGCAAGCTTCG-CCCGACTAGTAGGAAACAGTTA-3'.

Constructs for *apm-2* tissue-specific rescue assay

Multisite Gateway three-fragment construction vectors were used (Invitrogen). Promoter entry vectors, including pENTRY4-1 *Pdpy-30* and pENTRY4-1 *Ppdi-2*, were ordered from Open Biosystems. The *Ppdi-2* construct (Open Biosystems) lacks 373 nt (–808 to approximately –435 from the start codon of *pdi-2*) in the promoter region; however, skin expression was still observed using this construct. pENTRY4-1 *Prab-3* was generated by BP reaction. ORF entry vector pENTRY1-2 *apm-2* (ORF) was ordered from Open Biosystems. 3'UTR entry vector pENTRY2-3 GFP::*unc-54* 3'UTR was made by a Gateway BP reaction. The final constructs, *apm-2*::GFP fusion gene driven by different promoters, were generated by Gateway LR reactions.

Constructs for AP2: assembly immunoprecipitation

pMG16, *apa-2* translational GFP. *apa-2* 6-kb genomic sequence, including 2.3 kb of promoter and 3.7 kb of coding sequence without stop codon, was cloned between KpnI and XbaI sites in pGEM-3zf(+). Primers used were 5'-ACTCGGTACCGCATCTTGATGGAAAACCCGCTC-3' and 5'-AGCCTCTAGAAAATTGGTTGCCCAATAAGTCTAC-3'.

GFP with the *unc-54* 3'UTR was cloned from Fire laboratory vector pPD 95.77 with an XbaI site at one end and a HindIII site at the other end. This fragment was inserted after the *apm-2* coding sequence to make a GFP fusion protein. Primers used were 5'-ACCGTCTAGAGGGGTAGAAA-AAATGAGTAAAGGA-3' and 5'-AGCGAAGCTTCGGCCGACTAGTAGG-AAACAGTTA-3'.

pMG28, HA-tagged translational APS-2. *aps-2* 2-kb genomic sequence, including 1 kb of promoter and 1 kb of coding sequence (stop codon is replaced by HA tag), was cloned between EcoRI and PstI sites in pGEM-3zf(+). Primers used were 5'-AGCGGAATTCGGTTAGTCTT-GAGTGGCTTG-3' and 5'-ACAGCTGCAGAGCGTAATCTGGAACATCG-TATGGGTATTCCAGGGAAGTAAGCATGAGCA-3'.

unc-54 3'UTR was cloned from Fire laboratory vector pPD 95.77 with a PstI site at one end and a HindIII site at the other end. This fragment was inserted after the *aps-2*::HA coding sequence. Primers used were 5'-AGGCCTGCAGTAGCATTCGTAGAATTCCAAGTCA-3' and 5'-AGG-CAAGCTCCCATAGACACTACTCCACTTTC-3'.

Microinjection

The total DNA concentration of injection mix is 100 ng/ μ l. The injection marker was 50 ng/ μ l *Punc-122*::GFP, if not specified. 1-kb DNA

ladder (Fermentas) was used to make the injection mix final concentration 100 ng/ μ l.

Rescue experiment. 10 ng/ μ l of 12-kb *apm-2* genomic PCR fragment was injected into N2 to overexpress APM-2 in wild-type animals. 0.25 ng/ μ l of 12-kb *apm-2* genomic PCR fragment was injected into N2. The extra-chromosomal array was then crossed into *apm-2(e840)* and *apm-2(gm17)* mutants to evaluate rescue.

Tissue-specific rescue. 1 ng/ μ l each of pMG10 *Pdpy-30*::*apm-2*::GFP, pMG8 *Ppdi-2*::*apm-2*::GFP, and pMG9 *Prab-3*::*apm-2*::GFP DNAs were injected, respectively, into *apm-2(e840)*. For the skin and nervous system double rescue experiment, 1 ng/ μ l each of pMG8 and pMG9 were injected together. 10 ng/ μ l pMG9 and 50 ng/ μ l *lin-15(+)* were coinjected into *lin-15(n765ts)* to overexpress APM-2::GFP only in the nervous system. 1 ng/ μ l pMG8 and 2 ng/ μ l *Pmyo-2*::GFP were coinjected into *apm-2(e840)* to rescue *apm-2* mutant in skin with a different injection marker.

AP2 complex immunoprecipitation. pMG16 was injected at 10 ng/ μ l into the wild type. The array was crossed into *apm-2(e840)*. pMG16 and pMG28 were coinjected at 10 ng/ μ l into the wild type and the same array was then crossed into *apm-2(e840)*.

Western blot analysis

Worm samples were prepared by boiling 1 vol of the worm pellet in 9 vol of 1 \times loading buffer for 5 min. Samples were run on a 10% SDS-PAGE gel then transferred to polyvinylidene fluoride transfer membrane (Immobilon). Primary antibody for α adaptin was a rabbit polyclonal APA-2 antibody at a dilution of 1:500 (provided by B. Grant). Primary antibody incubation was done in 1% milk at room temperature for 4 h. Primary antibody for the standard control tubulin was a 12G10 mouse monoclonal antibody (Developmental Studies Hybridoma Bank) at a dilution of 1:5,000. Primary antibody incubation was done in 1% milk at room temperature for 1 h, and then the membrane was washed three times in 10 ml of 1 \times PBS plus Tween 20 (PBST). Secondary antibodies were anti-rabbit and mouse IgG fragment conjugated with HRP (GE Healthcare). Secondary incubations were done in 10% milk at room temperature for 45 min, and then the membrane was washed five times in 10 ml of 1 \times PBST. Detection reagent used was Lumigen PS-3 (GE Healthcare).

AP2 complex immunoprecipitation

250 μ l (\pm 50 μ l) of worm pellet was harvested. The pellet was suspended in 2 ml of ice-cold lysis buffer [5% Triton X-100, 50 mM Hepes, pH 7.3, 50 mM NaCl, and 1 tablet of protease inhibitor cocktail (Roche)]. The sample was lysed by a bead beater (NMB) for 10 s, three times, and was spun, and the supernatant was recovered. The supernatant was pushed through a 0.22- μ m filter. 15 μ l of agarose-conjugated rat anti-GFP IgG2a beads (MBL International) were added to the lysate. The mixture was incubated at 4°C for 2 h. Beads were harvested and washed with 1 ml of lysis buffer three times. 100 μ l of loading buffer was added on the bead pellet and boiled for 15 min.

Samples were run on 7, 10, or 15% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Primary antibody for GFP was a mouse monoclonal antibody at a dilution of 1:5,000 (Clontech Laboratories, Inc.). Primary antibody for HA was a mouse monoclonal antibody (12CA5; Santa Cruz Biotechnology, Inc.) at a dilution of 1:5,000. Primary antibody for β adaptin was a mouse monoclonal antibody (Thermo Fisher Scientific) at a dilution of 1:5,000. Primary antibody incubation was performed in 5% BSA at 4°C overnight, and then the membrane was washed three times in 10 ml of 1 \times PBST. Secondary antibodies were goat anti-mouse IgG fragment conjugated with HRP (GE Healthcare). Secondary incubations were performed in 5% BSA at 22.5°C for 2 h, and then the membrane was washed five times in 10 ml of 1 \times PBST. Detection reagent was SuperSignal West Dura kit (Thermo Fisher Scientific).

Thrashing assay

A single worm was put into a 50- μ l drop of M9 solution. The worm was allowed to adapt to the liquid environment for 2 min. The number of body bends was counted for 90 s for each genotype ($n = 7$). We analyzed lines of *apm-2(e840)* rescued by skin APM-2::GFP with two different injection markers [EG4029 *Punc-122*::GFP and EG4093 *Pmyo-2*::GFP]. The results are the same.

Aldicarb resistance assay

Each agar plate was seeded with bacteria and weighed to top spread plates with the appropriate amount of aldicarb. Plates with six different aldicarb final concentrations were prepared (0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 mM). Aldicarb was allowed to soak in overnight at room temperature.

For each genotype, 20 worms were put onto plates containing each different aldicarb concentration. The plates were blinded and the percentage of paralyzed worms were scored after 4 h of exposure to aldicarb. The same experiment was repeated five times for each genotype except *apm-2(e840)* *oxEx753[Prab-3::apm-2::GFP, Punc-122::GFP]*, which had only 10 worms on each plate and the experiment was repeated four times because of fewer transgenic animals.

Confocal microscopy

Worms are immobilized by using 2% phenoxy propanol and imaged on a confocal microscope (Pascal LSM5; Carl Zeiss, Inc.) with a plan-Neofluar 10x 0.3 NA, 20x 0.5 NA, or 40x 1.3 NA or plan-apochromat 63x 1.4 NA oil objectives (Carl Zeiss, Inc.).

Electron microscopy

Wild-type (N2), *apm-2(e840)*, EG4029 *apm-2(e840)*; *oxEx753[Ppdi-2::apm-2::GFP, Punc-122::GFP]*, and EG4213 *apm-2(e840)*; *oxEx789[Prab-3::apm-2::GFP, Punc-122::GFP]* adult nematodes were prepared in parallel for transmission electron microscopy as previously described (Hammarlund et al., 2007). In brief, 10 young adult hermaphrodites were placed onto a freeze chamber (100- μ m well of type A specimen carrier) containing space-filling bacteria, covered with a type B specimen carrier flat side down, and frozen instantaneously in the HPM 010 (Leica). This step was repeated for animals of all genotypes. The frozen animals were fixed in the EM AFS system (Leica) with 1% osmium tetroxide and 0.1% uranyl acetate in anhydrous acetone for 2 d at -90°C and for 38.9 more hours with gradual temperature increase ($6^{\circ}\text{C}/\text{h}$ to -20°C over 11.7 h, constant temperature at -20°C for 16 h, and $10^{\circ}\text{C}/\text{h}$ to 20°C over 4 h). The fixed animals were embedded in araldite resin following the infiltration series (30% araldite/acetone for 4 h, 70% araldite/acetone for 5 h, 90% araldite/acetone overnight, and pure araldite for 8 h). Mutant and control blocks were blinded. Ribbons of ultra-thin (33-nm) serial sections were collected using an Ultracut 6 microtome (Leica) at the level of the anterior reflex of the gonad. Images were obtained on an electron microscope (H-7100; Hitachi) using a digital camera (Gatan). 250 ultra-thin contiguous sections were cut, and the ventral nerve cord was reconstructed from two animals representing each genotype. Image analysis was performed using ImageJ software. The numbers of synaptic vesicles (~ 30 nm), dense-core vesicles (~ 40 nm), and large vesicles (>40 nm) in each synapse were counted and their distance from presynaptic specialization and plasma membrane as well as the diameter of each were measured from acetylcholine neurons VA and VB and the GABA neuron VD. A synapse was defined as the serial sections containing a dense projection as well as sections on either side of that density, which contain synaptic vesicle numbers above the mean number of synaptic vesicles per profile.

Electrophysiology

C. elegans were grown at room temperature ($22\text{--}24^{\circ}\text{C}$) on agar plates with a layer of OP50 *Escherichia coli*. Adult hermaphrodite animals were used for electrophysiological analysis. Miniature and evoked postsynaptic currents (mPSCs and ePSCs) at the neuromuscular junction were recorded as previously described (Liu et al., 2007) using a technique originally developed by Richmond and Jorgensen (1999). In brief, an animal was immobilized on a sylgard-coated glass coverslip by applying a cyanoacrylate adhesive along the dorsal side. A longitudinal incision was made in the dorsolateral region. After clearing the viscera, the cuticle flap was folded back and glued to the coverslip, exposing the ventral nerve cord and two adjacent muscle quadrants. A microscope (Axioskop; Carl Zeiss, Inc.) equipped with a 40x water immersion lens and 15x eyepieces was used for viewing the preparation. Borosilicate glass pipettes with a tip resistance of $\sim 3\text{--}5$ M Ω were used as electrodes for voltage clamping. The classical whole-cell configuration was obtained by rupturing the patch membrane of a gigaohm seal formed between the recording electrode and a body wall muscle cell. The cell was voltage clamped at -60 mV to record mPSCs and ePSCs. ePSCs were evoked by applying a 0.5-ms square wave current pulse at a supramaximal voltage (25 V) through a stimulation electrode placed in close apposition to the ventral nerve cord. Postsynaptic currents were amplified (EP10; HEKA) and acquired with Patchmaster software (HEKA). Data were sampled at a rate of 10 kHz after filtering at 2 kHz. The recording pipette solution contained the following: 120 mM KCl, 20 mM KOH, 5 mM TES, 0.25 mM CaCl_2 , 4 mM MgCl_2 , 36 mM sucrose, 5 mM EGTA, and 4 mM Na_2ATP ; pH adjusted to 7.2 with KOH and osmolality at $\sim 310\text{--}320$ mosM. The standard external solution included the following: 150 mM NaCl, 5 mM KCl, 5 mM CaCl_2 , 1 mM MgCl_2 , 5 mM sucrose, 10 mM glucose, and 15 mM Hepes; pH adjusted to 7.35 with NaOH with osmolality $\sim 330\text{--}340$ mosM.

Amplitude and frequency of mPSCs were analyzed using MiniAnalysis (Synaptosoft). A detection threshold of 10 pA was used in initial automatic analysis, followed by visual inspections to include missed events (≥ 5 pA) and to exclude false events resulting from baseline fluctuations. Amplitudes of ePSCs were measured with Fitmaster (HEKA). The amplitude of the largest peak of ePSCs from each experiment was used for statistical analysis. Data were imported into Origin, version 7.5 (OriginLab), for graphing and statistical analysis. Unpaired *t* test was used for statistical comparisons. A value of $P < 0.05$ is considered statistically significant. All values are expressed as mean \pm SEM. *n* is the number of worms that were recorded.

Quantification

ImageJ 1.36b was used for the pixel intensity analysis of α adaptin Western blot.

Online supplemental material

Fig. S1 shows rescue of the *apm-2* mutant phenotype. Fig. S2 shows that APM-2 is not mislocalized in endocytic mutants. Fig. S3 shows *apm-2* aldicarb assay. Fig. S4 is a cartoon structure of *apm-2::GFP* DNA constructs. Fig. S5 depicts the assembly of AP2 with or without $\mu 2$. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200806088/DC1>.

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