

Differential requirements for clathrin in receptor-mediated endocytosis and maintenance of synaptic vesicle pools

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Clathrin is a coat protein involved in vesicle budding from several membrane-bound compartments within the cell. Here we present an analysis of a temperature-sensitive (ts) mutant of clathrin heavy chain (CHC) in a multicellular animal. As expected *Caenorhabditis elegans chc-1(b1025ts)* mutant animals are defective in receptor-mediated endocytosis and arrest development soon after being shifted to the restrictive temperature. Steady-state clathrin levels in these mutants are reduced by more than 95% at all temperatures. Hub interactions and membrane associations are lost at the restrictive temperature. *chc-1(b1025ts)* animals become paralyzed within minutes of exposure to the restrictive temperature because of a defect in the nervous system. Surprisingly synaptic vesicle number is not reduced in *chc-1(b1025ts)* animals. Consistent with the normal number of vesicles, postsynaptic miniature currents occur at normal frequencies. Taken together, these results indicate that a high level of CHC activity is required for receptor-mediated endocytosis in nonneuronal cells but is largely dispensable for maintenance of synaptic vesicle pools.

Caenorhabditis elegans | synapse

Clathrin is thought to promote vesicle formation at several steps in membrane trafficking (1). Clathrin forms a triskelion, composed of 3 heavy and light chains. Interactions between triskelia drive coat formation, with interdigitation of triskelia creating repeating hexagonal and pentagonal units (2). According to this model, clathrin is a key component of vesicle formation; specifically, it provides a template that can drive membrane curvature. In the presynaptic membranes of neurons, it has been proposed that clathrin-mediated endocytosis maintains synaptic vesicle pools (3, 4). The definitive test for this model is to assay synaptic vesicle formation after eliminating clathrin function by mutation. Unfortunately, loss of clathrin heavy chain (CHC) is embryonically lethal in metazoans (5, 6), precluding assays of presynaptic function in such mutants. A solution to the chronic lethality of clathrin mutants is to analyze temperature-sensitive (ts) alleles of the protein in which the effects of acute loss of the protein function can be analyzed. In this work, we analyze a ts allele of CHC in an animal and demonstrate a differential requirement for clathrin in receptor-mediated endocytosis in oocytes and the maintenance of synaptic vesicle pool in neurons.

Results

rme-3 Encodes *Caenorhabditis elegans* Clathrin Heavy Chain. Analysis of 2 endocytosis mutants, *rme-3(b1024)* and *rme-3(b1025ts)*, identified in our previous genetics screens, demonstrated that they are alleles of the only CHC gene in *C. elegans* (*chc-1*) (6). The oocytes of these mutants fail to endocytose a GFP-tagged yolk protein, YP170-GFP. In wild-type animals, YP170-GFP is cleared efficiently from the body cavity by yolk receptors expressed on the surface of oocytes (Fig. 1A). In *chc-1(b1024)* animals uptake of YP170-GFP was reduced significantly, resulting in the accumulation of YP170-GFP in the body cavity (Fig.

1B). *chc-1(b1025ts)* exhibited a ts defect in yolk uptake with variable uptake at the permissive temperature and uniformly blocked uptake at the restrictive temperature (Fig. 1C and D and data not shown). *chc-1(b1025ts)* mutant animals also arrested development soon after being shifted to the restrictive temperature [supporting information (SI) Fig. S1].

chc-1(b1024) contains a 3-kb Tc5 transposable element inserted within the predicted promoter region of *chc-1*, 292 bases upstream of the predicted start codon (Fig. 1E). In *chc-1(b1025ts)*, we identified a 9-bp deletion centered around the stop codon of the *chc-1* gene. This lesion results in the deletion of the C-terminal methionine, a residue that is identical in worm, mouse, and human CHC. Furthermore, the loss of the stop codon is predicted to result in an extension of the ORF, adding 22 novel amino acids to the C terminus. Thus, this allele of *chc-1* is similar to ts yeast CHC mutants in which the C terminus is deleted or altered (7, 8). The C-terminal region of CHC projects from the vertex of the triskelion and interacts with neighboring triskelia in clathrin lattices (9). The loss of the terminal methionine and the addition of 22 amino acids is likely to interfere with triskelion interactions (Figs. S2 and S3) (9).

Recent reports indicate that clathrin light chain (CLC) is not required for endocytosis but rather contributes to other clathrin-mediated trafficking steps (10). Consistent with these reports, we found that wild-type worms depleted of *C. elegans* CLC (CLIC-1) by RNAi displayed normal YP170-GFP endocytosis and animal viability (Fig. S1 H and I). However, RNAi of *chc-1* in the *chc-1(b1025ts)* mutant background at the permissive temperature of 15°C resulted in fully penetrant embryonic lethality, indicating that CLC does contribute to at least one clathrin-mediated function in vivo (Fig. S1I).

Endogenous CHC Protein Is Severely Reduced in the *chc-1(b1025ts)* Mutant. In wild-type oocytes, endogenous CHC is localized largely to small punctate structures associated with the plasma membrane of oocytes, as expected for clathrin localized to coated pits and vesicles (Fig. 2A–B'). In *chc-1(b1025ts)* oocytes, CHC protein was barely detectable and lacked cortical enrichment (Fig. 2C–D'). α -Adaptin is one of the components of the adaptor protein 2 complex that participates in the formation of clathrin-coated vesicles, as well as in the selection of cargo molecules for incorporation

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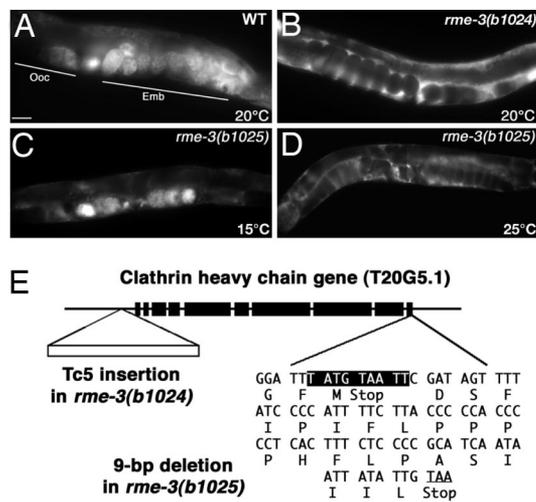


Fig. 1. *rme-3* encodes *C. elegans* CHC. (A–D) YP170-GFP endocytosis by oocytes of adult hermaphrodites. (A) In wild type, YP170-GFP is taken efficiently up by oocytes (Ooc) and after fertilization is found in embryos (Emb). (Scale bar, 30 μ m.) (B) In *rme-3(b1024)* mutants, very little YP170-GFP is endocytosed by oocytes; instead, YP170-GFP accumulates in the body cavity. (C) In *rme-3(b1025ts)* mutants, YP170-GFP uptake is mildly reduced in oocytes at 15°C. (D) At 25°C YP170-GFP uptake is blocked, and most YP170-GFP remains in the body cavity in *rme-3(b1025ts)* mutants. (E) In *rme-3(b1024)* a Tc5 transposable element is present in the *chc-1* promoter. In *rme-3(b1025ts)*, there is a 9-bp deletion spanning the stop codon. Black boxes indicate exons of *chc-1*.

into such vesicles (11). Like clathrin, α -adaptin is localized to small puncta on the plasma membrane of oocytes (Fig. 2 E and F). However, α -adaptin was enriched further in cortical puncta in *chc-1(b1025ts)* mutant oocytes, as has been observed previously when endocytosis is blocked in other organisms (Fig. 2 G and H) (12). Consistent with the localization data, anti-CHC-1 Western blots displayed strongly reduced band intensity at 15°C (4.5% of wild-type CHC) and 25°C (2.0% of wild-type CHC), suggesting that CHC-1 expression or stability is strongly impaired (Fig. 2I). Transgenic expression of GFP-tagged CHC-1(b1025) replicated the anti-CHC-1 immunofluorescence results (Fig. S2); the tagged mutant form of the protein expressed at an abnormally low level compared with wild-type controls and at the restrictive temperature failed to localize correctly to the plasma membrane in the gonad, intestine, and synaptic regions of neurons (Figs. S2 and S4 A–D’).

We also sought to examine the effects of the *b1025* mutation on clathrin assembly. The C-terminal one-third of CHC is often referred to as the “hub domain.” This region of CHC is necessary and sufficient for clathrin self-assembly into triskelions and also provides the binding interface for CLC (13). In a yeast 2-hybrid assay, wild-type CHC hub domains (residues 1075–1680) interacted with one another strongly both at 30°C and 37°C (Fig. S3). *b1025* mutant hub domains interacted with one another more weakly than wild-type hubs at 30°C and displayed a further weakened interaction at 37°C, suggesting that the *b1025* mutation tends to impair clathrin assembly, especially at higher temperatures (Fig. S3). These results suggest that the 2–5% of CHC remaining in *chc-1(b1025ts)* mutants is probably conditionally defective in assembly, a prerequisite for its association with membranes and endocytic vesicle formation. We note, however, that the permissive and restrictive temperatures for *C. elegans* are lower than those of yeast, so some caution is required in extending these interpretations to the *in vivo* situation in the worm.

CHC Is Concentrated in Presynaptic Regions of the Neuron. Particularly high rates of endocytosis are thought to be required at synapses to regenerate synaptic vesicles (14). Synaptic vesicle endocytosis is,

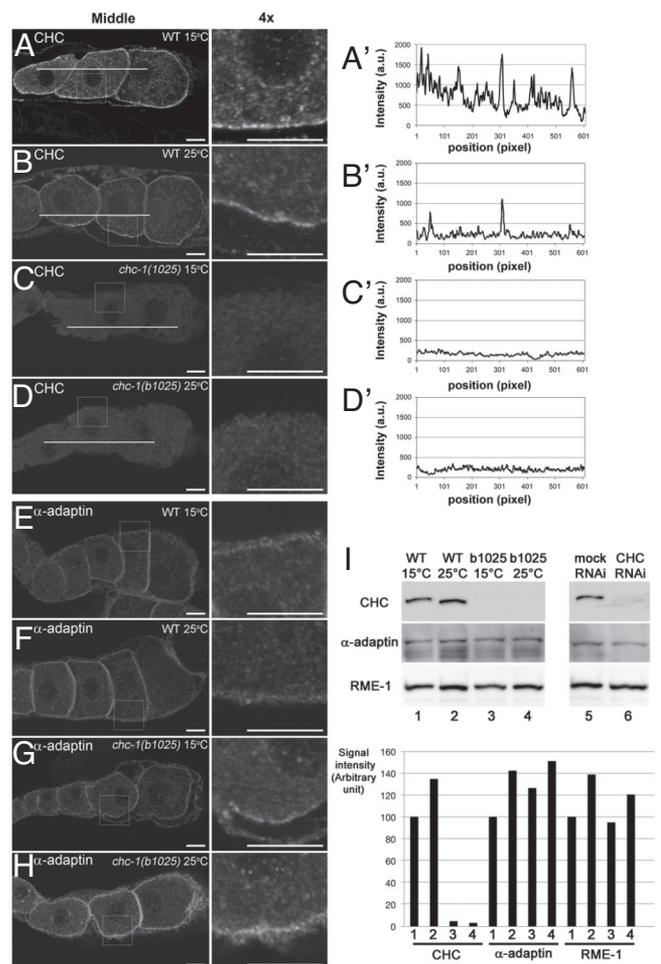


Fig. 2. Endogenous CHC levels are severely reduced in the *chc-1(b1025ts)* mutant. Gonads were dissected and immunostained with anti-CHC-1 (A–D) and anti- α -adaptin (E–H) antibodies. (A, E) wild type, 15°C; (B, F) wild type, 25°C; (C, G) *chc-1(b1025ts)*, 15°C; (D, H) *chc-1(b1025ts)*, 25°C. (A–H) Middle planes (Left) and enlarged images ($\times 4$) of boxed regions in the middle planes (Right) are shown. (Scale bar, 10 μ m.) (A’–D’) Fluorescence intensity of CHC staining as a function of position was graphed along a line through the center of the oocytes. (I) CHC protein level is dramatically reduced in *chc-1(b1025ts)* mutant animals. Total lysates were prepared from the indicated strains and were probed with anti-CHC, anti- α -adaptin, and anti-RME-1 antibodies in Western blots. Each lane is numbered. The signal intensity of each protein band was quantified and graphed (Bottom).

at least in part, mediated by clathrin, because synaptic vesicle components copurify with clathrin (4), and because clathrin-coated pits can be observed at synapses in electron micrographs (3). We analyzed clathrin localization *in vivo* in transgenic worms expressing mRFP (monomeric red fluorescent protein)-CHC in GABA neurons. mRFP-CHC was enriched in puncta in the neuronal processes with significant spatial overlap with GFP-synaptobrevin (GFP-SNB-1), a synaptic vesicle marker, demonstrating localization of CHC to the presynaptic varicosities *in vivo* (Fig. 3A–A’). We further analyzed GFP-CHC localization in neurons by immunostaining of GFP-CHC-expressing neurons with anti-SYD-2 (synaptic defective-2) antibodies. SYD-2/liprin is an active zone component that is required for active zone morphology and function (15). GFP-CHC-labeled puncta generally were adjacent to but not directly overlapping with structures labeled for SYD-2 (Fig. 3 B–B’), suggesting that clathrin-dependent endocytosis occurs in a region of the synapse distinct from, but adjacent to, the active zone. This result is in agreement with clathrin localization in the nervous system of other organisms (16).

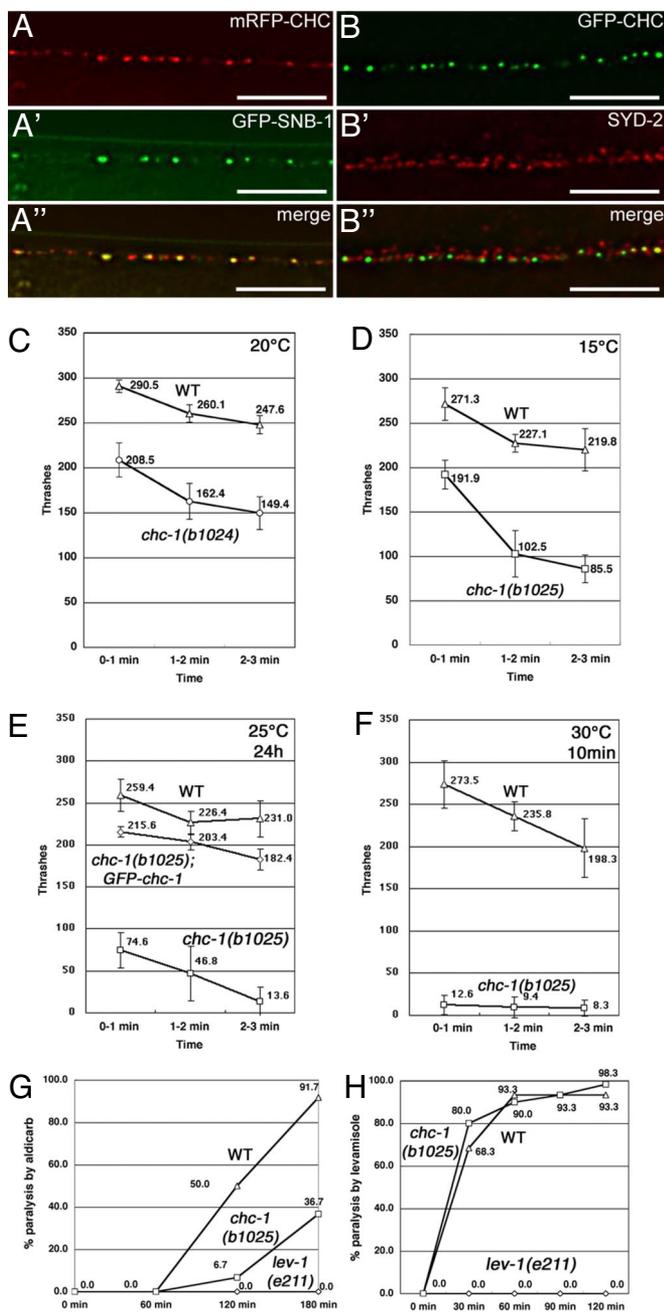


Fig. 3. Clathrin is required for neuromuscular function. (A–B'') CHC is concentrated presynaptically but is excluded from the active zone. (A–A'') Live worms co-expressing mRFP-CHC (A, A'') and the synaptic vesicle protein GFP-SNB-1 (A', A'') in GABA neurons were examined by fluorescence microscopy. (B–B'') Transgenic animals expressing GFP-CHC (B, B'') in the GABA neurons were stained with anti-GFP and anti-SYD-2 antibodies. Note that anti-SYD-2 antibody labels active zones in all synapses of the nerve cord (B'), whereas GFP-CHC labels only synapses in GABA motor neurons (B). (Scale bars, 10 μ m.) (C–F) Thrashing assays. (C) Wild-type L4 hermaphrodites ($n = 8$) and *chc-1(b1024)* ($n = 8$) were incubated at 20°C for 24 h and then transferred to M9 buffer. Head thrashes were counted. (D) Wild-type L4 hermaphrodites ($n = 8$) and *chc-1(b1025ts)* mutants ($n = 8$) were incubated at 15°C for 24 h and examined. (E) Wild-type L4 hermaphrodites ($n = 5$), *chc-1(b1025ts)* ($n = 5$), and *chc-1(b1025ts)* mutant animals expressing GFP-CHC (*dkEx6* [*P_{rgef-1}::GFP::chc-1*]) under the control of a neuron-specific promoter ($n = 5$) were incubated at 25°C for 24 h before examination. (F) Wild-type young adult worms ($n = 4$) or *chc-1(b1025ts)* mutants ($n = 8$) were incubated at 30°C for 10 min and examined. (G) Aldicarb resistance. Thirty young adult hermaphrodites of the wild type, *chc-1(b1025ts)*, and *lev-1(e211)* were placed on aldicarb plates (0.5 mM) at the semi-permissive temperature of

Clathrin Is Required for Neuromuscular Function. To assess aspects of synaptic function in clathrin mutants at the level of behavior, we analyzed swimming. When placed in a drop of buffer, normal worms perform rapid body bends with a reproducible periodicity. When we transferred wild-type worms to buffer, they continued thrashing actively for 3 min with only a slight decrement in rate (triangle, Fig. 3 C–F and *SI Text* and *Movie S1*). The weak mutant *chc-1(b1024)* thrashed at a slightly decreased rate compared with the wild type (circles, Fig. 3C). At the permissive temperature of 15°C, *chc-1(b1025ts)* worms also exhibited reduced thrashing activity compared with the wild-type control worms but continued to thrash slowly after 3 min (squares, Fig. 3D). After incubation at 25°C for 24 h, *chc-1(b1025ts)* animals initially displayed strongly reduced thrashing behavior and became almost completely paralyzed after 3 min, suggesting a progressive loss of synaptic function (squares, Fig. 3E, and *Movie S2*). We also observed a defect in pharyngeal pumping behavior in *chc-1(b1025ts)* animals consistent with defective neuronal function (Table S1).

To determine whether the thrashing defects in *chc-1(b1025ts)* animals were neuronal in origin, we expressed GFP-CHC under the control of a panneuron- (*rgef-1*) or muscle-specific promoter. *rgef-1* (F25B3.3) encodes the *C. elegans* orthologue of a Ca^{2+} -regulated ras guanine nucleotide exchange factor (*rgef-1*), which is expressed throughout the nervous system in *C. elegans* (17). The neuronal transgene restored the thrashing behavior of *chc-1(b1025ts)* animals to almost wild-type levels, indicating that most of the defects observed were caused by the reduced function of clathrin in the nervous system and not the musculature (diamonds, Fig. 3E). Expression of GFP-CHC in muscle did not improve the phenotype (data not shown). Expression of GFP-CHC-1(b1025) mutant protein in neurons did not complement the thrashing defect of the *chc-1(b1025ts)* animals and did not affect thrashing rates of wild-type animals, suggesting that the protein does not interfere with wild-type clathrin function (Fig. S4E). To determine whether there is an acute defect in synaptic transmission in *chc-1(b1025ts)* mutants, we raised worms at 15°C and then shifted them to 30°C for 10 min. Indeed, the defect was acute, because even this short exposure to restrictive temperature produced a significant thrashing defect in *chc-1(b1025ts)* animals compared with wild type (Fig. 3F). It is also possible that clathrin dysfunction unmasks an intrinsic temperature sensitivity of endocytosis or some other aspect of neurotransmission as reported for *ehs-1/eps15* deletion mutants (18).

As an additional test of whether *chc-1(b1025ts)* mutants have defects in pre- or postsynaptic transmission, we measured sensitivity to aldicarb and levamisole. Aldicarb is an inhibitor of acetylcholine esterase and causes a toxic accumulation of secreted acetylcholine at neuromuscular synapses (19). Aldicarb toxicity can be ameliorated by mutations that decrease neurotransmitter release at the synapse or that reduce the response to acetylcholine postsynaptically. *chc-1(b1025ts)* animals were less sensitive to aldicarb than wild-type worms, a phenotype that could be reversed by neuron-specific GFP-CHC expression (Figs. 3G and 5E). We also measured sensitivity to levamisole, an acetylcholine receptor agonist in nematodes (20). Levamisole sensitivity is thought to be purely postsynaptic. We found no significant difference in sensitivity to levamisole between the wild-type and *chc-1(b1025ts)* animals (Fig. 3H). We also found no significant difference in the postsynaptic muscle whole-cell membrane currents and resting membrane potential

22°C and checked for their ability to move at the indicated time-points in response to prodding. *lev-1(e211)* mutants that lack a subunit of the muscle inotropic ACh receptor were resistant to both aldicarb and levamisole and used as a control. (H) Levamisole sensitivity of *chc-1(b1025ts)*. Thirty young adult hermaphrodites of the wild type, *chc-1(b1025ts)*, and *lev-1(e211)* were placed on levamisole plates (60 μ M) and assayed for movement in response to prodding.

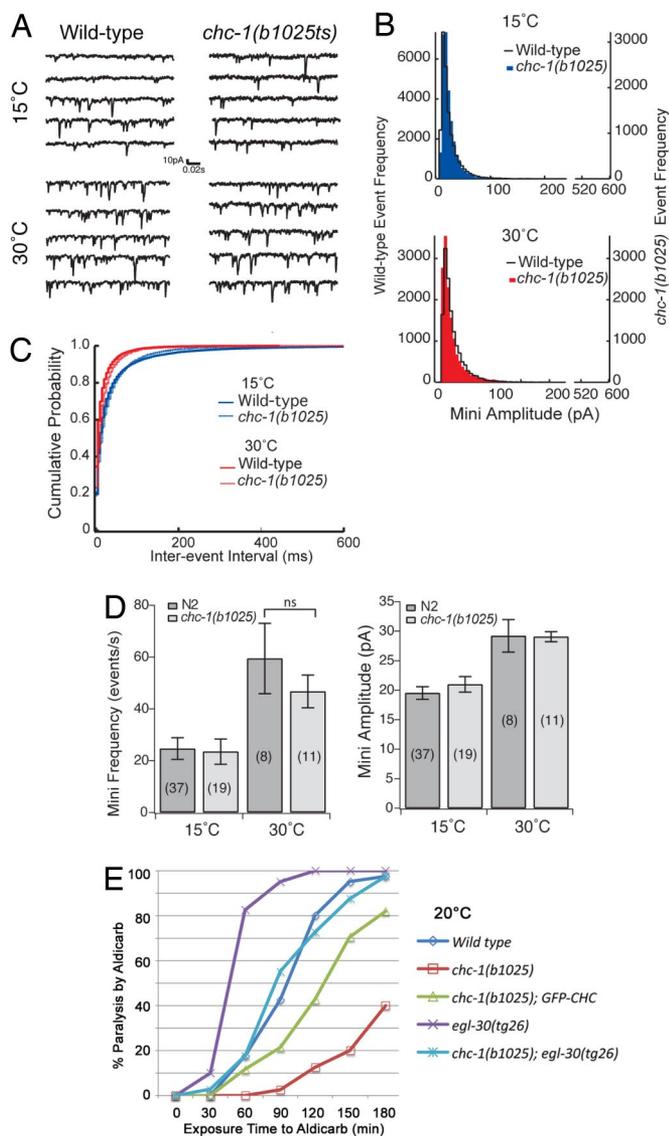


Fig. 5. Normal synaptic transmission in neurons of *chc-1(b1025ts)* animals. *chc-1(b1025ts)* mini frequency and amplitude are statistically indistinguishable from wild type at 15°C and 30°C. (A) Event records from animals raised at 15°C or animals exposed to 30°C for 1 h. (B) Histograms of pooled event mini amplitude for each genotype and condition. At 30°C a slight increase in the distributions is seen for both wild type and *chc-1(b1025ts)* (5-pA bins). (C) Cumulative probability of pooled inter-event intervals showing mini intervals decreases with increased temperature for both wild type and *chc-1(b1025ts)* (5-ms bins). (D) Quantification of average mini amplitude and frequency from individual animals from each genotype and at each condition. (E) Aldycarb sensitivity assay. Forty L4 hermaphrodites of wild-type, *chc-1(b1025ts)*, *egl-30(tg26)*, and *chc-1(b1025ts) egl-30(tg26)* animals were incubated at 15°C and then placed on aldycarb plates (0.5 mM) at 20°C. The *chc-1(b1025ts)* mutant animals expressing GFP-CHC (*dkEx6[Prgef-1::GFP::chc-1]*) under the control of a neuron-specific promoter also were examined. These animals were checked for their ability to move at the indicated time-points in response to prodding.

5). A likely remaining possibility is that probability of vesicle release is reduced in vivo, but this inhibition of release cannot be detected in a dissected preparation. Feedback inhibition, possibly mediated by acetylcholine itself through G protein-coupled metabotropic receptors (22), may be disrupted in our semi-intact preparation by superfused extracellular buffers. Inefficient receptor internalization in the clathrin mutant could lead to a build-up of inhibitory receptors on the motor neuron surface. To test whether G protein-

mediated inhibition of release could be overcome by a constitutively active stimulatory G protein, we introduced the *egl-30(tg26)* gain-of-function mutation $G\alpha_q$ into the clathrin mutant background. This mutant form of $G\alpha_q$ is known to increase the rate of acetylcholine release and confers hypersensitivity to aldicarb (23). Consistent with increasing vesicle release in the clathrin mutant background, the constitutively active $G\alpha_q$ suppressed the aldicarb resistance of *chc-1(b1025ts)* (Fig. 5E).

Discussion

Here we identify a temperature-sensitive mutant of CHC in a multicellular animal and show that clathrin is essential for receptor-mediated endocytosis and development. Although reduction of clathrin function blocks receptor-mediated endocytosis in oocytes, synaptic vesicle formation appears to be normal, albeit with altered morphology.

Several recent studies have reported that perturbing clathrin function interferes with synaptic vesicle recycling upon intense stimulation. Specifically, photoinactivation of both CLC and CHC using a fluorescein-assisted light inactivation (FAsH-FALI) technology disrupts synaptic vesicle recycling at *Drosophila* synapses upon high stimulation (24, 25). FAsH-FALI generates reactive oxygen species on the targeted molecule that can damage nearby molecules within a radius of at least 40 Å (26). It is possible that photolytic destruction generates protein fragments that may have antimorphic activity at the synapse. In addition, photolysis of clathrin may inactivate clathrin-interacting proteins, including regulators of membrane-associated actin assembly such as huntingtin-interacting protein 1-related protein (27, 28). Thus photolysis studies underscore the importance of clathrin-associated proteins for endocytosis of synaptic vesicles, but photolysis may in fact generate a more severe phenotype than a simple loss of clathrin.

In contrast to photoinactivation, RNAi reduces protein levels without generating protein fragments. Chronic RNAi of clathrin in hippocampal synapses causes a loss of synaptic vesicle endocytosis immediately after stimulation (29). These treated nerve terminals possessed normal levels of releasable vesicles before stimulation even though clathrin function had been disrupted for 3 d before stimulation. These data suggest that functional synaptic vesicles exist even when clathrin function has been disrupted. These data are consistent with the observations in *Drosophila*. When clathrin was photoinactivated at the fly neuromuscular junction, but synapses were not hyperstimulated, normal numbers of synaptic vesicles were observed (24), and neurotransmission was not affected upon mild stimulation (25). It is possible that an alternative mechanism for endocytosis can maintain vesicle pools when clathrin activity is impaired.

Taken together these studies indicate a surprising ability to maintain significant synaptic vesicle pools with little or no clathrin activity. This result also is consistent with mouse dynamin knockout mutants; these animals have surprisingly mild defects in synaptic transmission and synaptic vesicle number. Dynamin1 is largely dispensable for the biogenesis and endocytic recycling of synaptic vesicles; defects are most severe in inhibitory synapses, which might have very high rates of exocytosis (30, 31).

In our study we did not observe any significant defect in synaptic vesicle biogenesis in CHC mutants. Perhaps the residual clathrin (<2%) in the *chc-1(b1025ts)* animals is sufficient for the maintenance of synaptic vesicle pools in neurons under most physiological conditions. However, overexpression of the CHC-1(b1025) mutant protein cannot rescue the mutant phenotype, suggesting that the phenotype is not simply the result of the decrease in abundance of clathrin. Rather, it is more likely that the phenotype is the result of a failure of clathrin assembly and function. The mutant C termini display strongly reduced interaction at the restrictive temperature in a 2-hybrid assay, suggesting that triskelia cannot form. In fact, receptor-mediated endocytosis is severely blocked at the restrictive temperature in *chc-1(b1025ts)*, demonstrating that this mutation

severely reduces clathrin function. Together these data suggest that the mutant protein is likely to represent a loss of clathrin cage formation at high temperatures.

How can synaptic vesicles be generated with clathrin activity so diminished? In kiss-and-run recycling, synaptic vesicles are thought to fuse partially with the plasma membrane, forming a fusion pore large enough to release neurotransmitter. The synaptic vesicles then are pulled back into the cytoplasm without a need for clathrin (32, 33). This model predicts that endocytosis should take place at the active zone. A clathrin-independent mechanism that occurs at the active zone has been suggested in *Drosophila* (34). Alternatively, it is possible that clathrin is important for vesicle morphology but is not an essential component in synaptic vesicle formation. Recent studies in yeast have demonstrated that endocytosis takes place at sites enriched for clathrin and actin but that clathrin itself is not required for endocytosis at these sites (35). The large ensemble of secondary players may be capable of generating vesicles in the absence of clathrin.

Materials and Methods

General Methods and Strains. *C. elegans* strains were derived from the wild-type Bristol strain N2. Worm cultures, genetic crosses, and other *C. elegans* methods were performed according to standard protocols (36). RNAi was performed by the feeding method (37). *b1024* and *b1025* mutants were isolated in a screen described previously (6). The methods are described further in *SI Text*.

Electron Microscopy. Fixations were performed using a high-pressure freezing apparatus followed by substitution of solvent and fixative at -90°C . Two hundred fifty ultrathin (33 nm) contiguous sections were cut, and the ventral nerve cord was reconstructed from 2 animals representing each genotype.

Electrophysiology Methods. Adult animals were dissected as described previously (38) to expose the muscles of neuromuscular junctions to a patch-clamp pipette. Dissected animals were bathed in an external saline (in mM): 150 NaCl, 5 KCl, 1 MgCl₂, 5 CaCl₂, 15 Hepes, and 10 glucose (pH 7.35) and adjusted to 340 mOsm with sucrose. The pipette solution contained 120 KCl, 20 KOH, 4 MgCl₂, 5 N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 0.25 CaCl₂, 4 NaATP, 36 sucrose, 5 EGTA (pH 7.2). Pipettes were pulled and fire-polished to a tip resistance of 3–5 M Ω . Tight-seal whole-cell voltage clamp recordings of miniature current events were acquired with an EPC-10 amplifier (HEKA) in voltage-clamp mode at a holding potential of -60 mV. Analog signals were filtered at 2 kHz and sampled at 10 kHz. Events were analyzed with Mini-Analysis (Synaptosoft) with an event detection threshold 5 times the RMS baseline noise (range 1–4 pA). Animals—wild-type N2 and *chc-1(b1025ts)*—used for recordings were maintained at 15°C . Animals recorded under permissive conditions were dissected immediately and recorded in an air-conditioned room where the bath temperature averaged $20 \pm 1^{\circ}\text{C}$. Animals recorded under restrictive conditions first were pretreated by placing animals in a 30°C air incubator for 1 h. Animals then were dissected immediately in a heated room (23°C) and recorded on a warmed stage (average bath temperature of $26 \pm 2^{\circ}\text{C}$).

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