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 triskelon, 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. L4). 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 Tcl 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 triskelon 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 (CLC-1) by RNAi displayed normal YP170-GFP endocytosis and animal viability (Fig. S1 H and I). However, RNAi of clic-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. 2 C–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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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 (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. 2 I). Transgenic expression of GFP-tagged CHC-1(b1025) recapitulated 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 triskelia 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, 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 and components copurify with clathrin (4), and because clathrin-coated pits can be observed at synapses in electron micrographs (3).

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 (Ooo) and after fertilization is found in embryos (Emb). (Scale bar, 30 µm.) (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.
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. 3C–F and SI Text and Movie S1). The weak mutant \textit{chc-1(b1024)} thrashed at a slightly decreased rate compared with the wild type (circles, Fig. 3C). At the permissive temperature of 15°C, \textit{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, \textit{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 \textit{chc-1(b1025ts)} animals consistent with defective neuronal function (Table S1).

To determine whether the thrashing defects in \textit{chc-1(b1025ts)} animals were neuronal in origin, we expressed GFP-CHC under the control of a panneuron- (\textit{rgef-1}) or muscle-specific promoter. \textit{rgef-1} (F25B3.3) encodes the \textit{C. elegans} orthologue of a Ca2+-regulated ras guanine nucleotide exchange factor (\textit{rgef-1}), which is expressed throughout the nervous system in \textit{C. elegans} (17). The neuronal transgene restored the thrashing behavior of \textit{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 in \textit{chc-1(b1025ts)} mutant protein in neurons did not complement the thrashing defect of the \textit{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 \textit{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 \textit{chc-1(b1025ts)} animals compared with wild type (Fig. 3F). It is also possible that clathrin dysfunction unmask an intrinsic temperature sensitivity of endocytosis or some other aspect of neurotransmission as reported for \textit{ehs-1} mutant worms (18).

As an additional test of whether \textit{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. \textit{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 \textit{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. \textit{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 \textit{chc-1(b1025ts)}. Thirty young adult hermaphrodites of the wild type, \textit{chc-1(b1025ts)}, and \textit{lev-1(e211)} were placed on levamisole plates (60 μM) and assayed for movement in response to prodding.
between wild-type and chc-1(b1025ts) animals (data not shown). These results also indicate that the primary neuromuscular defect in chc-1 mutants is presynaptic.

A High Level of Clathrin Function Is Dispensable for Synaptic Vesicle Formation. In the mutant animals, a possible source of fatigue in the thrashing assays and of resistance in the aldicarb assays is that synapses at the neuromuscular junction are depleted of synaptic vesicles because of the loss of functional clathrin. We analyzed the number of synaptic vesicles using electron microscopy at neuromuscular junctions in wild-type and in chc-1(b1025ts) animals that had been kept at 15°C or had been shifted to 30°C for 2 h (Fig. 4A). Unexpectedly, the numbers of synaptic vesicles were not significantly different between the wild-type and chc-1(b1025ts) animals at either 15°C or at 30°C (Fig. 4B). For example, sections at GABA synapses in wild-type or mutant animals shifted to 30°C contained a similar number of synaptic vesicles: 39 per section in wild-type vs. 36 per section in chc-1(b1025ts) animals (Table S2). These data suggest that synaptic vesicles are still formed under conditions of acute clathrin depletion at nonpermissive temperatures. We also determined the number of synaptic vesicles for the wild-type and for chc-1(b1025ts) animals raised at 15°C, shifted to 25°C for 24 h, and allowed to thrash in liquid for 5 min. Under these conditions, the number of synaptic vesicles were somewhat reduced for chc-1(b1025ts) compared with the wild type, especially in GABA neurons (Fig. 4C), suggesting that synaptic vesicle recycling is affected mildly by chronic dysfunction of CHC. The subcellular localization of several synaptic proteins was unaffected in chc-1(b1025ts) animals (Figs. S5 and S6). Interestingly, synaptic vesicles were smaller (24 nm) in chc-1(b1025ts) animals that had been shifted to 30°C for 2 h than in mutants that had been kept at 15°C (28 nm, P < 0.0001) or the wild type at 30°C (28 nm, P < 0.0001) or 15°C (28 nm, P < 0.0001) (Figs. 4D and S7). These results suggest that clathrin is required for the normal morphology of synaptic vesicles. The functional significance of the smaller vesicles is not clear because the size of postsynaptic miniature current events (minis) representing the fusions of individual vesicles were not significantly different at 30°C (Fig. 5).

To demonstrate that the vesicles present at synapses were functional synaptic vesicles, we recorded the frequency of post synaptic minis in the muscle to assay whether these vesicles were competent for fusion (Fig. 5 A–D). In short, synaptic transmission appears normal in the chc-1(b1025ts) mutant. Wild-type animals raised at 15°C and dissected and recorded at 20°C exhibited an average mini frequency of 24.6 ± 4.2 fusions/sec (n = 37). chc-1(b1025ts) raised at 15°C had a similar mini frequency of 23.5 ± 4.9 fusions/sec (n = 19). We challenged the animals with an elevated temperature to increase fusion rates acutely (21). Animals were incubated at 30°C for 1 h, then dissected and recorded at 26 ± 2°C; at this elevated temperature the wild-type mini rate increased to 59.4 ± 13.6 fusions/sec (n = 8, P = 0.0012 vs. wild-type at 20°C, Mann–Whitney test). Similarly, mini rates in chc-1(b1025ts) mutants (n = 7) increased at elevated temperature to 46.7 ± 6.3 fusions/sec. This value was slightly less than that in the wild type, but the difference was not significant (P = 0.7 vs. WT at restrictive temperature, Mann–Whitney test) and is consistent with the slight reduction in synaptic vesicles observed in electron micrographs. Just as in wild-type animals, vesicle turnover is accelerated at higher temperatures in the chc-1(b1025ts) mutant, suggesting that even under the added temperature challenge the chc-1(b1025ts) mutants are able to keep pace with wild-type fusion rates. Given that CHC levels are reduced by 95% to 98% in C. elegans mutant individuals, and that the remaining CHC is likely to be defective in assembly at the restrictive temperature, we conclude that a high level of clathrin is not required for synaptic vesicle formation.

If the synaptic vesicle pool is not significantly reduced in chc-1(b1025ts) animals, then why are the mutant animals resistant to the paralyzing effects of aldicarb and uncoordinated at high temperature? The simplest explanation for these behavioral results is that excitatory cholinergic transmission is reduced. In the mutant, muscle sensitivity to acetylcholine is normal (Fig. 3H), and our ultrastructural and electrophysiological studies could not detect a significant decrease in the size of the vesicle pool or in the neurotransmitter content of the vesicles in the mutant (Figs. 4 and 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 Gaq into the clathrin mutant background. This mutant form of Gaq 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 Gaq 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, photoactivation of both CLC and CHC using a fluorescein-assisted light inactivation (FlaSH-FALI) technology disrupts synaptic vesicle recycling at *Drosophila* synapses upon high stimulation (24, 25). FlaSH-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 photoactivation, 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. Dynamin 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 activity (2%–3%) 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 function.

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 following 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 Heps, 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 MΩ. Tight-seal whole-cell voltage clamp recordings of nAChR currents 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 MiniAnalysis (Snyapsoft) with an event detection threshold 5 times the RMS baseline noise (range 1–4 pA). Animals–wild-type N2 and chc-1(b1025ts2)–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 ± 1°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 ± 2°C).

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Supporting information

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SI Text

Additional General Methods and Strains. Strains expressing transgenes in germline cells were grown at 25 °C. Other strains were grown at 15 °C or 20 °C. Transgenic strain bbs1 [vit-2::GFP] expressing a fusion of YP170, a yolk protein, and GFP was used to monitor yolk uptake by oocytes (1). A transgenic strain nls32[Punc-25::SNB-1::GFP, lin-15(+)] was kindly provided by Dr. Yishi Jin at the University of California, San Diego. The other strains were obtained from Caenorhabditis Genetics Center. A genomic clone of clathrin light chain was amplified by PCR and subcloned into RNAi vector L4440. For RNAi knockdown, larval stage 4 (L4) worms were placed onto RNAi plates, and P0 adults or F1 progeny were subsequently scored for phenotype.

Genetic Mapping and Molecular Cloning. b1024 was isolated using the mutator strain DH1069 mut-7 (pk204); nls1 [vit-2::GFP, rol-6 (su1006)]. For the isolation of the b1025 temperature-sensitive mutant, DH1066 bbs1[vit-2::GFP, rol-6 (su1006)] was mutagenized with N-ethyl-N-nitrosourea, and the F1 and F2 generations were grown at 15 °C. After incubation at 25°C for 12–24 h, the F2 generation was screened for animals exhibiting little or no GFP signal in oocytes and embryos but showing bright GFP signal in body cavity. Both rme-3 mutations are recessive. The rme-3 mutations were mapped to the middle of LGIII by SNP mapping (2). To identify a candidate for rme-3, we surveyed predicted genes in the C. elegans genomic sequence in this region and found chc-1 (T20G5.1). RNAi of chc-1 was known to produce a strong receptor-mediated endocytosis (RME) phenotype and severe embryonic lethality. Expression of GFP::CHC-1 complemented the temperature-sensitive embryonic lethality of rme-3(b1025ts). Genomic regions from chc-1 were amplified from each rme-3 mutant by PCR for sequencing.

Plasmids and Transgenic Strains. We used the bbs5[GFP::chc-1, rol-6(d1)] strain expressing GFP-CHC-1 under the control of the chc-1 promoter for complementation testing (3). For cell-specific expression we cloned the chc-1 ORF into Gateway destination vectors (Invitrogen). Genomic DNAs containing the ORFs of chc-1(+) and chc-1(b1025ts) were amplified by PCR and cloned into the Entry vector pDONR221. To drive expression of the GFP-fusion genes in the maternal germ line, we used a destination vector pID3.01B, which contains pie-1 5' UTR-GFP-Gateway cassette-pie-1 3' UTR sequences with the cotransformation marker C. elegans unc-119 (4). The ORF of chc-1 or chc-1(b1025) in pDONR221 then was transferred into pID3.01B by Gateway recombination cloning technology. These plasmids were introduced into unc-119(ed3) by microparticle bombardment (5) to generate the integrants pws147[Ppie-1::GFP::chc-1] and pws90[Ppie-1::GFP::chc-1(b1025)].

For intestinal expression of transgenes, we used a destination vector containing the vha-6 promoter-GFP-Gateway cassette-let-858 terminator with C. briggsae unc-119 (6). For expression of the transgenes in GABA neurons, we constructed a Gateway destination vector pKS7, which contains the unc-25 promoter-mRFP-Gateway cassette-let-858 terminator with C. briggsae unc-119. The ORF of chc-1 or chc-1(b1025) on pDONR221 was transferred into these vectors by Gateway recombination cloning technology. These plasmids were introduced into unc-119(ed3) by the bombardment method to generate the integrants dkls8[Pvha-6::GFP::chc-1], dkls14[Pvha-6::GFP::chc-1(b1025) and pws122[Pvha-6::mRFP::chc-1]. For pan-neuronal expression of N-terminal GFP fusion protein, we constructed a destination vector, pKS12, which contains rgef-1/ F25B.3 promoter, followed by GFP, a Gateway cassette, and the transcriptional let-858 terminator. The chc-1 ORF was transferred into pKS12 and microinjected into chc-1(b1025ts). This strain was used to examine complementation of the thrashing defect of chc-1(b1025ts) by neuron-specific expression of CHC-1. We also created a strain expressing GFP-CHC-1 in GABA neurons under unc-47 promoter control, ods-164[Punc-47::GFP::chc-1] by the microinjection method for colocalization analysis.

Western Blotting. To examine the amount of endogenous CHC-1, α-adaptin, and RME-1, 50 adult hermaphrodites were incubated at 15 °C or 25 °C and subjected to Western blotting as described previously (1). To determine the amount of GFP-CHC-1 proteins, 80 adult hermaphrodites expressing GFP-CHC-1 or GFP-CHC-1(b1025) under the control of vha-6 promoter were incubated at 15°C, 25 °C, or 30°C and subjected to Western blotting. Rabbit anti-CHC polyclonal antibody, rabbit anti-α-adaptin polyclonal antibody, and rabbit anti-RME-1 polyclonal antibody were used as described previously (5, 7, 8). Rat anti-GFP monoclonal antibody (GF090R) was purchased from NACARA-I tesque, Inc. and used at a 1:500 dilution.

Microscopy and Immunostaining. Fluorescence images were obtained using an Axiovert 200M (Carl Zeiss MicrOImaging, Inc.) microscope equipped with a digital CCD camera (C4742-95-12ER, Hamamatsu Photonics) and deconvolved with AutoDeblur software (AutoQuant Imaging Inc.). Confocal images were obtained using an Olympus confocal microscope system FV1000 (Olympus Corp.). Examination of images was performed with Metamorph software (Universal Imaging Co). To observe live worms expressing transgenes, worms were mounted on agarose pads with 10 mM levamisole in M9 buffer. Immunostaining of dissected gonads was performed as described previously (1). Whole-mount immunofluorescent staining of neurons was performed as described (9). Anti-SYD-2 polyclonal antibody was kindly provided by Dr. Mei Zhen of the University of Toronto. Mouse anti-GFP monoclonal antibody (3E6), Alexa Fluor 488-conjugated goat anti-mouse IgG, and Alexa Fluor 568-conjugated goat anti-rabbit IgG were obtained from QIBIOGene Inc. and Molecular Probes Corporation, respectively. For immunostaining of RAB-3, SNT-1, SNB-1, and UNC-64, animals were fixed and prepared for immunocytochemistry using a modified Bouin’s fixative or 2% paraformaldehyde (10, 11). The following antibodies were used: mouse anti-RAB-3 (11), rabbit anti-synaptotagmin (SNT-1) (10), rabbit anti-synaptobrevin (SNB-1) (12), rabbit anti-UNC-64 (13), and mouse GFP; (Roche Applied Science). Appropriate secondary Alexa Fluor 488 and Alexa Fluor 555-conjugated antibodies were used (Molecular Probes).

Immunostaining of dissected gonads were performed as described previously (1). The following antibodies were used: rabbit anti-CHC polyclonal antibody (8), rabbit anti-α-adaptin polyclonal antibody (5), and mouse anti-GFP (Roche Applied Science). Appropriate secondary Alexa Fluor 488 and Alexa Fluor 555-conjugated antibodies were used (Molecular Probes).

Thrashing movies were made using PixelINK capture software and a PixelINK PL-A661 firewire CCD camera attached to 10× ocular of an Olympus dissecting microscope.

Thrashing Assay. The thrashing assay was used to investigate the frequency of contraction of body-wall muscles in worms. L4 hermaphrodites of wild-type (N2) and chc-1 mutant worms were
grown synchronously to the young adult stage at 15 °C, 20 °C, or 25 °C. For 30 °C incubation, L4 hermaphrodites were grown at 15 °C for 24 h to the young adult stage and then transferred onto a 30 °C prewarmed OP50-seeded NGM (normal growth medium) plate. These worms were transferred into 200 µL of 15 °C, 20 °C, 25 °C, or 30 °C prewarmed M9 buffer on a depression slide to count their thrashes for 3 min. A thrash is defined as a change in the direction with the body bending in the middle.

Pharmacological Assays. Resistance of wild-type and mutant worms to aldicarb was performed as described (14). Briefly, young adult animals were placed on plates containing 0.5 mM aldicarb and assayed for paralysis at 30-min or 60-min intervals over a 3-h time-period indicated by a lack of response upon poking with a metal wire. To test for sensitivity to levamisole, 30 young adult hermaphrodites were placed on levamisole plates (60 µM), and their movement was checked by poking. lev-1(e2117) was used as a control of aldicarb and levamisole resistance. The number of paralyzed worms was counted at different time-points following exposure to aldicarb or levamisole, and the data were analyzed as the percent of animals that were paralyzed at each time-point.

Electron Microscopy. Fixations were performed using a high-pressure freezing apparatus followed by substitution of solvent and fixative at −90 °C. For animals raised at the permissive temperature in experiment #1 (Supporting information SI Table S2), broods of wild-type and chc-1(b1025s) worms were maintained at 15 °C and were exposed to ambient temperature for less than 2 min; in experiment #2, mutant worms were exposed to ambient temperature for less than 10 min. Note that 10-min exposure to room temperature was sufficient to change the diameter of synaptic vesicles. For animals raised at the nonpermissive temperature, animals in experiment #1 were raised at 15 °C and incubated at 30 °C for 2 h before freezing; animals in experiment #2 were raised at 15 °C and incubated at 25 °C for 24 h before freezing. Fixations were performed for the most part as described previously (15). Briefly, 10 animals were placed into a freeze chamber (a 100-µm well of a type A specimen carrier) containing space-filling bacteria, covered with a type B specimen carrier flat side down, and frozen instantaneously in the BAL-TEC HPM 010 (BAL-TEC). Then, the frozen animals were fixed in a Leica EM AFS system with 0.5% glutaraldehyde and 0.1% tannic acid in anhydrous acetone for 4 h well of a type A specimen carrier flat side down, and frozen instantaneously in the BAL-TEC HPM 010 (BAL-TEC). Then, the frozen animals were fixed in a Leica EM AFS system with 0.5% glutaraldehyde and 0.1% tannic acid in anhydrous acetone for 4 days at −90 °C, followed by 2% osmium tetroxide in anhydrous acetone for 39 h with gradual temperature increase (held at constant temperature at −90 °C for 7 h, increased by 5 °C/h to −25 °C over 13 h, held at constant temperature at −25 °C for 16 h, and raised by 10 °C/h to 4 °C over 2.9 h). The fixed animals were embedded in araldite resin following the infiltration series (30% araldite/acetoine for 4 h, 70% araldite/acetoine for 5 h, 90% araldite/acetoine overnight, and pure araldite for 8 h). Mutant and control blocks were blinded. Ribbons of ultrathin (33 nm) serial sections were collected using an Ultracut E microtome (Leica). Images were obtained on a Hitachi H-7100 electron microscope using a Gatan slow-scan digital camera (Gatan). Two hundred fifty ultrathin (33 nm) contiguous sections were cut, and the ventral nerve cord was reconstructed from 2 animals representing each genotype. Image analysis was performed using Image J software (National Institutes of Health). 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 and the diameter of each was measured from the cholinergic neurons VA and VB and the GABA neuron VD. Genotypes were unblinded only after completion of the morphometric analysis.

Additional Electrophysiology Methods. To warm the stage, a method commonly used by mammalian cell biologists to maintain cells at elevated temperatures was used (16). Briefly, warm air from a hair dryer was directed through heating duct to the underside of the microscope stage. The distance of the end of the duct from the stage controls the stage temperature. This method of heating the stage did not introduce electrical or mechanical contamination and was stable for the duration of the recording. Bath temperature was monitored by a thermostor probe inserted in the bath and read out on a Warner TC-324B temperature display.

Two-Hybrid Assay. The DupLEX-A 2-hybrid system (OriGene Technologies, Inc.) was used according to manufacturer’s instructions. A cDNA fragment corresponding to the C-terminal hub region of CHC-1 (residues 1075–1680) or CHC-1(b1025) was cloned into pEG202 (the bait vector) and pJG4–5 (the prey vector). These plasmids were introduced into reporter strains EGY48 or EGY194 included with the system. To assess the expression of the LEU2 reporter, transformants were grown on plates lacking leucine, histidine, tryptophan, and uracil and containing 2% galactose/1% raffinose at 30 °C or 37 °C for 3 days. The amount of the expressed protein in each yeast cell lysate (30 µg) was estimated by Western blotting using rat anti-HA monoclonal antibody 3F10 (Roche Applied Science).

Clathrin is essential for embryogenesis and development. After incubation at 25°C for 24 h or 48 h, rme-3/chc-1 (b1025ts) animals accumulated yolk in the body cavity (arrowheads, B, C). In addition, embryonic debris was often found in the uterus (arrow, D). (Scale bars, 10 μm.) (E) Progeny number in wild type (N2) and chc-1 mutants. L4 larvae were incubated at 15 °C and 25 °C, and the number of larvae and dead embryos in the next generation were counted. rme-3(b1025ts) produced progeny at 15 °C but not at 25 °C. Even at the permissive temperature of 15 °C, chc-1(b1025ts) mutant worms (n = 4) produced a reduced number of embryos (108 ± 23 embryos) compared with the wild-type (n = 4, 297 ± 56 embryos). chc-1(b1025ts) hermaphrodites (n = 4) transferred from 15 °C to 25 °C during the L4 stage progressed to adulthood but accumulated yolk in the body cavity and produced only dead embryos (16.5 ± 9 dead embryos/adult).

Eighty-eight percent of chc-1(b1025ts) embryos raised at 15°C hatched and displayed normal development. However, if freshly laid chc-1(b1025ts) embryos (n = 119) were shifted to 25 °C, none hatched. When chc-1(b1025ts) L1 larvae (n = 66) were shifted to 25 °C, 85% were arrested during the early larval stages, and only 12% progressed to adulthood. Adults became sluggish within 10 min after transfer to 30 °C. Adults maintained at 25 °C died within 3 to 4 days. These data suggest that CHC is required for the functions of differentiated cells in addition to being a requirement during development. (F–H) The effects of RNAi-based gene knockdown of chc-1 and clic-1 on yolk uptake by oocytes. YP170-GFP uptake by oocytes was examined in (F) a control, (G) chc-1 (RNAi), and (H) clic-1 (RNAi) worms at 20 °C. Scale bar represents 30 μm. (I) The phenotypic effects of clic-1 (RNAi) in wild-type or chc-1(b1025ts) mutant backgrounds were assayed at 15 °C. L4 larva of wild type (WT, n = 2) and chc-1(b1025ts) worms (n = 4) were placed on RNAi plates and the phenotype of F1 progeny was scored. RNAi of clic-1 caused fully penetrant lethality in the chc-1(b1025ts) strain (total 339 dead embryos, 2 L1 arrested larva, no adults from 4 worms).
Subcellular localization of GFP-CHC-1. (A–C) GFP-CHC-1 is localized to the plasma membrane and punctate structures in oocytes (A, B). As the most mature oocyte began to ovulate, GFP-CHC-1 lost its punctate localization and appeared diffuse (arrow). (C) Nomarski image. (D–F) Gonads expressing GFP-CHC-1 or GFP-CHC-1(b1025) were dissected and observed by wide-field deconvolution fluorescence microscopy. GFP-CHC-1, and to a lesser degree GFP-CHC-1(b1025), were localized to the plasma membrane when expressed in a wild-type strain (D, E). When GFP-CHC-1(b1025) was expressed in chc-1(b1025ts) mutants, where it cannot form mixed oligomers with wild-type CHC-1, it lacked most plasma membrane localization (F). It is not clear if the residual punctate appearance of the mutant protein reflects association with internal membranes or some kind of protein aggregation. (Scale bars, 10 μm.) (G–M) GFP-CHC-1(b1025) is unstable at high temperature. (G–I) GFP-CHC-1 (G, J) or GFP-CHC-1(b1025) (H, I, K, L) were expressed in the intestine under the control of the vha-6 promoter. L4 larvae were incubated at 15°C (G–I) or 25°C (J–L) for 24 h and observed by confocal laser scanning microscopy. The images in G, H, J, and K were taken using the same conditions. I and L are overexposed images of H and K, respectively. (Scale bars, 10 μm.) GFP-CHC-1 was localized predominantly to the apical membrane and punctate structures in the cytoplasm at 15°C and 25°C (G, J). On the other hand, the expression level of GFP-CHC-1(b1025) was quite low even at the permissive temperature (H, I) and was reduced further at the restrictive temperature (K, L). (M) GFP-CHC-1(b1025) is unstable at restrictive temperature. Worms expressing GFP-CHC-1 or GFP-CHC-1(b1025) in the intestine were incubated at 15°C or 25°C for 24 h or at 30°C for 10 min or 120 min and subjected to Western blotting. GFP fusions and RME-1 were detected using anti-GFP antibody and anti-RME-1 antibody, respectively. The level of GFP-CHC-1(b1025) was reduced in a temperature-dependent manner compared with GFP-CHC-1.
**Fig. S3.** Interaction of CHC-1(b1025) hub domains is impaired at high temperature. (A) A yeast 2-hybrid assay between the C-terminal hub domains of CHC-1 or CHC-1(b1025). The C-terminal hub region of CHC-1 (residues 1075–1680) or CHC-1(b1025) was expressed in a yeast reporter strain as fusions with the transcriptional activation domain of B42 (prey) or the DNA binding domain of LexA (bait). Interaction between bait and prey was tested at 30 °C (permissive temperature) and 37°C (restrictive temperature) using β-galactosidase reporter assays. The wild-type CHC-1 hub domains interacted with each other at 30 °C and 37 °C. However, the hub domains of CHC-1(b1025) showed reduced interaction compared with wild type at 30 °C and was reduced further at 37 °C. (B) The amount of the expressed protein in each yeast cell was estimated by Western blotting using an anti-HA antibody. The expression level of the CHC-1(b1025) mutant hub domains was nearly identical to that of the wild-type CHC-1 hubs.
Fig. S4. (A–D) Neuronal expression of GFP-CHC-1(b1025) mutant protein does not inhibit neuromuscular function. GFP-CHC-1 (A, A'/B, B'/C) or GFP-CHC-1(b1025) (C, C', D, D') were expressed in the chc-1(b1025) mutant under the control of the pan-neuronal rgef-1 promoter. GFP-CHC-1 was expressed at a high level in neurons under these conditions, but GFP-CHC-1(b1025) was not. L4 larvae were incubated at 15 °C (A, C) or 25 °C (B, D) for 24 h and observed by confocal laser scanning microscopy. VNC, ventral nerve cords; DNC, dorsal nerve cords. (Scale bar, 10 μm.) (E) Thrashing assays. L4 hermaphrodites of wild-type or chc-1(b1025) mutant animals expressing GFP-CHC-1 or GFP-CHC-1(b1025) under the control of the neuron-specific rgef-1 promoter were incubated at 25 °C for 24 h and then transferred to M9 buffer, and the number of head thrashes was counted. Note that overexpression of GFP-CHC-1 in neurons slightly affected thrashing ability. Expression of GFP-CHC-1(b1025) in neurons did not affect thrashing activity in wild-type animals.
Subcellular localization of α-adaptin in neurons. Wild-type and chc-1(b1025ts) animals expressing GFP-SNB-1 in the GABA neurons were stained with anti-GFP and anti-α-adaptin antibodies. α-Adaptin localizes to punctate structures on neurons both in the wild-type and chc-1(b1025ts) animals. (Scale bars, 10 μm.)

Subcellular localization of GFP-SNB-1 is normal in chc-1(b1025ts) neurons. Wild-type and chc-1(b1025ts) animals expressing GFP-SNB-1 (synaptobrevin) in GABA neurons were incubated at 15 °C for 24 h, 25 °C for 24 h, or 30 °C for 2 h and observed by fluorescence microscopy. (Scale bars, 10 μm.)

Subcellular localization of UNC-46 and UNC-47 is not affected in chc-1(b1025ts) neurons. Wild-type and chc-1(b1025ts) animals expressing GFP-UNC-46 (GABA transporter sorting factor) or GFP-UNC-47 (vesicular GABA transporter) in GABA neurons were incubated at 15 °C for 24 h or 25 °C for 24 h and observed by fluorescence microscopy. (Scale bars, 10 μm.)
Subcellular localization of synaptic proteins is unchanged in chc-1(b1025ts) animals. Transgenic worms expressing the synaptic vesicle protein GFP-SNB-1 in GABA neurons were stained with anti-GFP and anti-RAB-3, anti-SNT-1 (synaptotagmin), anti-UNC-10 (active zone protein), or anti-UNC-64 (syntaxin) antibodies. The localization of these synaptic proteins was largely unchanged in chc-1(b1025ts) mutants even at restrictive temperature. (Scale bars, 10 μm.)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Wild-type 15°C</th>
<th>Wild-type 30°C</th>
<th>chc-1(b1025) 15°C</th>
<th>chc-1(b1025) 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNB-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAB-3</td>
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</tr>
<tr>
<td>SNB-1</td>
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<tr>
<td>SNT-1</td>
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<tr>
<td>SNB-1</td>
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<td>UNC-64</td>
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</tbody>
</table>

Fig. S6. Subcellular localization of synaptic proteins is unchanged in chc-1(b1025ts) animals. Transgenic worms expressing the synaptic vesicle protein GFP-SNB-1 in GABA neurons were stained with anti-GFP and anti-RAB-3, anti-SNT-1 (synaptotagmin), anti-UNC-10 (active zone protein), or anti-UNC-64 (syntaxin) antibodies. The localization of these synaptic proteins was largely unchanged in chc-1(b1025ts) mutants even at restrictive temperature. (Scale bars, 10 μm.)
Fig. S7. Representative images of neuromuscular junctions. (Left) Wild-type adult hermaphrodites at 30°C. (Right) chc-1(b1025ts) adult hermaphrodites at 30°C.
Movie S1. Thrashing of wild-type and chc-1(b1025ts) worms. L4 larvae of wild-type (Movie S1) and chc-1(b1025ts) (Movie S2) worms were incubated at 25 °C for 24 h and transferred into a pre-warmed M9 buffer. Movies show worm behavior between 15 sec and 35 sec after transfer to buffer.

Movie S1 (MOV)
Movie S2.
Table S1. Pharyngeal pumping assay: pharyngeal pumps/min
\((n = 8)\)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>15°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>290 ± 7</td>
<td>313 ± 9</td>
</tr>
<tr>
<td>chc-1(b1024)</td>
<td>237 ± 8</td>
<td>227 ± 30</td>
</tr>
<tr>
<td>chc-1(b1025ts)</td>
<td>243 ± 12</td>
<td>166 ± 24</td>
</tr>
<tr>
<td>chc-1(b1025ts) GFP-CHC-1</td>
<td>N.T.</td>
<td>302 ± 12</td>
</tr>
</tbody>
</table>

Although the pharynx pumps at basal rates in the absence of neuronal input, pharyngeal muscle contraction is greatly stimulated by cholinergic motor neurons. Wild-type animals pump rapidly in the presence of food. The weak mutant chc-1(b1024) exhibited a slight defect in pharyngeal pumping at either temperature. In contrast, the temperature-sensitive mutant chc-1(b1025ts) exhibited a slight defect at 15°C, but pumping was reduced to half the normal rate compared with the wild type when incubated at 25°C for 24 h. The residual ability of the chc-1 mutants in pharyngeal pumping may be caused by clathrin-independent peptidergic secretion or reflect the neuronal input-independent contraction ability of the pharynx. Neuronal expression of GFP-CHC-1 rescued the pharyngeal pumping ability of the chc-1(b1025ts) mutants at 25°C. These data indicate that there is a neuronal defect in the pharynx of clathrin mutants.
Table S2. Clathrin mutants display reduced synaptic vesicle (SV) diameter but normal synaptic vesicle number

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature</th>
<th>SV/ACh Profile</th>
<th>No. n = synapses/no. worms</th>
<th>Mean SV diameter</th>
<th>No. n = SV/GABA Profile</th>
<th>n = No. synapses/no. worms</th>
<th>Mean SV diameter</th>
<th>No. SV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>15°C</td>
<td>24 ± 2</td>
<td>11/2</td>
<td>28.2</td>
<td>788</td>
<td>9/2</td>
<td>27.5</td>
<td>1369</td>
</tr>
<tr>
<td>chc-1(b1025ts)</td>
<td>15°C</td>
<td>24 ± 2</td>
<td>10/2</td>
<td>27.9</td>
<td>841</td>
<td>9/2</td>
<td>27.7</td>
<td>1170</td>
</tr>
<tr>
<td>Wild type</td>
<td>30°C</td>
<td>25 ± 3</td>
<td>8/2</td>
<td>27.6</td>
<td>444</td>
<td>8/2</td>
<td>27.5</td>
<td>718</td>
</tr>
<tr>
<td>chc-1(b1025ts)</td>
<td>30°C</td>
<td>22 ± 3</td>
<td>9/2</td>
<td>23.4</td>
<td>641</td>
<td>8/2</td>
<td>24.1</td>
<td>730</td>
</tr>
<tr>
<td>Wild type</td>
<td>15°C</td>
<td>22 ± 2</td>
<td>5/1</td>
<td>29.3</td>
<td>475</td>
<td>4/1</td>
<td>28.9</td>
<td>381</td>
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<tr>
<td>chc-1(b1025ts)</td>
<td>15°C</td>
<td>16 ± 2</td>
<td>5/1</td>
<td>24.1</td>
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<tr>
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<td>25 ± 1</td>
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<td>27.7</td>
<td>367</td>
<td>4/1</td>
<td>27.5</td>
<td>397</td>
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<tr>
<td>chc-1(b1025ts)</td>
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<td>16 ± 1</td>
<td>4/1</td>
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<td>301</td>
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<tr>
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<td>25°C</td>
<td>16 ± 1</td>
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<td>chc-1(b1025ts)</td>
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<td>16 ± 1</td>
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<td>24.0</td>
<td>224</td>
<td>4/1</td>
<td>24.7</td>
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</table>

For synaptic vesicle numbers per synaptic profile, the p value was examined by Student’s t-test where n = synapses examined in experiment #1: (ACh: N2 15°C vs. chc-1(b1025ts) 15°C, P = 0.92; N2 15°C vs. N2 30°C, P = 0.80; N2 30°C vs. chc-1(b1025ts) 30°C, P = 0.46; chc-1(b1025ts) 15°C vs. chc-1(b1025ts) 30°C, P = 0.45); (GABA: N2 15°C vs. chc-1(b1025ts) 15°C, P = 0.44; N2 15°C vs. N2 30°C, P = 0.91; N2 30°C vs. chc-1(b1025ts) 30°C, P = 0.52; chc-1(b1025ts) 15°C vs. chc-1(b1025ts) 30°C, P = 0.18). In experiment #2 there is a visible decrease in the synaptic vesicle number in chc-1(b1025ts) strains vs. the wild type. However, when combined with the experiment #1 data set, there is not a statistically significant difference between the genotypes: (ACh: N2 permissive vs. chc-1(b1025ts) permissive, P = 0.49; N2 restrictive vs. chc-1(b1025ts) restrictive, P = 0.97); (GABA: N2 permissive vs. chc-1(b1025ts) permissive, P = 0.95; N2 restrictive vs. chc-1(b1025ts) restrictive, P = 0.21). For synaptic vesicle diameters, the p value was examined by Student’s t-test where n = mean diameter of vesicles in a profile: (N2, 15°C vs. chc-1(b1025ts), 15°C, P = 0.99; N2, 15°C vs. N2, 30°C, P = 0.31; N2, 30°C vs. chc-1(b1025ts), 30°C, P < 0.0001; chc-1(b1025ts), 15°C vs. chc-1(b1025ts), 30°C, P < 0.0001).