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

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Abstract

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 CHC 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 (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. 2 A–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.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0809541106/DCSupplemental.

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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 (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. 2 I). Transgenic expression of GFP-tagged CHC-1(b1025ts) rescued 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 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).

**Fig. 1.** rme-3 encodes *C. elegans* CHC. (A–D) YP170-GFP endocytosis by oocytes of adult hermaphrodites. (A) Wild type, YP170-GFP is taken efficiently up by oocytes (Ooc) 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.

**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 (×4) of boxed regions in the middle planes (Right) are shown. (Scale bar, 10 μm.) (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).

**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 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. 3 A–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).
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 Ca2+-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. (F) 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 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 postsynaptic 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 added temperature challenge the wild-type animals, vesicle turnover is accelerated at higher temperatures (21).

![Figure 4](https://www.pnas.org/cgi/doi/10.1073/pnas.0809541106)

**Fig. 4.** The number of synaptic vesicles at chc-1(b1025ts) synapses. (A) Images of ACh neuromuscular junctions of wild-type (WT) or chc-1(b1025ts) mutant adults raised at 15°C or raised at 15°C and shifted to 30°C for 2 h. Arrowheads, small vesicles; arrow, dense core vesicle (DCV); open arrow, large vesicle (LV); mt, mitochondria. (B–C) Analysis of synaptic vesicles (SV) per section of a neuromuscular junction. (B) Shifting adult worms to 30°C for 2 h does not affect the number of vesicles in either wild-type or in chc-1(b1025ts) worms. (C) The number of synaptic vesicles in wild-type and chc-1(b1025ts) adult worms after shifting to 25°C for 24 h. (D) The number of synaptic vesicles in wild-type and chc-1(b1025ts) adults after shifting the temperature from 15°C to 30°C for 2 h.
mediated inhibition of release could be overcome by a constitutively active stimulatory G protein, we introduced the egl-30(tg26) gain-of-function mutation Gq into the clathrin mutant background. This mutant form of Gq 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 Gq 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 fluorescent-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 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. 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 is enough to allow vesicle recycling to occur. In contrast, low levels of clathrin function may not be sufficient to maintain synaptic vesicle pools. It is possible that clathrin activity is required 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

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-

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 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 mini frequency and amplitude are statistically indistinguishable from wild type under the control of a neuron-specific promoter also were examined. These animals were checked in our study we did not observe any significant defect in synaptic vesicle biogenesis in CHC mutants. Perhaps the residual clathrin activity is enough to allow vesicle recycling to occur. In contrast, low levels of clathrin function may not be sufficient to maintain synaptic vesicle pools. It is possible that clathrin activity is required 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

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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 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 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 neurones cultured in vitro 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-Analyses (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 °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).

ACKNOWLEDGMENTS. We thank Y. Jin (University of California, San Diego), M. Zhen (Mount Sinai Hospital), M. Nonet (Washington University), and K. Schuske (University of Utah) for reagents; R. Y. Tsien (University of California, San Diego) for mRFP plasmids; and the Caenorhabditis Genetics Center for strains. We thank J. L. Bessereau (INSERM, France) for high-pressure freezing samples during the start of this project. We thank A. Harada and Y. Kidokoro (Gunma University, Japan) for support and discussion. This work was supported by National Institutes of Health Grants GM067237 (to B.D.G.) and NS034307 (to E.M.J.). E.M.J. is a Howard Hughes Investigator. This research was supported by the Ministry of Education, Sports, Science, and Technology Japan (MEXT) Grant-in-Aid for Scientific Research on Priority Areas 2007 (Protein Community); by Scientific Research (C), 2008, and by Bioarchitect Research Projects II of RIKEN (K.S.). This work also was supported by grants-in-aid and by the Global Center of Excellence Program from the Japanese MEXT (M.S. and K.S.).