Synapse-Assembly Proteins Maintain Synaptic Vesicle Cluster Stability and Regulate Synaptic Vesicle Transport in *Caenorhabditis elegans*

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ABSTRACT The functional integrity of neurons requires the bidirectional active transport of synaptic vesicles (SVs) in axons. The kinesin motor KIF1A transports SVs from somas to stable SV clusters at synapses, while dynein moves them in the opposite direction. However, it is unclear how SV transport is regulated and how SVs at clusters interact with motor proteins. We addressed these questions by isolating a rare temperature-sensitive allele of *Caenorhabditis elegans* *unc-104* (KIF1A) that allowed us to manipulate SV levels in axons and dendrites. Growth at 20° and 14° resulted in locomotion rates that were ~3 and 50% of wild type, respectively, with similar effects on axonal SV levels. Corresponding with the loss of SVs from axons, mutants grown at 14° and 20° showed a 10- and 24-fold dynein-dependent accumulation of SVs in their dendrites. Mutants grown at 14° and switched to 25° showed an abrupt irreversible 50% decrease in locomotion and a 50% loss of SVs from the synaptic region 12-hr post-shift, with no further decreases at later time points, suggesting that the remaining clustered SVs are stable and resistant to retrograde removal by dynein. The data further showed that the synapse-assembly proteins *SYD-1*, *SYD-2*, and *SAD-1* protected SV clusters from degradation by motor proteins. In *syd-1*, *syd-2*, and *sad-1* mutants, SVs accumulate in an *UNC-104*-dependent manner in the distal axon region that normally lacks SVs. In addition to their roles in SV cluster stability, all three proteins also regulate SV transport.

KEYWORDS *Caenorhabditis elegans*; KIF1A; axonal transport; Cdk5; liprin; SAD-A; dynein

NEURONS have a unique requirement to transport synaptic vesicles (SVs) long distances along microtubule tracks in axons to establish synapses for the regulated secretion of neurotransmitters. Microtubules have an intrinsic plus- and minus-end polarity. Axonal microtubules are oriented with their plus-ends pointing toward the synaptic region (Burton and Paige 1981; Heidemann *et al.* 1981; Baas and Lin 2011). The plus-end-directed kinesin motor KIF1A carries SVs outward to the stable SV clusters at synapses (Hall and Hedgecock 1991; Okada *et al.* 1995; Pack-Chung *et al.* 2007). However, for reasons not immediately clear, the transport is bidirectional, alternating between minus-end-directed retrograde transport and plus-end-directed anterograde transport, with plus-end transport ultimately dominating to achieve SV accumulation at synapses (Miller *et al.* 2005; Wagner *et al.*

2009; Kumar *et al.* 2010; Ou *et al.* 2010). Dynein mediates the minus-end-directed transport of SVs (Koushika *et al.* 2004; Ou *et al.* 2010). Neurons also use KIF1A to transport neuropeptide-carrying dense core vesicles (DCVs) from the cell soma to the synaptic region (Jacob and Kaplan 2003; Pack-Chung *et al.* 2007), again in a bidirectional manner in concert with dynein (Barkus *et al.* 2008; Goodwin *et al.* 2012).

Although null alleles of KIF1A are lethal, forward genetic studies in *Drosophila* and *Caenorhabditis elegans* have generated many useful reduction-of-function alleles for investigating the consequences of impaired SV and DCV transport and/or for studying the functions of KIF1A's various domains (Hall and Hedgecock 1991; Pack-Chung *et al.* 2007; Barkus *et al.* 2008; Kumar *et al.* 2010; Maeder *et al.* 2014). Recent *C. elegans* studies have also identified rare gain-of-function *unc-104* (KIF1A) alleles (Wu *et al.* 2013; Zheng *et al.* 2014). However, despite the wealth of KIF1A alleles, the genetic toolkit for investigating SV and DCV transport would benefit from a conditional KIF1A allele, which would allow transport to be acutely impaired in adult animals after synapses have developed. One major question such an allele could

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address is what happens when synapses are allowed to develop and then anterograde transport is acutely impaired? Do all SVs go back to the soma by retrograde transport or are SV clusters stably maintained at synaptic sites? Are DCVs, which are not strongly clustered at synaptic sites (Hammarlund *et al.* 2008), affected in the same way as SVs?

Additionally, one of the most important goals in the synaptic vesicle transport field is to determine how the activity of KIF1A is regulated. The finding that null alleles of *C. elegans unc-104* (KIF1A) are lethal and essentially have a “nervous system null” phenotype (Hall and Hedgecock 1991) showed that no other motor protein can substitute for KIF1A. It also suggested that the regulation of KIF1A activity, along with external cues, provides the basis for synaptogenesis. Identifying regulators of KIF1A, which should have synaptic vesicle distribution phenotypes that resemble *unc-104* reduction-of-function mutants, is thus critical for understanding how synapses develop. Surprisingly, the first identified regulator of KIF1A-driven SV transport was *SYD-2* (liprin- α) (Shin *et al.* 2003; Miller *et al.* 2005; Wagner *et al.* 2009). *SYD-2* also regulates KIF1A-driven DCV transport (Goodwin and Juo 2013). Originally identified in a genetic screen for mutations causing diffuse localization of a synaptic vesicle protein at synapses (Zhen and Jin 1999), *SYD-2* has a critical function in regulating the assembly of presynapses in some, but not all, neurons (Dai *et al.* 2006; Patel *et al.* 2006). It is also a structural component of the dense projection at the active zone of the synapse, where it contributes to the clustering of SVs (Zhen and Jin 1999; Stigloher *et al.* 2011; Kittelmann *et al.* 2013). *SYD-2* does not require *UNC-104* to accumulate at synapses (Patel *et al.* 2006), and most SVs are not cotransported with *SYD-2* (Wu *et al.* 2013), suggesting that it may exert its SV transport regulatory effects before SVs enter the axon or act on selected populations during transport.

It is unclear whether the dual functions of *SYD-2* represent a special case or whether other synapse-assembly proteins also regulate SV and DCV transport. In the current study we used a forward genetic screen to discover a conditional mutation in the *UNC-104* motor domain that, for the first time, allows the use of temperature to alter plus-end-directed KIF1A motor activity and thus control SV and DCV levels in axons. We used growth and behavioral analyses, quantitative imaging of neuron-wide vesicle distributions, and time-lapse analyses of vesicles in living animals to investigate how temperature affects growth, locomotion, and vesicle transport in the mutant. These studies led us to discover a major difference between SV and DCV transport with respect to their entry into dendrites when *UNC-104* activity is reduced. The mutant also contributed to our discovery that multiple synapse-assembly proteins, and not just *SYD-2*, have dual roles in maintaining synaptic vesicle cluster stability and in regulating SV transport. An accompanying article shows that these same synapse-assembly proteins have an even broader role in regulating the active transport of cell soma organelles in neurons (Edwards *et al.* 2015).

Materials and Methods

Worm culture and strains

Worm culture and manipulation essentially followed previously described methods (Stiernagle 2006; Brenner 1974). Briefly, culture media was modified NGM (referred to as NGM-LOB) (Hoover *et al.* 2014). Prior studies define the culture plate types “spread plates,” “streak plates,” and “locomotion plates” (Miller *et al.* 1999; Edwards *et al.* 2008). We produced “spot plates” by dropping 6 μ l of OP-50 culture onto an unseeded plate and growing 32 hr at 20°. To produce 96-well solid-media culture plates we prepared NGM-LOB media supplemented with three times the normal amount of peptone and 1g/liter of yeast extract. We used a WellMate Microplate Dispenser (Matrix) to dispense 375 μ l of media to each well using standard bore tubing. Water for prewarming and priming the input lines and the molten media were kept in a 73° water bath while dispensing to prevent media solidification in the lines. After solidification of the wells we dispensed 10 μ l of OP-50 bacteria culture to each well using small-bore tubing hooked to the WellMate Dispenser. We then dried the plates with their lids off for 1 hr 15 min in a 37° room before putting their lids back on, repacking and sealing them in their original packaging, and incubating them at 37° for 64 hr. At the end of this time, we unpackaged the plates, removed the condensation from each lid with a Kimwipe, repackaged them, and stored them at 4° for up to 1 month before use. [Supporting Information, File S1](#) lists the genotypes and molecular lesions of all strains in this study.

INS-22-Venus forward genetic screen

We mutagenized 9000 L4's of the strain KG4247 *ceIs201* (*unc-17::INS-22-Venus*, *unc-17::mCherry*, *unc-17::ssmCherry*) (Hoover *et al.* 2014) with 27.6 mM EMS in M9 supplemented with OP-50 bacteria for 4 hr at 20°. After growing this P₀ generation 72 hr at 14° on 2 spread plates, we rinsed all adults and larvae from the plates and allowed the F1 generation eggs to hatch overnight at 20°. We collected F1 hatchlings, counted them, plated 3000 on each of six spread plates, and grew them for 3 days at 20°. After rinsing all adults and larvae from these plates with sterile M9 buffer, we scraped the eggs off using a glass pipet and sterile M9, transferred them to a 15-ml conical, and centrifuged for 2 min at 1500 RPM in a clinical centrifuge (Dynac). We then washed the pellet with 12 ml of M9 before recentrifuging and removing the supernatant to 200 μ l. We plated the egg suspension on unseeded NGM-LOB culture plates, incubated them overnight at 20° to allow the F2 eggs to hatch, and then collected F2 hatchlings, counted them, and plated 5000 on each of 18 spread plates. We grew half of these plates for 32 hr at 20° + 16 hr at 14° and half for 8 hr at 20° + 64 hr at 14° so that screening could be done on 2 successive days. These growth times produced mid-L4 stage synchronous F2 grandprogeny. For each half day of screening, we harvested animals from three of these plates using

5 ml of sterile PBS per plate and adding the suspension to 8 ml of sterile PBS stirring slowly in a 50 ml beaker. We counted 12 50- μ l aliquots of this suspension, obtained an average, and adjusted the suspension volume to 24 worms/50 μ l. At 20 min intervals, we pipetted 50 μ l (~24 worms) from the stirring suspension into each of the 12 center wells of a 96-well Mat-Tek glass bottom plate (MatTek Corporation, Ashland, MA; P96G-1.5-F-F) that had been preloaded with 50 μ l of 300 μ M Levamisole per well. Ten minutes after loading the first well we screened the animals in the 12 wells for 20 min using a 0.75 N.A. 20 $\times$ dry objective on a Nikon TE-2000E inverted microscope with a universal stage containing an inset sized for 96-well plates. In each half day of screening, over 2 successive days per week, we screened 9 such rows of 12 wells. We screened animals in each well for decreased fluorescence in the dorsal cord and/or increased fluorescence in cell somas. At the end of each 20-min screening session, after noting wells containing such mutants, we pipetted the contents of each mutant-bearing well onto a predried streak plate using a Pasteur pipet, rinsed the well with 100 μ l of M9, and then, immediately after the liquid dried in, clonally distributed up to 24 animals to a 96-well solid media culture plate containing a thick layer of OP-50 bacteria (see *Worm culture and strains*). Depending on the relative size of the mutant animal, we often plated fewer animals that were outside the relative size range observed for the mutant. After 3 days at 20 $^{\circ}$ + 1 day at 14 $^{\circ}$, we used a sterile toothpick to pick about six L4 stage animals from each well to Mat-Tek plate wells containing 150 μ M Levamisole. After screening on the TE-2000E microscope, we noted wells with 100% mutant phenotype and used the corresponding well on the 96-well plate to score behavioral and other phenotypes and to set up stocks. We screened 31,104 F2 animals for a calculated 4.8-fold genomic coverage, taking into account an experimentally determined 23% loss of animals during recovery of the mutant.

Isolation of other mutants used in this study

We isolated *sad-1(ce749)* and *syd-2(ce759)* in an unrelated forward genetic screen (Edwards *et al.* 2015). We isolated *unc-104(ce515)* in an unrelated forward genetic screen (K. G. Miller, unpublished results).

Mapping, identification, and outcrossing of new mutations

We tested *ce782* and *ce515* for allelism with *unc-104(e1265)* using a complementation test by crossing *ce782/+* or *ce515/+* males to *unc-104(e1265)* hermaphrodites and scoring male cross progeny for the *Unc-104* growth and locomotion phenotypes. Edwards *et al.* (2015) describes the mapping and whole-genome sequencing methods that we used to identify *sad-1(ce749)* and *syd-2(ce759)*. We outcrossed the strains bearing the *ce515*, *ce749*, *ce759*, *ce782*, and *ok217* mutations one, two, two, two, and three times to N2, respectively, before using them for the experiments in

this article. File S1 lists the methods used for genotyping and verification of all of the mutations we used in strain constructions.

Plasmids

File S1 lists all of the DNA constructs used in this study along with their sources and/or construction details. In all constructs involving the cloning of PCR fragments, we sequenced the inserts and used clones containing no mutations in the fragment of interest to make the final plasmid stock.

Production of transgenes and genomic insertions

We prepared plasmids for micro-injection using the Qiagen Tip-20 system according to the manufacturer's instructions, except that we added a 0.1 M potassium acetate/2 vol ethanol precipitation step after resuspending the isopropanol-precipitated pellet. We produced transgenic strains bearing extrachromosomal arrays by the method of Mello *et al.* (1991). For the *syd-2* rescue experiment, the host was KG4526 *syd-2(ok217)*; *wyls85*. For all other injection experiments, N2 was the host. We used pBluescript carrier DNA to bring the final concentration of DNA in each injection mixture to 175 ng/ μ l and integrated transgenes into the genome as described (Reynolds *et al.* 2005) using 9100 rad of gamma rays. File S1 lists all of the transgenic arrays in this study, their DNA contents, and the injection concentration of each DNA. We mapped the insertion sites of *ceIs255* and *ceIs264* by crossing the integrant through CB4856, reisolating and cloning homozygous insertion animals in the F2 generation, and using the resulting mapping lines to map the integration sites relative to SNPs as described (Schade *et al.* 2005).

CRISPR homologous recombination

To reproduce the G105E mutation via homologous recombination, we used the CRISPR oligo-templated *dpy-10(cn64)* co-conversion method (Arribere *et al.* 2014). We cloned the Cas9 target sequence GAAATCATATACAATGAT into the pRB1017 gRNA expression cassette as described (Arribere *et al.* 2014) to make the *unc-104* gRNA plasmid. The mutation changed the middle base of the NGG motif from G to A. The *unc-104(ce813)* oligo-template included 50 bases on each side of this mutation for 101 bases total. The injection mixture was pDD162 (Cas9 plasmid; 50 ng/ μ l) (Dickinson *et al.* 2013), pJA58 (*dpy-10* gRNA plasmid; 25 ng/ μ l) (Arribere *et al.* 2014), KG#784 (*unc-104* gRNA plasmid; 25 ng/ μ l), and the *dpy-10(cn64)* and *unc-104(ce813)* oligo-templates (500 nM each). We injected 40 *ceIs263* [unc-129::GFP-RAB-3] animals with this mixture and cloned 67 F1 rollers. Four of the lines (6%) yielded *unc-104(ce813) dpy-10(cn64)* progeny. Note that because *unc-104* and *dpy-10* are tightly linked (0.21 cM), and because co-conversion occurs in the same gamete, all of the converted animals were *unc-104 dpy-10* doubles with a *Dpy-10* phenotype (*cn64/cn64* animals are *Dpy* non-Rol). We therefore separated the two mutations

by crossing this double mutant to *ceIs263* males and screening the F2's for non-Dpy-10 *unc-104* mutants.

C. *elegans* strain constructions

To cross an integrated transgene into a mutant background, we typically crossed a male integrant strain made by the heat-shock method (Sulston and Hodgkin 1988) to the mutant, although in some cases we used integrant/+ heterozygous males. After incubating 3 days at 20°, we cloned five L4-stage cross progeny carrying the fluorescently marked transgene. After 4 days at room temperature, we cloned 12 bright-fluorescent (putatively homozygous for the transgene) mutant adult hermaphrodites and grew them 4 days at room temperature, before choosing one homozygous mutant line that was also homozygous for the transgene to make the stock. To construct strains carrying two mutations plus a transgene, we typically first crossed the insertion into each single mutant, and then used homo- or hemizygous integrant males for the first cross to mutant A, allowing the entire construction to be done in a background that is homozygous for the insertion. We constructed these strains, as well as the double mutants used for the locomotion assays (the latter lacking transgenes), using the standard method of crossing heterozygous males of mutant A with homozygous hermaphrodites of mutant B and cloning virgin F1 cross progeny. From plates segregating mutant A in their F2 progeny, we cloned mutant A and/or mutant B animals and looked for segregation of the double mutant in the next generation using behavioral phenotypes or otherwise genotyping as described in [Supporting Information](#). After making a strain composed of two or more mutations, we confirmed the homozygosity of each mutation using the methods described in [File S1](#) (see *Mutations and Methods Used for Genotyping*).

Quantitative fluorescence imaging and image analysis of dense core vesicle and synaptic vesicle distributions

[File S1](#) provides details of the procedures used for quantitative fluorescence imaging and image analysis, including growth and mounting of strains, image acquisition, processing images, quantifying images, and producing representative images.

Time-lapse video microscopy of dense core vesicle and synaptic vesicle active transport in live animals

[File S1](#) provides details of the procedures used for time-lapse imaging, including growth and mounting of strains, temperature control, image acquisition, processing time-lapse images, quantifying movements from kymographs, pause analysis, and producing time-lapse movies.

Behavioral assays and videos

Previous studies described the basal locomotion rate assays (Miller *et al.* 1999; Reynolds *et al.* 2005). For assaying heterozygotes, L4 hermaphrodite cross progeny were plated at three per plate on 10 locomotion plates at 23.5° and assayed 20–24 hr later as described (Miller *et al.* 1999; Reynolds *et al.* 2005), except each of 10 animals was assayed for

6 min. The basal locomotion rate assays were modified as follows to assay animals at room temperature (defined as 22.0°–23.5°) that had been hatched and grown to young adulthood at different temperatures: we plated 12 L2-stage larvae on each of 12 spread plates (source plates) labeled 1–12 and grew them at the desired temperature until they had produced F1 mature adults. Sixteen hours before starting the assay, we equilibrated 12 locomotion plates (assay plates) to room temperature, wiped any condensation off the underside of the lids, and stored them inverted and unbagged in an incubator set at 23.5° for 16 hr. We then started a countdown timer for 8 min, removed the 1 assay plate from 23.5° and the 1 source plate from the growth incubator and picked a single day 1 gravid adult from the source plate onto the assay plate. We then immediately returned the assay plate to 23.5° and discarded the source plate. When the 8-min timer beeped, we reset it at 8 min and restarted the 8-min countdown, removed the 2 set of plates from each incubator and loaded them as above, and then transferred the 1 assay plate from the 23.5° incubator to a stereomicroscope stage at room temperature, at which point we located the animal on the plate (leaving the lid on the plate) and counted its body bends for 6 min using the standard definition of a body bend (Miller *et al.* 1999; Reynolds *et al.* 2005). When the 8-min timer beeped again, we repeated the above steps until we had loaded and assayed all 12 animals. To assay animals for locomotion after growth at 20° and recovery at 13.5°, we produced 12 source plates by transferring 20 L2-stage *unc-104(ce782)* to each of 12 spread plates and grew them 8 days at 20° to produce a growing culture of F2 adults. We then performed the same locomotion assay as above, returning each plate to the 13.5° incubator for recovery before repeating the assay on the same set of plates after 24 and 48 hr at 13.5°. To assay animals for locomotion after growth at 13.5° + 24 hr at 22.5° + 24 hr at 13.5°, we produced 12 source plates by transferring 20 L2-stage *unc-104(ce782)* to each of 12 spread plates and grew them 14 days at 13.5° to produce a growing culture of F2 adults. We then performed the same locomotion assay as above, repeating the assay on the same set of plates after 24 hr, and then moving plates to 13.5° and assaying them 24 hr later on preequilibrated 22.5° plates. For the time-course locomotion assays we used the same growth and assay methods. We assayed the same set of 12 animals in the same order at each time point. We captured and converted videos of worms on culture plates as described (Schade *et al.* 2005).

Growth assays

To measure the growth time to adulthood and percent larval arrest, we first produced cultures at the desired growth temperature that contained gravid adults. We then picked 28 (N2) or 35 (*ce782*) gravid adults to each of two spot plates and incubated them at the growth temperature for 37 hr (if 13.5°) or 16 hr (all other temps). We then picked ~20 late-stage (three- to fourfold stage) embryos from the plates to each of four locomotion plates (removing and

replacing each plate one at a time from the growth incubator) and incubated them for 2 hr (13.5°), 1 hr (20°), 52 min (23.5°), or 50 min (25°). We then removed unhatched eggs from each plate, redistributing hatchlings between plates such that there were 12 hatchlings on each of three locomotion plates and nine hatchlings on the fourth plate. We then put all four plates at the growth temperature for 96 hr (13.5°), 48 hr (20°), 42.5 hr (23.5°), or 40 hr (25°) before beginning regular observations for young adults. We screened the plates by removing them one at a time from the growth temperature to room temperature, counting and picking off any young adults on the plate, and then returning the plate to the growth temperature. The intervals between observations (after the initial growth periods specified above) were 4 hr (13.5°), 2 hr (20°), 1 hr 45 min (23.5°), or 1 hr 40 min (25°). For temperatures at which the *unc-104* mutants were very slow growing or larval arrested, we checked for young adults every 12 or 24 hr.

Statistical analysis

We performed all statistical comparisons using the unpaired *t*-test, Welch corrected (for comparisons between two selected groups), or ANOVA followed by the Tukey Kramer post-test (for comparisons involving three or more groups) or Fisher's exact test (as indicated) using Graphpad Instat 3 (Graphpad Software, Inc.). The *P*-value cutoff for statistical significance was ≤ 0.05 .

Data availability

Strains and reagents are available upon request. The *unc-104(ce782)* mutant may be obtained from the Caenorhabditis Genetics Center (<http://www.cbs.umn.edu/research/resources/cgc>). File S1 contains Supplemental Materials and Methods and a detailed description of all of the strains, plasmids, and transgenes produced in this study.

Results

In the *UNC-104 (KIF1A)* motor domain mutant *ce782*, DCVs are depleted from axons and accumulate in cell somas

To identify proteins that regulate the distribution of DCVs between cell somas and axons, we expressed the fluorescently tagged neuropeptide *INS-22-Venus* as a genomically integrated transgene in *C. elegans* cholinergic motor neurons and performed a forward genetic screen for mutations that altered its distribution. In a screen of ~31,000 F2 animals (approximately fivefold genomic coverage) we isolated, among other mutants, one mutant, *ce782*, in which DCVs are strongly depleted from axons and concentrated in cell somas (Figure 1, A and B). Genetic mapping and sequencing revealed that *ce782* contains a missense mutation, G105E, in the motor domain of *UNC-104 (KIF1A)*. The fact that G105 is conserved in mammals allowed us to map the *ce782* mutation onto the previously determined crystal structure of the mouse KIF1A motor domain complexed with Mg-ADP

(Kikkawa *et al.* 2001). G105 (mouse G109) is part of a beta bridge and is immediately adjacent to the ADP-interacting residue K106 (mouse K110) (Figure 1, C and D). Nucleotide exchange from ADP to ATP is critically important for both the regulation and motility of kinesin motors (Endow and Barker 2003; Cross 2004; Hirokawa and Takemura 2005; Block 2007; Hirokawa and Noda 2008).

The *UNC-104 (KIF1A)* motor domain mutation confers temperature-sensitive allele-specific effects on locomotion

While working with *unc-104(ce782)*, we noted that its growth and locomotion phenotypes appeared strongly sensitive to the temperature at which the animals were grown. When we measured the time required for it to grow from hatching to adulthood at various temperatures we found that, at the relatively low temperature of 13.5°, 100% of the animals tested reached adulthood in a time that was only 1.24-fold longer than that of wild type (Figure 2A). In contrast, at 25°, only $8 \pm 3\%$ of *ce782* mutants reached adulthood before the experiment was stopped at 13.6 days (mean $\pm$ SEM of four populations of 9–12 animals). Since a previous study found that most *unc-104* null homozygotes arrest their growth as larvae (Hall and Hedgecock 1991), *unc-104(ce782)* mutants grown at 25° appear to be null by this phenotype. However, much of this temperature sensitivity for growth is not unique to the *unc-104(ce782)* allele. The strong loss-of-function alleles *unc-104(e1265)*, which has the missense mutation D1497N that prevents cargo binding (Kumar *et al.* 2010), and *unc-104(ce515)*, which produces a stop codon at Q1418 (this study) also showed significantly more larval arrest at 25° than at 13.5° (Figure 2B). Since 25° is near the maximum temperature at which wild-type *C. elegans* can grow, the temperature effects on *unc-104* loss-of-function mutants may be caused by the stress of growth at higher temperatures combined with the growth-limiting effects of the mutations.

The locomotion rates and postures of *unc-104(ce782)* mutants also varied widely with the growth temperature. When grown from hatching to adulthood at 13.5°, and then transferred to 23.5° plates for 8 min, *ce782* had a locomotion rate that was 39% of wild type and often exhibited near wild-type posture and sinusoidal movement. In contrast, when the growth temperature was 20°, *ce782* mutants moved only at 3.2% of the wild-type rate and were often tightly coiled, similar to *unc-104* loss-of-function mutants (Figure 2, B and C; movies in File S2, File S3, File S4, and File S5). These temperature-sensitive phenotypes are unique to the *ce782* allele: the loss-of-function mutants *unc-104(e1265)* and *unc-104(ce515)* showed no temperature sensitivity for locomotion (Figure 2B) or posture (data not shown).

To determine whether the *ce782* mutation is the sole cause of the temperature-sensitive locomotion phenotype, we used CRISPR oligo-templated homologous recombination to reproduce the *ce782* mutation in a wild-type background. This strain, *unc-104(ce813)*, phenocopied the *ce782* mutant in

Figure 1 In the *unc-104(ce782)* motor domain mutant, DCVs are depleted from axons and accumulate in cell somas. Boxes in top drawing show the dorsal and ventral regions imaged in A and B. (A and B) Representative identically scaled images and quantification of INS-22-Venus neuropeptide cargo in dorsal axons (A) and ventral somas (B) in animals with the indicated genotypes. INS-22-Venus is expressed from the integrated transgene *cels201* in cholinergic motor neurons. Dashed lines outline cell soma boundaries in B. Graph data are means and standard errors from 12–13 animals each. ***, $P < 0.0001$ when compared to wild type. (C) Sequence and secondary structure of mouse KIF1A, as determined by Kikkawa *et al.* (2001), near the *unc-104(ce782)* mutation site. Purple arch, turn; orange wave, alpha helix; tan arrow at G109, beta bridge. Green circles indicate residues interacting with ADP, including K110. The location of the *ce782* mutation (G109 in mouse; G105 in *C. elegans*) is circled red. (D) Location of the *unc-104(ce782)* mutation. Shown is the crystal structure of the motor domain of mouse KIF1A complexed with Mg-ADP as determined by Kikkawa *et al.* (2001). The model was generated from the RCSB Protein Data Bank (PDB ID: 1I55) using RCSB PDB Protein Workshop 4.1.0. The locations of ADP (ball and stick model), Mg²⁺ (green sphere), glycine 109, and lysine 110 are indicated.

its temperature-sensitive effects on locomotion: in assays performed as above, animals grown at 13.5° or 20° had locomotion rates that were 71% $\pm$ 15% and 6.4% $\pm$ 0.74% of the

wild-type control strain, respectively (mean $\pm$ SEM, $N = 12$). We conclude that the G105E mutation causes the temperature-sensitive locomotion phenotype.

Figure 2 The UNC-104 (KIF1A) motor domain mutation confers temperature-sensitive allele-specific effects on locomotion. (A) Temperature sensitivity of the *unc-104(ce782)* mutant revealed by growth rates at various temperatures. Eggs hatched and grown at the indicated temperatures were monitored until they reached adulthood or until the experiment was stopped at 13.6 days. Data are means and SEMs from populations of 9 animals. ***, $P < 0.0001$ when comparing to wild type at the same temperature. (B) Temperature sensitivity of the indicated *unc-104* alleles for larval arrest. Animals hatched at the indicated temperatures were monitored for development to the adult stage for 18 days (13.5°) or 7.5 days (25°). Data are means and SEMs from 3 populations of ≥ 50 animals each for each genotype. ***, $P < 0.0001$ when comparing to the same strain at 13.5°. (C) Effects of growth temperature on locomotion rate for the indicated *unc-104* alleles. After growth at the specified temperatures, day 1 adults were transferred to plates equilibrated at 23.5° and allowed to adjust to the assay temperature for 8 min before quantifying locomotion rate. Data are means and SEMs from populations of 12 animals. ***, $P = 0.0003$ when comparing to the same strain at 13.5°. (D) Temperature sensitivity of the *unc-104(ce782)* mutant revealed by posture and appearance on plates after growth at permissive and restrictive temperatures. See also Movies in File S2, File S3, File S4, and File S5.

The similarity of the *ce782* mutant phenotypes to those of loss-of-function *unc-104* mutants suggests that the *ce782* mutation causes a temperature-sensitive loss of UNC-104 function. Consistent with this, the *ce782* mutation is recessive: *ce782/+* heterozygotes grown and assayed at the restrictive temperature of 23.5° had wild-type locomotion rates (26.6 ± 4.0 body bends/min for *ce782/+* vs. 28.4 ± 3.3 for wild type, $N = 10$ animals each). This indicates that the mutant protein is not maintained at significant levels at this temperature and/or that wild-type UNC-104 effectively outcompetes the mutant protein.

In the UNC-104 (KIF1A) motor domain mutant, temperature-sensitive defects result in increased stalling and longer retrograde movements

To determine how the *ce782* mutation affects vesicle transport at low and high temperatures, we used time-lapse imaging to record the movements of INS-22-Venus - tagged

DCVs in motor neuron axon commissures in living animals. For these experiments we allowed animals to hatch and grow to adulthood at 13.5° and then either acquired the time lapses at 13.5° or acutely shifted the animals to 25°-equilibrated plates for ~20 min during animal selection and mounting before starting the time lapses (25°–20 min). To investigate longer-term effects of temperature, we also collected time lapses from animals shifted to 25° as L3–L4 larvae and grown the remaining 12 hr to young adulthood at 25° (25°–12 hr). For temperature control, we moved the imaging equipment between controlled temperature rooms so that all equipment and the mounting area were the desired temperature.

In *ce782* mutants that were grown at 13.5° or only briefly incubated at 25° (25°–20 min), we were able to capture and record DCV movements in both the ventral part of the axon commissures (proximal to the cell soma) and the dorsal part

of the commissures (distal to the cell soma and just prior to the synaptic region of the dorsal cord). DCVs were also readily detectable in the dorsal cord at levels that were ~40% of wild type, as shown below as part of Figure 10. However, in *ce782* mutants that were incubated for 12 hr at 25°, we were unable to detect any DCVs in the distal region of the commissure or in the dorsal cord, although these regions were detectable through the soluble RFP marker coexpressed in the same neurons by the integrated transgenic array. Thus, we collected all of the time lapses from the 25°–12 hr *ce782* mutants from the ventral regions of the commissures.

Figure 3A shows representative kymographs produced from the wild-type and *ce782* time lapses at 13.5° and 25°. Kymographs allow vesicle movements to be viewed and measured for various parameters on a distance vs. time plot. DCVs in motor neuron commissures move bidirectionally. Movements outward from the cell soma toward the synaptic region (mediated by *UNC-104*) are designated as anterograde whereas movements toward the cell soma (presumably mediated by dynein) are designated as retrograde.

The data showed that DCVs in wild type increased their antero- and retrograde velocities by 2.8- and 2.7-fold, respectively, when shifted from 13.5° to 25°, which is consistent with temperature effects on biological activity within physiological ranges. At 13.5° the anterograde velocity of *ce782* DCVs was about half the velocity of wild type (53%; $P < 0.0001$). This anterograde velocity defect was not significantly enhanced after 20 min at 25° (25°–20 min) (Figure 3C). However, after 12 hr at 25°, the anterograde velocity showed a significant drop to 34% of wild type (Figure 3C). The retrograde velocity of DCVs in *ce782* at 13.5° was only slightly reduced to 78% of wild type (not quite significant, $P = 0.055$); however, at 25°, the retrograde velocity of DCVs in *ce782* was more strongly reduced, falling to ~60% of wild type for both the 25°–20 min and the 25°–12 hr conditions ($P \leq 0.006$) (Figure 3C). The finding that retrograde minus-end-directed velocity is affected by a mutation in the motor domain of a plus-end-directed motor suggests a close regulatory connection between minus- and plus-end-directed transport in bidirectionally moving DCVs.

To determine if a lower ratio of anterograde to retrograde movements could contribute to the temperature sensitivity of *ce782* we compared the percentage of anterograde movements at each temperature. The data showed only a small decrease when shifting *ce782* from 13.5° to 25° (56–~50%) that was not statistically significant (Figure 3D).

To further test if differences in antero- vs. retrograde movements could contribute to the temperature sensitivity of *ce782*, we compared the mean run lengths in the antero- and retrograde directions. At both 13.5° and 25°, the data revealed shorter run lengths in the *ce782* mutant, but the shorter distances closely paralleled the slower velocities in each direction (*i.e.*, when compared to wild type, they were decreased by about the same percentage as the velocities; Figure 3E). Since retrograde velocity at 25° appears less affected by the mutation than anterograde velocity (60%

of wild for retrograde vs. 34% of wild for anterograde after 25°–12 hr), we tested whether there was a significant difference in retrograde vs. anterograde run length that could explain the absence of DCVs in *ce782* mutant axons after 12 hr at 25°. Consistent with the significant drop in anterograde velocity after 12 hr at 25° (compared to 20 min at 25°), the data showed that, in the 25°–12 hr *ce782* mutants, the mean retrograde run length was 67% greater than the mean anterograde run length ($P = 0.0085$; Figure 3E). When we performed the same comparisons for wild type and *ce782* at 13.5° and 25°–20 min, there was no significant difference in the run length in one direction vs. the other (Figure 3E).

To determine whether a defect in the initiation of DCV movement also contributes to the temperature sensitivity of *ce782*, we compared the percentage of mobile puncta per time course at each temperature. At 13.5°, *ce782* had a lower percentage of mobile puncta than wild type (37% vs. 53%; $P = 0.0043$; Figure 3F). For wild type acutely shifted to 25° (25°–20 min) there was no significant change in the percentage of mobile puncta; however, when *ce782* mutants were acutely shifted to 25° we observed a significant decrease in the percentage of mobile puncta (from 37 to 23%, $P = 0.002$; Figure 3F). Thus, at 13.5° wild-type animals have a 1.4-fold higher percentage of mobile puncta than *ce782* (53%/37%), and at 25° this ratio increases to 2.8-fold (64%/23%).

In summary, the time-lapse data show that the *ce782* mutant has a temperature-independent effect on DCV velocity that results in anterograde velocities that are about half of wild type at both 13.5° and after 20 min at 25°. A second decrease in anterograde velocity that is temperature dependent occurs sometime between 20 min and 12 hr at 25°. Retrograde velocity of DCVs is significantly reduced at 25°–20 min compared to wild type, but unlike anterograde velocity, does not further decrease after 12 hr at 25°. Correlating with the skewed effects on directional velocity, the data showed that after 12 hr at 25° *ce782* mutant DCVs move greater distances in the retrograde direction than the anterograde direction, thus providing an explanation for the absence of DCVs in *ce782* mutant axons after 12 hr at 25°. The mutant also shows a temperature-dependent decrease in the percentage of mobile DCVs, suggesting that, in addition to the directional bias, the mutation causes a temperature-sensitive defect in the initiation of DCV transport.

unc-104(ce782) mutants show an abrupt, irreversible impairment of locomotion after 12 hr at the restrictive temperature

To further investigate how long *unc-104(ce782)* mutants must spend at the higher restrictive temperature to maximize impairment of the anterograde vesicle motor, we performed a time-course locomotion assay by allowing *ce782* mutants to hatch and develop at 13.5°, shifting them to 23°-equilibrated plates and quantifying the locomotion rates of the same set of animals every 4 hr for 24 hr. Under these conditions, the wild-type control animals showed a strong ~50% reduction in locomotion rate at the first two time

Figure 3 Active transport properties of wild-type and *unc-104(ce782)* mutant animals at 13.5° and 25°. (A) Representative kymographs of DCV movements in motor neuron commissures of animals with the indicated genotypes carrying the *celS201* transgene that expresses the tagged neuropeptide INS-22-Venus in cholinergic motor neurons. Top two kymographs: from animals kept at 13.5° from hatching through image acquisition; the bottom two kymographs: from animals hatched and grown to adults at 13.5°, and then shifted to 25° for 23 min before starting the time lapse. The left part of each image is closest to the cell soma. Inset illustrates anterograde vs. retrograde movements. See also Movies in File S6, File S7, File S8, and File S9. (B) Table summarizing the sample sizes used to produce the graphs in C–G. (C–F) Graphs plotting various indicated parameters extracted from the kymographs and time-course analyses. “13.5°” indicates that animals were kept at 13.5° from hatching through image acquisition. “25.0°” indicates that animals were hatched and grown at 13.5° and then shifted to 25.0° for 23 min before

points (4 and 8 hr), but then recovered to rates that were not significantly different from the 0-hr rate for all remaining time points (Figure 4A). This apparent temperature-induced lethargus may be related to recent findings that heat shock can induce quiescence or a sleep-like state in *C. elegans* (Hill *et al.* 2014; Nelson *et al.* 2014). Unlike wild type, the *ce782* mutant maintained its 0 hr locomotion rate (~50% of wild type) at the 4- and 8-hr time points, suggesting that this effect requires a threshold of neuronal activity that is not met in the *ce782* mutant. Between the 8- and 12-hr time points, however, the locomotion rate of *ce782* showed an abrupt decrease to ~50% of its 0-hr rate. This rate did not significantly decrease at subsequent time points, but instead remained about fivefold higher than *ce782* mutants that had been hatched and grown at 20° with no temperature shift (Figure 4A). The 12-hr time point was also the first time point at which we noted postural changes in the animals (a shift from sinusoidal to coiled postures; data not shown).

The temperature-induced transition that occurs at 12 hr is irreversible: when we allowed *ce782* mutants to hatch and develop at 13.5°, shifted them to 23° for 24 hr, and then shifted them back to 13.5° for an additional 24 hr, their locomotion rate did not significantly change during the final 13.5° incubation (Figure 4B).

In combination with the time-lapse data, the behavioral data suggest that, in addition to the acute effects of the temperature change on vesicle transport, exposure of the *unc-104(ce782)* mutant to the restrictive temperature for somewhere between 8 and 12 hr causes an additional, irreversible change in the mutant motor, such as a destabilization of the motor, that further impairs its function.

Retrograde movement bias and increased pausing of synaptic vesicles in the *unc-104(ce782)* mutants

Defects in SV transport, as opposed to DCV transport, are likely the major cause of the strong growth and locomotion defects in *unc-104(ce782)* mutants. We therefore used time-lapse imaging to record the movements of GFP-RAB-3-tagged SVs in DA6/DB6 motor neuron axon commissures, in the region proximal to the cell soma, using a new transgene that we made, *celS263*, which expresses GFP-RAB-3 at low levels in these neurons (Figure 5, drawing). We quantified directionality, velocity, percentage of time spent paused, and mean run lengths in each direction after growth at 13.5° followed by 20 min at 25°. The *unc-104* mutant had a significantly lower percentage of its SVs moving in the anterograde direction significantly lower than that of wild type (~40% vs. 70% in wild type; Figure 5, A and B). The effects of the *unc-104* mutation on SV velocities was similar

collecting the time lapses. “25° + 12h” indicates that animals shifted to 25° as mid- to late-stage larvae for 12 hr before acquiring the time lapses. Error bars are standard errors of the means, derived from individual values that are means for each time course. *P*-values for selected comparisons are shown in blue.

Figure 4 *unc-104(ce782)* mutants show an abrupt, irreversible impairment in locomotion after 12 hr at the restrictive temperature. (A) The restrictive temperature affects behavior of the *unc-104(ce782)* mutant at ~12 hr. Animals of the indicated genotypes were hatched and developed to the young adult stage at the permissive temperature of 13.5° and then were transferred to 22.5°-equilibrated plates for 8 min before counting body bends/min for 6 min at 23°. Each point represents the mean and standard error of 12 animals. (B) After temperature decreases the locomotion rate of *unc-104(ce782)*, there is no recovery when switching back to 13.5°. Shown are locomotion rates of *unc-104(ce782)* mutants after hatching and growth to adulthood at 13.5°, after a 24-hr incubation at 23° and after an additional 24-hr incubation at 13.5°.

to its effects on DCV velocities (shown in Figure 3). The mean anterograde velocity was reduced to ~50% of wild type, and the mean retrograde velocity was also significantly reduced, to 76% of wild type vs. 60% of wild type for DCVs (Figure 5C). The SVs in the *unc-104* mutant spent most of their time (85%) in a paused state. In contrast, wild-type SVs spent only 49% of their time in a paused state (Figure 5D). Wild type's mean anterograde run length (6.5 μ m) was significantly longer than its mean retrograde run length (5.0 μ m). In contrast, *unc-104*'s mean anterograde run length (2.4 μ m) was significantly shorter than its mean retrograde run length (3.1 μ m; Figure 5E). In summary, compared to wild type, SVs in the *unc-104(ce782)* mutant spend most of their time in a paused state and, when moving, have a bias toward retrograde movements in both directionality and run length.

In *unc-104(ce782)* mutants, growth temperature determines how synaptic vesicles are distributed between synaptic and dendritic regions

We next tested how growth temperature affects the global distribution of SVs between the cell soma, dendrite, and

axonal synaptic region of a cholinergic motor neuron in *unc-104(ce782)*. For these experiments we visualized SVs using the genomically integrated transgene *wyls85*, which expresses the synaptic vesicle protein GFP-RAB-3 in the DA9 cholinergic motor neuron (Figure 6A; Klassen and Shen 2007). We imaged animals at 20° after allowing them to hatch and grow to young adulthood at the desired temperature.

The analysis of the synaptic region paralleled the growth and behavior results in Figure 2. For *ce782* mutants grown at 11° and 13.5°, the SV density was 50 and 59% of wild type, respectively, and there was no significant difference between these two lower temperatures. However, for animals grown at 20°, the SV density was reduced to only 1.7% of wild type (Figure 6B). This is similar to the locomotion rate reduction to 3.2% of wild type (Figure 2C). In contrast, the loss-of-function alleles *ce515* (nonsense allele) and *e1265* (cargo binding missense allele) had similarly low SV densities in their axons whether animals were grown at 13.5° or 20° (~2% of wild type; Figure S1). Thus, the temperature-sensitive SV transport phenotype of *ce782* is allele specific.

The cell soma analysis revealed the expected accumulation of SVs. *ce782* mutants grown at 13.5° had about three-fold higher cell soma SV density than that of wild type; however, this ratio was not significantly changed in *ce782* mutant animals grown at 20° (Figure 6C). Instead the bulk of the “missing” SVs accumulated in *ce782* mutant dendrites in a temperature-dependent manner. At 13.5°, *ce782* mutant dendrites had a 10-fold higher SV density than that of wild type at the same temperature, whereas at 20° this ratio increased to 24-fold (Figure 6C).

Dynein-mediated transport prevents synaptic vesicles from accumulating in the synaptic region and drives accumulation in dendrites when UNC-104 function is reduced

In *C. elegans* neurons, including cholinergic motor neurons, microtubules in the axon are oriented with their plus ends out (i.e., toward the synaptic region), whereas microtubules in the dendrite are predominantly oriented with their minus ends out (Goodwin *et al.* 2012; Maniar *et al.* 2012; Yan *et al.* 2013). Ou *et al.* (2010) found that eliminating the cyclin-dependent kinase pathways CDK-5 (cyclin-dependent kinase 5) and PCT-1 (Pctaire kinase) in the DA9 motor neuron causes a redistribution of SVs from the synaptic region to the cell soma and dendrite similar to *unc-104* loss-of-function mutants and that this redistribution requires components of the dynein minus-end-directed motor complex, such as DHC-1 (dynein heavy chain) and NUD-2 (known as NUDEL in vertebrates). NUDEL is a substrate of Cdk5 (Niethammer *et al.* 2000) that forms a complex with the dynein regulator LIS1 and dynein heavy chain (Niethammer *et al.* 2000; Sasaki *et al.* 2000). To determine the extent to which the dynein motor complex contributes to the dramatic redistribution of SVs in *unc-104(ce782)* we compared DA9

Figure 5 Synaptic vesicle transport defects in *unc-104(ce782)* mutants. Drawing illustrates the location and anatomy of the cholinergic motor neuron commissures imaged in this experiment. (A) Representative kymographs of synaptic vesicle movements in motor neuron commissures proximal to the DA6/DB6 cell somas of animals with the indicated genotypes carrying the *cel263* transgene that expresses GFP-RAB-3-tagged SVs. Inset illustrates antero-grade vs. retrograde movements. See also *Movies* in *File S10* and *File S11*. (B–E) Graphs plotting various indicated parameters extracted from the kymographs and time-course analyses. All animals were hatched and grown to young adults at 13.5° and imaged after ~23 min at 25° (mounting time). Error bars are standard errors of the means for the indicated number of commissures (B) or combined synaptic vesicle puncta (C–E). (*, **, and ***) *P*-values <0.05, 0.01, and 0.0001, respectively. Black asterisks compare the marked bar to the wild-type value; red asterisks compare the marked retrograde value to the anterograde value for the same strain.

synaptic vesicle distributions at 13.5° and 20° in *ce782* mutants carrying the null deletion mutation *nud-2(ok949)*. At 13.5°, we found that eliminating *NUD-2* in *ce782* increased SV density in the synaptic region from 59 to 105% of wild type and, at 20°, eliminating *NUD-2* increased synaptic SV density >13-fold, from 1.7 to 23% of wild type (Figure 7). Correspondingly, in the dendrites of animals grown at 13.5° or 20°, eliminating *NUD-2* in *ce782* reduced SV density by 16- and 7-fold, respectively. For animals grown at 13.5°, this level is below that of wild type (Figure 8). The cell soma SV densities in these double mutants were either not significantly changed for animals grown at 13.5°

or slightly increased for animals grown at 20° (Figure 8). In summary, the data suggest that the minus-end-directed motor dynein prevents SVs from accumulating in the synaptic region and drives their accumulation in dendrites when *UNC-104* function is reduced.

Eliminating the dynein regulator *NUD-2* (*Nudel*) suppresses the sluggish locomotion caused by reducing *UNC-104* function

If the above redistribution of GFP-RAB-3 in the neurons of *unc-104(ce782); nud-2(ok949)* double mutants represents a redistribution of actual SVs in most or all neurons, these

Figure 6 In the *unc-104(ce782)* mutant, growth temperature determines how synaptic vesicles are distributed between synaptic, somatic, and dendritic regions. (A) Drawing illustrates the location and anatomy of the DA9 cholinergic motor neuron imaged in this experiment. The soma and the dendrite are on the ventral side of the animal. An axonal commissure carries SVs to the synaptic region on the dorsal side. A long axonal region anterior to the synaptic region lacks SVs and synapses in wild type. (B and C) Representative images and quantification of GFP-RAB-3 SVs in the dorsal synaptic region (B) or soma and dendrite (C) of the DA9 motor neuron in animals with the indicated genotypes that were hatched and grown at the indicated temperatures before imaging at room temperature. GFP-RAB-3 is expressed from the integrated transgene *wyls85*. Graph data are means and standard errors from 12–13 animals each. *P*-values for B: at 11.0° and 20.0°, *P* < 0.0001 when comparing to wild type at the same temperatures, whereas the *P*-value at 13.5° is 0.026. *P*-values for C: at 11.0°, the cell soma difference between wild type

and *unc-104(ce782)* is not statistically significant, but the dendrite *P*-value at 11.0° is 0.0028. All other *P*-values are <0.0001 when comparing wild type and the mutant at the same temperatures. n.s., not significant.

double mutants should also show improved locomotion relative to *unc-104(ce782)* single mutants and that is the case. Similar to the results with SV density in the synaptic region and dendrites, eliminating *nud-2* increased the locomotion rate of *ce782* animals grown at 13.5° from 39% of wild type to a level that was not significantly different from wild type. Similarly, for *ce782* animals grown at 20°, eliminating *nud-2* increased their locomotion rate by about fivefold, which roughly parallels the sevenfold increase in SV density in the synaptic region. Despite these strong effects on locomotion rate of the *ce782* mutant, the locomotion rate of *nud-2* single mutants was not significantly different from wild type (Figure 9).

Unlike synaptic vesicles, dense core vesicles do not accumulate in dendrites in a mutant with an impaired UNC-104 (KIF1A) motor

Although DCVs and SVs both use UNC-104 for transport to axons, a previous study found differences in the membrane trafficking pathways that operate on these two kinds of vesicles before they exit the cell soma (Hoover *et al.* 2014). To determine if DCVs, like SVs, also accumulate in dendrites when UNC-104 function is reduced, we produced

genomically integrated transgenes for imaging DCVs and SVs in the same neurons, a subset of 9 DA and DB class cholinergic motor neurons. One strain expresses the synaptic vesicle marker GFP-RAB-3, and the other strain expresses the DCV neuropeptide cargo INS-22-Venus. Both transgenic strains also express soluble RFP under the same promoter for use as an internal control for expression. A quantitative analysis of this marker showed that the *unc-104(ce782)* mutation did not significantly affect the cell soma levels of soluble RFP (data not shown).

Our analysis of SVs in these neurons largely paralleled the results presented in Figure 6 for the DA9 neuron. For *ce782* mutants grown at 13.5°, the synaptic region SV density was 55% of wild type. At 20°, synaptic SV density in the mutant was reduced to 2.7% of wild type (Figure 10A). In cell somas, *ce782* mutant animals grown at 13.5° and 20° had ~20- and 27-fold higher SV densities than that of wild type, respectively (Figure 10A). As in the DA9 neuron, SVs accumulated at high levels in *ce782* mutant dendrites in a temperature-dependent manner. At 13.5°, *ce782* mutant dendrites had ~14-fold higher SV densities than that of wild type at the same temperature, and at 20° this ratio increased to ~25-fold (Figure 10A). To determine whether the *ce782*

Figure 7 The dynein regulator NUD-2 (NUDEL) prevents synaptic vesicles from accumulating in the synaptic region when UNC-104 (KIF1A) function is reduced. Drawing illustrates the location and anatomy of the DA9 cholinergic motor neuron. The boxed synaptic region was imaged. Bottom: representative images and quantification of GFP-RAB-3 SVs in strains with the indicated genotypes that were hatched and grown at the indicated temperatures. GFP-RAB-3 is expressed from the integrated transgene *wyls85*. Images are identically scaled relative to wild type at the same temperature. Graph data are means and standard errors from 12–13 animals each. *P*-values for selected comparisons are shown in blue. n.s., not significant.

mutation is the sole cause of the temperature-dependent synaptic vesicle redistribution phenotype, we used CRISPR-generated allele *unc-104(ce813)*, which contains the same mutation as *ce782* in a background containing the GFP-RAB-3 integrated transgene. The *ce813* mutant phenocopied the *ce782* mutant: SV levels in *ce813* mutant axons, somas, and dendrites at 13.5° and 20° did not differ significantly from SV levels in *ce782* mutant axons, somas, and dendrites at the same temperatures (Figure 10A). We conclude that the G105E mutation causes the temperature-sensitive synaptic vesicle redistribution phenotype.

Our analysis of the DCV marker in the same neurons showed similar effects of the *ce782* mutation with respect to the DCV distribution between cell somas and the synaptic regions. For *ce782* mutants grown at 13.5°, the synaptic region DCV density was 36% of wild type and, at 20°, it was reduced to <2% of wild type (Figure 10B). In cell somas, *ce782* mutant animals grown at 13.5° and 20° had about ~3- and 2.3-fold higher DCV densities, respectively, than that of wild type (Figure 10B). In dendrites, wild-type animals showed very low levels of the DCV marker, on the order of one to two vesicle puncta every 30 μm. However, in a striking difference between DCV and SV transport, the DCV marker did not accumulate in the dendrites of *ce782* mutant animals grown at either 13.5° or 20°. At both growth temperatures the DCV density in *ce782* mutant dendrites was not significantly different from wild type (Figure 10B). This is unlikely to be an allele-specific trait of the *ce782* mutant because a previous study found that the loss-of-function mutant *unc-104(e1265)* had close to wild-type levels of dendritic DCVs, although that study did not analyze dendritic SV accumulation in the same neurons (Goodwin *et al.* 2012).

CDK-5 (cyclin-dependent kinase 5) inhibits dense core vesicles from accumulating in dendrites in mutants with an impaired UNC-104 (KIF1A) motor

The dramatic difference between dendritic DCV and SV densities in an *unc-104* loss-of-function background suggests a difference in how microtubule minus-end-directed transport of these two kinds of vesicles is controlled in a background of reduced UNC-104 activity. Previous studies have found that CDK-5 and the synapse-assembly protein SYD-2 inhibit the dynein-mediated dendritic accumulation of DCVs (Goodwin *et al.* 2012; Goodwin and Juo 2013). CDK-5 and SYD-2 also act together in the same system to inhibit the dendritic accumulation of lysosomes in mutants lacking UNC-16 (Edwards *et al.* 2015). To determine if CDK-5 and/or SYD-2 inhibit DCVs from accumulating in dendrites in *unc-104(ce782)* mutants, we quantified dendritic and cell soma DCV densities in *cdk-5* and *syd-2* single mutants and the corresponding doubles with *unc-104(ce782)*. For these experiments we used the null deletion alleles *cdk-5(gm336)* (Juo *et al.* 2007) and *syd-2(ok217)* (Wagner *et al.* 2009; Kittelmann *et al.* 2013), and we analyzed all strains after growth at 14°. In both *cdk-5* and *syd-2* single mutants, DCVs accumulated in dendrites at levels that were about nine- and threefold higher, respectively, than that of wild type (Figure 11A), consistent with previous findings (Goodwin *et al.* 2012; Goodwin and Juo 2013). We also quantified DCVs in *cdk-5* and *syd-2* mutant cell somas and observed ~1.5- and 2.5-fold significant increases, respectively (Figure 11B). However, in double-mutant combinations of *cdk-5* and *syd-2* with *unc-104*, only *unc-104; cdk-5* double mutants accumulated DCVs in their dendrites. The accumulation in the distal dendrite region was ~14-fold higher than that of wild type.

Figure 8 The dynein regulator NUD-2 (NUDEL) mediates synaptic vesicle accumulation in dendrites when UNC-104 (KIF1A) function is impaired. Drawing illustrates the location and anatomy of the DA9 cholinergic motor neuron. The boxed region was imaged. Bottom: representative images and quantification of GFP-RAB-3 in strains with the indicated genotypes that were hatched and grown at the indicated temperatures. GFP-RAB-3 is expressed from the integrated transgene *wyls85*. Images are identically scaled relative to wild type at the same temperature. Graph data are means and standard errors from 12–13 animals each. *P*-values for selected comparisons are shown in blue. n.s., not significant.

This fold increase in dendrites is similar to what we observed for SVs in *unc-104* single mutants grown at 14° (Figure 10A), and it was accompanied by a ~50% decrease in cell soma DCVs in *unc-104; cdk-5* double mutants compared with *unc-104* single mutants (Figure 11B). In contrast the DCV densities in *unc-104; syd-2* double mutants were significantly lower than that of wild type in both their somas and dendrites. Although the reason for this is not immediately clear, it is not caused by mutant-specific changes in the expression level, since levels of the soluble mCherry internal expression control that is also part of the transgenic array were not significantly different from wild type for any of the single- or double-mutant combinations (Figure 11C). In summary, the results suggest that CDK-5 inhibits DCVs from accumulating in dendrites in mutants with decreased UNC-104 activity.

Synaptic stability under conditions of excessive retrograde transport

The time-lapse data, along with the data showing strong rescue of *ce782* mutant phenotypes by impairing minus-end-directed dynein transport in axons, and as much as a 24-fold accumulation of SVs in *ce782* mutant dendrites containing minus-end-out microtubules, suggest that the *ce782* mutant

defect ultimately translates into a disruption in the balance of minus- vs. plus-end-directed transport such that there is excessive retrograde transport of SVs in the axon. The temperature sensitivity of the *ce782* mutant allowed us to investigate an interesting question: How stable are mature synaptic vesicle clusters under conditions of excessive retrograde transport? Are they completely disassembled or is a population of SVs in the cluster resistant to redistribution by molecular motors?

The locomotion data in Figure 4A showed an abrupt 50% decrease in locomotion rate after 12 hr of exposure of the *unc-104(ce782)* mutant to the restrictive temperature. However, this rate did not significantly decrease at subsequent time points, but instead remained about fivefold higher than *ce782* mutants that had been hatched and grown at 20° with no temperature shift. One possible explanation for this behavioral data is that significant numbers of SVs are not removed from *ce782* mutant synapses until sometime between 8 and 12 hr after the temperature shift, and the remaining SVs are stably maintained, even at the restrictive temperature. We tested this by allowing *ce782* mutants to produce mature synapses at the growth temperature of 13.5°, shifting them to 25°, and then quantifying GFP-RAB-3 at DA9 motor neuron synapses every 4 hr for 24 hr. The imaging

Figure 9 Eliminating the dynein regulator NUD-2 suppresses the sluggish locomotion caused by reducing UNC-104 function. After growth at the indicated temperatures, day 1 adults were transferred to plates equilibrated at 23.5° and allowed to adjust to the assay temperature for 8 min before counting body bends for a 6 min interval. Data are means and SEMs from 12 animals. *P*-values for selected comparisons are shown in blue. n.s., not significant.

results paralleled the behavioral results: the synaptic SV density remained unchanged 4 and 8 hr after switching from 13.5° to 25°. However, the 12 hr time point showed a 50% drop in synaptic SV density. This level did not further decrease at the remaining time points (16, 20, and 24 hr), but instead remained at 18–23% of wild type (Figure 12, which is >10-fold higher than the synaptic SV density of *ce782* mutants that had been hatched and grown at 20° with no temperature shift (~1.7%; as shown in Figure 6). We note that this is not the case with DCVs, since *ce782* mutants that had been shifted to 25° for 12 hr to prepare for the time-lapse imaging in Figure 3 had no detectable DCVs in their dorsal commissures or axons (data not shown).

Taken together, the behavioral and imaging data suggest that a population of ~50% of the SVs at mature synapses are resistant to being removed by the synaptic vesicle retrograde motor when the anterograde motor is impaired.

Synapse-assembly proteins prevent the anterograde transport of synaptic vesicles beyond the synaptic region

One possible explanation for stability of mature SV clusters in the context of an impaired anterograde motor is that the synapse-assembly protein SYD-2, required to tether vesicles to the active zone dense projection and known to inhibit dissociation of SVs from mature synapses (Stigloher *et al.* 2011; Kittelmann *et al.* 2013; Wu *et al.* 2013), protects a distinct population of ~50% of the SVs from loss by the minus-end-directed motor dynein. Since directly testing this hypothesis would require conditionally inactivating *syd-2*, we looked for additional evidence that SYD-2 and/or other synapse-assembly proteins protect synaptic vesicle clusters from dissociation by a molecular motor. If synapse-assembly proteins inhibit dissociation of synaptic vesicle clusters, then, in synapse-assembly mutants, the anterograde motor should carry SVs or clusters of vesicles beyond the synaptic region to the asynaptic region in neurons that have distal asynaptic

regions. To test this, we imaged GFP-RAB-3 in the DA9 motor neuron synaptic and asynaptic regions in wild-type and synapse-assembly mutants. DA9's asynaptic region extends well beyond the synaptic region to cover nearly half of the adult animal's length (Figure 13 drawing). Previous studies have found that SYD-1 and SYD-2 act together in the same pathway to regulate synapse assembly (Dai *et al.* 2006; Patel *et al.* 2006). *sad-1* mutants show similar defects in synaptic vesicle clustering (Crump *et al.* 2001; Patel *et al.* 2006), and the synaptic localization of CFP-tagged SAD-1 is dependent on SYD-2 (Patel *et al.* 2006), suggesting that it may act downstream of SYD-2. We found that null deletion mutations in *syd-1* and *syd-2* reduced the SV density in the synaptic region to ~65% and 40% of wild type, respectively, while a *sad-1* nonsense mutant showed a nonsignificant reduction to 80% of wild type (Figure 13A). Corresponding with the loss of the synaptic vesicle marker from the synaptic region, we found SVs or SV clusters in the asynaptic region in all of these mutants. Wild type's asynaptic region contains almost no detectable SVs, whereas each of the synapse-assembly mutants contain SV clusters throughout their length (Figure 13, representative images). To quantify this, we defined a minimum threshold of GFP-RAB-3 intensity for a wild-type synaptic vesicle cluster, applied this to wild type and each of the mutants, and then measured the length of the region containing SV clusters that equaled or exceeded the threshold. By this metric, *syd-1*, *syd-2*, and *sad-1* mutants had synaptic region lengths that were 3.8-, 4.0-, and 3.0-fold longer than that of wild type, respectively, with *P*-values of significance at <0.0001 (Figure 13B). For *syd-2*, we demonstrated that this is a cell autonomous effect, as opposed to, for example, a developmental cue from another neuron that causes synapses to form in the asynaptic region, because a transgene expressing the *syd-2* gene solely in the DA9 neuron significantly rescued *syd-2*'s SV phenotypes in the synaptic and asynaptic regions (Figure 13, A and B). The total axonal GFP-RAB-3 fluorescence *syd-1*, *syd-2*, and *sad-1* null mutants, calculated by adding the total integrated intensity within the synaptic and asynaptic regions, was not significantly different from wild type, with the exception of one of the two *syd-2* mutants, which was slightly lower (Figure 13C; *P* = 0.04).

Synapse-assembly proteins are required to produce synaptic vesicle clusters at synapses when anterograde synaptic vesicle transport is compromised

To investigate genetic interactions between synapse-assembly mutants and UNC-104 (KIF1A) and to confirm that UNC-104 is the motor responsible for the anterograde accumulation of SV clusters in the asynaptic region in the synapse-assembly mutants, we crossed the synapse-assembly mutants *syd-2(ok217)*, *syd-2(ce759)*, and *sad-1(ce749)* into the *unc-104(ce782)* mutant background. This revealed a synthetic genetic interaction in which double mutants that were hatched and grown at the permissive temperature of 13.5° resembled *unc-104(ce782)* single mutants that had been hatched and

A

B

Figure 10 Unlike synaptic vesicles, dense core vesicles do not accumulate in dendrites in mutants with an impaired UNC-104 (KIF1A) motor. (A and B) As indicated, graphs and representative images of GFP-RAB-3 SVs expressed from the *cels263* integrated transgene and INS-22-Venus dense core vesicle cargo expressed from the *cels255* integrated transgene in strains with the indicated genotypes that were hatched and grown at the indicated temperatures before imaging the indicated regions at room temperature. Images from the mutants are scaled identically to wild type at the same temperature. Data are means and standard errors from 12–15 animals each. (A) *unc-104(ce782)* (produced by random EMS mutagenesis) and *unc-104(ce813)* (produced by CRISPR homologous recombination) are identical mutations in *unc-104*. *P*-values comparing wild type and *ce782* at the same temperatures are <0.0001 for all comparisons except the dendrite 13.5°C data (*P* = 0.001). The two *unc-104* alleles do not differ significantly from each other.

grown at the restrictive temperature of 20° with respect to posture, growth, and locomotion rates. While none of the three synapse-assembly single mutants had locomotion rates that were significantly lower than that of wild type, the two *unc-104(ce782); syd-2* double mutants, hatched and grown at 13.5°, often showed coiled postures (data not shown) and had locomotion rates that were ~5% of wild type (Figure 14A) and not significantly different from *unc-104(ce782)* mutants hatched and grown at 20°. While this manuscript was in preparation, another study also reported a strong synthetic effect on locomotion rate when combining a weak *unc-104* mutation and a *syd-2* null mutation (Zheng *et al.* 2014).

To determine the cellular basis of the synthetic behavioral phenotypes, we crossed the genomically integrated SV marker into the *syd-2(ce759)* single mutant and the *unc-104(ce782); syd-2(ce759)* double mutant and quantified SV density in the DA9 motor neuron axon in animals grown at the permissive temperature of 13.5°. *unc-104* and *syd-2* single mutants grown at this temperature had synaptic SV densities that were ~60 and 70% of wild levels (Figure 14B). However, in *unc-104; syd-2* double mutants grown at 13.5°, synaptic GFP-RAB-3 was nearly absent (~5% of wild type; Figure 14B). The synaptic region length of the *unc-104; syd-2* double mutant was shortened to $10.2 \pm 5.0\%$ of wild type (mean $\pm$ SEM; $N = 14$ axons), and we detected no GFP-RAB-3 signal in the asynaptic region (data not shown). We conclude that UNC-104 is the anterograde motor that carries synaptic vesicle clusters to the distal asynaptic region in *syd-2* single mutants and that synapse-assembly proteins are required to produce synaptic vesicle clusters at synapses when anterograde synaptic vesicle transport is reduced.

Synapse-assembly mutants accumulate synaptic vesicles in their somas and dendrites due to a defect in synaptic vesicle transport

The synthetic genetic interactions between *unc-104* and *syd-2* mutants may result, in part, from the inability of SVs to be retained at synaptic sites due to reduced SV clustering in *syd-2* mutants (Stigloher *et al.* 2011; Kittelmann *et al.* 2013; Wu *et al.* 2013). However, previous studies in flies and worms have found that, in addition to impaired SV clustering, *syd-2* loss-of-function mutants also have defects in SV and DCV transport (Shin *et al.* 2003; Miller *et al.* 2005; Wagner *et al.* 2009; Goodwin and Juo 2013; Zheng *et al.* 2014). Thus, the strong genetic interaction that prevents SVs from accumulating at synaptic sites in *unc-104(ce782); syd-2* double mutants grown at the permissive temperature may result from an earlier defect (*i.e.*, preceding synapse formation), in which a double “hit” to synaptic vesicle transport prevents SVs from reaching the synaptic region.

Since *sad-1* mutants also showed a strong genetic interaction with *unc-104(ce782)* grown at the permissive temperature, we wanted to determine if SAD-1 and other synapse-assembly proteins also have a role in SV transport. We therefore tested whether *syd-1*, *syd-2*, and *sad-1* null mutants have an increased net ratio of minus- to plus-end-directed transport as assayed by a neuron-wide SV distribution analysis. If synapse-assembly mutants have an increased net ratio of minus- to plus-end-directed transport, SVs should accumulate in the dendrite since most dendritic microtubules in *C. elegans* cholinergic motor neurons are oriented with their minus-ends out (Goodwin *et al.* 2012; Yan *et al.* 2013). Previous studies have reported increased levels of SV markers in the dendrites of the synapse-assembly mutants *syd-1* and *sad-1* (Hallam *et al.* 2002; Hung *et al.* 2007). This could result from an axon–dendrite polarity/identity defect or, alternatively, it could result from a SV transport defect. If it is a transport defect, then both the cell soma and the dendrite should have increased levels of the synaptic vesicle marker, as is the case when UNC-104 function is reduced. If it is an axon–dendrite polarity/identity defect, then there is no reason to expect that the synaptic vesicle marker should accumulate in the cell somas of synapse-assembly mutants. Indeed, the hypothesis proposed for *sad-1* and *syd-1* mutants is that the axon maintains its identity, but the dendrite also takes on the identity or partial identity of an axon (Hallam *et al.* 2002; Hung *et al.* 2007). In *C. elegans* cholinergic motor neurons, this would produce a motor neuron with two axons. If this were the case, then SVs should be driven in two directions away from the cell soma to populate the two axons. Thus, if there is a conversion or partial conversion of the dendrite into an axon in the mutants, eliminating *sad-1* should, if anything, decrease SV density in somas.

We first quantified SV density in the DA9 dendrite and cell soma of synapse-assembly single mutants and found increased levels at both locations in all of the mutants. Dendritic SV density in *syd-1*, *syd-2*, and *sad-1* mutants was increased by ~4-, 4-, and 7-fold over wild type, respectively, and cell soma SV density was increased by 2- to 2.75-fold over wild type (Figure S2). All of the differences were highly significant. However, because the synapse-assembly mutants had wild-type total levels of GFP-RAB-3 in their axons (Figure 13C) and increased levels in their somas and dendrites we could not rule out that the mutations indirectly affected expression of the transgene. We therefore repeated the experiment using the *cel-263* integrated transgene, which expresses GFP-RAB-3 in a subset of DA- and DB-type cholinergic motor neurons and contains an internal expression control of soluble mCherry expressed from the same transgene promoter. Using this new transgene, the synaptic region SV density was significantly lower than that of wild

other for any of the parameters. (B) For *unc-104(ce782)* at 20°, N is 3 for the axon images and 5 for the soma and dendrite images. P -values comparing wild type and *ce782* at the same temperatures are <0.0001 for both sets of axon data and the 13.5° soma data. $P = 0.014$ when comparing wild type and *ce782* soma levels at 20°. Differences between wild type and *ce782* in the dendrite are not significant.

Figure 11 CDK-5 (Cyclin-dependent kinase 5) inhibits dense core vesicles from accumulating in dendrites in mutants with an impaired UNC-104 (KIF1A) motor. (A–C) As indicated, graphs and representative images of INS-22-Venus-labeled DCVs (A and B) or soluble mCherry (C; internal expression control; graph only) expressed from the *cels255* integrated transgene in strains with the indicated genotypes that were hatched and grown at 13.5° before imaging the indicated regions at room temperature. Images from the mutants are scaled identically to wild type at the same temperature. Data are means and standard errors from 13–15 animals each. *** and **, *P*-values of <0.001 and <0.01, respectively, for comparisons with wild type. *P*-values for other comparisons are indicated in blue. Unmarked bars do not differ significantly from wild type.

type for the *syd-2* null mutant (~70% of wild type), but was not significantly different from wild type for *sad-1* or *syd-1* null mutants (Figure 15A). The somas of *syd-2*, *sad-1*, and *syd-1* mutants had SV densities that were 6-, 6-, and 3-fold higher than that of wild type, respectively, while dendritic levels were increased by 8-, 9-, and 3-fold over wild type, respectively (Figure 15B). These strong increases in the somas and dendrites did not result from the mutations

affecting expression of the transgene: levels of the soluble mCherry expression control were not significantly different from wild type for *syd-2* and *sad-1* mutants. The *syd-1* mutant did show a significantly increased level of soluble mCherry, but this level was only ~1.5-fold higher than that of wild type (Figure 15C). To determine if the soma/dendrite accumulation of SVs is dynein dependent we tested the extent to which a *nud-2* null mutation altered the SV distribution in the *syd-2*

Figure 12 Synaptic stability under conditions of excessive retrograde synaptic vesicle transport. Animals of the indicated genotypes were hatched and developed to various larval stages at the permissive temperature of 13.5° and then were shifted to 25° for the indicated times to complete development to young adulthood followed by imaging of GFP-RAB-3 expressed from the integrated transgene *wyls85*. Graph points represent the means and standard errors of 6–14 animals. Representative images (bottom) are identically scaled at each time point.

null mutant. The *nud-2* null mutation significantly increased SV density in the synaptic region of the *syd-2* null mutant, to a level that was not significantly different from wild type (Figure 15A). Correspondingly, eliminating *nud-2* lowered soma and dendritic SV density in the *syd-2* null mutant from 6- and 8-fold higher than that of wild type, respectively, to ~2.5-fold higher than that of wild type, without affecting the transgene expression level (Figure 15, B and C). The data suggest that the high levels of SVs in cholinergic motor neuron somas and dendrites of synapse-assembly mutants result from a transport defect rather than a problem with axon–dendrite identity in adult animals.

Discussion

Using a forward genetic screen we discovered a mutation in the *UNC-104* motor domain that, for the first time, allows the use of temperature to alter plus-end-directed KIF1A motor activity and thus control SV and DCV levels in axons. In this study, we used growth and behavioral analyses, quantitative imaging of neuron-wide vesicle distributions, and time-lapse analyses of vesicles in living animals to investigate how temperature affects growth, locomotion, and vesicle transport in the mutant. These studies led us to discover a major difference between SV and DCV transport with respect to their entry into dendrites when *UNC-104* activity is reduced. The mutant also contributed to our discovery that multiple synapse-assembly proteins, and not just *SYD-2*, have dual roles in determining SV cluster stability and in regulating SV transport.

G105E: a rare temperature-sensitive mutation in the UNC-104 (KIF1A) motor domain

To the best of our knowledge, this is the first reported conditional mutation in a kinesin motor domain. Mutants hatched and grown at the restrictive temperature of 25° essentially have the *unc-104* null phenotype of >90% larval arrest. This suggests that mutant *UNC-104* protein produced at this temperature is nonfunctional. Mutants hatched and grown at the intermediate temperature of 20° are viable, but have a strong reduction-of-function phenotype in which both locomotion rate and SV content at synapses are reduced to <5% of wild type. Mutants hatched and grown at the permissive temperature of 13.5° have locomotion rates and SV content at synapses that are ~50% of wild type and often exhibit wild-type sinusoidal movement.

The time-lapse and locomotion time-course studies suggest that mutant protein produced at the permissive temperature is relatively functional and is not immediately and completely inactivated by switching to the restrictive temperature. The increased temperature does acutely (within 20 min) decrease the fraction of DCVs that are mobile and the retrograde velocity. However, after 12 hr at the restrictive temperature, correlating with a lack of detectable DCVs in axons at this time point, we see a significant drop in anterograde velocity (that is not accompanied by a corresponding drop in retrograde velocity) that results in significantly longer retrograde movements relative to anterograde movements. Our time-course experiments revealed an abrupt 50% decrease in both locomotion rate and SV content at synapses that occurred ~12 hr after shifting to

Figure 13 Synaptic assembly proteins inhibit the anterograde accumulation of synaptic vesicle clusters beyond the synaptic region. (A) At 20°, synapse-assembly mutants have reduced levels of SVs in the synaptic region. Quantification of GFP-RAB-3 SVs in the synaptic region of the DA9 motor neuron in strains with the indicated genotypes that were hatched and grown at 20°. GFP-RAB-3 is expressed from the integrated transgene *wyls85*. Bottom: straightened, identically scaled images of the synaptic regions from strains with the indicated genotypes. Data are means and SEMs from samples of 12–14 animals. Except where indicated by lines between bars, *P*-values shown in blue or indicated with asterisks are comparisons of each strain to wild type. (***, $P < 0.0001$. n.s., not significant). (B) In synapse-assembly mutants, synaptic vesicle clusters aberrantly move into the asynaptic region. Threshold fluorescence intensity values were used to standardize the beginning and end of the synaptic region in wild type, and then the same values were applied to the other strains to determine the length of each strain's synaptic region. GFP-RAB-3 is expressed from the integrated transgene *wyls85*. Bottom right: straightened, identically scaled images of the asynaptic regions from strains with the indicated genotypes. Data are means and SEMs from samples of 12–14 animals. Except where indicated by lines between bars, *P*-values shown in blue or indicated with asterisks are comparisons of each strain to wild type. (***, $P \leq 0.0001$. (C) In synapse-assembly mutants, the total synaptic + asynaptic level of axonal SVs is similar to wild type. Quantification of GFP-RAB-3 SVs in the synaptic and asynaptic regions of the DA9 motor neuron in strains with the indicated genotypes that were hatched and grown at 20°. GFP-RAB-3 is expressed from the integrated transgene *wyls85*. Data are means and SEMs from samples of 12–14 animals. *P*-values shown in blue or indicated with n.s. (not significant) are comparisons of each strain to wild type.

the restrictive temperature. This suggests that the mutant protein undergoes a transition to a stronger loss-of-function state after 8–12 hr at the higher temperature. One possibility is that it becomes destabilized after a certain number of step cycles at the higher temperature. Although it is clear from the time-lapse experiments that this transition does not produce the null state of the protein, the persistence of stable SV clusters at synapses after this point should not be taken as evidence that new SVs are reaching the synaptic region (see below). Indeed, the lack of DCVs in axons at this time point suggests that axons no longer have the anterograde transport activity necessary to move DCVs to the distal commissure or synaptic region.

Our quantitative analysis of SV distribution in the mutant revealed a striking buildup of SVs in motor neuron dendrites (10- and 24-fold higher than that of wild type at the permissive and restrictive temperatures, respectively). Previous

studies using the *unc-104(e1265)* allele also reported this (Yan *et al.* 2013; Maeder *et al.* 2014); however, our *nud-2* behavioral and imaging experiments are the first evidence that the dendritic accumulation of SVs in an *unc-104* loss-of-function mutant and the accompanying effects on locomotion are dynein dependent. The *e1265* mutation impairs the binding of UNC-104 to SVs and is on the opposite end of the protein from the motor domain (Kumar *et al.* 2010). This indicates that the strong dendritic accumulation of SVs is a general consequence of reduced plus-end-directed SV transport, rather than a phenotype specific to the *unc-104(ce782)* allele.

Our time-lapse analyses also revealed a significant decrease in the retrograde velocities of both DCVs and SVs in the *unc-104* mutant. Prior studies found that a *unc-104* reduction-of-function mutation in the cargo-binding domain had no effect on SV retrograde velocity, despite causing

Figure 14 Synaptic assembly proteins are required to produce synaptic vesicle clusters at synapses when anterograde synaptic vesicle transport is compromised. (A) Synthetic genetic interactions between *unc-104* and the synaptic assembly mutants *syd-2* and *sad-1*. Animals developed and grown at the permissive temperature of 13.5° were transferred to plates equilibrated at 23.5° and allowed to adjust to the assay temperature for 8 min before counting body bends for a 6-min interval. The locomotion rate of *syd-2(ok217)* is not significantly different from that of wild type. By comparison, the locomotion rates of the two *unc-104; syd-2* double mutants that were grown at 13.5° are not significantly different from an *unc-104(ce782)* single mutant grown at the restrictive temperature of 20° (the latter of which is shown in Figure 2B). Data are means and SEMs from populations of 12 animals. *P*-values are shown in blue for selected comparisons. (B) Boxed region in drawing indicates region imaged. Quantification and representative images of GFP-RAB-3 SVs in the synaptic

a decrease in anterograde velocity (Kumar *et al.* 2010; Maeder *et al.* 2014). Since the *ce782* mutation is in the motor domain, which is on the opposite end of the protein from the cargo binding domain, it is possible that the *ce782* mutation also indirectly affects dynein motor activity in these bidirectionally moving vesicles.

Why don't DCVs accumulate in dendrites when the *UNC-104* motor is impaired?

One of the findings to come out of our study is that entry of SVs and DCVs into dendrites is differentially affected under conditions of reduced *UNC-104* activity. No prior study has compared the dendritic accumulation of SVs and DCVs in the same neurons under conditions of reduced *UNC-104* function. We found that, unlike SVs, which accumulate in *unc-104* mutant dendrites at levels up to 20-fold higher than that of wild type, DCVs do not accumulate in dendrites when *UNC-104* function is reduced. One possibility is that the DCV neuropeptide cargo we tagged (*INS-22-Venus*) is degraded in, or secreted from, the soma/dendrite regions.

However, although the data do not allow us to determine the extent to which somatic neuropeptide secretion occurs under conditions of reduced *UNC-104* function, two observations suggest that differences in the way cholinergic motor neurons regulate the transport of DCVs and SVs into dendrites contribute to the lack of DCV accumulation in *unc-104* mutant dendrites. First, when *UNC-104* activity is reduced, the fold increases of SVs in DA9 somas and DCVs in DA6/DB6 somas are similar (about threefold), and yet the dendritic levels are dramatically different (14-fold for SVs and 0-fold for DCVs). Second, in *unc-104; cdk-5* double mutants grown at 13.5°, DCVs accumulate in dendrites at levels that are 14-fold higher than that of wild type. If dendrites were unable to store DCVs under conditions of reduced *UNC-104* activity (e.g., due to secretion or degradation), then we would not expect to see this stable increase. Indeed, the fold increase of DCVs in *unc-104; cdk-5* mutant dendrites is about the same as the fold increase seen with SVs in *unc-104* single mutant dendrites. Thus, the data suggest that the relative absence of DCVs in *unc-104* mutant dendrites results from *CDK-5* inhibiting their transport to dendrites rather than enhanced secretion after transport to dendrites. *CDK-5* could perform this function by negatively regulating dynein, by positively regulating *UNC-104*, or by regulating both motors.

The finding that SVs in the *unc-104* mutant dendrites are present at levels that are as high, or higher than, the

region of the DA9 motor neuron in strains with the indicated genotypes that were hatched and grown at the permissive temperature of 13.5°. GFP-RAB-3 is expressed from the integrated transgene *wyls85*. Images are identically scaled relative to wild type at the same temperature. Graph data are means and standard errors from 14 animals each. The *unc-104(ce782); syd-2(ce759)* double mutant has significantly lower levels of GFP-RAB-3 in the synaptic region than either single mutant. *P*-value shown in blue.

A Synaptic region GFP-RAB-3 synaptic vesicles

Synaptic region GFP-RAB-3 fluorescence/ μm

B Soma and dendrite GFP-RAB-3 synaptic vesicles

GFP-RAB-3 fluorescence/ μm (sq. μm for soma)

C Soma RFP (expression control)

Soma RFP fluorescence/ sq. μm (expression)

Figure 15 Synaptic assembly mutants accumulate synaptic vesicles in both their somas and their dendrites. Drawing illustrates the regions imaged. The somas are the DA6 and DB6 cholinergic motor neurons, and the dendrite region contains the proximal dendrites that overlap between the two somas. (A–C) Representative images and quantification of the indicated markers in strains with the indicated genotypes. GFP-RAB-3 and soluble RFP (expression control) markers are expressed from the integrated transgene *ceIs263*. Images are identically scaled relative to wild type. Graph data are means and standard errors from 12–14 animals each. Unmarked bars are not significantly different from wild type. (***) *P*-values for comparisons to wild that are <0.0001 and <0.01, respectively. *P*-values for comparisons of *syd-2* (ok217) to the *syd-2*; *nud-2* double are 0.015 (synaptic region), <0.0001 (somas), <0.0001 (dendrite), and >0.05 (not significant; RFP expression).

levels in *unc-104* mutant somas suggest that wild-type CDK-5 has limited or no capacity to prevent the dendritic accumulation of SVs in the *unc-104* mutant. Adding to this puzzle is the finding that both DCVs and SVs use CDK-5 and SYD-2 to prevent their dynein-dependent loss to dendrites in *unc-104*(+) backgrounds (this study; Ou *et al.* 2010; Goodwin *et al.* 2012; Goodwin and Juo 2013), so

both SV and DCV transport are normally regulated by CDK-5 and SYD-2. It is unclear why this difference between DCVs and SVs is apparent only in an *unc-104* reduction-of-function background. One possibility is that reduced *unc-104* function reproduces a physiologically relevant condition that triggers the differential regulation of the two vesicle types.

Synapse stability under conditions of excessive retrograde transport

Electron microscopy studies using high-pressure freezing or cryo-EM of synapses in vertebrates, *Drosophila*, and *C. elegans* have revealed a filamentous network that clusters SVs near the active zone, as well as an elaborate electron-dense projection centered on the active zone. This dense projection contacts vesicles in the cluster and extends thin filaments deep into the cluster to anchor the cluster to the plasma membrane (Siksoo *et al.* 2007; Fouquet *et al.* 2009; Fernandez-Busnadiego *et al.* 2010; Jiao *et al.* 2010; Stigloher *et al.* 2011).

The ability to conditionally control SV transport using *unc-104(ce782)* enabled us to investigate the stability of these mature SV clusters under conditions of excessive retrograde transport. Our imaging time-course data suggest that, after 12 hr at the restrictive temperature of 25°, a population of ~50% of the SVs is abruptly lost; however, no further loss occurs after this. The timing of the SV loss correlates with a 50% decrease in locomotion rate that also does not further decrease with time. In contrast to SVs, the DCV marker is undetectable in *unc-104(ce782)* axons after 12 hr at the restrictive temperature. DCVs are only slightly enriched at synapses (Hammarlund *et al.* 2007) and, although some can be connected to the SV cluster via filaments, they tend to localize to the outer edge of the cluster when they are present at synapses (Stigloher *et al.* 2011). This lack of clustering of most DCVs at synapses as well as their presence on the outer edge of the cluster (*i.e.*, with potentially fewer connections) may make DCVs more vulnerable to dynein-mediated loss when *UNC-104* is impaired. Although our results do not identify the population of SVs that was lost during the time course, we hypothesize that it may be a population with no filamentous connections to the cluster and/or SVs that have fewer connections because they are on the outer edge of the cluster. However, if motors are capable of breaking off tethered vesicles from the outer edge of the cluster, then it must be only a subset of weakly tethered vesicles, because no further SV loss occurs after the initial 50% loss even though a new outer cluster edge would be formed. Alternatively, it is possible that the resistant population has lost its ability to serve as a cargo for the dynein motor complex irrespective of whether it is tethered to the cluster, or that tethering to the cluster is also associated with loss of transport capacity. Because SV clusters stabilized at 50% of the preshift levels for hours after the temperature shift, the data also suggest that recycling SVs (which may be briefly untethered) are not vulnerable to loss by the retrograde motor.

We also considered the alternative hypothesis that the decrease in axonal SV content after the temperature upshift might result from SV turnover/degradation at synapses; however, if this were the case, then the loss would continue at later time points, and it does not. Furthermore, the axonal SV content mirrors the effects on locomotion rate since there is an abrupt 50% decrease in locomotion rate between 8 and

12 hr postshift, with no further decrease at later time points. This indicates that the remaining axonal SVs in the ventral cord motor neurons are functional.

The role of synapse-assembly proteins in anchoring synaptic vesicles to the synaptic region

The ability to express GFP-RAB-3-tagged SVs in a single identified motor neuron allowed us to determine how synapse-assembly proteins affect the distribution of SVs between the synaptic and asynaptic regions. Both *syd-1* and *syd-2* mutants showed reduced levels of the synaptic vesicle marker in the synaptic region, which has also recently been demonstrated for *syd-2* mutants using an EM analysis (Kittelmann *et al.* 2013). Although *syd-2* mutants have a SV transport defect, the reduced SV density at *syd-2* mutant synapses is not the result of a transport defect (*i.e.*, SVs not reaching the synaptic region) because we found synaptic vesicle clusters in the distal asynaptic region of *syd-2* mutants that brought its total axonal GFP-RAB-3 to wild-type levels. We found similar levels of SVs in the distal asynaptic regions of *syd-1* and *sad-1* mutants.

A study using high-pressure freezing EM and tomographic reconstructions of active zones found that *syd-2* mutants have a redistribution of vesicles away from the dense projection and reduced numbers of undocked vesicles contacting the dense projection (Stigloher *et al.* 2011). Consistent with *syd-2* mutant dense projections having a reduced capacity to tether SVs, another recent EM reconstruction study found that *syd-2* null mutants have significantly smaller dense projections (Kittelmann *et al.* 2013). In complementary studies, time-lapse imaging of GFP-RAB-3-tagged SVs revealed that a *syd-2* null mutation increased the dissociation rate of SVs from mature synapses in the synaptic region of the DA9 neuron, which is the same neuron we imaged in this study (Wu *et al.* 2013). The distal SV clusters we observed may result from *UNC-104* transport of untethered or loosely tethered vesicles from the synaptic region to the distal asynaptic region. While this manuscript was in preparation, a study in *Drosophila* also reported the accumulation of SV markers in the distal asynaptic regions of motor neuron axons in fly *Syd-1* and *liprin-α* (the ortholog of *SYD-2*) mutants, but *sad-1* mutants were not tested (Li *et al.* 2014). The same study also showed distal accumulation of the active zone proteins Bruchpilot and RIM (*UNC-10*); however, Li *et al.* (2014) also found no evidence that the distal clusters were undergoing exo-/endocytosis cycles as indicated by FM1-43 dye-loading experiments. Since the smaller size of the dense projection in *syd-2* mutants (Kittelmann *et al.* 2013) might reflect an underlying instability of the dense projection in *syd-2* mutants, it is possible that the distal clusters also contain detached pieces of the dense projection with active zone proteins. Alternatively, active zone proteins might be present as a consequence of being cotransported with ~23% of SV clusters from the cell soma to the synaptic region, as was observed in wild-type motor neurons (Wu *et al.* 2013).

A second distinct role for synapse-assembly proteins in regulating the transport of synaptic vesicles and dense core vesicles

The finding that *SYD-2*, *SAD-1*, and *SYD-1* regulate SV and DCV transport contrasts oddly with their functions in synapse assembly and stability. Although prior studies have demonstrated a role for *SYD-2* in SV and DCV transport in *Drosophila* (Miller *et al.* 2005) and *C. elegans* (Wagner *et al.* 2009; Goodwin and Juo 2013; Zheng *et al.* 2014), our study provides the first evidence that multiple synapse-assembly proteins, in addition to *SYD-2*, also have roles in vesicle transport. Past studies of *SAD-1* and *SYD-1* have reported SV accumulation (but not DCV accumulation) in *sad-1* and *syd-1* mutant dendrites; however, the proposed cause was that the dendrite took on the identity of an axon, referred to as a “polarity defect” (Hallam *et al.* 2002; Hung *et al.* 2007; Kim *et al.* 2008; Kim *et al.* 2010). Although a disruption of dendrite identity would lead to the observed accumulation of SVs in dendrites, such a defect should, if anything, decrease SV density in the cell somas of *sad-1* and *syd-1* mutants because the neuron would now have two axons to fill with SVs. However, if a transport defect is the major cause of the vesicle redistribution in *sad-1* and *syd-1* mutants, then both the cell soma and the dendrite should have increased levels of the SV marker, as is the case when *UNC-104* function is reduced. Our neuron-wide analysis of the SVs in adult animals confirms that *sad-1*, *syd-1*, and *syd-2* mutants, like *unc-104* mutants, all have high SV densities in their somas. In the accompanying study (Edwards *et al.* 2015), our analysis of cell soma organelle trafficking in the same motor neurons supports a general organelle transport function for *SAD-1*, *SYD-1*, *SYD-2*, and *CDK-5*, as well as *STRD-1* (*STRAD* α), a protein shown to directly interact with *SAD-1* (Kim *et al.* 2010). The demonstration that *sad-1* and *syd-2* null mutants have wild-type rates of coordinated locomotion (Edwards *et al.* 2015) also suggests that the axons and dendrites in the cholinergic motor neurons that mediate this locomotion maintain their proper identity despite the transport defect.

Despite the lack of evidence for an axon-identity defect in the synapse-assembly mutants in *C. elegans* cholinergic motor neurons, prior studies have demonstrated a clear role for *SAD-1*'s mammalian orthologs *SAD-A* and *SAD-B* in specifying axon identity in forebrain neurons (Kishi *et al.* 2005), where *SAD-A/B* act in a pathway downstream of the *LKB1* kinase (Barnes *et al.* 2007). However, neurons outside the cortex, including motor neurons and many other types of central neurons, do not require the *SAD* kinases or *LKB1* for axon formation or identity (Lilley *et al.* 2013). *SAD-1* and its mammalian orthologs also regulate axon termination (Crump *et al.* 2001) and terminal branching (Lilley *et al.* 2013) in certain classes of sensory neurons. It is unclear whether this latter function could be related to the transport function of *SAD-1*.

Since the synapse-assembly mutants have somatic and dendritic vesicle accumulation (*i.e.*, minus-end-directed accumulation), the wild-type role of these proteins could be to

stimulate *UNC-104*, to inhibit dynein, or both. A prior study found that *SYD-2* physically interacts with *UNC-104* and increases the anterograde velocity of *UNC-104*-GFP and a SV marker, resulting in an overall bias toward minus-end transport in a *syd-2* null mutant, although data from the same study also shows reduced net transport of a YFP-tagged dynein subunit (Wagner *et al.* 2009). A recent study found that DCV trafficking in the axon and dendrite was decreased in both directions in a *syd-2* null mutant with no change in the ratio of anterograde to retrograde movements, although plus-end-directed velocity was reduced in the dendrite (Goodwin and Juo 2013). Another recent study found that *SAM-4* acts in the same pathway as *SYD-2* and, consistent with the findings of Goodwin and Juo (2013), found reduced processivity (*i.e.*, reduced numbers of moving vesicles, reduced run lengths, and increased frequency of pauses) of *sam-4* null mutants in both the plus- and minus-end directions (Zheng *et al.* 2014).

It is not immediately apparent why *SAD-1*, *SYD-1*, and *SYD-2* have dual roles in active transport and synapse assembly/stability. One possibility is that neurons have developed an elegant and efficient process that uses some of the proteins that aid in transporting vesicles to their final locations to establish concentrated clusters of SVs at those locations. In this hypothesis, the synapse-assembly proteins would be cotransported with SVs, stimulating their transport along the way, and then deposited at the active zone. However, past studies have found that only ~23% of SVs are cotransported with *SYD-2* (Wu *et al.* 2013) and that neither *SAD-1* nor *SYD-1* nor *SYD-2* requires *UNC-104* to accumulate at synapses (Patel *et al.* 2006). This suggests that *SYD-2*'s regulation of *UNC-104* may occur prior to SVs entering the axon, or perhaps only during the initial stages of transport, or that it may act on a subpopulation of SVs. *SYD-2* may be especially well adapted to fulfill both a regulatory role (in transport) and a structural role (at the active zone) since it appears to belong to a class of “intrinsically unstructured proteins” that are able to bind several partners in a structurally adaptive process (Wagner *et al.* 2009).

The generality of the active transport functions of synapse-assembly proteins is supported by the accompanying study, which shows that *SAD-1*, *SYD-1*, *SYD-2*, and *CDK-5*, as well as *STRD-1* (*STRAD* α), a protein known to directly interact with *SAD-1* (Kim *et al.* 2010), regulate the active transport of multiple cell soma organelles in a gatekeeper system that controls entry of those organelles into axons and dendrites (Edwards *et al.* 2015).

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Note added in proof: See Edwards *et al.* 2015 (pp. 117–141) in this issue for a related work.

Literature Cited

- Arribere, J. A., R. T. Bell, B. X. Fu, K. L. Artiles, P. S. Hartman *et al.*, 2014 Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in *Caenorhabditis elegans*. *Genetics* 198: 837–846.
- Baas, P. W., and S. Lin, 2011 Hooks and comets: the story of microtubule polarity orientation in the neuron. *Dev. Neurobiol.* 71: 403–418.
- Barkus, R. V., O. Klyachko, D. Horiuchi, B. J. Dickson, and W. M. Saxton, 2008 Identification of an axonal kinesin-3 motor for fast anterograde vesicle transport that facilitates retrograde transport of neuropeptides. *Mol. Biol. Cell* 19: 274–283.
- Barnes, A. P., B. N. Lilley, Y. A. Pan, L. J. Plummer, A. W. Powell *et al.*, 2007 LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. *Cell* 129: 549–563.
- Block, S. M., 2007 Kinesin motor mechanics: binding, stepping, tracking, gating, and limping. *Biophys. J.* 92: 2986–2995.
- Brenner, S., 1974 The genetics of *C. elegans*. *Genetics* 77: 71–94.
- Burton, P. R., and J. L. Paige, 1981 Polarity of axoplasmic microtubules in the olfactory nerve of the frog. *Proc. Natl. Acad. Sci. USA* 78: 3269–3273.
- Cross, R. A., 2004 The kinetic mechanism of kinesin. *Trends Biochem. Sci.* 29: 301–309.
- Crump, J. G., M. Zhen, Y. Jin, and C. I. Bargmann, 2001 The SAD-1 kinase regulates presynaptic vesicle clustering and axon termination. *Neuron* 29: 115–129.
- Dai, Y., H. Taru, S. L. Deken, B. Grill, B. Ackley *et al.*, 2006 SYD-2 Liprin- α organizes presynaptic active zone formation through ELKS. *Nat. Neurosci.* 9: 1479–1487.
- Dickinson, D. J., J. D. Ward, D. J. Reiner, and B. Goldstein, 2013 Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nat. Methods* 10: 1028–1034.
- Edwards, S. L., N. K. Charlie, M. C. Milfort, B. S. Brown, C. N. Gravlin *et al.*, 2008 A novel molecular solution for ultraviolet light detection in *Caenorhabditis elegans*. *PLoS Biol.* 6: e198.
- Edwards, S. L., L. M. Morrison, R. M. Yorks, C. M. Hoover, S. Boominathan, and K. G. Miller, 2015 UNC-16 (JIP3) acts through synapse assembly proteins to inhibit the active transport of cell soma organelles to *Caenorhabditis elegans* motor neuron axons. *Genetics* 201: 117–141.
- Endow, S. A., and D. S. Barker, 2003 Processive and nonprocessive models of kinesin movement. *Annu. Rev. Physiol.* 65: 161–175.
- Fernandez-Busnadiego, R., B. Zuber, U. E. Maurer, M. Cyrklaff, W. Baumeister *et al.*, 2010 Quantitative analysis of the native presynaptic cytomatrix by cryoelectron tomography. *J. Cell Biol.* 188: 145–156.
- Fouquet, W., D. Oswald, C. Wichmann, S. Mertel, H. Depner *et al.*, 2009 Maturation of active zone assembly by *Drosophila* Bruchpilot. *J. Cell Biol.* 186: 129–145.
- Goodwin, P. R., and P. Juo, 2013 The scaffolding protein SYD-2/liprin- α regulates the mobility and polarized distribution of dense-core vesicles in *C. elegans* motor neurons. *PLoS One* 8: e54763.
- Goodwin, P. R., J. M. Sasaki, and P. Juo, 2012 Cyclin-dependent kinase 5 regulates the polarized trafficking of neuropeptide-containing dense-core vesicles in *Caenorhabditis elegans* motor neurons. *J. Neurosci.* 32: 8158–8172.
- Hall, D. H., and E. M. Hedgecock, 1991 Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in *C. elegans*. *Cell* 65: 837–847.
- Hallam, S. J., A. Goncharov, J. McEwen, R. Baran, and Y. Jin, 2002 SYD-1, a presynaptic protein with PDZ, C2 and rhoGAP-like domains, specifies axon identity in *C. elegans*. *Nat. Neurosci.* 5: 1137–1146.
- Hammarlund, M., M. T. Palfreyman, S. Watanabe, S. Olsen, and E. M. Jorgensen, 2007 Open syntaxin docks synaptic vesicles. *PLoS Biol.* 5: e198.
- Hammarlund, M., S. Watanabe, K. Schuske, and E. M. Jorgensen, 2008 CAPS and syntaxin dock dense core vesicles to the plasma membrane in neurons. *J. Cell Biol.* 180: 483–491.
- Heidemann, S. R., J. M. Landers, and M. A. Hamborg, 1981 Polarity orientation of axonal microtubules. *J. Cell Biol.* 91: 661–665.
- Hill, A. J., R. Mansfield, J. M. Lopez, D. M. Raizen, and C. Van Buskirk, 2014 Cellular stress induces a protective sleep-like state in *C. elegans*. *Curr. Biol.* 24: 2399–2405.
- Hirokawa, N., and R. Takemura, 2005 Molecular motors and mechanisms of directional transport in neurons. *Nat. Rev. Neurosci.* 6: 201–214.
- Hirokawa, N., and Y. Noda, 2008 Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics. *Physiol. Rev.* 88: 1089–1118.
- Hoover, C. M., S. L. Edwards, S. C. Yu, M. Kittelmann, J. E. Richmond *et al.*, 2014 A novel CaM kinase II pathway controls the location of neuropeptide release from *Caenorhabditis elegans* motor neurons. *Genetics* 196: 745–765.
- Hung, W., C. Hwang, M. D. Po, and M. Zhen, 2007 Neuronal polarity is regulated by a direct interaction between a scaffolding protein, Neurabin, and a presynaptic SAD-1 kinase in *Caenorhabditis elegans*. *Development* 134: 237–249.
- Jacob, T. C., and J. M. Kaplan, 2003 The EGL-21 carboxypeptidase E facilitates acetylcholine release at *Caenorhabditis elegans* neuromuscular junctions. *J. Neurosci.* 23: 2122–2130.
- Jiao, W., S. Masich, O. Franzen, and O. Shupliakov, 2010 Two pools of vesicles associated with the presynaptic cytosolic projection in *Drosophila* neuromuscular junctions. *J. Struct. Biol.* 172: 389–394.
- Juo, P., T. Harbaugh, G. Garriga, and J. M. Kaplan, 2007 CDK-5 regulates the abundance of GLR-1 glutamate receptors in the ventral cord of *Caenorhabditis elegans*. *Mol. Biol. Cell* 18: 3883–3893.
- Kikkawa, M., E. P. Sablin, Y. Okada, H. Yajima, R. J. Fletterick *et al.*, 2001 Switch-based mechanism of kinesin motors. *Nature* 411: 439–445.
- Kim, J. S., W. Hung, P. Narbonne, R. Roy, and M. Zhen, 2010 *C. elegans* STRAD α and SAD cooperatively regulate neuronal polarity and synaptic organization. *Development* 137: 93–102.
- Kim, J. S., B. N. Lilley, C. Zhang, K. M. Shokat, J. R. Sanes *et al.*, 2008 A chemical-genetic strategy reveals distinct temporal requirements for SAD-1 kinase in neuronal polarization and synapse formation. *Neural Dev.* 3: 23.
- Kishi, M., Y. A. Pan, J. G. Crump, and J. R. Sanes, 2005 Mammalian SAD kinases are required for neuronal polarization. *Science* 307: 929–932.
- Kittelmann, M., J. Hegermann, A. Goncharov, H. Taru, M. H. Ellisman *et al.*, 2013 Liprin- α /SYD-2 determines the size of dense projections in presynaptic active zones in *C. elegans*. *J. Cell Biol.* 203: 849–863.
- Klassen, M. P., and K. Shen, 2007 Wnt signaling positions neuromuscular connectivity by inhibiting synapse formation in *C. elegans*. *Cell* 130: 704–716.
- Koushika, S. P., A. M. Schaefer, R. Vincent, J. H. Willis, B. Bowerman *et al.*, 2004 Mutations in *Caenorhabditis elegans* cytoplasmic dynein components reveal specificity of neuronal retrograde cargo. *J. Neurosci.* 24: 3907–3916.

- Kumar, J., B. C. Choudhary, R. Metpally, Q. Zheng, M. L. Nonet *et al.*, 2010 The *Caenorhabditis elegans* Kinesin-3 motor UNC-104/KIF1A is degraded upon loss of specific binding to cargo. *PLoS Genet.* 6: e1001200.
- Li, L., X. Tian, M. Zhu, D. Bulgari, M. A. Bohme *et al.*, 2014 *Drosophila* Syd-1, liprin-alpha, and protein phosphatase 2A B' subunit Wrd function in a linear pathway to prevent ectopic accumulation of synaptic materials in distal axons. *J. Neurosci.* 34: 8474–8487.
- Lilley, B. N., Y. A. Pan, and J. R. Sanes, 2013 SAD kinases sculpt axonal arbors of sensory neurons through long- and short-term responses to neurotrophin signals. *Neuron* 79: 39–53.
- Maeder, C. I., A. San-Miguel, E. Y. Wu, H. Lu, and K. Shen, 2014 In vivo neuron-wide analysis of synaptic vesicle precursor trafficking. *Traffic* 15: 273–291.
- Maniar, T. A., M. Kaplan, G. J. Wang, K. Shen, L. Wei *et al.*, 2012 UNC-33 (CRMP) and ankyrin organize microtubules and localize kinesin to polarize axon-dendrite sorting. *Nat. Neurosci.* 15: 48–56.
- Mello, C. C., J. M. Kramer, D. Stinchcomb, and V. Ambros, 1991 Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* 10(12): 3959–3970.
- Miller, K. E., J. DeProto, N. Kaufmann, B. N. Patel, A. Duckworth *et al.*, 2005 Direct observation demonstrates that liprin-alpha is required for trafficking of synaptic vesicles. *Curr. Biol.* 15: 684–689.
- Miller, K. G., M. D. Emerson, and J. B. Rand, 1999 G_{α} and diacylglycerol kinase negatively regulate the G_{α} pathway in *C. elegans*. *Neuron* 24: 323–333.
- Nelson, M. D., K. H. Lee, M. A. Churgin, A. J. Hill, C. Van Buskirk *et al.*, 2014 FMRFamide-like FLP-13 neuropeptides promote quiescence following heat stress in *Caenorhabditis elegans*. *Curr. Biol.*
- Niethammer, M., D. S. Smith, R. Ayala, J. Peng, J. Ko *et al.*, 2000 NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. *Neuron* 28: 697–711.
- Okada, Y., H. Yamazaki, Y. Sekine-Aizawa, and N. Hirokawa, 1995 The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. *Cell* 81: 769–780.
- Ou, C. Y., V. Y. Poon, C. I. Maeder, S. Watanabe, E. K. Lehrman *et al.*, 2010 Two cyclin-dependent kinase pathways are essential for polarized trafficking of presynaptic components. *Cell* 141: 846–858.
- Pack-Chung, E., P. T. Kurshan, D. K. Dickman, and T. L. Schwarz, 2007 A *Drosophila* kinesin required for synaptic bouton formation and synaptic vesicle transport. *Nat. Neurosci.* 10: 980–989.
- Patel, M. R., E. K. Lehrman, V. Y. Poon, J. G. Crump, M. Zhen *et al.*, 2006 Hierarchical assembly of presynaptic components in defined *C. elegans* synapses. *Nat. Neurosci.* 9: 1488–1498.
- Reynolds, N. K., M. A. Schade, and K. G. Miller, 2005 Convergent, RIC-8 dependent G_{α} signaling pathways in the *C. elegans* synaptic signaling network. *Genetics* 169: 650–670.
- Sasaki, S., A. Shionoya, M. Ishida, M. J. Gambello, J. Yingling *et al.*, 2000 A LIS1/NUDEL/cytoplasmic dynein heavy chain complex in the developing and adult nervous system. *Neuron* 28: 681–696.
- Schade, M. A., N. K. Reynolds, C. M. Dollins, and K. G. Miller, 2005 Mutations that rescue the paralysis of *C. elegans ric-8* (Synembryo) mutants activate the G_{α} pathway and define a third major branch of the synaptic signaling network. *Genetics* 169: 631–649.
- Shin, H., M. Wyszynski, K. H. Huh, J. G. Valtschanoff, J. R. Lee *et al.*, 2003 Association of the kinesin motor KIF1A with the multimodular protein liprin-alpha. *J. Biol. Chem.* 278: 11393–11401.
- Siksou, L., P. Rostaing, J. P. Lechère, T. Boudier, T. Ohtsuka *et al.*, 2007 Three-dimensional architecture of presynaptic terminal cytomatrix. *J. Neurosci.* 27: 6868–6877.
- Stiernagle, T., 2006 Maintenance of *C. elegans*, pp. *WormBook*, ed. The *C. elegans* Research Community WormBook, pp. 1–11.
- Stigloher, C., H. Zhan, M. Zhen, J. Richmond, and J. L. Bessereau, 2011 The presynaptic dense projection of the *Caenorhabditis elegans* cholinergic neuromuscular junction localizes synaptic vesicles at the active zone through SYD-2/liprin and UNC-10/RIM-dependent interactions. *J. Neurosci.* 31: 4388–4396.
- Sulston, J., and J. Hodgkin, 1988 Methods, pp. 596–597 in *The Nematode Caenorhabditis elegans*, edited by W. B. Wood. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Wagner, O. I., A. Esposito, B. Kohler, C. W. Chen, C. P. Shen *et al.*, 2009 Synaptic scaffolding protein SYD-2 clusters and activates kinesin-3 UNC-104 in *C. elegans*. *Proc. Natl. Acad. Sci. USA* 106: 19605–19610.
- Wu, Y. E., L. Huo, C. I. Maeder, W. Feng, and K. Shen, 2013 The balance between capture and dissociation of presynaptic proteins controls the spatial distribution of synapses. *Neuron* 78: 994–1011.
- Yan, J., D. L. Chao, S. Toba, K. Koyasako, T. Yasunaga *et al.*, 2013 Kinesin-1 regulates dendrite microtubule polarity in *Caenorhabditis elegans*. *eLife* 2: e00133.
- Zhen, M., and Y. Jin, 1999 The liprin protein SYD-2 regulates the differentiation of presynaptic termini in *C. elegans*. *Nature* 401: 371–375.
- Zheng, Q., S. Ahlawat, A. Schaefer, T. Mahoney, S. P. Koushika *et al.*, 2014 The vesicle protein SAM-4 regulates the processivity of synaptic vesicle transport. *PLoS Genet.* 10: e1004644.

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Supporting Information

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Synapse-Assembly Proteins Maintain Synaptic Vesicle Cluster Stability and Regulate Synaptic Vesicle Transport in *Caenorhabditis elegans*

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Figure S1 Other *unc-104* loss-of-function mutants show a strong loss-of-function SV transport phenotype after growth at either 13.5° or 20°.

Drawing illustrates the location and anatomy of the DA9 cholinergic motor neuron. The boxed region shows the region imaged. Lower panels show representative images and quantification of SNB-1-YFP in the indicated genotypes. SNB-1-YFP is expressed from the integrated transgene *wyls109*. Images are identically scaled relative to wild type. Graph data are means and standard errors from 14 animals each. n.s., not significant.

Figure S2 High levels of synaptic vesicles in the dendrites and somas of synapse assembly mutants.

Drawing illustrates the location and anatomy of the DA9 cholinergic motor neuron. The boxed region shows the region imaged. Lower panels show representative images and quantification of GFP-RAB-3 in strains with the indicated genotypes. GFP-RAB-3 is expressed from the integrated transgene *wyls85*. Images are identically scaled relative to wild type. Graph data are means and standard errors from 12-13 animals each. ***, **: $P \leq .001$, $P \leq .005$ for comparisons of each mutant to wild type.

Supplemental Materials and Methods

Quantitative fluorescence imaging of live animals and image analysis

Growth of Strains: For strains with wild type growth, we plated 12 L2-stage larvae on each of five locomotion plates and grew them 1 d at 14° + 5 d at 20° or 12 d at 14° or 17 d at 11°, as specified, to produce next generation young adult progeny that had grown at developed at specific temperatures. Growth times and plating numbers were modified as determined in pilot experiments for slow growing or lower fertility strains, such as strains containing *unc-104(ce782)* or *nud-2(ok949)*. ~55 - 60 young adults were selected and transferred to an unseeded plate immediately prior to mounting as described below.

Agarose pad slide production: We cleaned the glass slides before applying the agarose pad by placing sets of slides in slide staining dishes and shaking them for 30 min in dilute dishwasher soap followed by 1 min rinses in tap water and house distilled water, and 5 min rinses in 70% EtOH + 1% HCl and house distilled water followed by drying in a 60° oven. Cleaned slides were stored between layers of Kimwipes in a plastic container. We produced ~18-19 mm diameter 2% agarose pads using the method of (Sulston and Hodgkin 1988), with the agarose made in M9 buffer. We produced agarose pad slides in batches and stored them with the pads covered with a 24 x 30 mm coverslip to keep the surface smooth for up to 3-4 weeks in humidified containers at 4°. The protective coverslip was removed and discarded to allow the pad to dry slightly for the final 4 min of the incubation period before applying the coverslip containing the worms.

Mounting animals on agarose pad slides: For imaging synaptic vesicles, we transferred the pre-picked animals into a 30 μ l drop of 6 mM Levamisole (Sigma L-9756; freshly prepared in M9 buffer from a powder stock) in one pick-full and incubated them for 6 min 10 sec (from the time the animals first touched the drop) on a water-moistened 1.5 cm square pad of folded Kimwipe tissue under a Petri plate lid. For imaging dense core vesicles, we used 30 mg/ml BDM (2, 3-Butanedione monoxime; Sigma B0753) in M9 buffer as the anesthetic, and we incubated them for 10 min. After the incubation, we removed ~27 μ l of the solution using a P20 microinjection tip (Eppendorf 5242 956.003), leaving the worms behind in the remaining anesthetic and inverted the coverslip onto an 18-19 mm diameter 2% agarose pad. After straightening the coverslip slightly and nudging it ~1 mm to possibly help with dorsal/ ventral orientation, we sealed two diagonal corners with a dab of clear nail polish and imaged animals over the next 35 - 55 min.

Image Acquisition: We acquired images using a Nikon Eclipse Ti-E inverted microscope equipped with a Nikon CFI Apo TIRF 100X/ 1.49 N.A. objective, a Nikon motorized high resolution z-drive, and a motorized filter turret containing GFP, YFP, and Texas Red filter cubes (Semrock). Our illumination source was a SOLA Light Engine LED source (Lumencor). We acquired images with an ORCA Flash 4.0 16-bit camera (Hamamatsu) controlled by Metamorph v. 7.7 software. We controlled exposure times by using Metamorph to turn the LEDs on and off rather than using a shutter. We only collected images from animals with their ventral or dorsal surfaces facing the objective. For images collected from strains with the *cel/s255* and *cel/s263* transgenes, we located and focused on the target region using the mCherry signal before collecting images with the corresponding vesicle fluorophore to reduce variability due to photobleaching. The ventral target region for *cel/s255* and *cel/s263* strains was the region centered on the DA6/ DB6 cell soma pair; the dorsal target region was the synaptic region centered on where the DA6/ DB6 posterior commissures merge with the dorsal cord for *cel/s255* and the region posterior to where the DB7 commissure merges with the dorsal cord for *cel/s263*. Z-series interval sizes, plane numbers, and exposure times were as follows: *wyl/s85* strains: 0.312 μ m X 14, 100 msec GFP light; *cel/s255* strains: 0.312 μ m X 14, 75 msec YFP light; *cel/s263* strains: 0.312 μ m X 16, 20 msec GFP light. The LEDs on the SOLA Light Engine were turned off via software control between successive planes. Before imaging each strain, we measured the light power of the peak emission wavelength at the objective plane using an XR2100 power meter (Lumen Dynamics) and an XP750 objective plane light sensor (Lumen Dynamics) with the stage position set at a standard distance (z-position) from the objective. We then adjusted the percent power of the SOLA Light Engine to produce the targeted mW power for the experiment, which typically was ~42% or 25% of maximum power for GFP and Venus imaging, respectively. The percent power required to reach the target value typically varied by $\leq 1\%$ over the course of each experiment.

Processing Images: We used AutoDeblur Gold CWF (Media Cybernetics) to deconvolve the image stacks using the Adaptive PSF blind method and 10 iterations at the low noise setting. After deconvolving, we used Metamorph to make maximum intensity projections of each image stack. Images referred to as "straightened" were produced using Image J with the "Straighten" plug-in (line width 20 pixels; no spine fit).

Quantifying Images: We used Metamorph 7.7 for all analysis and quantification. To quantify dorsal axon synaptic vesicle fluorescence intensities per micron, we first used the multi-line tool to mark a 40 micron region that paralleled the brightest part of the synaptic region. We then used the trace region tool to trace the outline of this synaptic region and obtained the total integrated fluorescence in the region, which, after subtracting an identical "on animal" background region, we then normalized per μ m. To measure synaptic region length we first reviewed wild type (*wyl/s85*) synaptic region images and set a minimum threshold for synaptic puncta intensity in the synaptic region of DA9. We applied this threshold to all strains in the experiment. We measured the length of the region from the first to the last puncta that equaled or exceeded the threshold. To compute "total synaptic fluorescence", we traced the synaptic region defined by the above threshold and obtained the total integrated fluorescence of the region, from which we subtracted an identical "on animal" background region. When quantifying axonal DCV fluorescence intensities, we traced from edge to edge of the field of view. We then copied the region and shifted it onto a region of the animal immediately adjacent to the cord to obtain the background value. We logged data to an Excel spreadsheet, which subtracted the background and computed the total fluorescence per micron of cord length. We produced regions for the DA9 cell soma and background similarly. For strains in which we quantified DA6 and DB6 cell somas, we traced each soma separately and added them together. To trace the DA9 dendrite region, we traced from the point where the dendrite begins widening as it leaves the cell soma to a point ~20 μ m distant (or until it went out of focus if it did so), measuring the exact

distance with the multi-line tool. For images quantifying the distal dendrite regions around DA6 and DB6, we used the multi-line tool to trace 20 μm along the dendrite starting at one of the cell soma boundaries that faces the other cell soma. If the region between and including the two somas was $>20 \mu\text{m}$, we deleted these lines and started each of the two dendrite regions at the outer edge of each soma, proceeding outward to each edge of the image. If the region between and including the two somas was $\leq 20 \mu\text{m}$, we made a second line that followed the dendrite in the opposite direction starting at the inner boundary of the other cell soma and proceeding in the opposite direction. If we came to the other cell soma before reaching 20 μm , we continued measuring across the soma to the dendrite on the other side of the soma until we reached 20 μm . We then started our dendrite traces at the end of each of these two lines, proceeding outward to each edge of the image using the Trace Region tool and combining the data from the two dendrite regions. For images quantifying the proximal dendrites around DA6 and DB6 we used the trace region tool to trace the region between the two cell somas, stopping at 20 microns if the region between the two somas was >20 microns.

Producing Representative Images: After quantifying an image set we produced representative images for display by saving 8-bit versions of an image that was close to the mean $\pm$ standard error for the set. All representative images were scaled identically to the reference strain, which was usually wild type at a specific temperature.

Time lapse video microscopy of dense core vesicle active transport in live animals

Growth of Strains: Animals were grown to the young adult stage as described above for quantitative fluorescence imaging, except we used more locomotion plates (30 for wild type; 60 for *ce782*) to provide sufficient numbers of animals for the many time lapse mountings.

Agarose Pad Slide Production: To avoid variability in agarose pad dryness, which affects animal compression and can affect active transport activity, we standardized a protocol for agarose pad slide production for time lapse imaging. After melting 0.2 g of agarose in 10 mls of M9 in a 50 ml glass bottle, we poured 0.5 ml of the molten agarose into several 1.5 ml microcentrifuge tubes in a 100° Isotemp block (Fisher). We inserted a blunt P200 pipet tip into each of 4 microcentrifuge tubes filled with distilled water and heated them to 100° in the Isotemp block. We preheated 8 clean slides (see above protocol for cleaning slides) along the front and back plastic surfaces of the Isotemp block outside of the metal tube-holding blocks. We then arranged 4 pairs of spacer slides (slides with 1 layer of lab labeling tape + 1 layer of $\frac{3}{4}$ inch Scotch tape to raise them off the bench), tape side up, in front of the Isotemp block. To make the agarose pads, we placed one of the warm bottom slides between the first pair of spacer slides. We then moved one of the warm top slides to a Sardstedt Styrofoam block that was in front of the first pair of spacer slides such that the slide was in perpendicular orientation relative to the bottom slide. We removed the first hot blunt P200 tip from its hot water tube in the Isotemp block, shook it to get rid of excess water, and attached it to a P100 set on 50 μl . We then pipetted 50 μl from the open tube of molten agarose, lifting the tube out of the block briefly during pipetting. We began pipetting immediately when the tip touched the agarose, and then set the tube back in the block and pipetted the agarose onto the bottom slide between the spacers. After hovering the top slide over the agarose drop for ~ 1 sec, we then applied the top slide perpendicular to the bottom slide, from one edge of the drop to the other. We adjusted the amount of finger pressure to give an average size pad of 18-19 mm in diameter. After the 4 slides were made, we pre-warmed another set of 8 slides and 4 blunt P200 tips for the next set. We then removed the top slide from the first slide pair and immediately applied a 24 X 30 mm coverslip to the agarose pad such that the long dimensions of the coverslip and slide are perpendicular to each other and the coverslip hangs off the edge of the slide, and then transferred this to a humidified plastic container. Slides were stored at 4° in a humidified plastic container until they were used the next day.

Temperature control: To mount and image animals at 13.5° we transferred our entire imaging system to a constant temperature room set at 13.5°, monitoring the temperature with CheckTemp digital thermometers (Hanna). To mount and image animals at 25°, we used portable heaters to control the temperature of the microscope room and CheckTemp thermometers in the mounting and imaging areas of the room to monitor the temperature.

Mounting animals on slides: We first equilibrated the agarose pad slides at the intended temperature (in their humidified container) and prepared fresh 6 mM Levamisole in M9 from a powder stock of Levamisole (Acros Organics; AC187870100; <6 months old). To mount animals on a pad, we pre-picked 30-40 young adults to an unseeded plate, applied 30 μl of 6 mM levamisole to a 24 X 30 mm coverslip, picked the 30 young adults to the droplet in one pick-full, and incubated them for 6:10 min on a moist Kimwipe square under a Petri plate lid. Immediately at the start of this incubation we removed an agarose pad slide from the humidified container, removed its protective coverslip, applied 10 μl of 6 mM Levamisole in M9 to the pad, and re-applied the same coverslip. With ~ 40 seconds remaining in the 6:10 min incubation time, we removed the coverslip from the slide, taking care to remove as much of the 10 μl of liquid as possible along with the coverslip and blotting around the edge of the agarose pad with a Kimwipe. At the end of the 6:10 min, we used a Kimwipe to dry the moisture from the bottom of the coverslip containing the worms, and, while supporting the coverslip on a Petri plate lid, removed 23.5 – 25.5 μl of liquid using a P20 and bent gel loading tip while viewing the animals under the stereomicroscope. We then inverted the coverslip onto the agarose pad and sealed two diagonal corners with nail polish. The goal was to have only a very small amount of liquid overflow around the edges of the pad, and the coverslip relatively loose, but not floating on liquid.

Image Acquisition: We acquired images using the same microscope, camera, and computer system described above for use in collecting static images. For dense core vesicle imaging in strains carrying *ceIs201*, we adjusted the light power of the SOLA LED light engine to 23% (a good level for reducing bleaching during the time lapse without compromising signal). We mounted the slide on the microscope and scanned the pad left to right, top to bottom using transmitted light to find the first animal oriented with its dorsal or ventral cord facing the objective. Using the YFP filter, we then looked for motor neuron commissures with 3 or more vesicles visible, and ideally containing 2 or more commissures in the same field of view. At 5:30 min after applying the coverslip, we started the first time lapse and, if we could find another animal within 2 min of completing the first, we also collected a second time lapse. Each time lapse consisted of 100 center-quad frames collected at 40 msec exposure times at 1 sec intervals (13.5°) or 394 msec intervals (25°). For synaptic vesicle imaging in strains carrying *ceIs263*, we used similar methods, except we set the SOLA light engine at 63% and the exposure time at 50 msec, and we collected 121 frames at 333 msec intervals. For synaptic vesicle imaging, the target region was the DA6/ DB6 commissures proximal to the cell somas in animals oriented with their ventral surface toward the objective, which we located using the soluble mCherry signal in that transgenic array.

Processing Time Lapse Images and Converting them to Kymographs: We used the Review Multidimensional Data Metamorph plug-in to convert the time lapse images into a multi-image TIFF file. After adjusting the scaling and sliding through the file to determine the point in the commissure that was closest to the cell soma at which an INS-22-Venus or RFP-RAB-3 punctum could be detected, we opened the Kymograph plug-in and used the multi-line tool to trace the commissure, starting at this point and proceeding anterogradely. During tracing we moved back and forth between tracing and the slider such that we could visualize the precise commissure path as DCVs or SVs moved through sparsely populated regions of the commissure (i.e. following the puncta by clicking as they move along the commissure). After ending the trace, we used the Kymograph plug-in to set the line width at 15 - 40, depending on the extent to which the animal moved. If necessary, we additionally shifted the position of the line slightly to compensate for animal movement. For some movements that were too large to be compensated for by changing the line width, we manually measured the distance moved using the multi-line tool on a single frame, but scrolling back and forth through multiple frames to determine the starting and ending point of the movement relative to other landmarks in the commissure such as kinks, turns, or stationary vesicles. In other cases where animal movement prevented us from making a full 100 frame kymograph, we made kymographs that only included the segments during which vesicles moved and used those for analysis, noting that no vesicle movements occurred in the frames not included if that was the case. Frames were excluded from the total frame count if vesicle movements occurred that could not be quantified manually or via kymographs. We then created, reviewed, and saved the Kymograph, used the Save Regions plug-in to save the line traces associated with the file, and noted the optimal line width for each trace.

Quantifying DCV Movements from Kymographs: After opening the multi-plane tiff file, using the Load Regions plug-in to re-load the line traces, and setting the line width at the above determined optimum, we re-created the kymograph (previously saved kymographs can't be used to log data). We then used the Line tool to trace each anterograde movement and logged the Distance and Time data for each movement before repeating for the retrograde movements. We defined movements at 25° (or 13.5° in parentheses) as events with velocities of ≥ 0.35 (0.137) $\mu\text{m}/\text{sec}$ for times of ≥ 0.90 (2.0) sec (for short movements lasting 0.90 (2.0) – 3.0 (8.0) sec) or events with velocities of ≥ 0.075 (0.029) $\mu\text{m}/\text{sec}$ for times of ≥ 3.0 (8.0) sec. The movement ends when it pauses for ≥ 2 (5) sec, when it reverses direction, or when it reaches the end of the time course. Movements that changed their velocity without pausing or changing direction were treated as single point-to-point straight line movements, with one end of the line at the beginning of the movement and the other end at the end of the movement (thus creating an average distance and time from point A to point B). For synaptic vesicle movement analysis, we also quantified the fraction of time each vesicle spent in a paused state. Pauses were analyzed on all movies having at least 60 frames for movement analysis (out of a maximum of 121 frames). These 60-121 – frame sets were observed for movements/ pauses both frame-by-frame and using kymographs. Because of the occasional movement of puncta out of the field of view, each punctum's time spent paused was compared only to its own total time visible – quantified as a proportion of time visible spent paused (i.e. 0.00 indicating that the punctum never paused and 1.00 indicating that the punctum was paused for the entire time it was visible).

Time lapse movies: We annotated multi-plane TIFF files using Metamorph Display > Graphics > Text or Arrow. We converted selected multi-plane TIFF files to movies using Metamorph Stack > Make Movie > AVI, specifying 12/30ths for each frame (which is real time for the 25° acquisitions collected at 394 msec intervals). Movies were saved as full frames/ uncompressed and converted to Quicktime using Wondershare Video Converter Platinum.

C. elegans Non-wild type Strains

Strain name	Genotype (origin and/ or first use cited if not produced in this study)
KG4724	<i>cdk-5(gm336); cels255</i>
KG4247	<i>cels201 [unc-17::INS-22-Venus, unc-17::mCherry, unc-17::ssmCherry]</i> (Hoover <i>et al.</i> 2014)
KG4664	<i>cels255 [unc-129::INS-22-Venus, unc-129::mCherry, unc-129::ssmCherry]</i>
KG4678	<i>cels263 [unc-129::GFP-RAB-3, unc-129::mCherry]</i>
RB1022	<i>nud-2(ok949)</i> (<i>C. elegans</i> gene knockout consortium) (Ou <i>et al.</i> 2010)
KG4732	<i>nud-2(ok949); cels263</i>
KG4736	<i>nud-2(ok949); syd-2(ok217); cels263</i>
KG4503	<i>nud-2(ok949); unc-104(ce782)</i>
KG4527	<i>nud-2(ok949); unc-104(ce782); wyls85 [itr-1::GFP-RAB-3]</i>
KG4506	<i>nud-2(ok949); wyls85 [itr-1::GFP-RAB-3]</i>
KG4400	<i>sad-1(ce749) 2X outcrossed</i>
KG4610	<i>sad-1(ce749); wyls85 [itr-1::GFP-RAB-3]</i>
KG4735	<i>sad-1(ce749); cels263</i>
KG4734	<i>syd-1(tm6234); cels263</i>
KG4615	<i>syd-1(tm6234); wyls85 [itr-1::GFP-RAB-3]</i>
KG4401	<i>syd-2(ce759) 2X outcrossed</i>
KG4560	<i>syd-2(ce759); wyls85 [itr-1::GFP-RAB-3]</i>
KG4599	<i>syd-2(ok217) 3X outcrossed</i> (<i>C. elegans</i> gene knockout consortium) (Wagner <i>et al.</i> 2009)
KG4725	<i>syd-2(ok217); cels255</i>
KG4733	<i>syd-2(ok217); cels263</i>
KG4526	<i>syd-2(ok217); wyls85 [itr-1::GFP-RAB-3]</i>
KG4660	<i>syd-2(ok217); wyls85 [itr-1::GFP-RAB-3]; ceEx454 [itr-1::syd-2 gene]</i>
KG4386	<i>unc-104(ce782) 2X outcrossed</i>
KG4727	<i>unc-104(ce782); cdk-5(gm336); cels255</i>
KG4472	<i>unc-104(ce782); cels201</i>
KG4645	<i>unc-104(ce782); cels255 [unc-129::INS-22-Venus, unc-129::mCherry, unc-129::ssmCherry]</i>

KG4704	<i>unc-104(ce782); cels263 [unc-129::GFP-RAB-3, unc-129::mCherry]</i>
KG4700	<i>unc-104(ce782); sad-1(ce749)</i>
KG4621	<i>unc-104(ce782); syd-2(ce759)</i>
KG4620	<i>unc-104(ce782); syd-2(ok217)</i>
KG4726	<i>unc-104(ce782); syd-2(ok217); cels255</i>
KG4413	<i>unc-104(ce782); wyls85 [itr-1::GFP-RAB-3]</i>
KG4716	<i>unc-104(ce813); cels263 [unc-129::GFP-RAB-3, unc-129::mCherry]</i>
RM977	<i>unc-104(e1265)</i>
KG2279	<i>unc-104(e1265); wyls109</i>
KG1935	<i>unc-104(ce515)</i>
KG2116	<i>unc-104(ce515); wyls109</i>
TV1229	<i>wyls85 [itr-1::GFP-RAB-3]</i> (Klassen and Shen 2007)
TV2436	<i>wyls109 [mig-13::CFP-RAB-3, mig-13::SNB-1-YFP, mig-13::SNG-1-mCherry]</i> (Poon <i>et al.</i> 2008)

Mutations and Methods Used for Genotyping

Mutation	Description of molecular lesion	Effect on protein	Method(s) used for genotyping	References for mutant isolation and/ or mutation description
<i>cdk-5(gm336)</i>	760 bp deletion that starts in proximal promoter and deletes exons 1, 2, and most of exon 3 (>60% of coding region)	Putative null	PCR with primers inside deleted region	(Juo <i>et al.</i> 2007)
<i>nud-2(ok949)</i>	1109 bp deletion and a 1 bp insertion. Starts at the predicted ATG of nud-2 and deletes the entire open reading frame except the last part of the last exon.	Putative null	PCR with primers inside deleted region	<i>C. elegans</i> Gene Knockout Consortium and (Fridolfsson <i>et al.</i> 2010)
<i>sad-1(ce749)</i>	Q57Stop (out of 835 or 914 amino acids, depending on the isoform). Mutation position is the same in both isoforms.	Putative null	Make 250 bp PCR product centered on the mutation, followed by restriction digest with Mse I (the mutation creates an Mse I site).	Edwards <i>et al.</i> , co-submitted
<i>syd-1(tm6234)</i>	1266 bp deletion starting in the intron between exons 7 and 8 and ends in the middle of exon 11 of the “b” isoform. The deletion eliminates R249 – Y471 (out of 942 amino acids total) and results in a frame shift predicted to prevent translation of the remaining protein.	Putative null	Behavioral phenotypes and PCR with primers inside deleted region	Japanese National Bioresource Project for the Experimental Animal “Nematode <i>C. elegans</i> ” and this study.
<i>syd-2(ce759)</i>	Q387Stop (out of 1139 amino acids total)	Putative null	Behavioral phenotypes and PCR followed by sequencing	Edwards <i>et al.</i> , co-submitted
<i>syd-2(ok217)</i>	~2 Kb deletion covering most of the N-terminal coiled coil domains. Results in a frame shift and stop codon at amino acid 200 (out of 1139 amino acids total).	Putative null	Behavioral phenotypes and PCR with primers inside deleted region	<i>C. elegans</i> Gene Knockout Consortium and (Wagner <i>et al.</i> 2009; Kittelmann <i>et al.</i> 2013)
<i>unc-104(ce782)</i>	G to A missense mutation causing G105E amino acid change	Temperature sensitive mutation	Behavioral phenotypes and PCR followed by sequencing	This study
<i>unc-104(ce813)</i>	G to A missense mutation causing G105E amino acid change	Temperature sensitive mutation	Behavioral phenotypes and PCR followed by sequencing	This study
<i>unc-104(e1265)</i>	D1497N missense mutation PH domain of cargo binding domain	Strong loss-of-function	Not crossed in this study	(Kumar <i>et al.</i> 2010)
<i>unc-104(ce515)</i>	Q1418Stop (out of 1584 amino acids total)	Strong loss-of-function	Not crossed in this study	This study

Plasmids

KG#230	unc-129:: expression vector	(Edwards <i>et al.</i> 2009)
KG#240	unc-129::____-mCherry expression vector	(Edwards <i>et al.</i> 2009)
KG#255	ttx-3::RFP	(Edwards <i>et al.</i> 2009)
KG#374	unc-129::____-Venus expression vector	(Edwards <i>et al.</i> 2009)
KG#470	rab-3:: [Sbf I] expression vector	Synthesized 2 complementary oligos containing Nhe I and Age I sticky ends and Sbf I and Kpn I sites in between. Hybridization of the 2 oligos to each other produces Nhe I and Age I sticky ends. After hybridization, we cloned this synthetic insert (non-phosphorylated on its 5' termini) into Nhe I/ Age I cut KG#59.
KG#471	mig-13:: expression vector	Used BamH I/ Apa I to cut out the ~825 bp MCS + unc-54 3' control region from KS#1 mig-13, leaving the 6.1 kb vector fragment containing the mig-13:: promoter. To this vector fragment, we ligated the 1000 bp BamH I/ Apa I fragment from KG#470.
KG#481	unc-17::rab-3 cDNA	Used AffinityScript Multiple Temperature Reverse Transcriptase and a primer engineered with a restriction site to make the full length rab-3 cDNA minus its stop codon (0.66 Kb). Used Herculase II polymerase and primers engineered with restriction sites to amplify and clone the cDNA into Nhe I/ Age I cut KG#94 unc-17:: expression vector (6.9 Kb).
KG#502	unc-129:: [Asc I] expression vector	Used Herculase II polymerase and primers engineered with restriction sites to amplify the 1100 bp Msc I/ Apa I fragment from KG#230 unc-129:: expression vector and cloned it into Msc I/ Apa I cut KG#230 unc-129::____ (5.4 Kb) [5' primer contains sequential Msc I and Asc I sites].
KG#503	unc-129:: [Asc I/ Sbf I] expression vector	Used Asc I/ Apa I to cut out the ~1100 bp MCS and unc-54 3' control region from KG#502, leaving the 5.4 kb vector fragment containing the unc-129 promoter. To this vector fragment, we ligated the 1100 bp Asc I/ Apa I fragment (identical except it has an Sbf I site) cut from KG#471 mig-13:: expression vector.
KG#504	unc-129::mCherry-____	Used Herculase II polymerase and primers engineered with restriction sites to amplify the 870 bp RFPa from KS#3 (mig-13::RFP-rab-3) and cloned it into Asc I/ Sbf I cut KG#503 unc-129::____ (6.4 Kb).
KG#563	unc-129::mCherry-RAB-3	Used Herculase II polymerase and primers engineered with restriction sites to amplify the 0.66 Kb rab-3 cDNA from KG#481 mig-13::GFP-RAB-3 and cloned it into Sbf I cut KG#504 (6.4 Kb).
KG#59	rab-3:: expression vector	(Schade <i>et al.</i> 2005)
KG#648	unc-17β::ssmCherry	(Hoover <i>et al.</i> 2014)
KG#687	unc-129::ssmCherry	Used Nhe I/ Age I to cut out the 0.9 Kb ssmCherry insert from KG#648 (unc-17 ::ssmCherry) and cloned it into the like-digested unc-129::vector KG#230 (6400 bp).
KG#691	unc-129::INS-22-Venus	Used Herculase II polymerase and primers engineered with restriction sites to amplify the 333 bp ins-22 gene (without its stop codon and with reading frame adjusted to allow fusion to downstream Venus) from N2 genomic DNA and cloned it into Nhe I/ Age I cut KG#374 (unc-129::____-Venus; 7.2 Kb).
KG#730	itr-1:: expression vector	Used Herculase II and primers engineered with restriction sites to amplify the 2.3 Kb itr-1 promoter from N2 genomic DNA and clone it into Pst I/ Bam HI cut pPD96.52 (3.7 Kb after removal of the myo-3:: promoter). The boundaries of this itr-1 promoter were described in (Klassen and Shen 2007).
KG#731	itr-1::SYD-2 gene	Used Herculase II and primers engineered with restriction sites to amplify the 5.4 Kb SYD-2 gene from N2 genomic DNA and cloned it into Nhe I/ Kpn I cut KG#730 (6.0 Kb).
KG#776	unc-129::GFP-RAB-3	Used Herculase II Polymerase and engineered primers to produce overlapping PCR fragments followed by Gibson Assembly Master Mix (NEB) to insert the GFP fragment (PCRed from pPD94.81) into a PCR fragment derived from KG#563 (unc-129::mCherry-RAB-3). The GFP exactly substitute for mCherry such that the start codon of GFP immediately follows the AAAA that preceded the mCherry start codon.
KG#94	unc-17:: expression vector	(Edwards <i>et al.</i> 2009)
KS#1	Pmig-13::delta pSM (mig-13:: expression vector)	Gift of Kang Shen, Stanford University (Klassen and Shen 2007)
KS#3	mig-13::RFP-RAB-3	Gift of Kang Shen, Stanford University (Klassen and Shen 2007)
KS#5	odr-1::GFP	Gift of Kang Shen, Stanford University (Klassen and Shen 2007)
pPD94.81	unc-54::GFP	Gift of Andrew Fire, Stanford University
pPD96.52	myo-3:: expression vector	Gift of Andrew Fire, Stanford University

Transgenic Arrays and Genomically Integrated Insertions

Array name	Insertion location	Experimental contents and injection concentrations	Co-transformation markers and injection concentrations	References for transgene or integrated insertion (if not made in this study)
ceEx454	extrachromosomal array	KG#731 [itr-1::syd-2 gene] – 2.5 ng/ μl	KS#5 [odr-1::GFP] – 20 ng/ μl	
cels201	I: 0.00 +/- 0.45	KG#686 (unc-17::INS-22-Venus) – 6 ng/ μl	KG#654 (unc-17::ssmCherry) - 4 ng/ μl KG#381 (myo-2::RFP) – 1.5 ng/ μl KG#248 (unc-17::RFP) - 4 ng/ μl	(Hoover <i>et al.</i> 2014)
cels255	I: -23 or +21	KG#691 [unc-129::INS-22-Venus] – 1.5 ng/ μl KG#240 [unc-129::mCherry] – 2.5 ng/ μl KG#687 [unc-129::ssmCherry] – 2.5 ng/ μl	KG#255 (ttx-3::RFP) – 15 ng/ μl	
cels263	III: near -0.92	KG#776 [unc-129::GFP-RAB-3] – 0.75 ng/ μl KG#240 [unc-129::mCherry] – 1.5 ng/ μl	KG#255 (ttx-3::RFP) – 15 ng/ μl	
wyls85	V: near 4.63	itr-1::GFP-RAB-3 – 10 ng/ μl	odr-1::dsRed - 20 ng/ μl	(Klassen and Shen 2007)
wyls109	V	Pmig-13::CFP-RAB-3 Pmig-13::SNB-1-YFP Pmig-13::SNG-1-mCherry	odr-1::GFP - 20 ng/ μl	(Poon <i>et al.</i> 2008)

Supplemental References

- Edwards, S. L., N. K. Charlie, J. E. Richmond, J. Hegermann, S. Eimer *et al.*, 2009 Impaired dense core vesicle maturation in *Caenorhabditis elegans* mutants lacking Rab2. *J Cell Biol* 186: 881-895.
- Fridolfsson, H. N., N. Ly, M. Meyerzon and D. A. Starr, 2010 UNC-83 coordinates kinesin-1 and dynein activities at the nuclear envelope during nuclear migration. *Dev Biol* 338: 237-250.
- Hoover, C. M., S. L. Edwards, S. C. Yu, M. Kittelmann, J. E. Richmond *et al.*, 2014 A Novel CaM Kinase II Pathway Controls the Location of Neuropeptide Release from *Caenorhabditis elegans* Motor Neurons. *Genetics* 196: 745-765.
- Juo, P., T. Harbaugh, G. Garriga and J. M. Kaplan, 2007 CDK-5 regulates the abundance of GLR-1 glutamate receptors in the ventral cord of *Caenorhabditis elegans*. *Mol Biol Cell* 18: 3883-3893.
- Kittelmann, M., J. Hegermann, A. Goncharov, H. Taru, M. H. Ellisman *et al.*, 2013 Liprin-alpha/SYD-2 determines the size of dense projections in presynaptic active zones in *C. elegans*. *J Cell Biol* 203: 849-863.
- Klassen, M. P., and K. Shen, 2007 Wnt signaling positions neuromuscular connectivity by inhibiting synapse formation in *C. elegans*. *Cell* 130: 704-716.
- Kumar, J., B. C. Choudhary, R. Metpally, Q. Zheng, M. L. Nonet *et al.*, 2010 The *Caenorhabditis elegans* Kinesin-3 motor UNC-104/KIF1A is degraded upon loss of specific binding to cargo. *PLoS Genet* 6: e1001200.
- Ou, C. Y., V. Y. Poon, C. I. Maeder, S. Watanabe, E. K. Lehrman *et al.*, 2010 Two cyclin-dependent kinase pathways are essential for polarized trafficking of presynaptic components. *Cell* 141: 846-858.
- Poon, V. Y., M. P. Klassen and K. Shen, 2008 UNC-6/netrin and its receptor UNC-5 locally exclude presynaptic components from dendrites. *Nature* 455: 669-673.
- Schade, M. A., N. K. Reynolds, C. M. Dollins and K. G. Miller, 2005 Mutations that Rescue the Paralysis of *C. elegans ric-8* (Synembryn) Mutants Activate the $G\alpha_s$ Pathway and Define a Third Major Branch of the Synaptic Signaling Network. *Genetics* 169: 631-649.
- Sulston, J., and J. Hodgkin, 1988 Methods, pp. 596-597 in *The Nematode Caenorhabditis elegans*, edited by W. B. Wood. Cold Spring Harbor Laboratory.
- Wagner, O. I., A. Esposito, B. Kohler, C. W. Chen, C. P. Shen *et al.*, 2009 Synaptic scaffolding protein SYD-2 clusters and activates kinesin-3 UNC-104 in *C. elegans*. *Proc Natl Acad Sci U S A* 106: 19605-19610.

File S2. Wild type locomotion after growth at 13.5°.

File S2 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is an L4-stage N2 (wild type) animal hatched and grown at 13.5° and then equilibrated for 20 min on a 23.5° plate. The ~30 sec movie shows spontaneous movement through a thin lawn of *E. coli* bacteria followed by tail stimulation with a wire pick.

File S3. *unc-104(ce782)* locomotion after growth at 13.5°.

File S3 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is an L4-stage *unc-104(ce782)* mutant hatched and grown at 13.5° and then equilibrated for 20 min on a 23.5° plate. The ~30 sec movie shows spontaneous movement through a thin lawn of *E. coli* bacteria followed by tail stimulation with a wire pick.

File S4. Wild type locomotion after growth at 20.0°.

File S4 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is an adult N2 (wild type) animal grown at 20.0° and then equilibrated for 20 min on a 23.5° plate. The ~30 sec movie shows spontaneous movement through a thin lawn of *E. coli* bacteria followed by tail stimulation with a wire pick.

File S5. *unc-104(ce782)* locomotion after growth at 20.0°.

File S5 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is an adult *unc-104(ce782)* mutant hatched and grown at 20.0° and then equilibrated for 20 min on a 23.5° plate. The ~30 sec movie shows the animal on a thin lawn of *E. coli* bacteria and includes tail stimulation with a wire pick to show the strong state of paralysis of the animal.

File S6. Time lapse of dense core vesicle movements in wild type at 13.5°.

File S6 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is a region of a cholinergic motor neuron commissure from a wild type adult that was hatched, grown, mounted, and imaged at 13.5°. The actual length of the time lapse was 100 seconds (1 frame/ sec), but the movie has been sped up ~2.5 – fold (to 39 sec) to compensate for slower vesicle movements at the lower temperature.

File S7. Time lapse of dense core vesicle movements in *unc-104(ce782)* at 13.5°.

File S7 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is a region of a cholinergic motor neuron commissure from an *unc-104(ce782)* mutant adult that was hatched, grown, mounted, and imaged at 13.5°. The actual length of the time lapse was 100 seconds (1 frame/ sec), but the movie has been sped up ~2.5 – fold (to 39 sec) to compensate for slower vesicle movements at the lower temperature.

File S8. Time lapse of dense core vesicle movements in wild type at 25°.

File S8 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is a region containing two cholinergic motor neuron commissures feeding into the dorsal nerve cord. This wild type adult was hatched and grown at 13.5°, and then acutely shifted to 25° (on pre-equilibrated plates) for 23 min during picking and mounting before acquiring the time lapse. Frames were acquired every 394 msec, and the movie frame rate is real time.

File S9. Time lapse of dense core vesicle movements in *unc-104(ce782)* at 25°.

File S9 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is a region of a cholinergic motor neuron commissure. This *unc-104(ce782)* mutant adult was hatched and grown at 13.5°, and then acutely shifted to 25° (on pre-equilibrated plates) for 23 min during picking and mounting before acquiring the time lapse. Frames were acquired every 394 msec, and the movie frame rate is real time.

File S10. Time lapse of synaptic vesicle movements in wild type at 25°.

File S10 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is a region of a cholinergic motor neuron commissure. This wild type adult was hatched and grown at 13.5°, and then acutely shifted to 25° for 23 min before acquiring the time lapse. Frames were acquired every 333 msec, and the movie frame rate is in real time.

File S11. Time lapse of synaptic vesicle movements in *unc-104(ce782)* at 25°.

File S11 is available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177337/-/DC1. Shown is a region of a cholinergic motor neuron commissure. This *unc-104* mutant adult was hatched and grown at 13.5°, and then acutely shifted to 25° for 23 min before acquiring the time lapse. Frames were acquired every 333 msec, and the movie frame rate is in real time.