Kinesin Mutations Cause Motor Neuron Disease Phenotypes by Disrupting Fast Axonal Transport in Drosophila

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ABSTRACT

Previous work has shown that mutation of the gene that encodes the microtubule motor subunit kinesin heavy chain (*Khc*) in Drosophila inhibits neuronal sodium channel activity, action potentials and neurotransmitter secretion. These physiological defects cause progressive distal paralysis in larvae. To identify the cellular defects that cause these phenotypes, larval nerves were studied by light and electron microscopy. The axons of *Khc* mutants develop dramatic focal swellings along their lengths. The swellings are packed with fast axonal transport cargoes including vesicles, synaptic membrane proteins, mitochondria and prelysosomal organelles, but not with slow axonal transport cargoes such as cytoskeletal elements. *Khc* mutations also impair the development of larval motor axon terminals, causing dystrophic morphology and marked reductions in synaptic bouton numbers. These observations suggest that as the concentration of maternally provided wild-type KHC decreases, axonal organelles transported by kinesin periodically stall. This causes organelle jams that disrupt retrograde as well as anterograde fast axonal transport, leading to defective action potentials, dystrophic terminals, reduced transmitter secretion and progressive distal paralysis. These phenotypes parallel the pathologies of some vertebrate motor neuron diseases, including some forms of amyotrophic lateral sclerosis (ALS), and suggest that impaired fast axonal transport is a key element in those diseases.

THE asymmetric organization and long projections of neurons place special demands on cytoplasmic motility processes. The axon of a motor neuron can be a meter in length and contain >99% of the cell's total cytoplasm. Because the axon supports little synthesis of proteins or membrane, materials must be constantly imported from the synthetically active cytoplasm of the cell body. This anterograde transport is balanced by retrograde transport that returns old, damaged components and endocytic material to the cell body for recycling. Axonal transport is essential for axon development and the maintenance of neuronal function. When it is blocked by disease or experimental manipulation, axons lose their signalling capacity and eventually degenerate.

Axonal transport proceeds in two rate components. Cytoskeletal elements such as neurofilaments, tubulin and actin move in the slow component (0.5–2 mm/day), while membrane-bounded organelles and synaptic membrane proteins move in the fast component (50–200 mm/day) (BRADY 1991; VALLEE and BLOOM 1991; OCHS and BRIMIJOIN 1993; HIROKAWA 1996). The molecular mechanism of slow axonal transport is not well understood. However, a general understanding of the mechanism of fast axonal transport has evolved from studies of microtubule polarity within axons (HEIDEMANN *et al.* 1981) and from the characterization of families of microtubule motor proteins (reviewed by BLOOM and ENDOW 1994; HIROKAWA 1996; SCHOLEY 1996). Axonal microtubules, which are oriented with their minus ends toward the cell body and their plus ends toward the axon terminal, serve as polarized tracks for the ATP-driven movement of various kinesins and cytoplasmic dyneins. Plus-end directed (anterograde) kinesins carry cargo toward the axon terminal. Minus-end directed (retrograde) motors, such as cytoplasmic dynein and perhaps NCD-related kinesins, carry cargo back toward the cell body.

The likelihood that multiple members of the kinesin superfamily participate in fast anterograde transport has raised the issue of which kinesin-related proteins (KRPs) transport what cargoes. The possibility that different kinesins transport different axonal cargoes is supported by the observation that the predicted cargo-binding regions of kinesin superfamily members have little sequence similarity (GOLDSTEIN 1993). Genetic studies of UNC-104, a nematode KRP, suggest that it associates with and moves synaptic vesicle precursors (HALL and HEDGECOCK 1991). Cell fractionation and localization studies of its homologue from mouse (KIF1A) support that conclusion (OKADA *et al.* 1995). Further, localization and *in vitro* motility studies suggest that KIF1B and perhaps its homologues associate with and transport mitochondria (NANGAKU *et al.* 1995). In contrast, studies of conventional kinesin suggest that it can bind a variety of organelles in neurons and other

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cell types. Furthermore, experiments designed to disrupt kinesin function using antibodies and antisense oligonucleotides affect the movements of a wide range of organelles and proteins (reviewed by BLOOM and ENDOW 1994).

To study the roles of microtubule motors and cytoplasmic transport in a complex organism, we have used a genetic approach to disrupt kinesin function in Drosophila. Kinesin is abundant and ubiquitously expressed in Drosophila (SAXTON *et al.* 1988, 1991). Null mutations in the gene that encodes kinesin heavy chain (*Khc*), the force-producing subunit of kinesin, cause lethality during the second larval instar. This observation does not address the question of whether or not kinesin has important functions at earlier stages of development because mutant oocytes from heterozygous mothers contain maternally provided wild-type kinesin. The lethal phase of the null mutations suggests that protein turnover and dilution eliminate most of the maternal load of wild-type kinesin by the second larval instar.

Before dying, *Khc* mutant larvae become paralyzed. The paralysis is more severe in posterior segments than in anterior segments. This is particularly evident with hypomorphic *Khc* genotypes that allow development to progress through the third larval instar (SAXTON *et al.* 1991). Electrophysiological tests of *Khc* mutant larvae and interactions between mutations in *Khc* and mutations in ion channel genes indicate that the distal paralysis is due to two separate defects in neuron function (GHO *et al.* 1992; HURD *et al.* 1996). Impaired sodium channel activity reduces compound action potential amplitudes by a factor of four and makes them difficult to stimulate. Independent of the action potential defect, excitatory junctional current (EJC) amplitudes are reduced by a factor of three in anterior segments and by a factor of five in posterior segments. These EJC data indicate that the capacity of motor axon terminals to secrete neurotransmitter is reduced, and that the terminals of the long axons that innervate posterior segments are most severely affected. The distal paralysis behavioral phenotype and the length-dependent neurosecretion defect support the hypothesis that kinesin is a motor for axonal transport.

This report describes the effects of *Khc* mutations on the structure and ultrastructure of larval neurons. *Khc* mutations cause axonal swellings that are filled with the cargoes of fast axonal transport, including many membrane-bounded organelles and synaptic membrane proteins. In addition, *Khc* mutations inhibit motor axon terminal development. It appears that impaired kinesin function causes a general disruption of fast axonal transport that in turn leads to dystrophic neuron development, length-dependent defects in neurotransmission and progressive distal paralysis. These effects are similar to those caused by some motor neuron diseases in vertebrates.

MATERIALS AND METHODS

Drosophila culture and mutant and control genotypes: Flies were cultured at 25° with a 12 hr light and 12 hr dark cycle on standard soft medium (0.5% agar, 7% molasses, 6% cornmeal, and 0.8% killed yeast) seeded with live yeast. To obtain the primary hemizygous test genotype (y^1w^1; *Khc*^{6}/*Df(2R)Jp6*), males with the genotype y^1w^1; *Khc*^{6}/*CyO* P{y^+} were mated to females with the genotype y^1w^1; *Df(2R)Jp6/CyO* P{y^+}. The use of y^1 on the first chromosome and a y^+ transgene on the second chromosome balancer allowed the recognition of larvae carrying the mutant genotype based on light pigmentation of their mouthparts rather than on behavioral phenotypes. All of the mutant animals used in this study were y^1w^1; *Khc*^{6}/*Df(2R)Jp6,* except in tests for alelle specificity. Three types of controls were used: wild-type Oregon R, heterozygous siblings of the hemizygous test animals, and animals carrying the test genotype with the addition of a wild-type *Khc* transgene on chromosome *3* (transgenic rescue). The transgenic rescue genotype was constructed by mating y^1w^1; *Khc6/CyO* P{y^+} males to y^1w^1; *Df(2R)Jp6;* P{w^+, *Khc*$^+$}/*T(2, 3) CyO, TM6b Tb* females. Larvae bearing this control genotype were then picked based on light mouthpart pigmentation and normal body shape (nontubby). The genotypes of the controls shown in the various experiments are noted in the figure legends. Detailed descriptions of the genetics of the *Khc* mutations used can be found in SAXTON *et al.* (1991) and HURD *et al.* (1996). The other markers and chromosomes used are described by LINDSLEY and ZIMM (1992).

Immunocytochemistry and confocal microscopy: Larvae were dissected in Ca^{++} free dissection buffer (128 mM NaCl, 36 mM sucrose, 2 mM KCl, 1 mM EGTA, 5 mM HEPES, 4 mM $MgCl_2$) in a Sylgard coated depression dish using microdissection scissors and spines from a ball cactus (Notocactus). Fixation was done by rapidly exchanging the dissecting buffer with 4% formaldehyde in dissecting buffer. The fixative was replaced several times over 30–60 min. Samples were then rinsed rapidly with three changes of 0.2% Triton X-100 in standard phosphate buffered saline (PBST), followed by several more changes over 30–60 min. Antibodies were diluted in PBST. The types, concentrations, and sources of the primary antibodies used were as follows: rabbit polyclonal anti-horseradish peroxidase at 1:250 (Jackson Immunoresearch), rabbit polyclonal anti-synaptotagmin at 1:500 (LITTLETON *et al.* 1993), rabbit polyclonal anti-KHC at 1:500 (SAXTON *et al.* 1988), affinity-purified rabbit polyclonal anti-KHC at 1:500 (RODIONOV *et al.* 1993), mouse monoclonal anti-β-tubulin at 1:10 (DETTMAN *et al.* 1996), mouse monoclonal anti-cysteine-string protein at 1:500 (ZINSMAIER *et al.* 1994), rabbit polyclonal anti-syntaxin at 1:500 (HATA *et al.* 1993), rabbit polyclonal anti-ankyrin at 1:500 (DUBREUIL and YU 1994), and mouse monoclonal anti-Fasciclin II at 1:10 (LIN *et al.* 1993). Fluorescein isothiocyanate- or TRITC-goat anti-rabbit or goat anti-mouse (Jackson Immunoresearch) secondary antibodies were typically used at 1:500. TRITC-phalloidin (Molecular Probes, Inc.) was used at 1:500 to stain F-actin. Primary antibodies were typically applied overnight at 4° and were followed by multiple rinses in PBST for a total of 60 min at room temperature. Secondary antibodies were typically applied for 30–60 min at room temperature and followed by multiple rinses in PBST. Samples were then mounted in buffered glycerol (10% 0.1 M NaH_2CO_3 pH 8.4) and examined using a Bio-Rad MRC-600 laser scanning confocal microscope. Extended focus images were collected at a miminal confocal aperature using Kalman averaging. They were prepared for publication using a Macintosh Quadra 800 (Apple Computer Co.) running Image v1.49b (W. RASBAND, National Institutes of Health), Photoshop v2.5.1 (Adobe Systems, Inc.) and Canvas v3.5 (Deneba Software). Final images were printed on a Phaser IISDX dye-sublimation printer (Tektronix, Inc.).

Electron microscopy and video image collection: Transmission electron microscopy was used to examine segmental nerves from four control and three mutant wandering third instar larvae. Controls consisted of two heterozygotes, one Oregon R and one transgenically rescued mutant. Larvae were dissected in Schneider's tissue culture medium. They were then fixed by multiple rapid exchanges with 2% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2–7.4) followed by four changes in a moist sealed container over 60 min. After rinsing in cacodylate buffer, postfixation was done in 0.5% OsO_4, 0.8% K_3 $Fe(CN)_6$ in cacodylate buffer. Samples were then rinsed in distilled water, stained in 2% aqueous uranyl acetate, dehydrated in an acetone series and flat-embedded in Epox-Araldite. Serial ultrathin (75–100 nm) transverse sections of larval segmental nerves were cut with a Leica UCT ultramicrotome and transferred to Formvar-coated single-slot grids. Grids were examined and photomicrographs were obtained using a JEOL JEM 1010 running at 60 kV. More detailed descriptions of these techniques can be found elsewhere (MCDONALD 1994). Larvae were prepared for scanning electron microscopy (SEM) using the same dissection and fixation methods. Further sample preparation and SEM were done by F. R. TURNER as described (WAKIMOTO *et al.* 1984).

Larval crawling behavior was videotaped as previously described (SAXTON *et al.* 1991) using a Dage 68 Newvicon camera fitted with a Nikon Micro-nikkor 55 mm 1:2.8 lens. Single frames were captured and digitized on a Macintosh Quadra 840AV (Apple Computer Co.) running Premier v4.2 (Adobe Systems, Inc.). They were processed and printed as described above.

RESULTS

Kinesin mutations cause posterior-ventral paralysis: The crawling motion of a Drosophila larva is driven by the contraction of segmentally repeated sets of bodywall muscles that are attached to the inner surface of the cuticle. The pattern of contractions is coordinated by motor neurons whose cell bodies reside in the ventral ganglion. These neurons extend axons to the appropriate muscles via compound segmental nerves, each of which contains 60–80 motor and sensory axons. Scanning electron microscopy shows that mutations in *Khc* do not cause major defects in the organization or morphology of the larval neuromuscular system (compare Figure 1A to Figure 3 of JAN and JAN 1976). Nevertheless, striking behavioral phenotypes appear in *Khc* mutant larvae. The anterior-posterior gradient of paralysis that was originally reported for *Khc* mutants (SAXTON *et al.* 1991) is preceded by a dorsal-ventral gradient of paralysis (Figure 1, B and C). Early loss of motor activity in the ventral posterior segments causes an imbalance in bodywall contractions such that larvae rhythmically flip their tails upward during locomotion. Various degrees of the tail-flipping phenotype are caused by most *Khc* mutant genotypes. Dorsal and ventral motor axons that innervate the same posterior segment are not very different in length. Therefore, the tail-flipping phenotype suggests that while axon length is important, other structural or physiological factors can also influence the sensitivity of neurons to a loss of kinesin function.

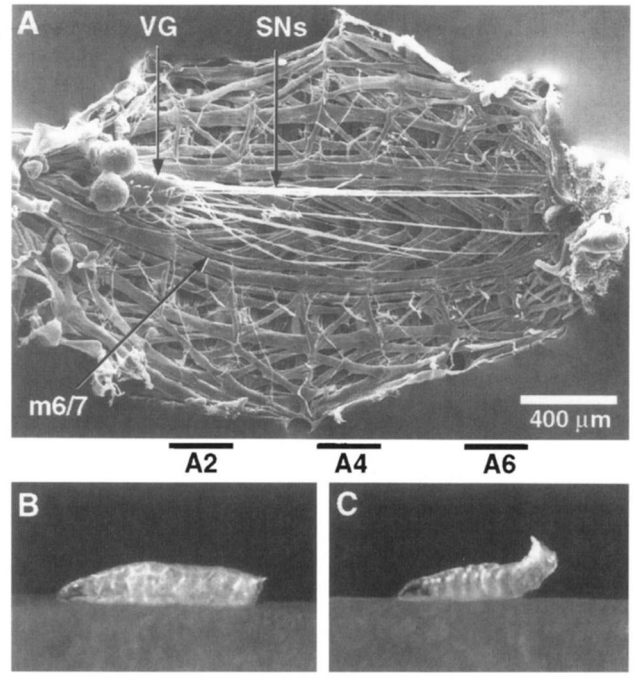

FIGURE 1.—Kinesin mutations cause early posterior ventral paralysis. (A) Scanning electron micrograph of a neuromuscular preparation from a mutant larva (*Khc*^{6}/*Df(2R)Jp6*). The ventral ganglion (VG), located in the anterior end, constitutes the larval central nervous system. Outside the ventral ganglion, most motor and sensory axons are bundled together in the major segmental nerves (SNs). The rectangular body wall muscles are arranged on either side of the ventral midline in a bilaterally symmetrical, segmentally repeated pattern. Each lateral half-segment is innervated by a single major segmental nerve. Motor neuron RP3 and a second motor neuron innervate the 6/7 pair of ventral longitudinal muscles, and they form synaptic contacts in and around the cleft between the muscles (m6/7: the arrow points to the cleft; see also Figures 4 and 5). Abdominal segments 2, 4, and 6 are indicated (A2, A4, and A6). While the general structure of the larval neuromuscular system is normal in kinesin mutants, its function is not. A heterozygous control (B) and a mutant (C) larva are shown in the act of crawling. The mutant periodically flips its tail upward indicating early paralysis of posterior-ventral body wall muscles.

Kinesin mutations cause organelle-filled axonal swellings: The behavioral phenotypes and the electrophysiological defects of kinesin mutants suggest that kinesin mutations primarily affect the nervous system (SAXTON *et al.* 1991; GHO *et al.* 1992; HURD *et al.* 1996). To determine if kinesin mutations cause visible defects in axonal transport, the ultrastructure of larval segmental nerves from control and *Khc* mutant larvae was compared. Transverse sections of control nerves show that each contains an inner bundle of unmyelinated axons lying among the cytoplasmic processes of glial support cells (Figure 2, A and C). This inner bundle is surrounded by a sheath of glial cells that is in turn enclosed by a layer of extracellular matrix. The diameters of axons vary from ~100 to 700 nm. The primary cytoskeleton of the axons consists of microtubules; Drosophila and other arthropods lack neurofilaments (LASEK *et al.* 1983). The number of microtubules seen in axons var-

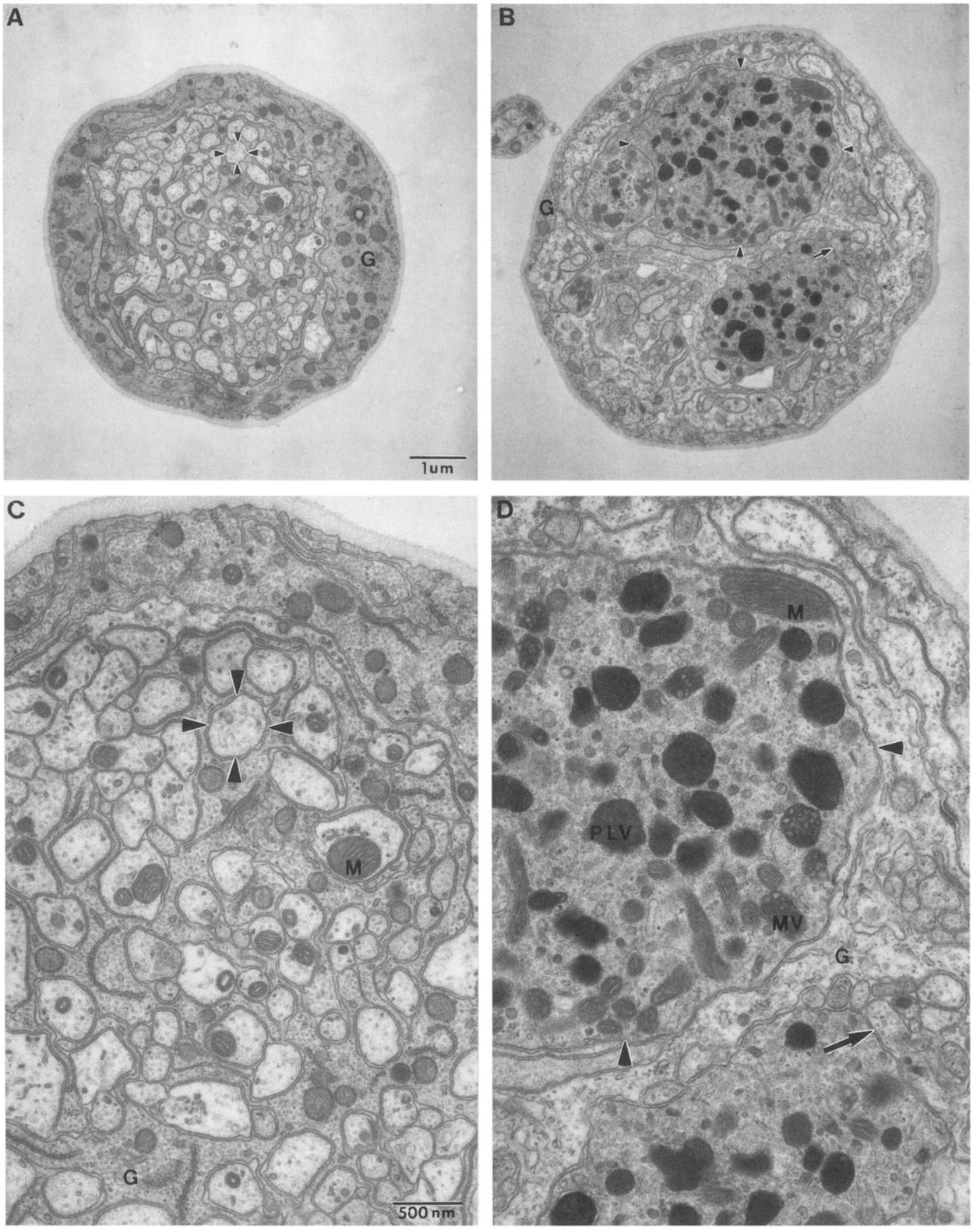

FIGURE 2.—Kinesin mutations cause organelle-filled axonal swellings. A and C show electron micrographs of a cross section through a heterozygous control segmental nerve. Antiparallel motor and sensory axons are bundled in the central part of the nerve among the cytoplasmic processes of glial support cells (G). Arrowheads point to the axonal plasma membrane of the same axon in the low and high magnification views. The inner bundle of axons is surrounded by a layer of glial cells that are enclosed by a layer of extracellular matrix. The diameters of axons in the control nerve vary from ~100 to 700 nm. Mitochondria (M) are seen in some axons, as are clear vesicles (~30–60 nm) and a few small multivesicular bodies. B and D show micrographs of a mutant segmental nerve. Although many of the axons appear normal (arrows), a few are swollen to many times a normal diameter. The arrowheads point to the plasma membrane of the same swollen axon in the low and high magnification views. The swelling is packed with mitochondria, clear vesicles, dark vesicles, large multi-vesicular bodies (MV), and large, dark prelysosomal vacuoles (PLV).

ies from one to 15 depending on axon diameter. Mitochondria and small, clear vesicles (30–60 nm) are often seen in axons. Irregular cisternal membranes and small mutivesicular bodies are occasionally seen.

In some sections of *Khc* mutant nerves all axons have normal diameters and appear to contain normal populations of membrane-bounded organelles. However, in other sections one or more axons are dramatically swollen and packed with membrane-bounded organelles (Figure 2, B and D). Serial section analysis revealed that the diameter of an axon of normal appearance could increase more than 10-fold within a micron. The organelles seen in the swellings include a multitude of mitochondria, small vesicles, cisternal membranes and multivesicular bodies. These types of membrane-bounded organelles are the normal cargoes of fast axonal transport.

Large, dark-staining organelles, including large multivesicular bodies and vacuoles (Figure 2, B and D) are a conspicuous feature of the swellings. Their morphologies suggest that they represent early stages in the lysosomal pathway (NIXON and CATALDO 1995). Prelysosomal organelles of this size have not been seen in unswollen regions of mutant axons or in control axons. Therefore, they probably represent products of the fusion and maturation of smaller prelysosomal organelles. Stress-driven autophagy of cytoplasm within the swellings may also contribute to the dark organelles (NIXON and CATALDO 1995).

Slow axonal transport does not make a substantial contribution to the swellings: Axonal swellings containing disorganized neurofilaments and membrane-bounded organelles are associated with some forms of amyotrophic lateral sclerosis (ALS) and other vertebrate motor neuron diseases (HIRANO 1991; DYCK *et al.* 1993; CLEVELAND 1996). Experiments with transgenic mice have shown that expression of a mutant neurofilament subunit or overexpression of wild-type subunits causes tangles of disorganized neurofilaments, axonal swellings, axonal dystrophy and motor neuron disease (reviewed by BRADY 1995; CLEVELAND 1996). Recent experiments suggest that the neurofilament disorder causes swellings by focal inhibition of the slow axonal transport of cytoskeletal proteins and perhaps of the fast axonal transport of membrane-bounded organelles (COLLARD *et al.* 1995).

To test the contribution of the slow component of axonal transport to the organelle-filled swellings caused by *Khc* mutations, the distributions of tubulin, ankyrin and actin were compared to that of the mutant fast transport motor itself (KHC). In control nerves KHC is evenly distributed (Figure 3A), and microtubules appear to lie parallel to the long axis of the segmental nerve (Figure 3C). Ankyrin and actin are also evenly distributed, although their faint staining patterns suggest that these proteins are not major components of the axonal cytoskeleton (not shown). In mutant nerves, KHC is concentrated in numerous elongated swellings (Figure 3B). Tubulin (Figure 3D), ankyrin and F-actin have normal distributions and clearly do not accumulate in axonal swellings. The *Khc* allele used (Khc^6) produces a partially functional full length KHC protein (SAXTON *et al.* 1991). By the third instar, little maternal KHC should remain, so the anti-KHC staining in mutants represents the distribution of the mutant protein. These staining patterns indicate that the swellings form with little contribution from the fibrous cytoskeletal elements that move in the slow component of axonal transport.

Kinesin mutations cause abnormal accumulation of nerve terminal proteins in axon swellings: Membrane proteins required in the axon terminal for the synthesis of synaptic vesicles and plasma membrane are synthesized in the cell body. It is thought that small transport vesicles bearing these proteins are formed by the trans-Golgi network and then are carried to the terminal by fast anterograde axonal transport. If the abundant vesicles in the axonal swellings of *Khc* mutants are deposited by fast anterograde transport, they should carry proteins that are normally destined for the axon terminal.

To determine if anterograde transport vesicles contribute to the axonal swellings of kinesin mutants, the distributions of a number of terminal membrane proteins were compared in control and *Khc* mutant third instar larvae. Synaptotagmin is an integral membrane protein of synaptic vesicles, which are concentrated in terminal boutons (neurite varicosities). Anti-synaptotagmin staining of control larvae shows the expected accumulation of synaptotagmin in terminal boutons (Figure 4C). Significant quantities of synaptotagmin are not found in control segmental nerves (Figure 4A). In contrast, striking accumulations of synaptotagmin are seen in the segmental nerves of *Khc* mutants (Figure 4B). Similar staining patterns were seen with antibodies to three other terminal membrane proteins: cysteine-string protein (synaptic vesicle membranes), syntaxin (intracellular, presynaptic plasma membrane), and Fasciclin II (extracellular, presynaptic plasma membrane) (HATA *et al.* 1993; LIN and GOODMAN 1993; ZINSMAIER *et al.* 1994; SCHULZE *et al.* 1995). Double-label experiments suggest that the distributions of these proteins and of KHC in the swellings are coincident. The accumulation of the various synaptic membrane proteins, mutant KHC and membrane-bounded organelles in axonal swellings contrasts with the lack of accumulation of cytoskeletal elements. These observations suggest that the axonal swellings of *Khc* mutants are caused by a direct inhibition of fast axonal transport.

The axonal swellings observed in Khc^6 hemizygotes are not allele specific. Three other alleles tested, including Khc^8 (amorphic), Khc^{1ts} (hypomorphic), and Khc^{BD} (mild hypomorphic), also cause accumulation of synaptotagmin in segmental nerves. The extent of synaptotagmin accumulation caused by each of these four alleles parallels the relative severities of the behavioral and other phenotypes that they cause (SAXTON *et al.* 1991;

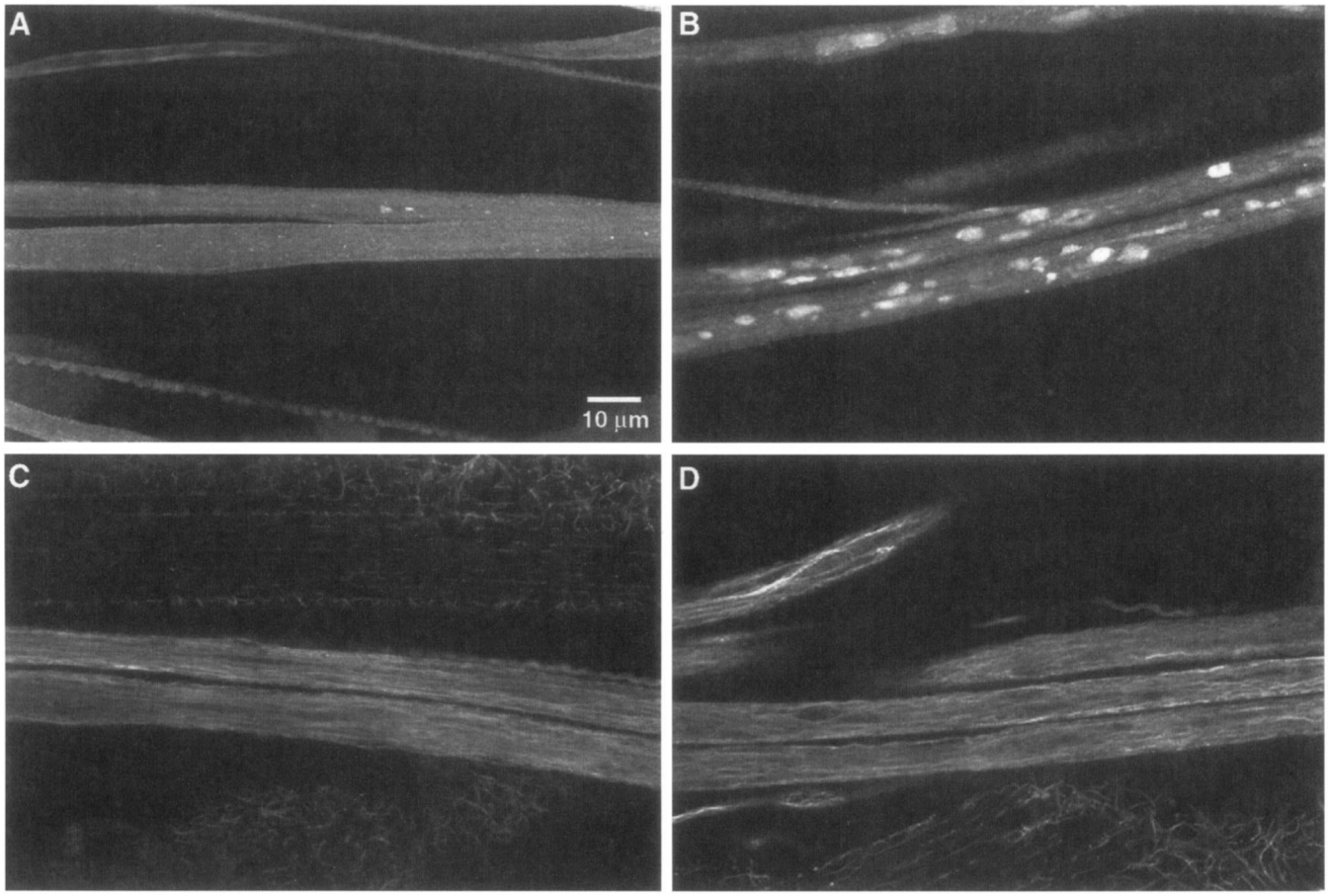

FIGURE 3.—Kinesin mutations cause novel accumulations of kinesin but not tubulin in segmental nerves. Heterozygous control (A and C) and mutant (B and D) third instar larvae were stained with antibodies to Drosophila KHC (A and B) or β-tubulin (C and D) and examined with a confocal microscope. Micrographs show representative larval segmental nerves passing through segment A4. Note that KHC accumulates abnormally in mutant nerves, but tubulin does not.

HURD *et al.* 1996). We have not observed synaptotagmin-containing axonal swellings in the segmental nerves of Oregon R larvae. In heterozygous and transgenic control animals, small anti-synaptotagmin reactive swellings have been observed, but they are exceedingly rare.

Axon terminals in kinesin mutants have fewer synaptic boutons: While synaptotagmin and the other terminal membrane proteins accumulate normally in the boutons of mutant terminals, the number of boutons per terminal appears reduced relative to wild-type (Figure 4, C and D). To characterize this defect, neuromuscular preparations of third instar larvae were incubated with anti-horseradish peroxidase antibodies to completely stain motor axon terminals rather than just the terminal boutons (JAN and JAN 1982; SNOW *et al.* 1987). We examined the junctions that form on the ventral longitudinal muscle pair 6/7 in segments A2 (anterior) and A6 (posterior) of many animals. A qualitative comparison of controls to mutants shows that mutant terminals branch less frequently, appear thin and have relatively few boutons (Figure 5).

To quantify the terminal phenotype, we counted the number of synaptic boutons per muscle 6/7 junction in segments A2 and A6 in control and mutant wandering third instar larvae (Figure 6). *Khc* mutations cause a threefold reduction in the number of boutons per junction in A2 and a fivefold reduction in A6 relative to controls. The more severe effect on the posterior terminal is consistent with the more severe reduction in its ability to secrete neurotransmitter (GHO *et al.* 1992), as well as with the distal paralysis behavioral phenotype (SAXTON *et al.* 1991). The terminal phenotype is completely rescued by a single copy transgene carrying wild-type *Khc* (Figures 6 and 7).

To determine how the terminal phenotype develops, boutons were counted in mutant and control larvae at a variety of developmental stages (Figure 7). Terminals in mutant early second instar larvae are found in the expected locations and have a normal appearance. Therefore, axonal pathfinding and synaptogenesis during embryonic stages proceed normally in *Khc* mutants. Early in the second instar, terminals in both mutant and control animals are small with only a few boutons each. In controls, terminal size and bouton numbers increase as larvae grow. In mutants, bouton numbers do not increase after the second instar despite continued growth of the larvae. Either no new boutons develop or the development of new boutons is balanced by the disappearance of old ones. In either case, it appears that there is little net increase in the number of boutons

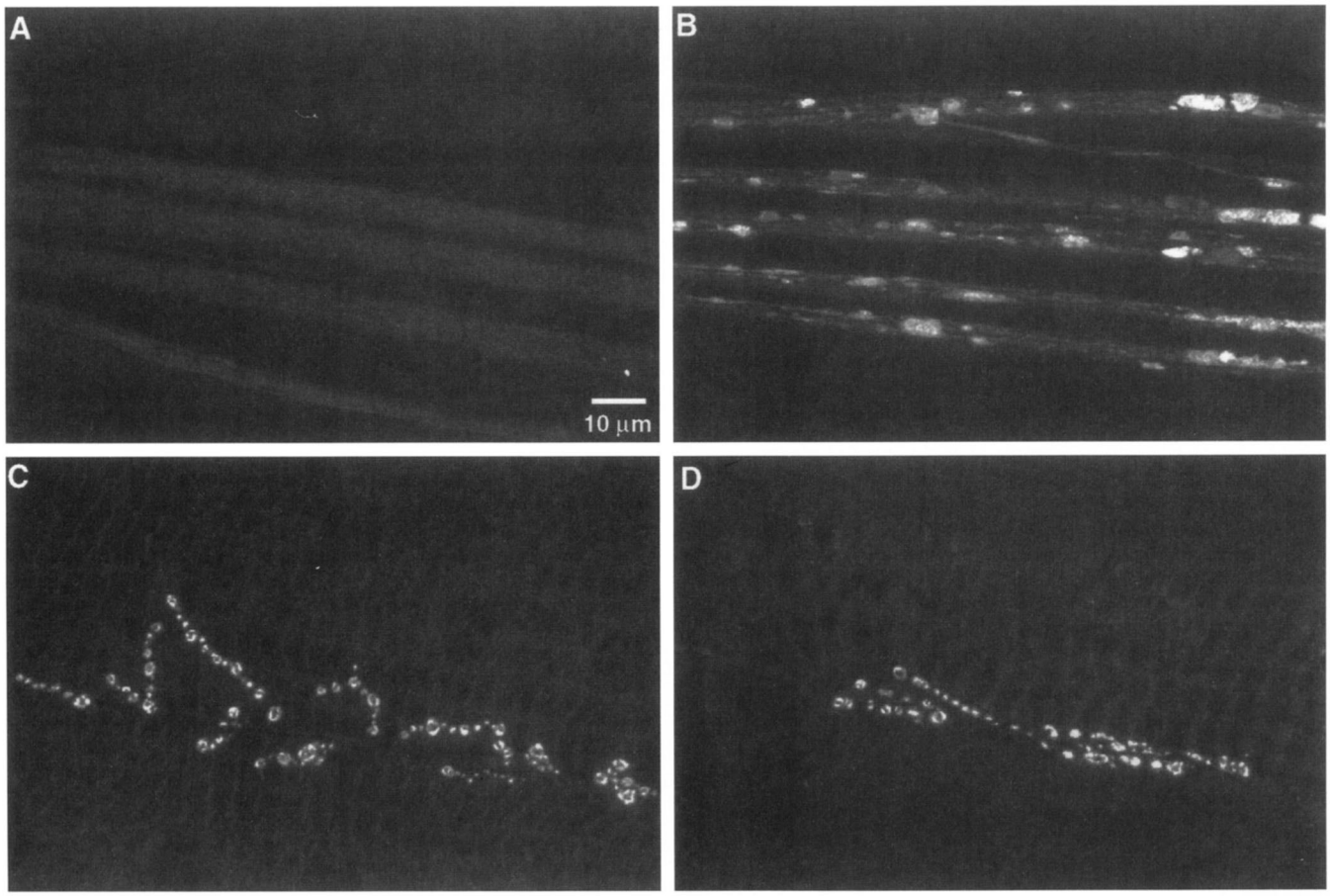

FIGURE 4.—Kinesin mutations cause accumulations of synaptic proteins in nerves. Heterozygous control (A and C) and mutant (B and D) third instar larvae were stained with antibodies to Drosophila synaptotagmin (anti-DSYT) and examined with a confocal microscope. Micrographs show representative larval segmental nerves passing through segment A4 (A and B) and m6/7 neuromuscular junctions from segment A2 (C and D). Note that while synaptotagmin's subcellular localization in mutant boutons appears normal, its accumulation in mutant nerves is highly abnormal.

after the second instar. Early in the second instar, before the motor terminal phenotype appears, anti-synaptotagmin reactive axonal swellings are observed in mutant segmental nerves (not shown). It is likely that the retention of membrane proteins and organelles in the axonal swellings reduces their availability for new terminal growth and bouton construction as the mutant animals grow.

DISCUSSION

Kinesin mutations inhibit anterograde and retrograde fast axonal transport: The organelles and proteins found in the axonal swellings caused by *Khc* mutations in Drosophila are similar to those that accumulate at nerve ligations in vertebrates. At a ligation, the organelles carried by fast anterograde axonal transport accumulate on the side proximal to the cell body. They include mitochondria, irregular cisternal membranes and small vesicles derived from the trans-Golgi network (TSUKITA and ISHIKAWA 1980; HIROKAWA *et al.* 1991; QUATACKER *et al.* 1995). Synaptic terminal membrane proteins are thought to be delivered to the axon terminal via Golgi-derived vesicles and fast anterograde transport driven by kinesin and kinesin-related motors. Consistent with this, synaptic membrane proteins, kinesin and related motors accumulate on the proximal but not on the distal sides of vertebrate nerve ligations (HIROKAWA *et al.* 1991; OKADA *et al.* 1995). The axonal swellings of kinesin mutants contain many small vesicles, high concentrations of terminal membrane proteins and mutant KHC. The accumulation of organelles and proteins indicates that impaired kinesin function inhibits fast anterograde axonal transport. This interpretation is consistent with studies showing that antisense inhibition of KHC expression in vertebrate neurons impairs the anterograde transport of a variety of axonal proteins (FERREIRA *et al.* 1992; AMARATUNGA *et al.* 1995).

Fast retrograde axonal transport carries mitochondria, cisternal membranes, vesicles and prelysosomal organelles from the axon to the cell body. These organelles accumulate in axons on the distal side of a nerve ligation (TSUKITA and ISHIKAWA 1980; HIROKAWA *et al.* 1991; QUATACKER *et al.* 1995). The axonal swellings of *Khc* mutants contain many prelysosomal organelles, including small and large multivesicular bodies and dark vacuoles. The appearance of the large organelles sug-

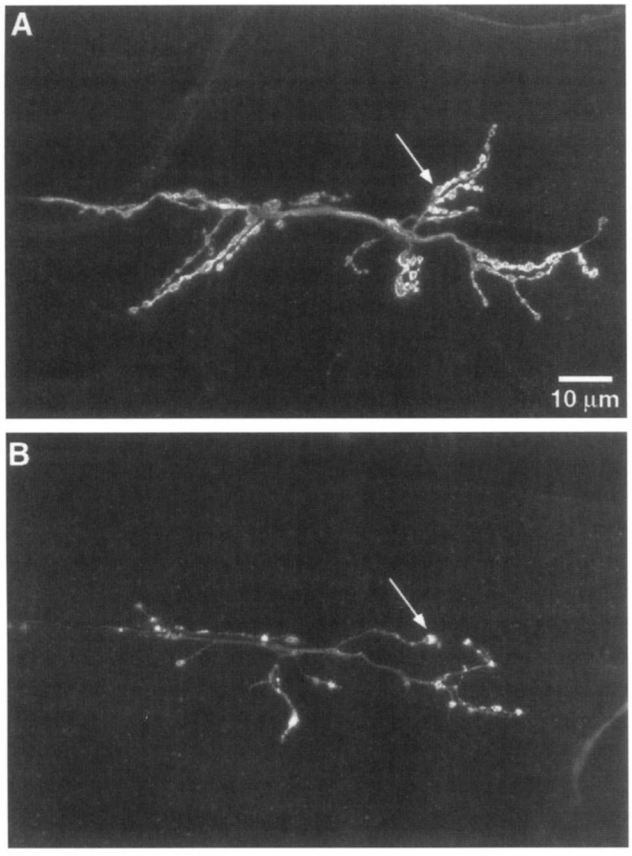

FIGURE 5.—Kinesin mutations cause dystrophic neuromuscular junctions. Heterozygous control (A) and mutant (B) wandering third instar larvae were stained with antibodies to horseradish peroxidase and examined with a confocal microscope. Each micrograph shows a representative m6/7 neuromuscular junction from segment A2. Note the reduction in the number of synaptic boutons (arrows) and the abnormal morphology of the kinesin mutant terminals.

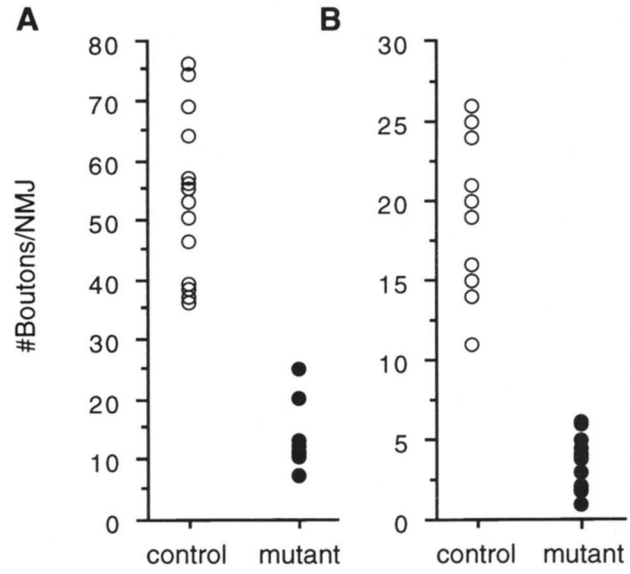

FIGURE 6.—Kinesin mutations cause a significant reduction in the number of synaptic boutons per neuromuscular junction in wandering third instar larvae. The total number of synaptic boutons per neuromuscular junction was determined for the 6/7 muscle pair in A2 (A) and A6 (B) of control and mutant wandering third instar larvae. Each point represents the number of boutons in a single neuromuscular junction. For quantification, a synaptic bouton was defined as a discrete swelling of the neurite with a diameter >1 μm. Segment A2 in controls possessed 54.0 $\pm$ 3.3 boutons per junction (mean $\pm$ SEM, n = 16), while A2 in mutants had 14.3 $\pm$ 1.8 boutons per junction (n = 9). Means were compared using an unpaired, two-tailed *t*-test and were determined to be significantly different with $P < 0.0001$. Segment A6 in controls possessed 19.6 $\pm$ 1.4 boutons per junction (n = 12), while A6 in mutants had 3.7 $\pm$ 0.4 boutons per junction (n = 17). These means were also significantly different with $P < 0.0001$. All three control genotypes were tested and their phenotypes were not distinguishable from one another (see Figure 7).

gests that they form by fusion of the smaller organelles that are normally carried by retrograde transport (HOLLENBECK 1993; NIXON and CATALDO 1995). The accumulation of the prelysosomal organelles in the axonal swellings leads to the surprising conclusion that impaired kinesin function inhibits retrograde as well as anterograde fast axonal transport.

The composition of swellings and their random distribution along larval axons suggest the following model for their stochastic formation. A mutant embryo contains a supply of wild-type KHC provided by its heterozygous mother. As the concentration of maternal KHC decreases during early larval development, the anterograde transport of kinesin's cargoes becomes less processive, and individual organelles periodically stall. A stalled organelle increases the probability that other kinesin cargoes will stall by physically impeding their passage. As the size of the organelle jam increases, it hinders the transport of organelles carried by kinesin-related proteins and cytoplasmic dyneins. Incoming organelles push stalled organelles away from their microtubule tracks, forcing a distension of the axon. At some point, even though stalled organelles should retain some functional motor proteins, dispersal of the jam becomes unlikely because most organelles are too distant from a microtubule to reattach and resume transport.

Fast axonal transport inhibition causes other *Khc* mutant phenotypes: The impaired compound action potentials reported by GHO *et al.* (1992) are caused by a reduction of the inward sodium conductance of axonal membranes. The reduced conductance is probably due to the impaired anterograde transport of ion channels and a consequent reduction in channel density in the axonal membrane (HURD *et al.* 1996). A lack of suitable antibodies or labeled toxins has prevented a direct examination of ion channel distribution in larval nerves. However, we expect that vesicles carrying ion channels accumulate in organelle jams along with those carrying synaptotagmin and the other membrane proteins.

The abnormal morphology of motor axon terminals in *Khc* mutants is also a consequence of the fast axonal transport inhibition. The morphology of a motor axon terminal can be influenced by excitation-coupled plasticity, by trophic interactions with its target cell and by

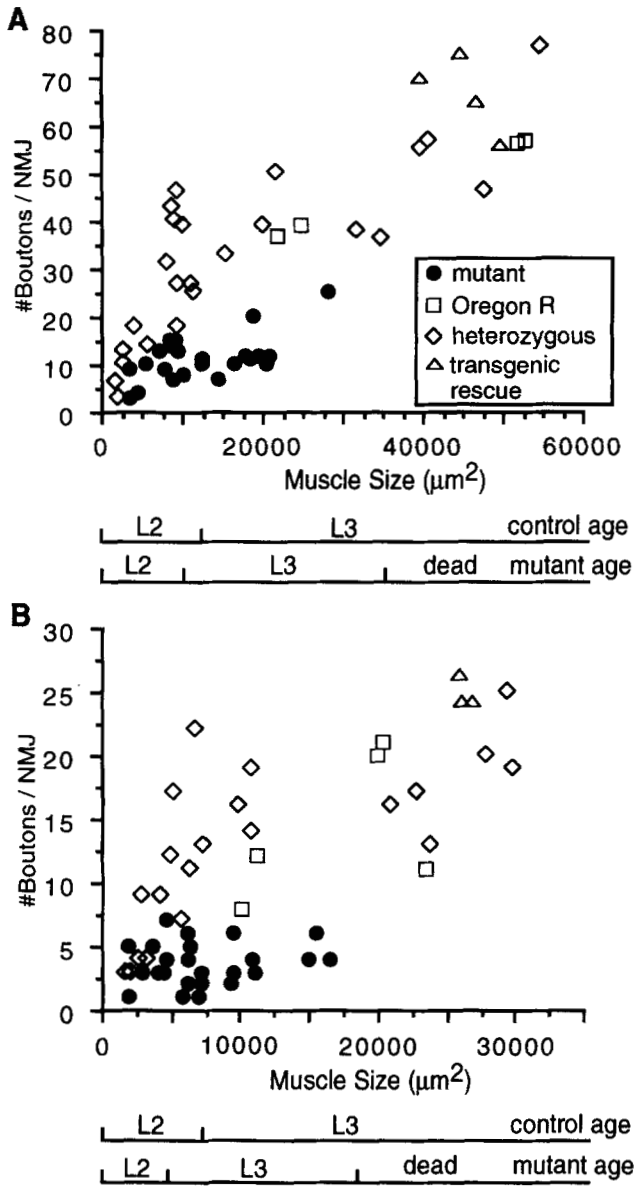

FIGURE 7.—Kinesin mutations cause abnormal growth of neuromuscular junctions. Scattergrams show the number of synaptic boutons per neuromscular junction (Y-axis) as a function of the growth of muscle 6 (X-axis) for segment A2 (A) and segment A6 (B). The sagittal section area of muscle 6 was used as an independent indicator of size; therefore, the unit for the X-axis is μm^2. Each point represents the number of boutons for one neuromuscular junction. Because mutant and control larvae grow at different rates, animals of the same size are not necessarily of the same age. Developmental stages for mutants and controls are indicated by extra X-axes. Data points contributed by each of the control genotypes are indicated on the graph.

the availability of structural proteins needed for bouton construction (BUDNIK *et al.* 1990; ZHONG *et al.* 1992; CONNOR and SMITH 1994; DAVIS and MURPHEY 1994; STEWART *et al.* 1996). In *Khc* mutants impaired ion channel activity could contribute to poor excitation-coupled terminal development, and impaired retrograde transport could interrupt the delivery of trophic signals to neuronal cell bodies. However, since the swellings inhibit anterograde axonal transport, we suspect that the main cause of abnormal terminal development is interruption of the delivery of components needed for bouton construction.

The *Khc* mutant genotype used here reduces bouton numbers 3.7-fold in the anterior segment A2 and 5.2-fold in the posterior segment A6. This same mutant genotype was previously tested for its effects on electrotonically stimulated EJC amplitude, which is a measure of motor terminal transmitter secretion capacity. EJC amplitudes were reduced 2.6-fold in segment A2 and 4.7-fold in segment A6 (GHO *et al.* 1992). Synapse formation and transmitter secretion occur primarily at synaptic boutons (ATWOOD *et al.* 1993). Since individual boutons in *Khc* mutants appear normal (this study and GHO *et al.* 1992) the decrease in bouton numbers probably causes the decrease in secretion capacity of *Khc* mutant terminals.

The distal paralysis displayed by *Khc* mutant larvae is the behavioral manifestation of an inhibition of axonal transport. It reflects the more severe effects of *Khc* mutations on the structure and function of the long axons that innervate posterior segments. Greater length provides more opportunities for the formation of organelle jams and an earlier or stronger inhibition of fast axonal transport. The differential sensitivity of dorsal and ventral neurons implied by the larval tail-flipping phenotype remains unexplained. Perhaps one or more motor neurons that innervate ventral muscles requires an unusually high volume of fast axonal transport.

The phenotypes caused by *Khc* mutations in Drosophila are due to a general inhibition of fast axonal transport by organelle jams. It should be possible to identify kinesin cargoes if the initial stages of organelle jam formation can be effectively observed. Preliminary studies with temperature-sensitive *Khc* alleles indicate that temperature shifts can be used to induce jam formation (D. HURD, unpublished results). Immunocytology and electron microscopy may reveal specific types of organelles that nucleate jams. Genetic screens based on enhancement of *Khc* axonal transport phenotypes may also identify kinesin's cargoes and specific proteins used for cargo linkage.

Impaired fast axonal transport may cause motor neuron disease: Axonal swellings are a feature of a variety of motor neuron diseases in humans and other vertebrates. The accumulation of disorganized neurofilaments in the swellings has led to suggestions that an inhibition of slow axonal transport contributes to the pathology of such diseases (HIRANO 1991; DYCK *et al.* 1993; BRADY 1995; BROWN 1995; CLEVELAND 1996). Studies of transgenic mice suggest that defective neurofilaments and the tangles that they form can trigger the formation of axonal swellings and cause ALS-like motor neuron disease by blocking slow axonal transport (COTE *et al.* 1993; XU *et al.* 1993; LEE *et al.* 1994; COLLARD *et al.* 1995). Since membrane-bounded organelles were found trapped in the masses of neurofilaments, it

has been suggested that a secondary inhibition of fast axonal transport may also contribute to the ALS-like phenotypes (BRADY 1995; COLLARD *et al.* 1995).

The axonal swellings in Drosophila *Khc* mutants are caused by a disruption of fast axonal transport. They are filled with membrane-bounded organelles and contain no masses of cytoskeletal filaments. This specific disruption of fast axonal transport causes motor neuron disease-like phenotypes. The parallels include defective compound action potentials, reduced transmitter secretion by motor terminals, axonal dystrophy and progressive distal paralysis (CORNBLATH *et al.* 1992; HAHN 1993; LAMBERT and DYCK 1993; MASELLI *et al.* 1993; ROWLAND 1994; BOSTOCK *et al.* 1995; EISEN 1995). This suggests that an inhibition of fast axonal transport is a major factor in some vertebrate motor neuron diseases. Furthermore, our data support the hypothesis that a secondary inhibition of fast transport is an important pathological effect of neurofilament tangles.

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