Increased neuromuscular activity causes axonal defects and muscular degeneration

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Summary

Before establishing terminal synapses with their final muscle targets, migrating motor axons form en passant synaptic contacts with myotomal muscle. Whereas signaling through terminal synapses has been shown to play important roles in pre- and postsynaptic development, little is known about the function of these early en passant synaptic contacts. Here, we show that increased neuromuscular activity through en passant synaptic contacts affects pre- and postsynaptic development. We demonstrate that in zebrafish twister mutants, prolonged neuromuscular transmission causes motor axonal extension and muscular degeneration in a dose-dependent manner. Cloning of twister reveals a novel, dominant gain-of-function mutation in the muscle-specific nicotinic acetylcholine receptor α-subunit, CHRNA1. Moreover, electrophysiological analysis demonstrates that the mutant subunit increases synaptic decay times, thereby prolonging postsynaptic activity. We show that as the first en passant synaptic contacts form, excessive postsynaptic activity in homozygous embryos severely impedes pre- and postsynaptic development, leading to degenerative defects characteristic of the human slow-channel congenital myasthenic syndrome. By contrast, in heterozygous embryos, transient and mild increase in postsynaptic activity does not overtly affect postsynaptic morphology but causes transient axonal defects, suggesting bidirectional communication between motor axons and myotomal muscle. Together, our results provide compelling evidence that during pathfinding, myotomal muscle cells communicate extensively with extending motor axons through en passant synaptic contacts.

Key words: Zebrafish, nic1, Motor axon, En passant terminals, Synaptogenesis, Acetylcholine receptor α-subunit, chnra1, Slow-channel congenital myasthenic syndrome, twister

Introduction

It is well-established that synaptic connections between motor axons and muscle are shaped by activity. This has been demonstrated extensively for processes occurring late during synaptogenesis, in which activity regulates synaptic maturation and refinement (Sanes and Lichtman, 1999a). In the absence of activity, neuromuscular junctions (NMJ) develop an aberrant morphology with a reduction of post-synaptic specializations (Duxson, 1982; Jarecki and Keshishian, 1995; Misgeld et al., 2002). Moreover, blockade of neural transmission leads to the loss of naturally occurring motorneuron cell death and synapse elimination, causing aberrant branching of motor axons and multiple innervation of muscle fibers (Broadie and Bate, 1993a; Dahm and Landmesser, 1988; Duxson, 1982; Misgeld et al., 2002; Pittman and Oppenheim, 1978; Zhao and Nonet, 2000). In addition to its role during these late stages, neuromuscular activity is also important for earlier steps of pre- and post-synaptic development, suggesting a role for neurotransmission during the initial formation of nerve-muscle contacts (Misgeld et al., 2002). Finally, neural transmission also plays a role in muscle growth, as well as in its structural and metabolic maturation (Fredette and Landmesser, 1991; Houenou et al., 1990; Misgeld et al., 2002). Thus, activity-dependent processes occurring during late and early synaptogenesis are essential for neuromuscular development.

Intriguingly, the first synaptic contacts between muscle and nerve form before growth cones reach their final synaptic targets. In contrast to well-characterized terminal synapses, in which the growth cone has stopped advancing and has transformed into a typical presynaptic terminus, en passant synaptic contacts form while the growth cone is still advancing towards its muscle target. In the central nervous system (CNS), en passant synaptic contacts, there known as en passant synapses, have been well described and are characterized by varicosities along the length of the axonal shaft (Claihorne et al., 1986; Hatada et al., 1999; Mason and Gregory, 1984). En passant synaptic contacts along the path of motor axons have also been reported in mammalian and teleost embryos (Sheard and Duxson, 1997; Westerfield et al., 1990). In embryonic rat intercostals muscle, en passant contacts, reminiscent of immature synapses, form transiently at non-terminal regions of the axon (Sheard and Duxson, 1997). To avoid confusion with en passant synapses typical of the CNS, we refer to early synaptic contacts emerging along the motoaxonal shaft, while
In the zebrafish embryo, acetylcholine receptors (AChR) cluster along the length of primary motor axons as growth cones pioneer a path on the medial surface of the myotome (Westerfield et al., 1990). Shortly after the appearance of clustered AChRs, muscle fibers start contracting coordinately, suggesting that these AChR clusters represent functional synapses (Liu and Westerfield, 1992; Melançon et al., 1997). Moreover, several studies have shown that spontaneous release of acetylcholine from growth cones induces a post-synaptic response within minutes after initial contact with muscle cells (Chow and Poo, 1985; Hume et al., 1983; Xie and Poo, 1986). Thus, en passant synaptic contacts probably represent active neuromuscular connections. Their function, however, has so far been unclear.

Blocking neuromuscular transmission does not overly affect axonal pathfinding or target selection (Broadie and Bate, 1993a; Westerfield et al., 1990), but causes axonal defasciculation and nerve branching, and also interferes with the normal cessation of axon growth (Dahm and Landmesser, 1988). Thus, neuromuscular transmission plays a role in modulating axonal behaviors, such as fasciculation and extension. One attractive idea is that during the process of pathfinding, en passant synaptic contacts enable activity-dependent, bi-directional communication between axons and muscle, thereby coordinating pre- and post-synaptic development. Although such activity-dependent retrograde and anterograde signaling is thought to be important for coordinating pre- and post-synaptic development, little is known about this process in vivo (Davis and Goodman, 1998; Fitzsimonds and Poo, 1998; Tao and Poo, 2001; Zhao and Nonet, 2000).

Here, we examine the effects of increased neuromuscular activity on pre- and post-synaptic development. We show that in twistet mutant embryos, neuromuscular transmission is prolonged because of a gain-of-function mutation in the α-subunit of the muscle-specific AChR (CHRNA1). Analysis of twistet mutant embryos reveals that the first defects become apparent during pathfinding, when the first en passant contacts between extending motor axons and the myotome form. In homozygous mutants, the high levels of synaptic activity cause excitotoxicity, leading to progressive disruption of pre- and postsynaptic development. In heterozygous twistet mutants, moderate increase in neuromuscular transmission does not overly affect muscle fiber integrity or the formation of en passant synaptic contacts, but causes altered growth cone morphology and axonal extension, consistent with the idea that signals at en passant synaptic contacts modulate pre-synaptic development. Whereas the evidence for activity-dependent retrograde signals has been proposed in several systems in which neuromuscular activity is reduced (Nick and Ribera, 2000; Zhao and Nonet, 2000), our results provide a clear example that increased neuromuscular transmission affects pre- and postsynaptic development in vivo.

Materials and methods

Zebrafish strains and breeding

A single twistet allele, nic<sub>twister</sub> dbn12 was identified in a dominant motility screen (van Eeden et al., 1999). Heterozygous and homozygous mutants obtained from mating crosses between carriers were identified between 17 and 26 hours post-fertilization (hpf) based on their trunk morphology and motility in response to touch. Heterozygous nic<sub>twister</sub> dbn12 were propagated in the TLF background, and were raised and maintained as described by Mullins et al. (Mullins et al., 1994). The nic<sub>thb107</sub> strain was generously provided by M. Westerfield (University of Oregon, Eugene, OR, USA).

Antibody staining and α-bungarotoxin (α-BTX) labeling

Embryos at 17-30 hpf were anesthetized (0.01% Tricaine), fixed in 4% paraformaldehyde with 1% DMSO for three hours at room temperature or overnight at 4°C, and then washed several times in 0.1 M phosphate buffer pH 7.4 (PBS). The following primary antibodies and dilutions were used: zn-p1 (1:200, Antibody Facility, University of Oregon) (Trevorrow et al., 1990); anti-slow-twitch myosin F59 (1:10, kindly provided by F. Stockdale) (Crow and Stockdale, 1986; Devoto et al., 1996); anti-fast-twitch myosin F310 (1:200, generously provided by N. Rubinstein) (Crow and Stockdale, 1986); and SV2 (1:50, Developmental Studies Hybridoma Bank, University of Iowa, USA). For zn1-stainings detected colorimetrically with DAB (Vector Laboratories, Burlingame, CA, USA), antibody stainings were performed as described by Zeller and Granato (Zeller and Granato, 1999). For labeling of myosins, fixed embryos were dehydrated through a methanol series and stored in 100% methanol at −20°C, until permeabilized with acetone for 10 (F59) or 45 (F310) minutes at −20°C. Embryos were washed thoroughly with incubation buffer (0.2% BSA, 0.5% Triton-X in 0.1 M PBS, pH 7.4), and incubated with diluted primary antibody overnight at 4°C. Stainings were detected by using AlexaFluor 488 or AlexaFluor 594 conjugated anti-mouse secondary antibodies diluted in incubation buffer (1:500; Molecular Probes, Eugene, OR, USA).

For double labeling of neuromuscular junctions, fixed embryos were digested with 0.1% collagenase (Sigma, St Louis, MO, USA) in PBS for 7-30 minutes at room temperature, and then washed several times with PBS. To label AChR clusters, embryos were incubated for 30 minutes at room temperature in AlexaFluor 594 conjugated α-BTX (10 μg/mL, Molecular Probes) diluted in incubation buffer with 1% normal goat serum. Axons were labeled with a 1:1 mixture of zn-p1 and SV2 antibodies overnight at 4°C, followed by incubation with secondary AlexaFluor 488 conjugated anti-mouse antibody. Fluorescent embryos were immersed and mounted in Vectashield mounting medium (Vector Laboratories). Embryos were viewed using Nomarski optics or epifluorescence on a Zeiss Axioplan microscope (Zeiss, Thornwood, NY, USA) or Leica MZFLIII stereomicroscope (Bannockburn, IL, USA). Images of non-fluorescent stainings were captured with a Progress 3012 digital camera (Kontron Elektronik). Fluorescent stainings were imaged using a LSM510 confocal microscope (Zeiss).

Pharmacological treatments

α-Bungarotoxin (2.5 mM stock in 0.1 M phosphate buffer, pH 7.4; Sigma) was diluted to 0.25 mM in injection buffer (0.1 M KCl: phenol red, 3:1) and injected directly into the yolk of live 12-14 hpf embryos. α-BTX-treated embryos were collected for further analyses if greater than 70% of injected embryos remained paralyzed through 26 hpf. Embryos were collected at 26-30 hpf, scored for trunk morphology, and then fixed and stained with zn-p1 and F59 for analyses of axonal and muscle morphology. Injected embryos were scored as wild-type/homozygous or as homozygous phenotype according to the axonal and muscle morphology.

Genetic mapping and linkage analysis

A three-generation mapping cross between a Tübingen (Tü) strain carrying the nic<sub>twister</sub> dbn12 mutation and the polymorphic WIK strain was established. Mapping procedure and the WIK line were described previously (Knapik et al., 1996; Rauch et al., 1997). F2 nic<sub>twister</sub> dbn12 embryos, heterozygous and wild-type siblings were collected.
separately and stored at –20°C. For bulk segregation analysis, pools of 25 embryos were used. Embryonic DNA extraction was performed as described in Gates et al. (Gates et al., 1999), and amplified using SSLP markers z17212, z6601 and z9738 (Research Genetics, Huntsville, AL, USA) with the following PCR conditions: 94°C for 2 minutes, then 35 cycles of 92°C for 1 minute; 55°C, 1 minute; 72°C, 1 minute, followed by 75°C, 5 minutes.

To genotype individual F2 mutant and wild-type embryos, we used the dCAPS method to generate polymorphic markers (Neff et al., 1998). The chrna1 3’UTR was amplified by PCR with primers nic13UTR-F: CCAAAATCCCCAACCAG and nic1/BseRI-R: GCACACGCGCATCTGGCATAAAAGA with the following PCR conditions: 94°C for 2 minutes, then 30 cycles: 92°C, 30 seconds; 64°C, 1 minute; 73°C, 1 minute, followed by 75°C, 5 minutes. The PCR product was digested with BseRI and separated on 2% Agarose: 2% MetaPhor agarose gel (Cambrex, Rockland, ME, USA).

Cloning of chrna1 cDNA and sequence analysis

Total RNA from wild-type homozygous mutant embryos was extracted with TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), and used as a template for first-strand cDNA using the Superscript PreAmplication System (Life Technologies). The chrna1 cDNA sequence of wild-type and mutant embryos was determined by amplifying two overlapping fragments with the following primers: chrna1-#1-F: CGTTCAGTCAGCTA TAAGGAC with chrna1-#1-R: GCAATGACCAGATGAGTG; chrna1-#2-F: CTGCATGCTG-TTCTCCITCC with chrna1-#2-R: GCGTGTGGTGGGATTTTGG. The following PCR conditions were used: 94°C for 2 minutes, then 35 cycles: 92°C, 1 minute; 58°C, 1 minute; 73°C, 1.5 minutes, followed by 75°C, 5 minutes. PCR products were subcloned into the pGEM-T Easy Vector System (Promega, Madison, WI, USA). Two to three independent clones from two independent PCR reactions were sequenced (Napcore, Children’s Hospital of Philadelphia, PA, USA). Sequence was analyzed using MacVector and Blast 2 software from the National Center for Biotechnological Information (www.ncbi.nlm.nih.gov/blast/bl2seq). Mutant embryos were genotyped by amplifying a portion of the chrna1 M2 domain with primers chrna1-#3-F: GAGAAGATGACCCCTCAGCAT and chrna1-#3-R: TCACAGTGGATTAGTGG, and digested with restriction enzyme MspI (New England Biolabs, Beverly, MA, USA).

In vivo recordings of synaptic currents

Whole cell recordings of synaptic currents were performed on muscle of 72 hpf wild-type and twister heterozygote fish as previously described (Ono et al., 2002). Spontaneous miniature end-plate currents (mEPCs) and stimulus evoked end-plate currents (EPCs) were recorded at holding potentials of –90 mV and –50 mV, respectively. The mEPCs were filtered at 2 kHz to reduce the noise, and data was sampled at 50 kHz. The EPCs were evoked by extracellular stimulation of the spinal cord using 20-30 V pulses of 300 μsec duration. In a separate set of experiments, we synthesized mRNA from clones containing the wild-type alpha or nic-Twister dbn12 alpha subunit in pC32+. mRNA (2 ng) was injected into single-cell nic-P107 embryos, and injected embryos were screened between 48-hpf to 72-hpf embryos to identify nic-P107 embryos lacking response to touch. These homozygous larvae were then subjected to whole-cell patch clamp recordings. Synaptic events were analyzed and fitted using the minianalys software (Synaptosoft).

Electron microscopy

Embryos at 26 hpf were fixed in 6% glutaraldehyde in either 0.1 M cacodylate or phosphate buffer pH 7.2-7.4 for at least 1 hour at room temperature, and used immediately or stored for up to several days at 4°C in the fixative. Head, yolk sac, yolk extension and most of the tail fin were removed within the first minutes of fixation to allow better penetration of the fixative. Tails were post-fixed in 2% OsO₄ in the same buffer, en-bloc stained with saturated aqueous uranyl acetate overnight, and embedded in Epon 812. To better preserve glycogen, some were post-fixed in 2% OsO₄, 0.8% K₃Fe(CN)₆ in 0.1 M cacodylate buffer. Thin sections were stained in 4% uranyl acetate in 50% ethanol and with a solution of lead salts (Sato, 1968). Sections were examined using a Phillips 410 electron microscope.

Results

Twister mutant embryos display severe motor axon defects

A single twister mutant allele, dbn12, was isolated in a genetic screen for dominant mutations affecting locomotor behavior (van Eeden et al., 1999). At 26 hpf, heterozygous twister embryos display uncoordinated movements. Instead of exhibiting alternating left-right tail flaps in response to tactile stimulation, heterozygous twister embryos contract and shorten along their body axis as though opposite axial muscles are activated simultaneously. Heterozygous twister larvae recover by 6 days post-fertilization (dpf) and grow up to viable and fertile adults, indistinguishable from wild-type animals. In contrast, homozygous mutant twister embryos do not respond to touch but are in an apparent state of muscle hypercontraction, presumably caused by persistent activation of left and right axial muscle. As a consequence, the notochord is buckled and the body axis shortened. Homozygous mutants do not recover and die at around 7 dpf. The locomotor behavior of heterozygous twister mutants is characteristic of recessive mutations of the ‘accordion’ group (Granato et al., 1996). This group includes the dwanka gene, which plays a central role in the guided migration of spinal motor axons (Zeller and Granato, 1999).

To determine whether the twister gene plays a similar role in axonal pathfinding, we examined the axonal trajectories of primary motor neurons. In 26 hpf wild-type embryos, the znpl-1 antibody labels the common axonal path of the primary motor neurons CaP, MiP and RoP, from the spinal cord to the choice point, as well as the cell-type-specific projections of CaP and MiP axons into ventral and dorsal somites, respectively; (Fig. 1A,B) (Eisen et al., 1986; Myers et al., 1986). In heterozygous twister embryos, motor axonal trajectories were only mildly affected. In 25% of the heterozygous mutant hemisegments, motor axons sprouted short, ectopic branches at the region of the choice point and along the path through the ventral myotome (n=340; Fig. 1C). Similar ectopic branches were also present in homozygous twister embryos, although these branches were longer and occurred at a much higher frequency (55%; n=340; Fig. 1D). Moreover, motor axons crossed into neighboring somites (4%), or completely failed to extend into the ventral somite (11%; Fig. 1D). Thus, mutations in the twister gene give rise to aberrant motor axon trajectories, suggesting an important role for the twister gene in motor neuron development.

Mutations in the Twister gene disrupt muscle fiber organization and ultrastructure

To determine whether the axonal phenotypes in twister mutant embryos are caused by obvious defects in the somitic environment through which motor axons migrate, we examined myotome patterning and differentiation. Fast muscle fibers make up the bulk of somitic tissue and they constitute the medial surface along which primary motor axons migrate...
Slow muscle cells are derived from adaxial cells, which are initially located on the medial side of the myotome (Devoto et al., 1996), and have been shown to play important roles for motor axon guidance (Zeller and Granato, 1999; Zhang and Granato, 2000). As primary motor neurons initiate axonogenesis, adaxial cells migrate radially to the lateral surface of the somites where they form a superficial layer of slow muscle fibers (Devoto et al., 1996). To determine whether the axonal phenotypes observed in twist mutants are caused by obvious defects in fast or slow muscle development, we examined both populations with fiber-type-specific antibodies.

In 26 hpf wild-type embryos, slow muscle fibers form a monolayer on the lateral surface of the myotome, whereas fast muscle constitutes a deeper mass of multinucleated fibers oriented obliquely within the segment (Devoto et al., 1996) (Fig. 2A,B). In heterozygous twist mutant embryos, primary motor axons develop ectopic branches at the region of the choice point (black arrows). In twist homozygous mutant embryos, motor axons form ectopic branches at the choice point (black arrow) or in the ventral myotome (yellow arrows). In addition, motor axons are stalled along the common path (blue arrows; all lateral views).

**Fig. 2.** In twist mutant embryos, slow and fast muscle fiber development is severely affected. Confocal micrographs of 26 hpf wild-type and mutant embryos stained with antibodies specific for slow muscle fibers (F59; A,C,E) or fast muscle fibers (F310; B,D,F; all lateral views). (A) In wild-type embryos, slow muscle forms a superficial monolayer of striated fibers arranged in parallel. (B) In wild-type embryos, fast muscle fibers are orientated obliquely and form the bulk of the myotome. (C,D) In twist heterozygous mutants, individual fibers are thinner, such that gaps appear between them (arrowheads), and somite boundaries are irregular (arrow). (E,F) twist homozygous mutants exhibit severely disrupted slow and fast myofiber organization. Some fibers have detached from the somite boundaries, which are irregular (white arrow). Many muscle fibers have contraction clots of myofibril material, as shown by densely stained clots (arrowheads). Scale bar: 50 μm.
Increased neuromuscular activity during development

Increased neuromuscular activity during development or even absent (Fig. 2E,F). Individual myofibers appeared splayed apart and some were detached from their substrate at the somite boundary (Fig. 2E).

To analyze these defects in more detail, we examined myofibril organization by electron microscopy. Sections of 26 hpf wild-type muscle revealed the characteristic organization of striated muscle: regularly arranged myofibrils surrounded by membranes of the sarcoplasmic reticulum (SR) and the T-tubules. In cross-sections, filaments were assembled in a hexagonal arrangement typical of mature myofibrils, with each thick filament encircled by six thin filaments (Fig. 3A, and inset). Corresponding longitudinal sections showed pronounced A and I bands and triads of SR and T-tubules at the Z lines, hallmarks of sarcomere organization (Fig. 3B). In heterozygous *twister* mutant muscle, the overall myofibril and membrane ultrastructure was preserved, albeit in a less organized fashion (Fig. 3C,D). The hexagonal array of thick and thin filaments appeared less regular, with individual thick filaments often surrounded by more than six thin filaments (Fig. 3C inset). Longitudinal sections confirmed that many myofibrils lacked the precise alignment between thick and thin filaments, as well as the stereotypic position of triads at the Z line (Fig. 3D). Analysis of homozygous *twister* muscle confirmed that myofibril organization was severely impaired (Fig. 3E,F). Myofibrils were thin and extended in various directions and the contractile filaments appeared disorganized. Similarly, membranes of the SR and the T-tubules were poorly developed. Moreover, we observed cytological evidence of cell death, which was confirmed by TUNEL analysis (data not shown). Together, the above analyses strongly suggest that *twister* activity is essential not only for motor axon growth, but also for the development and/or the integrity of somitic muscle. Similar to the axonal phenotype, the severity of the muscle defect increased with the number of mutant *twister* alleles present in the embryo. This suggests that the *twister* mutation acts in a dose-dependent fashion, and that tight regulation of wild-type *twister* activity is essential for nerve and muscle development.

**twister-related phenotypes are dependent on neuromuscular activity**

Given that *twister* mutants display defects in muscle and motoneuron development, we sought to determine the mechanism by which the *twister* gene controls both of these processes. One possibility is that the muscle phenotype is a consequence secondary to the axonal defect, and that the *twister* gene controls one defined aspect of axonal pathfinding, a process independent of neuromuscular activity (Westerfield...
et al., 1990). Alternatively, the pre- and post-synaptic defects are both secondary and the twistern gene acts at the site where the muscle and nerve interact, at the neuromuscular junction. For example, the twistern gene may control neuromuscular transmission and the twistern-related phenotypes could result from unregulated neuromuscular activity. To distinguish between these two possibilities, we blocked neuromuscular transmission in embryos by application of α-BTX, an inhibitor of AChR function (Berg et al., 1972; Pittman and Oppenheim, 1978). The expectation is that blocking neuromuscular activity would not affect activity-independent defects, such as lack of muscle differentiation or neuronal pathfinding, but would rescue activity-dependent defects caused by increased neuromuscular transmission.

We injected α-BTX into the yolk of ~13-14-hpf embryos, prior to the formation of neuromuscular junctions and consequently the acquisition of spontaneous movements (Liu and Westerfield, 1992; Saint-Amant and Drapeau, 1998). Nearly all injected embryos survived (>95%), and over 70% of injected embryos remained paralyzed until they were collected for analysis at 26 hpf. In control experiments, 25% of buffer-injected embryos derived from a heterozygous intercross displayed severe axonal and myofiber defects, identical to those observed in twistern homozygous mutants (Fig. 4A). Approximately 50% of buffer-injected embryos displayed axonal and muscle defects characteristic for heterozygous embryos, and the remaining 25% embryos appeared wild-type. In contrast, when injected with α-BTX, the percentage of embryos with wild-type and heterozygous phenotypes increased from 75% in the control to 88.1% (Fig. 4A). Concomitant with the increase of embryos with wild-type phenotypes, α-BTX treatment significantly reduced the proportion of embryos with severe muscle and axonal defects from 25% (in controls) to 11.9% (Fig. 4A). Moreover, genotyping of individual embryos confirmed that α-BTX treatment of homozygous twistern embryos rescued axonal and muscle morphology (data not shown). Thus, blockade of neuromuscular activity restores axonal and muscle morphology in twistern embryos, suggesting a role for the twistern gene in regulating activity-dependent processes at the neuromuscular junction.

**Fig. 4. twistern-related nerve and muscle defects are caused by prolonged synaptic transmission.** (A) Injecting buffer into embryos obtained from two twistern heterozygotes resulted in the expected distribution of axonal and muscle phenotypes, i.e. 75% displayed wild-type or heterozygous axonal and myofiber phenotypes (white bar), whereas 25% displayed severe axonal and myofiber defects (gray bar; n=261, from three experiments). In contrast, α-BTX injection increased the proportion of embryos displaying wild-type or heterozygous axonal and myofiber morphology (88.1±3.2%), and decreased the proportion of embryos displaying twistern homozygous defects (11.9±3.2%; n=475 from six experiments; P<0.0001 for Fisher’s exact test). (B-G) Comparison of spontaneous (mEPC) and evoked (EPC) synaptic currents obtained by whole-cell voltage clamp of 72 hpf wild-type (B) and heterozygous twistern (C) larvae. (B,C) The plot represents the average of 10 individual spontaneous currents, aligned at the peaks. (D,E) Frequency histograms of decay times for individual mEPCs from wild-type (D) and heterozygous twistern (E) muscle. Decay times were determined on the basis of 90% decay from peak amplitude. Note that in wild-type muscle, all events showed decay times shorter or equal to 10 mseconds, whereas in mutant muscle most of the events had decay times longer than 10 mseconds (black arrows). (F,G) Evoked end-plate currents obtained from wild-type (F) and heterozygous twistern (G) muscle in response to 50 Hz stimulation of the spinal cord. Ten consecutive trains were averaged. Time points of stimulation are indicated by filled circles and the broken line indicates the baseline holding current.

**In twistern mutants decay rates of neuromuscular currents are altered**

To determine the role of twistern in regulating neuromuscular transmission, we analyzed synaptic transmission at the neuromuscular junction. Spontaneous synaptic currents were measured by whole-cell recordings from 72-hpf axial muscle in intact larvae. We compared the profiles of spontaneous mEPCs in wild-type larvae with those in heterozygous twistern mutants (at this stage, twistern homozygous mutants display severe muscle degeneration, preventing efficient recordings). Rise times of spontaneous synaptic currents obtained from heterozygous twistern and wild-type muscle were similar (Fig. 4B,C). In contrast, synaptic decay times in heterozygous twistern mutants were significantly prolonged (Fig. 4B,C).
wild-type muscle, the decay phase was typically well-fitted by a single exponential curve with a decay time constant on the order of 5 to 10 mseconds, whereas in *twister* heterozygous muscle, the decay phase of mEPCs failed to conform to a single exponential decay. Instead, as many as three exponential components were required to describe the decay phase, consistent with the presence of mixed wild-type and mutant copies of the *twister* gene in heterozygous larvae. To quantify the differences in decay kinetics between wild-type and *twister* mEPCs, we computed the time required for each current to decay to 10% of its peak amplitude. The mean decay time for wild-type mEPCs was 9.4±6.4 mseconds, in contrast to 41.8±21.4 mseconds for *twister* heterozygous larvae (Fig. 4D,E). Thus, mutations in the *twister* gene significantly prolong the decay phase of spontaneous end-plate currents.

To determine whether *twister* mutations also prolong AChR openings in response to CNS stimulation, we recorded synaptic currents (EPCs) evoked by repeated stimulation of motor neurons. In wild-type larvae, a 50-Hz train of electric stimulation resulted in fast-activating synaptic currents that completely decayed between successive stimuli (Fig. 4F). Similarly, stimulation of heterozygous *twister* larvae evoked fast-activating synaptic currents. However, in heterozygous *twister* mutants EPCs failed to decay fully between successive stimuli, causing a summation of individual end-plate currents. (Fig. 4G). Thus, mutations in the *twister* gene do not overtly affect the rise times of spontaneous and evoked end-plate currents, but do extend their decay rates. We conclude that in *twister* mutants, synaptic activity is prolonged and that the *twister* gene is a central component of the machinery regulating neuromuscular activity.

**The *twister* phenotype is caused by a gain-of-function mutation in the muscle acetylcholine receptor α-subunit (chrna1)**

To identify the molecular nature of the *twister* gene, we combined molecular genetic mapping with a candidate gene approach. We first mapped the mutation to a small genetic interval, and then examined the corresponding region on a radiation hybrid map for possible candidate genes. To map the *twister* locus, we used bulk-segregation analysis of approximately 100 simple sequence length polymorphic (SSLP) markers distributed evenly throughout the genome (Knapik et al., 1998). We found that the *twister* mutant phenotype co-segregated with SSLP marker z6601 on linkage group 6 (Fig. 5A). Fine-resolution mapping of more than 700 individual F2 mutant embryos positioned the *twister* locus 0.77 cM centromeric to z6601 (Fig. 5B,C).

Examination of the LNS4 radiation hybrid panel revealed that marker z6601 maps in the vicinity of the α-subunit of the muscle nicotinic acetylcholine receptor (nic-1/chrna1) (Fig. 5C) (Sepich et al., 1998). The α-subunit is a component of the pentameric AChR (α3β2δ), a ligand-gated ion channel clustered at the neuromuscular junction to transmit the neural signal to the muscle. The α-subunit is expressed exclusively in muscle, and there functions cell autonomously (Sepich et al., 1994; Sepich et al., 1998). To further test the candidacy of chrna1, we identified a restriction length fragment polymorphism specific to the 3’UTR of chrna1 and examined the linkage between chrna1 and the *twister* mutant phenotype. No recombination was detected in 750 meioses examined, indicating that *twister* and chrna1 are tightly linked and providing further evidence that the *twister* phenotype may be caused by a mutation in chrna1 (data not shown).

To determine whether mutations in chrna1 cause the *twister* phenotype, we isolated cDNAs encoding the chrna1 gene from *twister* mutant embryos. Sequence analysis revealed that the *twister* cDNA coding for chrna1 has a T-to-C nucleotide transversion at the second base of codon 258, giving rise to a leucine (CTG) to proline (CCG) amino acid substitution (L258P) (Fig. 5D). Leucine 258 is located in the second transmembrane domain (M2), which is 100% conserved across all species examined (Fig. 5E,F). Moreover, sequence analysis revealed that the ENU-treated founder fish, which gave rise to the *twister*dbn12 allele, contains the wild-type sequence CTG at codon 258 (data not shown). This demonstrates that the C-to-T nucleotide substitution is not a polymorphism but a mutation presumably caused by the ENU treatment.

Mutations in the zebrafish chrna1 gene have previously been isolated (Sepich et al., 1998). The nic-1b107 mutation is a γ-ray-induced allele that segregates in a recessive manner (Westerfield et al., 1990). The nic-1b107 mutation prevents correct splicing of the pre-mRNA, thus completely blocking protein synthesis of the α-subunit and assembly of functional AChRs (Sepich et al., 1998). Consequently, no phenotypes are observed in heterozygous nic-1b107 embryos, whereas homozygous embryos are paralyzed but do not display any obvious defects in motor axon or muscle fiber morphology (Westerfield et al., 1990). To further confirm that *twister* is a mutation in the nic-1/chrna1 gene, we performed complementation analysis between *twister* and nic-1b107 mutants. As shown above, heterozygous embryos carrying one copy of the *twister*dbn12 allele only display mild muscle and motoneuronal phenotypes, whereas homozygous embryos carrying two copies of the *twister*dbn12 allele exhibit strong muscle and motoneuronal phenotypes (Figs 1, 2). In approximately 20% of the embryos derived from crosses between *twister*dbn12 and nic-1b107 heterozygous adults, we observed pathfinding and muscle defects typically observed in heterozygous mutant *twister* embryos (data not shown). Such non-complementation result is expected if the *twister*dbn12 and nic-1b107 mutations affect one and the same gene. Moreover, the phenotype of *twister*dbn12nic-1b107 embryos carrying one gain-of-function and one loss-of function allele is identical to embryos carrying two gain-of-function alleles. This, together with the synaptic recordings from heterozygous *twister*dbn12 embryos strongly indicates that *twister*dbn12 acts as a gain of function allele of the nic-1/chrna1 gene. From this point on, we will refer to the *twister* mutation as nic-1*twister* dbn12.

To demonstrate that the L258P mutation in the chrna1 gene causes the slowed synaptic decay, we expressed the wild-type and the L258P mutation in embryos lacking the chrna1 gene (nic-1b107). Recording from un.injected nic-1b107 homozygous larvae confirmed the complete absence of mEPCs, and thus the lack of AChRs (Westerfield et al., 1990). We compared mEPCs recorded between 48 and 72 hpf from nic-1b107 homozygous larvae expressing the wild-type or L258P mutant α-subunit. The mEPCs recorded from larvae expressing the mutant α-subunit rose and decayed slower than those expressing the wild-type α-subunit (Fig. 5G). Unlike heterozygous nic-1*twister* dbn12, which express both the mutant and the
wild-type α1-subunit and decay with multiple exponential components, mEPCs recorded from larvae expressing only the mutant α1-subunit decayed with a single exponential time course. By fitting mEPCs with single exponential functions, we found that synaptic decay times produced by the mutant channels were significantly longer. The average decay times for nic-1b107 larvae expressing the mutant α1-subunit ranged from 10.9 milliseconds to 45.9 milliseconds with an overall mean of 24.3 milliseconds (Fig. 5H). In contrast, the average decay times for nic-1b107 larvae expressing the wild-type receptor ranged from 1.4 milliseconds to 10.9 milliseconds with an overall mean of 6.2 milliseconds (Fig. 5H). In addition, we determined that the L258P mutation prolongs the rate of channel opening, as determined by measuring the 10 to 90% rise times (2.9 milliseconds for the mutant compared with 1.9 milliseconds for wild-type, data not shown). Thus, molecular-genetic mapping, sequencing and recordings from larvae expressing the mutant α1-subunit demonstrate that the L258P mutation in the chnral gene gives rise to prolonged neuromuscular activity, resulting in the dominantly transmitted pre- and postsynaptic defects observed in twister embryos.

**Excessive neuromuscular activity disrupts early aspects pre- and post-synaptic development**

By 26 hpf, slow and fast muscle fibers have differentiated and primary motor neurons have already extended their projections far into the periphery, thereby constituting a simple motor system mediating touch-inducible reflex behaviors. At this stage, homozygous nic-twister dbn12 mutant embryos display severe pre- and postsynaptic defects (Figs 1-3). However, wild-type zebrafish embryos display spontaneous movements as early as 16 hpf, when muscle cells are still maturing and primary motor axons are migrating into the somites (Liu and Westerfield, 1992; Myers et al., 1986). This suggests that as early as 16 hpf, functional neuromuscular connections are present. To determine whether excess neuromuscular activity impairs these early steps of neuromuscular development, we examined motor axon pathfinding, formation of clustered AChRs and muscle differentiation at 17hpf, when motor axons pioneer into the periphery and establish the first neuromuscular synapses.

At 17 hpf, wild-type muscle fibers expressed cell-type-specific myosins, but fibers were thin and only some exhibited their characteristic cross-striations (Fig. 6A, fast muscle data not shown). Although their muscle fibers are not yet fully
Increased neuromuscular activity during development

Increased neuromuscular activity during development. Confocal analysis of 17 hpf wild-type and nic^Twister dbn12 mutat embryos, stained with antibody F59 specific for slow muscle fibers (A-C), or double stained with motoneuron-specific antibodies (D,G,J,M-O), and with AlexaFluor 594-conjugated α-BTX to visualize clustered AChRs (E,H,K,M-O) at 17 hpf (A-L) or 26 hpf (M-O; all lateral views). (A,B) In 17 hpf wild-type and nic^Twister dbn12 heterozygous mutant embryos, muscle fibers are thin and elongated but few display striations. (C) In nic^Twister dbn12 homozygous mutant embryos, muscle fibers are less elongated with no visible striations. In homozygous mutant embryos, somites are compressed along the anterior-posterior axis and expanded along the dorso-ventral axis. (D-F) In 17 hpf wild-type embryos, motor axons have reached or just extended past the choice point (the level is indicated by white bars). Note the elaborate and fan-like morphology of the axonal tip, characteristic of advancing growth cones (white double arrowhead). As motor axons pioneer into the somites, clustered AChRs emerge and co-localize along the extending axon, reminiscent of en passant synaptic contacts. These dense AChR clusters decorate parts of the axon, except for the presumptive growth cone (white arrow) which precedes the distal limit of clustered AChR (white arrowhead). (G-I) In heterozygous embryos, most motor axons extend normally and their presumptive growth cones display a wild-type-like morphology (white arrow). However, a significant fraction of heterozygous motor axons stall before reaching the choice point, and presumptive growth cones appear smaller and less elaborate (red arrow). On those axons, dense AChR clusters are localized distal to presumptive growth cones (white arrow). Note that the morphology of these aneural AChR clusters is indistinguishable from those that co-localize with the axon. (J-L) In nic^Twister dbn12 homozygous mutant embryos, many motor axons (red arrows) are stalled before the choice point. Growth cones are less elaborate and appear collapsed (white double arrowhead), and AChR clusters are smaller and scattered throughout the somite (white arrowheads). (M) In 26 hpf wild-type embryos, AChR clusters are restricted along the lengths of the ventral and dorsal motor axons (yellow arrows) and along the somite boundaries (yellow arrowhead). (N) In nic^Twister dbn12 heterozygous embryos, AChR clusters co-localize along the lengths of the ventral and dorsal axons (yellow arrows) and along the somite boundary (yellow arrowhead). AChR clusters are also detected along aberrant branches (blue arrowhead). (O) In 26 hpf nic^Twister dbn12 homozygous mutants, smaller and fewer AChR cluster co-localize with axonal branches (blue arrowhead). Somite boundary localization of clustered AChRs is strongly reduced. Scale bar: 50 μm.

differentiated, 17 hpf wild-type embryos display well-characterized spontaneous movements (Saint-Amant and Drapeau, 1998). At this stage, muscle fibers in heterozygous nic^Twister dbn12 embryos appeared indistinguishable from those in wild-types (Fig. 6B). In contrast, homozygous nic^Twister dbn12 mutant embryos did not move spontaneously but appeared in a state of muscle hypercontraction. In these embryos, the somites were markedly compressed along the anterior-posterior axis and expanded along the dorso-ventral axis (Fig. 6C). Although expression levels and cellular localization of slow muscle myosin was comparable to wild-type embryos, fibers were shorter and less fasciculated. Over the next 4 hours, homozygous mutant muscle fibers became progressively splayed and detached from the somite boundary, similar to what we had observed at later stages (26 hpf, Fig. 3). Thus, excess postsynaptic activity in twister homozygous
mutants interferes with muscle fiber maturation already at a stage when the first neuromuscular synapses form.

Although excess neuromuscular activity affects muscle fiber development only in homozygous mutant embryos, it interferes with the early steps of pathfinding and synaptogenesis in both homozygous and heterozygous nic-1\textsuperscript{twister dbn12} mutants. At 17 hpf, the first wild-type motor growth cones have exited the spinal cord and pioneer a path along the medial surface of the myotome (Bernhardt et al., 1998; Eisen et al., 1986). As pioneering growth cones advance, dense AChR clusters emerge and co-localize along the extending axon, reminiscent of en passant synapses (Fig. 6D-F) (Hatada et al., 1999; Liu and Westerfield, 1990; Liu and Westerfield, 1992; Sheard and Duxson, 1997). Increased movements of the embryo coincide with the appearance of these dense AChR clusters, which have been shown to represent functional synapses (Melançon et al., 1997). By 26 hpf, CaP and MiP motoneurons have extended their cell-type-specific projections into the ventral and dorsal myotome, respectively, with AChR clusters localized along these trajectories (Fig. 6M).

In 25% of heterozygous nic-1\textsuperscript{twister dbn12} mutant hemisegments, we observed that motor axons failed to extend as far as wild-type axons, possibly because of stalling and/or retraction (n=32; Fig. 6G). Interestingly, in such affected hemisegments, AChR clusters were present extra-synaptically, as we observed α-BTX-positive clusters distal to the presumptive growth cone (Fig. 6H,I). This is significant because in wild-type embryos, clustered AChRs were always apposed to motor axons and behind the presumptive growth cone (Fig. 6D-F). Moreover, these nerve-free AChR clusters were indistinguishable from those in wild-type embryos. By 21 hpf, axonal extension defects were only detectable in 7% of heterozygous nic-1\textsuperscript{twister dbn12} hemisegments, and by 26 hpf, axonal stalling had given way to excessive axonal branches decorated by ectopic AChR clusters (Fig. 6N and data not shown). Thus, moderately increased neuromuscular transmission present in heterozygous mutants appears to influence motor axons in two different ways. Initially, increased activity transiently delays axonal growth, but then promotes aberrant axonal branching and formation of ectopic neuromuscular connections.

Similar to pioneering motor axons of heterozygous mutants, homozygous nic-1\textsuperscript{twister dbn12} growth cones displayed axonal stalling/retraction. However, these pre-synaptic defects were more severe, more persistent, and always associated with the failure to form and/or maintain clusters of AChRs. At 17 hpf, within hours of the onset of primary motor axon extension, most homozygous mutant growth cones stalled at the spinal cord exit point or at the choice point (Fig. 6J). Rather than displaying their typical fan-shaped morphology, presumptive growth cones appeared collapsed (double arrowhead in Fig. 6J). Only few and small AChR clusters were detectable, many of which were not associated with motor axons but were instead dispersed throughout the myotome (Fig. 6K,L). By 26 hpf, most homozygous mutant motor axons had projected further, although often along aberrant paths (Fig. 6O). Mutant motor axons formed long ectopic branches, along which AChR clusters occasionally co-localized (Fig. 6O, see also Fig. 1D).

Thus, analyses of heterozygous and homozygous mutants show that increased neuromuscular transmission results in two temporally distinct effects on pathfinding motor axons. Increased activity first transiently delays axonal growth, but then promotes aberrant axonal branching. Both of these effects appear to be dose-dependent, as the severity of axonal stalling and branching increased with the number of hyperactive receptors present in the embryos. Interestingly, in heterozygous mutants presynaptic defects occurred in the absence of overt morphological postsynaptic defects. Together, these results suggest that during pathfinding, axons and muscle communicate extensively through en passant synaptic contacts and that postsynaptic activity can modulate presynaptic growth.

**Discussion**

The **twister** mutant phenotype is caused by a gain-of-function mutation in the **chrna1** gene

Cloning revealed that the nic-1\textsuperscript{twister dbn12} allele contains a missense mutation in the **chrna1** gene, substituting a leucine with a proline, L258P, in the second transmembrane domain (M2; Fig. 5D). This domain, which lines the channel pore, is 100% conserved across vertebrate species, suggesting that the L258P substitution causes the **twister** mutant phenotype. Four additional lines of evidence lead to the same conclusion. First, the **twister** mutant phenotype maps to a chromosomal region containing the **chrna1** locus and co-segregates with a polymorphism in the 3’UTR of the **chrna1** gene. Second, results from the complementation analysis between the dominant nic-1\textsuperscript{twister dbn12} allele and loss-of-function nic-1\textsuperscript{b107} allele shows that both mutations affect the same gene and that nic-1\textsuperscript{twister dbn12} is a gain-of-function allele of the nic-1\textsuperscript{chrna1} gene. Third, the L258P substitution was not present in the ENU-treated fish from which the **twister** allele was derived. Thus, the L258P substitution does not represent a polymorphism in the parental strain, but reflects a mutation most probably introduced by ENU mutagenesis. Finally, expressing the L258P mutant subunit recapitulates the synaptic defects observed in nic-1\textsuperscript{twister dbn12} mutant larvae. Together, this data demonstrates that the L258P substitution in the M2 domain of the **chrna1** gene is responsible for the phenotypes observed in nic-1\textsuperscript{twister dbn12} mutant embryos.

The nic-1\textsuperscript{twister dbn12} L258P substitution in the AChR α-subunit alters neurotransmission at neuromuscular junctions. The embryonic muscle AChR channel is a pentameric membrane protein (α2βγδ). Each subunit contains four membrane-spanning segments (M1-M4), of which the M2 segments together form five rods that twist upon activation to allow flow of cations (Miyazawa et al., 1999; Unwin, 1995). Labeling and mutagenesis studies have shown that amino acid residues of the M2 domains line the channel lumen and contribute to the channel’s gate and ion selectivity filter (Akabas et al., 1994; Karlin, 2002; Leonard et al., 1988; Wilson and Karlin, 1998). Indeed, our recordings demonstrate that the L258P mutation in the M2 domain prolongs channel openings, thereby resulting in increased end-plate currents. Such prolonged end-plate currents are characteristic of an inherited human condition, slow-channel congenital myasthenic syndrome (SCCMS) (Engel et al., 2003). Fifteen slow-channel mutations have been reported, all of which are caused by dominant, gain-of-function mutations in various AChR subunits (Engel et al., 2003).

In neuromuscular preparations from SCCMS patients, end-
plate currents are 4 to 10-fold prolonged (Engel et al., 2003). Furthermore, several SCCMS mutations in the M2 segment have been shown to result in the normal expression and assembly of AChRs, but generate prolonged channel openings by stabilizing the open state and decreasing the rate of channel closure (Croxen et al., 1997; Engel et al., 1996; Milone et al., 1997; Ohno et al., 1995). These observations support our interpretation that the nic- \textit{L258P} mutation renders the AChR channel leaky, the severe myofibril degeneration, and by 6 dpf, mutant larvae die. Therefore, our analysis of homozygous mutants reveals that kinetic mutations resulting in a mild dominant phenotype in heterozygous mutants, can lead to massive and global degeneration of skeletal muscle in a homozygous situation. Recently, mutations in the zebrafish acetylcholinesterase gene (\textit{ache}) have been reported (Behra et al., 2002; Downes and Granato, 2004). In contrast to \textit{nic-}, \textit{L258P} substitution causes unusually high rates of spontaneous channel openings (Engel et al., 1996; Milone et al., 1997; Ohno et al., 1995). These observations support our interpretation that the \textit{nic-} channel defects might occur even before the first growth cones arrive, as the mutant AChR channel may open for extended periods in the absence of ACh ligand. In fact, mutations in the M2 segment of the human \(\alpha\), \(\beta\) and \(\epsilon\) subunits have been shown to cause unusually high rates of spontaneous channel openings (Engel et al., 1996; Milone et al., 1997; Ohno et al., 1995). Although we have not determined whether the \(\alpha\text{L258P}\) mutation renders the AChR channel leaky, the severe myofibril and AChR cluster defects observed in 17- \(\alpha\text{HP}\) homozygous mutants, despite the presence of only few synaptic contacts, are consistent with the possibility that \textit{nic-} \textit{channel} opens extensively in a ligand-independent manner. Regardless of the precise mechanism, our results demonstrate that the onset of progressive end-plate and myopathy can occur during embryogenesis, much earlier than previously reported in SCCMS patients or SCCMS mouse models (Engel et al., 1982; Engel et al., 2003; Gomez et al., 1997). Thus, as growth cones make their first contacts with the muscle, increased channel activity impacts ongoing pre- and post-synaptic development.

Unlike the characterized mutations giving rise to progressive symptoms in heterozygous SCCMS patients, the \(\alpha\text{L258P}\) substitution causes a dominant phenotype only transiently in heterozygous \textit{nic-} \textit{mutants}. Although heterozygous mutant AChR channels significantly prolong synaptic decay, they do not elicit postsynaptic degeneration observed in homozygous mutants and SCCMS patients. Instead, heterozygous embryos transiently exhibit an ‘accordion’ motility resulting from simultaneous contraction of left and right axial muscles, a behavior presumably caused by the prolonged activation of AChRs and summation of synaptic currents. Interestingly, heterozygous mutants recover within 6 dpf and growth up to viable adults. Although we have not examined end-plate structure and physiology in these adults, they do not display any of the motor impairments observed prior to 6 dpf. Therefore, heterozygous mutant AChRs cause mild but transient pre- and post-synaptic defects. One possible
Neural activity modulates motor axon behaviors through en passant synaptic contacts

Neurotransmission has been shown to play an important role in synapse maturation and refinement (Sanes and Lichtman, 1999b; Zhang and Poo, 2001). Loss of postsynaptic activity causes presynaptic defects but does not affect the ability of motor axons to grow towards their muscle targets (Broadie and Bate, 1993a; Misgeld et al., 2002; Westerfield et al., 1990). Most studies, however, have focused on terminal synapses, at sites where motile growth cones have transformed into non-motile synaptic termini. Although loss-of-activity experiments show that in the absence of postsynaptic activity axonal growth and presynaptic vesicle release occur correctly (Li et al., 2003; Westerfield et al., 1990), these experiments do not address the possible effects elevated postsynaptic activity might have on growth cone behaviors. Heterozygous nic-Twister dbn12 embryos provide a unique system in which to examine how increased levels of postsynaptic activity affect extending growth cones.

During the migration of wild-type motor axons to their target areas, clustered AChRs emerge along the length of the axon, proximal to the growth cone (Liu and Westerfield, 1992). Shortly after the first AChR clusters appear, muscle fibers start contracting, suggesting that these AChR clusters represent functional synapses on extending motor axons (Liu and Westerfield, 1992; Melançon et al., 1997). This is reminiscent of en passant synapses, characteristic of synapses between neurons (e.g. C. elegans, mammalian hippocampal neurons), but has also been reported between embryonic rat motoneurons and skeletal muscle (Dailey and Smith, 1993; Sheard and Westerfield, 1992). Shortly after the first en passant synaptic contacts form in heterozygous nic-Twister dbn12 embryos, motor axons appear stalled and/or retracted with a collapsed presumptive growth cone. In some cases, AChR clusters were present past the distal tip of these collapsed growth cones, a situation never observed in wild-type embryos (Liu and Westerfield, 1992). These aneural clusters probably result from appropriate clustering of AChRs below the growth cones, which subsequently retracted. Time-lapse analysis is required to distinguish between axon stalling and retraction and to describe in detail the effects of excess neuromuscular transmission on axon growth dynamics. Nevertheless, these results make the suggestion that excessive postsynaptic activity influences axonal growth by causing stalling and/or retraction of presynaptic growth cones.

One possible explanation for the presynaptic defect is that increased postsynaptic activity damages muscle fibers such that motor axons fail to grow on their surface, a situation clearly illustrated in homozygous mutants. However, several lines of evidence suggest that heterozygous muscle fibers are not overtly compromised in their ability to serve as a substrate for motor axons. First, at 17 hpf, muscle fiber morphology and expression of cell-type-specific myosin are indistinguishable from wild-type. Second, AChR clusters appeared indistinguishable in surface localization, position and size from those in wild-type segments, suggesting that the muscle fibers are suitable substrates for synaptic formation and maintenance. Finally, motor axons eventually resume their migration, and by 26 hpf display excessive rather than reduced growth. Thus, in heterozygous embryos, increased postsynaptic activity affects axonal extension without obviously disrupting muscle fiber integrity.

We cannot completely exclude the possibility that presynaptic defects are a consequence of subtle muscle cell damage or of impaired activity-independent processes such as interactions between axons with postsynaptic filopodial projections (Ritzenthaler et al., 2000; Uhm et al., 2001). Interestingly, dynamic behaviors of such myopodia have been proposed to be regulated by neuromuscular transmission (Misgeld et al., 2002). However, we favor a model that considers retrograde signaling from the muscle to the presynaptic terminal. Postsynaptic cells do not only receive information, but they also provide retrograde signals to the presynaptic neuron (reviewed by Fitzsimonds and Poo, 1998; Tao and Poo, 2001). Such reciprocal interaction is important for development and maintenance of the presynaptic cell. Although the existence of activity-dependent retrograde signals has been suggested from studies in which neuromuscular activity was reduced (Loeb et al., 2002; Nick and Ribera, 2000; Zhao and Nonet, 2000), our studies provide compelling evidence that increased post-synaptic activity influences presynaptic development in vivo. We propose that wild-type muscle fibers produce retrograde signals triggered by synaptic activity at en passant synaptic contacts, and that such signals modulate the rate of axonal extension. Thus, the en passant configuration of the earliest neuromuscular synapses may play a vital role in enabling the first reciprocal interactions between pre- and postsynaptic cells.

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