Characterization of a hypercontraction-induced myopathy in *Drosophila* caused by mutations in *Mhc*

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The Myosin heavy chain (*Mhc*) locus encodes the muscle-specific motor mediating contraction in *Drosophila*. In a screen for temperature-sensitive behavioral mutants, we have identified two dominant *Mhc* alleles that lead to a hypercontraction-induced myopathy. These mutants are caused by single point mutations in the ATP binding/hydrolysis domain of *Mhc* and lead to degeneration of the flight muscles. Electrophysiological analysis in the adult giant fiber flight circuit demonstrates temperature-dependent seizure activity that requires neuronal input, as genetic blockage of neuronal activity suppresses the electrophysiological seizure defects. Intracellular recordings at the third instar neuromuscular junction show spontaneous muscle movements in the absence of neuronal stimulation and extracellular Ca\(^{2+}\), suggesting a dysregulation of intracellular calcium homeostasis within the muscle or an alteration of the Ca\(^{2+}\) dependence of contraction. Characterization of these new *Mhc* alleles suggests that hypercontraction occurs via a mechanism, which is molecularly distinct from mutants identified previously in troponin I and troponin T.

**Introduction**

Muscle contraction is a highly complex and coordinated process, involving a molecular machine tightly regulated to provide ATP-dependent motion in response to neuronal stimulation. Activation of the muscle from the innervating neuron results in a postsynaptic action potential that stimulates stimulation. Activation of the muscle from the innervating neuron results in a postsynaptic action potential that stimulates the sarcomere in an ATP-dependent process. In addition to proteins required during contraction, numerous secondary proteins are required for its continued maintenance, structural support, and force transduction (Huxley, 2000; Lamb, 2000; Pollard, 2000; Poage and Meriney, 2002; Ruff, 2003). Characterization of this complex system may lead to molecular understanding of human diseases such as cardiomyopathies, which arise from perturbations in several known cardiac muscle proteins (Seidman and Seidman, 2001).

The genetic tractability of *Drosophila* has made it an ideal system to characterize mutations affecting neuromuscular function. Many of these mutant alleles are caused by single point mutations in the ATP binding/hydrolysis domain of *Mhc* and lead to degeneration of the flight muscles. Electrophysiological analysis in the adult giant fiber flight circuit demonstrates temperature-dependent seizure activity that requires neuronal input, as genetic blockage of neuronal activity suppresses the electrophysiological seizure defects. Intracellular recordings at the third instar neuromuscular junction show spontaneous muscle movements in the absence of neuronal stimulation and extracellular Ca\(^{2+}\), suggesting a dysregulation of intracellular calcium homeostasis within the muscle or an alteration of the Ca\(^{2+}\) dependence of contraction. Characterization of these new *Mhc* alleles suggests that hypercontraction occurs via a mechanism, which is molecularly distinct from mutants identified previously in troponin I and troponin T.
Szidonya, 1979; Deak et al., 1982; Homyk and Emerson, 1988; Beall and Fyrberg, 1991). Hypercontraction mutants can be genetically suppressed by specific mutant alleles of \textit{Mhc}. The mechanism of suppression has been suggested to be a potential \textit{Mhc}–Troponin I direct interaction (Kronert et al., 1999). However, additional data suggests that an overall decrease in actomyosin force is sufficient to explain suppression of hypercontraction by mutant \textit{Mhc} (Nongthomba et al., 2003). The identification of \textit{Mhc} alleles that directly cause hypercontraction and enhance the hypercontraction defects of other mutants may facilitate defining the role of myosin in the regulation of contraction.

To further understand the molecular and cellular processes underlying neuromuscular function, we performed a screen for \textit{Drosophila} TS behavioral mutants. One complementation group isolated in our screens, Samba, disrupts the \textit{Mhc} locus, leading to hypercontraction and muscle degeneration. Characterization of the \textit{Samba} mutants has revealed potential molecular mechanisms that lead to muscle degeneration through hypercontraction via distinct mechanisms from hypercontraction mutants characterized previously. In addition, these mutants give insight into the role of \textit{Mhc} in the regulation of the contractile process in addition to its role in ATP-dependent motor function.

### Results

**Isolation and characterization of the Samba mutants, Mhc\textsuperscript{S1} and Mhc\textsuperscript{S2}**

The \textit{Samba} (\textit{Samba\textsuperscript{1}} and \textit{Samba\textsuperscript{2}}) mutants were isolated in an ethyl methanesulfonate mutagenesis screen for X-linked and autosomal-dominant TS behavioral mutants. \textit{Samba} adults exhibit dominant TS behavioral defects that include a rapid onset of seizure-like behavior and TS loss of mesothoracic leg function. This behavior is readily evident in all flies by 1 min of exposure to 38°C (Fig. 1 A). \textit{Samba} mutants also display defects at permissive temperatures. \textit{Samba\textsuperscript{1}+} flies are flightless, with thoracic indentations similar to those found in \textit{ether-ago-go\textsuperscript{1}}, \textit{Shaker\textsuperscript{133}} (\textit{eag\textsuperscript{1}}, \textit{Sh\textsuperscript{133}}) mutant flies (Fig. 1 B). Thoracic indentations in \textit{eag\textsuperscript{1}}, \textit{Sh\textsuperscript{133}} flies occur through hypercontraction of the IFM thought to be induced by excessive neurotransmitter release in the presynaptic neuron, which is caused by loss of voltage-gated potassium channels and subsequent reduction in repolarization (Ganetzky and Wu, 1983; Wu et al., 1983). Although homozygous \textit{Samba} mutants are lethal, rare \textit{Samba\textsuperscript{1}} escapers with femur hypercontraction defects can be found. In addition to \textit{Samba}, two other complementation groups, \textit{Swing} (\textit{Swg\textsuperscript{X118}}) and \textit{Breakdance} (\textit{Brkd\textsuperscript{J29}}), were isolated in our screens that exhibited similar dominant TS behavior.

![Figure 1](image-url)
behavioral defects and showed genetic interactions with Samba. Double heterozygotes of Swg\textsuperscript{X118} and Samba\textsuperscript{1} or Samba\textsuperscript{2} and double heterozygotes of Swg\textsuperscript{X118} and Brkd\textsuperscript{J29} are semi-lethal with escapers having hypercontracted femurs (Fig. 1 C). Double heterozygotes of Samba\textsuperscript{1} or Samba\textsuperscript{2} and Brkd\textsuperscript{J29} are synthetic lethal (Fig. 1 D). These genetic interactions and similarities to eag\textsuperscript{1}, Sh\textsuperscript{133} double mutants suggest that Samba, Swing, and Breakdance define a genetic pathway required in the regulation of membrane excitability, and when disrupted, lead to abnormal muscle hypercontraction.

Segregation analysis of Samba revealed an autosomal-dominant mutation on the second chromosome, refined to 2–52 cM by recombination mapping. Deficiency mapping by lethality narrowed the cytological interval between 36A8 and 36C4 on the left arm of chromosome 2. To help identify the Samba locus, we screened for revertants of TS seizure behavior in a \textit{/H9253}-irradiation reversion screen in order to isolate potential loss of function mutations in the Samba locus. Three revertants were identified by loss of TS behavioral defects. These revertants were embryonic lethal with normal morphological development, but showed complete loss of muscle wave propagation in late stage embryos (unpublished results).

Noncomplementation to Mhc\textsuperscript{1} by both the TS mutants and the three revertants identified the Samba mutations as new alleles of the Mhc locus (Mogami and Hotta, 1981). We designated the Samba\textsuperscript{1} and Samba\textsuperscript{2} alleles Mhc\textsuperscript{S1} and Mhc\textsuperscript{S2}, respectively, and the revertants Mhc\textsuperscript{crv1}, Mhc\textsuperscript{crv2}, and Mhc\textsuperscript{crv3}.

The Mhc locus is complex, encoding all muscle-specific isoforms through the use of extensive alternative splice patterns (Rozek and Davidson, 1983; Bernstein et al., 1983). The locus contains 19 coding exons, 5 of which are alternatively spliced, and one that is either included or excluded (Wassenberg et al., 1987; George et al., 1989; Collier et al., 1990; Hess and Bernstein, 1991; Zhang and Bernstein, 2001). An allele isolated previously, Mhc\textsuperscript{5} (G200D), causes similar hypercontraction defects to the Samba mutants. Mhc\textsuperscript{5} introduces a point mutation in exon 4 of the Mhc locus, disrupting the ATPase domain of Mhc (Homyk and Emerson, 1988). Due to the phenotypic similarities with Mhc\textsuperscript{5}, exon 4 of these new alleles was sequenced. Both Mhc\textsuperscript{S1} and Mhc\textsuperscript{S2} were found to be point mutations (V235E and E187K, respectively) mapping to the ATP binding and hydrolysis site of the protein (Fig. 2, A–E).

Seizure activity in Samba flies is dependent upon neuronal activity

Extracellular dorsal longitudinal muscle (DLM) recordings were used to characterize the behavioral seizures at restrictive...
temperatures (Engel and Wu, 1992). Electrical activity was observed in adult flies at room temperature, shifted to 38°C, and returned to room temperature. Correlating with the behavioral defects, abnormal spiking activity in the DLMs was recorded at restrictive temperatures that was not observed at permissive temperatures, nor in Canton-S at 38°C (Fig. 3, A, B, D, and F). Similar seizure activity was observed in SwgX118 and Brkd20 (Fig. 3, H and J). This abnormal activity could be either muscle autonomous or dependent upon synaptic input. To address these possibilities, we used a mutation in the voltage-gated Na+ channel, paralytic (para2), which is required for action potential propagation in the motor neuron (Suzuki et al., 1971). This mutation specifically abolishes neuronal action potentials at elevated temperatures, as para expression is not detected in muscles (Hong and Ganetzky, 1994). In para/Y;Mhc5/+ (X indicating 5, S1, or S2) flies, we observed a suppression of the seizure activity at 38°C (Fig. 3, C, E, and G). Similarly, suppression of activity was observed with para/Y;SwgX118/+ and para/Y;Brkd20/+ (Fig. 3, I and K). These data suggest that mutant muscles are hyperexcitable at restrictive temperatures. This hyperexcitable state, however, cannot lead to autonomous muscle firing, but must be triggered by an initial input by the innervating motor neuron.

**Samba mutations lead to hypercontraction**

To further define how the Samba mutants affect Mhc function, we analyzed genetic interactions with known muscle mutants that increase or decrease the contractile state of the muscle. We characterized genetic interactions with mutations in Troponin I, Troponin T, and Tropomyosin 2 (Tm2). Troponin I is encoded by the wupA locus. A mutation in troponin I, heldup2 (wupAhel2), has been identified previously as a hypercontraction mutation (Deak et al., 1982; Beall and Fyrberg, 1991). A mutation in Troponin T, upheld/1 (up1), is similar to wupAhel2, also causing hypercontraction (Fekete and Szidonya, 1979; Homyk et al., 1980). Hemizygous flies for either wupAhel2 or up1, and heterozygous for Mhc5 or Mhc2 are synthetic lethal, whereas double heterozygous females have femur hypercontraction defects (unpublished results). A mutation in Tm2 (Tm2D53) suppresses the hypercontraction of both up1 and wupAhel2 (Naimi et al., 2001). Similarly, Tm2D53 suppresses the recessive lethality of Mhc5, increasing viability of the homozygotes from 2.38% to 80.92% (Mhc5; Tm2D53 n = 435, Mhc5; Tm2D53 n = 505). These results indicate that the Samba alleles of Mhc cause hypercontraction defects similar to wupAhel2 and up1. Interestingly, both up1 and wupAhel2 exhibit abnormal TS behavior similar to Mhc5 and Mhc2, suggesting that the behavioral defects are most likely a TS susceptibility resulting from an altered state of the muscle secondary to hypercontraction, as opposed to a specific TS dysfunction of the Mhc protein (Fig. 1 A). In contrast to the TS seizure behavior in hypercontraction mutants, Tm2D53 flies do not show abnormal behavior at 38°C, nor do heterozygotes of the Mhc null (Mhc1/1) and heterozygotes of a hypercontraction suppressor mutant (Mhc2D53); Fig. 1 A).

To confirm that the Samba phenotype results from hypercontraction, we analyzed the structure of the IFM in Mhc5/1 flies. The ordered array of filaments in muscles leads to birefringent properties, allowing muscle visualization under polarized light microscopy. In hypercontraction mutants characterized previously, the IFM exhibit one of two defects. Some show loss of birefringence in the middle of the muscles due to breakage or degradation, with the bulk of the muscle fiber at either one or both of the attachment sites. Others show separation from the attachment sites, with birefringence found only in the middle of the fiber (Nongthomba et al., 2003). Mhc5/1 flies exhibited the former defect, showing birefringence at the attachment sites, with loss of birefringence in the middle of the IFM. This defect was partially suppressed in the background of Tm2D53, as the IFM of dou-
ble mutants displayed less degradation despite the presence of indented thoraces compared with similarly aged Samba flies (Fig. 1, E–G). These data confirm that the Samba mutants lead to hypercontraction defects in the muscle.

**Samba mutant muscles do not alter synaptic function but move independently of neuronal input**

Hypercontraction in *Drosophila* muscles induced by the Samba mutants leads to progressive degradation of fibers, similar to degeneration observed in muscular dystrophies. In some animal models of muscular dystrophy, loss of acetylcholine receptor clustering results in the functional denervation of diseased fibers (Rafael et al., 2000). Because *MhcS1* and *MhcS2* were isolated by TS behavioral defects and abnormal extracellular DLM activity, we hypothesized that these mutations may cause functional or structural changes at the neuromuscular junction (NMJ).

Bouton number at the NMJ is tightly regulated and is sensitive to disruptions in both presynaptic and postsynaptic function. Though poorly understood, postsynaptic defects can alter presynaptic structural and functional properties through homeostatic regulatory pathways (Petersen et al., 1997; Davis et al., 1998). To analyze the morphology of the NMJ in Samba mutants, we stained third instar larvae with /H9251-synaptotagmin I antisera, a marker for presynaptic terminals, and analyzed muscle fibers 6 and 7 (Canton-S n = 18 larvae, 97 muscles; *MhcS1/+ n = 27, 157*). Type I innervation from glutamatergic motor neurons was not altered in *MhcS1/+* animals, suggesting little effect of dysfunctional muscles on excitatory innervation (Fig. 4 J). The number of muscles showing ectopic innervation, however, was found to be more frequent than wild type, increasing from 4.1% in control animals to 15.9% in *MhcS1/+* animals (Fig. 4, A–F and K). These were determined to be type II synapses due to their morphology and the absence of postsynaptic DLG staining (Gramates and Budnik, 1999). Increases in type II innervation have also been reported in mutants such as *tipE* and *nap*, which reduce nerve excitability (Jarecki and Keshishian, 1995). The increase in type II innervation suggests that alterations in muscle function can lead to altered neuromodulation. Similar data was obtained with *MhcS2/+* larvae (unpublished data).

To determine whether the altered innervation pattern correlated with abnormal synaptic transmission, we characterized miniature excitatory junctional potential amplitude (mEJP), excitatory junctional potential amplitude (EJP), and mEJP frequency from the muscle 6 NMJ in 0.4 mM Ca²⁺ using intracellular recording techniques (Fig. 5 A; Table I). Wild-type fibers were found to have a mEJP of 0.95 ± 0.04 mV and EJP of 39.6 ± 1.8 mV (n = 8, 23). In mutant fibers, these values were 0.79 ± 0.02 mV and 34.2 ± 1.7 mV, respectively (n = 11, 29). Although these differences are statistically significant and may reflect subtle changes in synaptic function, the change in release is small and the differences more likely reflect the complication caused by an apparent oscillation of the resting membrane potential due to spon-

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**Figure 4. Structural properties of the neuromuscular junction in *MhcS1/+* mutants.** Third instar larvae neuromuscular junctions from (A–C) CantonS and (D–I) *MhcS1/+* labeled with the indicated markers. Arrows indicate an axonal branch containing type II synapses as evidenced by the absence of postsynaptic DLG staining. Bar, 50 µm. (J) Excitatory type I glutamatergic innervation on muscles 6/7 does not change in mutant muscles despite altered muscular function. (K) Ectopic type II innervation increases in mutant muscles.
taneous muscle movement in *Samba* mutants (Fig. 5 B). Movements such as these can lead to apparent voltage changes due to electrode motion. Spontaneous muscle movements did not resemble action potential–induced contraction events. Instead, a slow, cyclic activity that did not use the full contractile potential of the muscle cell was continuously observed in the *Samba* mutants. Because contraction usually depends upon Ca$^{2+}$ influx through L-type calcium channels in the sarcolemma, we hypothesized that alterations of Ca$^{2+}$ influx through these channels may be responsible for the spontaneous contractions in *Samba* mutants. To test this, we recorded from mutant muscles in Ca$^{2+}$-free saline. Spontaneous contractions were still observed in Ca$^{2+}$-free saline similar to those in high extracellular Ca$^{2+}$ (Fig. 5 B). An alternative possibility is that spontaneous contractions may reflect altered signaling from the innervating neuron. Although these recordings are normally done in preparations in which the axon has been severed from the cell body, we further tested this possibility in brain-intact preparations in the presence or absence of 3 μM TTX. Spontaneous movements were seen regardless of neuronal activity (Fig. 5 B). These results indicate that hypercontraction in *Samba* mutants is not due to an increase in neuronal activity or neurotransmitter release, nor to multiple release events per axonal action potential, but can be explained by autonomous movement in the absence of neuronal input. This movement is not dependent upon extracellular Ca$^{2+}$, suggesting a defect in either intracellular Ca$^{2+}$ homeostasis or the Ca$^{2+}$ requirements of muscle contraction.

**Discussion**

Muscle contraction requires the coordinated action of many proteins under the regulation of defined stimuli. Dysregulation of this system leads to myopathy and dystrophy syndromes in humans. *Drosophila* provides an efficient model to dissect the molecular and cellular functions of the individual components and their roles in the coordinated machine that underlies this process. To this end, we have begun a molecular and cellular characterization of muscle dysfunction in *Drosophila* using the hypercontractive *Samba* mutants, *Mhc* and *Mhc*.

**Hypercontraction and Mhc**

Genetic perturbations can lead to hypercontraction in the *Drosophila* IFM by increasing actomyosin force through either a decrease in structural integrity of the sarcomere or an alteration in thin filament regulation of the cross-bridge cycle (Kronert et al., 1995; Reedy et al., 2000). Hypercontraction by the latter mechanism can be suppressed by mutations in *Mhc*, though it remains unclear whether suppression is obtained through an overall decrease in actomyosin force or by a direct role of Mhc on regulating thin filament dynamics (Kronert et al., 1999; Nongthomba et al., 2003). Here, we characterized two new alleles of *Mhc*. These alleles enhance the defects of *up101* and *wupA* and are partially suppressed by *Tm2* (Kronert et al., 1999; Nongthomba et al., 2003). Genetic interactions suggest that although *Mhc* and *Mhc* lead to hypercontraction defects that are similar to *up101* and *wupA*, they do so through a different molecular mechanism, as *up101* and *wupA* are fully suppressed by *Tm2* (Kronert et al., 1999). Using a simplified five state model of contraction based upon the allosteric/cooperative model described previously, we can hypothesize the molecular mechanisms behind hypercontraction (Fig. 6; Lehrer and Geeves, 1998).

In wild-type sarcomeres, the troponin complex remains in a closed state during rest (state A). If myosin–nucleotide complex binds this state, it is referred to as “blocked.” as it is not conducive to contraction. Neural stimulation shifts the equilibrium between state A and state B (open state) by allowing Ca$^{2+}$ to enter the sarcomere, which acts as an allosteric regulator of the troponin complex via troponin C. The equilibrium shift increases the probability of myosin binding the open state in a noncooperative manner (state C). If a sufficient number of myosins bind, cooperative mechanisms fa-
ciliate binding of multiple myosin heads (state D), a low-force state of the sarcomere. Release of P_i allows myosin to enter rigor, the high-force state of the sarcomere (state E), followed by ADP–ATP exchange to complete the cycle. During this transition, an estimated 20–30% of myosins remain bound to facilitate multiple cycles during a single contraction event, as this transition state is similar to state C (Lehrer and Geeves, 1998).

Hypercontraction in Mhc mutants can be caused by two different mechanisms. One mechanism leads to hypercontraction by decreasing structural integrity of the sarcomere (Mhc\textsubscript{6}, Mhc\textsubscript{7}). The other mechanism involves the mutations Mhc\textsubscript{S1}, Mhc\textsubscript{S2}, and Mhc\textsubscript{5}. Hypercontraction by Mhc\textsubscript{S1}, Mhc\textsubscript{S2}, and Mhc\textsubscript{5} may be caused by stabilizing actin–myosin interactions during the state E–state A transition, probably by preventing proper ADP–ATP exchange. This is consistent with the localization of the amino acid substitutions in the ATPase domain of the protein. Further stabilization during this transition could lead to contraction oscillations long after the nerve-stimulated Ca\textsuperscript{2+} transient, increasing actomyosin force during relaxation periods. The spontaneous contraction oscillations observed in third instar larvae support this model. However, mutants such as up\textsuperscript{101} and wup\textsuperscript{Ahdp2}, rather than calcium returning to intracellular stores, the calcium remains buffered in the sarcomere. This may be due to two possibilities. One possibility is that these mutations respond to lower calcium concentrations, where calcium ions are continually binding a mutant complex, transitioning to state B, released upon return to state A, and then repeating this binding cycle long after the large calcium transient has passed for regulated contraction. Although the [Ca\textsuperscript{2+}]\textsubscript{free} remains relatively low, there is an overall buffering of a significant amounts of calcium in the sarcomere by the troponin complex. The other possibility, though not mutually exclusive, is that these mutations lead to a lower activation energy for the A→B transition in the

Mhc\textsuperscript{S1} could occur through direct Mhc–Troponin I interactions, as proposed previously (Kronert et al., 1999). However, this seems unlikely as these mutants are single amino acid substitutions which map to the ATPase domain as opposed to surfaces more accessible to protein–protein interactions.

### Excitability and hypercontraction mutants

The TS seizure activity in Mhc\textsuperscript{5}, Mhc\textsuperscript{S1}, and Mhc\textsuperscript{S2} as well as up\textsuperscript{101} and wup\textsuperscript{Ahdp2} is likely to reflect a temperature-dependent defect caused by an alteration in the cellular state of a hypercontractive muscle, rather than direct temperature-dependent defects of mutant proteins. The model proposed for hypercontraction may account for the activity through a dysregulation of calcium homeostasis. In normal muscles, calcium levels dramatically increase in the sarcomere in order to increase the fraction of troponin complexes in state B during regulated contraction. However, in mutants such as up\textsuperscript{101} and wup\textsuperscript{Ahdp2}, rather than calcium returning to intracellular stores, the calcium remains buffered in the sarcomere. This may be due to two possibilities. One possibility is that these mutations respond to lower calcium concentrations, where calcium ions are continually binding a mutant complex, transitioning to state B, released upon return to state A, and then repeating this binding cycle long after the large calcium transient has passed for regulated contraction. Although the [Ca\textsuperscript{2+}]\textsubscript{free} remains relatively low, there is an overall buffering of a significant amounts of calcium in the sarcomere by the troponin complex. The other possibility, though not mutually exclusive, is that these mutations lead to a lower activation energy for the A→B transition in the

### Table I. Physiological properties of the NMJ

| Genotype   | Resting potential | Miniature frequency | mEJP amplitude | EJP amplitude |
|------------|-------------------|---------------------|----------------|--------------|
| CantonS\textsuperscript{b} | $-69.4 \pm 1.6$ | $2.10 \pm 0.21$ | $0.95 \pm 0.04$ | $39.6 \pm 1.8$ |
| MhcS1+/+  | $-62.6 \pm 1.0^a$ | $1.53 \pm 0.14^d$ | $0.79 \pm 0.02^e$ | $34.2 \pm 1.7^d$ |

\textsuperscript{a}Represented as value ± SEM.
\textsuperscript{b}n = 8.23.
\textsuperscript{c}n = 11.29.
\textsuperscript{d}P < 0.05.
\textsuperscript{e}P < 0.001.
absence of calcium. State B, having a higher affinity for calcium, allows binding of calcium away from endogenous muscle calcium buffers. This can also lead to an overall aberrant buffering of calcium. Likewise, Mhc\(^{C1}\), Mhc\(^{S1}\), and Mhc\(^{S2}\) lead to buffering because the sarcomere is continually cycling through states C-D-E, leading to various potential state(s) that are different from hypocontracted and normal states. Moreover, it is unclear how muscle hypercontraction would induce increased innervation of any, a subset, or all type II–like synapses in the adult (Rivlin et al., 2004). Furthermore, it is unknown whether muscle hypercontraction would induce increased innervation of any, a subset, or all type II–like synapses in the adult (Rivlin et al., 2004). Moreover, it is unclear how increases in type II innervation may alter excitability of muscles in a temperature-dependent fashion.

Although more experimentation will be required to discern between these possibilities as well as other potential mechanisms, it is clear that hypercontraction creates a distinct muscle state that is different from hypocontracted and normal muscles. In support of this, hypercontraction mutants that are not evident in hypocontraction mutants or in wild-type flies. In addition, TS behavioral defects are not likely due to mixtures of differentially active myosins being expressed in the IFM, as Mhc\(^{C1}\)/+ heterozygotes do not display TS behavioral defects such as those found in Mhc\(^{C1}\)/+, Mhc\(^{S1}\)/+, and Mhc\(^{C1}\)/+. Future studies in determining the components that contribute to this altered state will be critical in understanding the underlying causes of excitability defects in hypercontraction mutants. Characterization of genetic interactors of Mhc\(^{C1}\) and Mhc\(^{S1}\) mutants such as the Seeing and Breakdance loci described here may also provide insights into the molecular pathways underlying hypercontraction myopathies, as well as contribute to understanding of the mechanisms underlying human muscle diseases such as hypertrophic cardiomyopathy.

**Materials and methods**

**Fly strains and crosses**

Flies were cultured on standard medium at 22°C. All crosses using appropriate genotypes were cultured at 25°C. Mhc\(^{C1}\) and Mhc\(^{C2}\) were generated in an F1 EMS screen for X-linked and autosomal dominant TS behavioral defects. Samba mutants were recombination mapped to 2–52 cm on the second chromosome with Sp using marker chromosomes, deficiency mapped to 36A8–36C4, and tested for noncomplementation with P(\textit{SwI})\(^{V}\) to determine if Mhc\(^{C1}\) and Mhc\(^{C2}\) were isolated. In addition, revertants of Mhc\(^{C1}\) TS dysfunction were isolated by \(\gamma\)-irradiation. Mhc\(^{C1}\)/CyO males were paired with 6,000 yw, crossed to Gla/CyO, and F1 progeny were tested at 38°C for loss of TS behavior. Three revertants (Mhc\(^{C1}\), Mhc\(^{C2}\), and Mhc\(^{C3}\)) were isolated. All three were embryonic lethal with normal morphological development, but showed complete loss of muscle wave propagation in late stage embryos. In addition, all three revertant alleles showed noncomplementation to Mhc\(^{C1}\) transgression.

**Suppression of lethality was scored as live flies that were able to survive eclosion and feed with enough motor coordination to prevent becoming trapped in the media.**

**Adult behavior analysis**

10 flies were placed into a preheated glass vial at 38°C. Flies showing TS behavioral defects were scored in 15-s intervals. The analysis was done with 10 repetitions for each genotype and each repetition contained an independent set of 10 flies.

**Polarized light micrographs**

Polarized light micrographs of the adult flight muscles were analyzed as described previously with the modification of using Xylenes as a clearing agent (Fyrberg et al., 1994). Thoraces were mounted using Permound (Fisher Scientific) and analyzed under Nomarski optics.

**Mutation and crystal structure analysis**

Mutations were determined by PCR and sequencing. Genomic DNA was isolated from Canton-S and Mhc\(^{C1}\)/\(\text{Dr}^{2L} \text{H}20\) flies (Simpson, 1983). Exons 4–6 were amplified by PCR and the product was sequenced at the MIT Cancer Center sequencing facility. Genomic DNA from homozygous Mhc\(^{S2}\) embryos was isolated and similarly processed. Amino acids are numbered according to the Mhc-P11 sequence. Crystal structure analysis of the mutations was done using the Swiss PDB Viewer software available at http://us.expasy.org/spdbv/. Crystal structures 2MYS and 1MMG were downloaded from the National Center for Biotechnology Information (NCBI) and mutations mapped according to BLAST alignments done through the NCBI BLAST website (Fisher et al., 1995; Rayment et al., 1993, 1995).

**Antibodies and immunohistochemistry of third instar larvae**

Wandering third instar larvae were raised at 25°C, and then dissected and fixed by standard procedures. Affinity-purified rabbit \(\alpha\)-synt antibodies (Littleton et al., 1993) were used at 1:1,000 and Cy2-conjugated goat \(\alpha\)-rabbit secondary antibodies at 1:200 (Jackson ImmunoResearch Laboratories). Texas red–conjugated phalloidin was incubated simultaneously with the secondary antibody at 1:500 (Molecular Probes). Visualization and quantification was performed using NIKON Microphot-FXA microscope equipped with a 40×/1.3NA oil-immersion lens. Images were taken using confocal microscopy under similar conditions and processed with Zeiss PASCAL software.

**Electrophysiology**

**Extracellular DLM recordings.** Extracellular DLM recordings were done in male flies raised at 25°C. 1–5–ME electrodes were filled with 3 M KCl. The recording electrode was inserted into the lateral thorax with the ground electrode inserted into the eye. Basal activity was recorded for 2 min at 22°C. Temperature was then shifted to 38°C for 1 min, and returned to 22°C. Recordings were done using an Axoclamp-2B amplifier (Axon Instruments, Inc.) and digitized with a 500 MHz card at 4 kHz sampling rate. Data was digitized at a Digidata 1322, filtered at 10 kHz online, and analyzed using pCLAMP v8.0 software (Axon Instruments, Inc.). mEPSP amplitude and frequency were determined by manual analysis, analyzing representative samples from each muscle recording. EJP amplitude was similarly analyzed, using the maximal response to suprathreshold stimulation (determined for each individual muscle). Ca\(^{2+}\)-free recordings were done in a similar manner.

**Fail...**
ure to evoke release was used to verify that Ca$^{2+}$ was minimal in the external solution. Recordings with an intact central nervous system were done in 0.2 mM Ca$^{2+}$ to prevent substantial depolarization during central pattern activity in the presence or absence of 3 µM TTX.

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