Synaptic silencing of fast muscle is compensated by rewired innervation of slow muscle

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INTRODUCTION
It is generally accepted that the skeletal muscle in vertebrates consists of two types of muscle fibers: slow and fast (1). An intermediate type, with mixed characteristics of slow and fast fibers, is also reported in multiple species (2, 3). The functional roles of fast and slow muscle in locomotion have been conjectured based on classical studies (4). With the advent of molecular techniques, mutants that fail to develop fast or slow muscles were analyzed (5–7). These mutants enabled analysis of locomotor activities under free-moving conditions without recording electrodes. These studies (5–7) corroborated that fast muscles contribute to quick movement, while slow muscles underlie certain types of locomotion requiring endurance. However, a combination of anatomical and metabolic abnormalities accompanying genetic mutations complicated the interpretation of results. Moreover, these mutants died prematurely, and the long-term effects of silencing fast or slow muscle fibers remained unexplored.

In the present study, we addressed these problems using genome-editing techniques for genes expressed in synapses. Zebrafish as a model animal have various advantages in studying synapses in slow and fast muscles (8, 9). Slow and fast muscle cells in zebrafish are spatially segregated and can be easily distinguished by their anatomical and histological characteristics (10, 11). Acetylcholine receptors (AChRs) in the neuromuscular junction (NMJ) are nicotinic, as opposed to muscarinic AChR receptors that work through G proteins (12). Nicotinic AChRs are pentamers composed of 2α1s, β1, δ, and ε (or γ) subunits, which are all specific to skeletal muscles (13). In zebrafish as well as in mammals, γ subunits are expressed in larvae and change to ε as the animal matures (14, 15). Recent studies showed that AChRs in slow muscles of zebrafish lack e/γ subunits and are composed of only α, β, and δ subunits (13). To further explore the significance of these newly identified AChR compositions, we generated knockout (KO) zebrafish lines that lacked γ and ε subunits. By analyzing locomotion and synaptic traits in these mutants, we investigated the functional contribution of fast and slow muscles in both larval and adult zebrafish.

RESULTS
γ/ε: AChR subunit KO zebrafish lines
We generated a γ subunit KO zebrafish (γKO) using CRISPR-Cas9 (Fig. 1A) and an ε subunit gene KO zebrafish (εKO) using transcription activator–like effector nucleases (TALEN) (Fig. 1A). The γKO zebrafish did not show obvious phenotypes during development and matured in a fashion indistinguishable from wild-type (WT) siblings (fig. S1). In contrast, εKO fish generally failed to form swim bladders, and most of them died prematurely within 2 weeks after fertilization. However, a fraction of εKO fish (approximately 25%) survived to adulthood. A double KO (DKO) line was generated by crossing γKO and εKO lines. DKO larvae also failed to form swim bladders (Fig. 1B) and died within 2 weeks after fertilization.

We histologically analyzed the expression of AChRs in the trunk region of 6 days post-fertilization (dpf) larvae by using α-bungarotoxin (α-BTX) conjugated with Alexa Fluor 488, a toxin that specifically binds to the assembled AChR (Fig. 1C). AChR clusters in DKO were observed only in boundary regions between body segments (Fig. 1C), where slow muscles form NMJs (16). We initially expected that AChRs in fast muscles of DKO larvae would convert to the slow muscle–type AChRs, comprising only α, β, and δ subunits. This conversion of subunit composition would not cause a change in AChR distribution visualized by α-BTX, because both types of AChRs bind to α-BTX. However, α-BTX signals were absent in fast muscles, which suggested that fast muscles could not express AChRs composed of α, β, and δ subunits.

Synaptic transmission at NMJs of fast and slow muscles
To correlate the AChR expression pattern observed by the α-BTX staining with the synaptic function, we analyzed synaptic activities of fast and slow muscles in the DKO line at 6 dpf. We recorded spontaneous synaptic currents from muscle cells using the whole-cell patch clamp technique (Fig. 2, A to C). Traces show miniature endplate currents (mEPCs) from muscles of WT or DKO larvae (Fig. 2A). Slow muscles in the DKO line exhibited mEPCs. The

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A

\[ \gamma \text{ Gene (chrng)} \]

Target site
7-base pair insertion

WT
KO
CTGCCCTGATAAGACCTTGACGTGAGAACAGAGT
CTGCCCTGATAAGACCTTGACGTGAGAACAGAGT

\[ \varepsilon \text{ Gene (chrne)} \]

Target site
1-base pair insertion

WT
KO
TGCCCCTGATAAGACCTTGACGTGAGAACAGAGT
TGCCCCTGATAAGACCTTGACGTGAGAACAGAGT

Fig. 1. Generation of \( \gamma/\varepsilon \) DKO zebrafish. (A) Schematic diagram of targeted genes. Arrowheads indicate targeted regions of genome editing. Each box and line indicates an exon and an intron, respectively. Alignment of genomic DNA sequences of WT and KO lines showed a 7-base pair (bp) insertion in the AChR \( \gamma \) subunit gene chrng and a 1-bp insertion in the AChR \( \varepsilon \) subunit gene chrne. (B) Photograph showing WT and \( \gamma/\varepsilon \) DKO larva at 6 dpf. Notice the lack of swim bladder (arrowheads) in DKO. Scale bar, 1 mm. (C) Trunk regions of a WT larva (6 dpf) and a DKO larva (6 dpf) were stained with \( \alpha \)-BTX conjugated with Alexa Fluor 488 (green). In WT, AChRs were distributed in myoseptal regions (arrows) and in punctae in middle regions (arrowhead). DKO had \( \alpha \)-BTX signals only in myoseptal regions. Scale bars, 100 \( \mu \)m.

frequency (14.5 ± 3.1 Hz in WT, 15.5 ± 3.2 Hz in DKO) and the amplitude of slow muscle mEPCs (260.0 ± 74.1 pA in WT, 491.7 ± 105.2 pA in DKO) showed no differences between WT and DKO lines (Fig. 2, B and C). However, fast muscles in DKO failed to produce mEPCs. To confirm that the lack of mEPCs is caused by the absence of functional receptors, we recorded currents in muscles generated by puff application of ACh (Fig. 2D). While fast muscles in WT larvae showed ACh-induced currents (756.4 ± 138.6 pA), those in DKO failed to produce any response (0 ± 0 pA). These results, in conjunction with the \( \alpha \)-BTX staining (Fig. 1C), showed that fast muscles of DKO larvae do not express any AChRs and receive no synaptic input.

We performed in vivo Ca\(^{2+}\) imaging in the DKO larvae at 6 dpf to further support the result of synaptic current recordings. We designed a gene construct in which a pan-muscle promoter, \( \alpha \)-actin promoter, drives the expression of a Ca\(^{2+}\) indicator, GCaMP7a (17), and injected the construct into fertilized eggs (Fig. 2E). In DKOs, we recorded Ca\(^{2+}\) response associated with spontaneous locomotion activities, induced by the application of N-methyl-d-aspartate (50 \( \mu \)M) (18). The results showed that slow muscle cells exhibited Ca\(^{2+}\) transients, while fast muscle cells did not generate any Ca\(^{2+}\) response.

Considering that fast muscles do not allow composition of \( \alpha, \beta, \) and \( \delta \) subunits, we next examined whether slow muscles conversely allow incorporation of \( \varepsilon \) subunits in the AChR pentamer, by overexpressing the \( \varepsilon \) subunit in slow muscles. We designed a gene construct that expressed an \( \varepsilon \) subunit fused with enhanced green fluorescent protein (e-EGFP) under the regulation of a slow muscle–specific promoter, psmyhc (19). We injected the construct into fertilized WT eggs and observed the expression of EGFP at 3 to 4 dpf. EGFP signals typically filled the cytoplasm of the slow muscle cells and never colocalized with \( \alpha \)-BTX signals (Fig. 2F). In a control experiment, in which e-EGFP was driven by the pan-muscle promoter (\( \alpha \)-actin promoter), the e-EGFP signals made clusters in fast muscles, colocalizing with \( \alpha \)-BTX signals in deeper layers of the trunk region where fast muscles form NMJs. Together, fast muscles and slow muscles express specific types of AChR, and the alternate composition of subunits is prohibited.

Analysis of locomotion in larvae

To examine how silencing of synapses in fast muscles affect locomotion, we next analyzed swimming of WT and DKO larvae at 6 dpf. We induced escape responses by gentle tactile stimuli. Locomotion was recorded with a high-speed camera, and we measured angles between head and tail trajectories throughout each escape response (Fig. 3A and movie S1). WT fish turned their heads 120° to 140° in the initial stage of escape. The typical startle response of teleosts generally begins with a large turn of the head (termed C-bend), followed by a robust forward propulsion as described in previous studies (20).

The initial turns of the DKO larvae were in sharp contrast to WT. Averaged maximum head turn angles in DKOs were markedly smaller compared to WT larvae (116.0 ± 5.8° in WT, 20.2 ± 4.0° in DKO; \( P < 0.001 \)) (Fig. 3B), and time to reach the maximum angle was increased (8.7 ± 0.2 ms in WT, 15.8 ± 0.8 ms in DKO; \( P < 0.001 \)) (Fig. 3C). In addition to the absence of C-bends, the post-startle swimming speed of the DKO line was also notably slower (84.9 ± 8.1 mm/s in WT, 12.8 ± 1.3 mm/s in DKO; \( P < 0.001 \)) (Fig. 3D).

In addition to the escape response, we also analyzed spontaneous locomotion, which corresponds to the “slow swim” described by Budick and O’Malley (21) or “scout” reported by Burgess and Granato (22) (Fig. 3, E and F). Significant difference in swimming speed was not observed between WT and DKO (16.1 ± 1.60 mm/s in WT, 13.2 ± 0.9 mm/s in DKO; \( P = 0.20 \)) (Fig. 3F). Thus, the contribution...
Fig. 2. Synaptic functions of the DKO line. (A) mEPC traces from fast or slow muscles of WT and DKO larvae (6 dpf) by whole-cell patch-clamp recordings. Fast muscle cells in DKO failed to exhibit mEPCs. (B and C) Frequencies (B) and amplitudes (C) of mEPCs were plotted for each muscle (n = 8 cells). (D) Representative traces of voltage-clamped slow and fast muscles in DKO larvae in response to the application of 30 μM ACh. Calibration: 1 s, 500 pA. Amplitudes of ACh-induced currents in slow (n = 7 cells) and fast muscles (n = 7 cells) are shown. Each dot represents a muscle cell. (E) Construct used for Ca²⁺ imaging. Top: The GCaMP7a coding sequence was fused to the promoter region of the α-actin promoter pαact. Bottom: Schematic illustration showing the experimental procedure. The gene construct was injected into eggs of DKO at the one cell stage. Ca²⁺ response was analyzed at 6 dpf. Representative traces showing the increase of ΔF/F in a fast muscle (black line) and a slow muscle (red line) during spontaneous contractions. (F) Overexpression of the ε subunit fused with an EGFP (ε-EGFP) in WT (3 dpf). Top panels: ε-EGFPs were expressed under the control of a slow muscle–specific promoter, psmyhc. EGFP signals (green), expressed in the superficial slow muscles, filled the cytoplasm and did not colocalize with α-BTX (magenta) signals. Bottom panels: ε-EGFPs were expressed under the regulation of pαact. In deeper layer fast muscles, the clusters of EGFP and α-BTX colocalized (arrowheads). Scale bars, 50 μm.
of fast muscles in spontaneous swimming is relatively small. These results strongly suggest that fast muscles in larval zebrafish play a key role in executing quick escape responses including the C-bend and fast forward propulsion behaviors, which corroborate earlier studies (23).

**Locomotion in adults**

DKO fish die prematurely and do not develop into adults. However, ε KOs that reached the adult stage are expected to lack both γ and ε subunits, because γ subunit expression terminates early in development.

To dismiss the possibility of compensatory up-regulation of the γ subunit in adult ε KOs, we analyzed the expression of γ subunit mRNA with digital droplet polymerase chain reaction (ddPCR). γ Subunit mRNA was not detected in adult ε KOs, which were 3 to 5 months old (Fig. 4A). Interestingly, γ subunit mRNA was strongly up-regulated in larval ε KOs (Fig. 4B), which may account for
Fig. 4. AChR expression restricted to slow muscles of 3- to 5-month-old adult εKO zebrafish. (A) Quantification of γ or ε subunit mRNA in adult muscles. γ Subunit was not detected in WT. γ or ε subunit mRNA was not detected in εKO (n = 6 fish in WT, n = 5 fish in εKO). Sample numbers are shown in parentheses. (B) mRNA expression of γ subunit in 1-dpf larvae. γ Subunit was highly up-regulated in the εKO (n = 5 fish) compared to WT (n = 5 fish). Sample numbers are shown in parentheses. (C) Schematic illustration of a transverse section of the trunk region. The area shown in microphotograms is indicated with a box. The distribution of AChRs in adults, WT or εKO, was visualized by α-BTX conjugated with Alexa Fluor 488 (green). Broken lines indicate the boundary of fast muscle area (arrowheads). Fast muscles in the εKO fish lack α-BTX signals. (D) Sections of adult fast muscles of WT and εKO, stained with the fast muscle–specific F310 antibody. Fast muscles in εKO fish did not display atrophy. In the right panel, diameters of fast muscles in WT and εKO were calculated (87 fibers, n = 3 fish). There was no significant difference. Scale bars, 100 μm.
functional escape response behavior at 6 dpf (fig. S1). Thus, our findings suggest that compensation by the γ subunit expression occurs only in larval eKOs and not in adults.

The expression of AChR in adult eKO fish, visualized by α-BTX, was consistent with the lack of γ compensation (Fig. 4C). Transverse sections of the trunk region were labeled with α-BTX. Slow, intermediate, and fast muscles are spatially segregated (11). Slow muscles are located closest to the surface. WT fish displayed universally distributed, positive α-BTX signals. In sharp contrast, α-BTX signals in the eKO fish were detected only in shallow, lateral regions, and fast muscles of the adult eKO lacked AChR expression.

In spite of the absence of α-BTX–positive signals, fast muscle fibers in eKO fish unexpectedly lacked signs of prominent atrophy (24). A fast muscle–specific F310 antibody used via immunohistochemistry allowed the visualization and diameter measurements of fast muscle fibers. Statistical analysis revealed no difference between eKOs and WT fiber size (58.7 ± 0.5 μm in WT, 58.3 ± 0.7 μm in eKO; P = 0.945) (Fig. 4D).

We observed escape responses induced by objects dropping on water and subsequently analyzed C-bend angles and the swimming speed during escape (Fig. 5A) (25). We compared the maximum C-bend angles between the focal genetic lines. Similar to WT larvae (Fig. 3), WT adults start the escape response with the initial extreme head turn. Unexpectedly, we found that eKO adult fish also display robust C-bends (Fig. 5, A and B). Although smaller in amplitude (103.0 ± 7.5° in WT, 53.4 ± 2.5° in eKO), their time course did not exhibit any delay compared to WT. This is in sharp contrast to the complete loss of C-bend behavior observed in larval DKOs (Fig. 3). The duration of first turn also showed no significant difference between WT and eKOs (38.9 ± 3.8 ms in WT, 46.6 ± 4.9 ms in eKO).

Furthermore, the forward propulsion during escape of the eKO adult zebrafish was almost intact. When the distance traveled was plotted against the time after stimulation, the curves for WT and eKO nearly overlapped (Fig. 5C). The swimming speed (31.7 ± 1.3 cm/s in WT, 25.5 ± 3.0 cm/s in eKO; P = 0.08) and total distance traveled (4.0 ± 0.2 cm in WT, 3.2 ± 0.4 cm in eKO; P = 0.08) were similar between WT and eKO adults.

Suspecting that compensation of locomotion occurred at the level of neural projection, we examined the projections of motor neurons by retrograde labeling using a fluorescent tracer, dextran conjugated with Alexa Fluor 488 (Fig. 6, A to C). We injected the tracer into muscles of WT and eKO fish following a method described in a previous report (26). Spinal motor neurons in adult zebrafish are classified on the basis of morphological features. Dorso medial motor neurons with larger cell somas, which are called primary motor neurons (pMNs), specifically innervate fast muscles. Ventrolateral motor neurons with smaller somas, called secondary motor neurons (sMNs), are grouped in distinct populations depending on the innervation target: fast, intermediate, and slow muscles (27–29). We analyzed the location of motor neuron somas in the spinal cord (Fig. 6B) by measuring the distance from the center of spinal cord to cell somas. In WT adults, fast muscles were innervated mainly by dorso medial motor neurons (located close to the center), and slow muscles were innervated by ventrolateral motor neurons (Fig. 6, A and B).

Both the location and the size of motor neuron somas suggested that slow muscles in eKO adults were innervated by large motor neurons, which innervate only fast muscles in WT adults (Fig. 6C). Ventrolateral neurons did not seem to innervate slow muscles in eKOs, as they were absent in retrograde labeling (Fig. 6, B and C). When we injected the tracer into fast muscles of eKO adults, pMNs were not labeled (fig. S2). Motor neurons labeled in these preparations were presumably fast sMNs (26).

To rule out the possibility that pMN axons are inadvertently damaged by dye injections into slow muscles of eKO adults, we used another method of retrograde labeling using a lipophilic tracer Dil or DiIC18), which has a minimal possibility of causing pressure injection damage (30). After gently placing crystals of Dil onto slow muscles of eKO adults, we found that pMNs were labeled in spinal cords of eKO adults (Fig. 6D). We also analyzed the presynaptic input in muscles of WT and eKO adults using SV2A antibody to visualize presynaptic proteins (Fig. 6E). The results showed that positive signals within fast muscles were reduced in eKO compared to WT adults. Thus, fewer motor neurons innervated fast muscles in eKO fish.

The muscle cell type is determined by the motor neuron input (31). Suspecting the signals from pMNs may convert the properties of slow muscles into those of fast muscles in adult eKO fish, we examined the characteristics of slow muscle fibers. To do so, we analyzed the F310 antibody immunohistochemistry in adult eKO fish, which labels fast muscle–specific myosin (Fig. 6, F and G) (19). We also examined the α-glycerophosphate dehydrogenase (α-GPD) activity, which is a well-established method to visualize glycolytic muscles, i.e., fast muscles (Fig. 6, H and I) (32). Some tissue located in slow muscle regions stained positive for F310 (n = 3 fish; Fig. 6G) and α-GPD signals (n = 3 fish; Fig. 6I), suggesting that some slow muscles expressed the fast muscle–type isoform of myosin light chain and obtained glycolytic ability. Intermediate muscle fibers in eKO also showed higher glycolytic ability compared to WT (Fig. 6, H and I). Thus, a subpopulation of slow and intermediate muscles was converted to fast muscles, presumably due to the innervation of fast muscle motor neurons (31).

In summary, the absence of AChRs in developing eKOs is presumed to drive motor neuron axon innervation of fast muscles to instead reroute to slow muscles. These rewired pMNs presumably predominate over original axons in slow muscles, as a result of synaptic competition, and convert some slow and intermediate muscles to fast muscles (Fig. 6J).

**DISCUSSION**

This is the first report to analyze an adult animal whose synaptic contact on fast muscles is selectively silenced. Although overexpression of slow muscle–specific transcriptional regulators produced mice with an increased proportion of slow muscle cells, the conversion was not complete (33, 34). Synaptic silencing in this study showed that fast muscles are necessary for making C-bends in the larval stage. However, adult eKO fish displayed unexpected adaptation to synaptic silencing: rewiring of motor axons and conversion of slow and intermediate muscles to fast muscles.

An additional important finding was that fast muscle could not express AChRs of the slow muscle type, comprising α, β, and δ subunits; conversely, slow muscle AChRs could not incorporate the ε subunit. Although the distinct compositions between fast and slow muscle AChR subunits were recently described (13), the mechanism remains unresolved. Ahmed and Ali reported that slow muscles expressed mRNA of γ and ε subunits (35). The absence of alternate subunit composition reported here also strongly supports the idea
that the subunit composition is regulated at the posttranslational level. The conversion of slow muscle properties observed in εKO adults (Fig. 6, F to I) poses an interesting phenomenon. If the change to fast muscle is complete, AChRs will no longer express without γ and ε subunits, which contradicts the result of α-BTX staining in εKOs (Fig. 4). Therefore, the conversion seems partial; the myosin light chain type and metabolism are changed, while the AChR subunit composition is spared.

Motor neurons that innervate slow and fast muscles are distinct, in terms of both the synaptic drive from interneurons and the passive electrical properties (36). In larval zebrafish, pMNs innervate fast muscles, while a subgroup of sMNs called intermyotomal sMNs innervates slow muscles. These intermyotomal sMNs are silenced during fast speed swimming behavior (37, 38). In addition, the dorsoventrally projecting subgroup of sMNs innervates both fast and slow muscles (36).

In the adult spinal cord, fast pMNs purely innervate fast muscles. Whereas the fast, intermediate, and slow classes of sMNs innervate fast, intermediate, and slow muscles, respectively (26). Adult slow sMNs, in contrast to larval slow muscle–specific sMNs, are active in both slow and fast swimming (26).

The neural network underlying the escape response has been extensively studied (39). In response to tactile or acoustic stimuli, Mauthner neurons in the hindbrain activate and send signals down
Fig. 6. Retrograde labeling of motor neurons show changed innervation. (A) Schematic illustration of a transverse section of the trunk region showing the sites of dye injections. Right panels showing cell bodies of labeled motor neurons (arrowheads) in spinal cords. Broken lines indicating outlines of spinal cords. Scale bars, 50 μm. (B) A graph showing the distance from the center of the spinal cord to cell bodies of motor neurons. In WT, motor neurons located close to the center innervate fast muscles, and ventrolateral motor neurons innervate slow muscles. In eKO, slow muscles were innervated by motor neurons located close to the center. Numbers of labeled cells are shown in parentheses. (C) Graph showing the size of cell somas of motor neurons. In WT, large motor neurons innervate fast muscles, and smaller neurons innervate slow muscles. In eKO, slow muscles were innervated by large motor neurons. (D) Schematic illustration of a transverse section of the trunk region showing the locations of the DiI crystal insertion. The right panel displays cell body of labeled pMN (arrowhead) in the spinal cord. The broken line indicates the outline of the spinal cord. Scale bar, 50 μm. (E) Presynaptic structures were visualized by SV2A antibody. Broken lines indicate the boundary of slow muscle area (left side). Note the reduced signal in the fast muscles of the eKO fish. Scale bars, 100 μm. (F and G) Fast muscle–specific myosins labeled by F310 antibody in WT (F) and eKO (G). In (G), the boxed area is enlarged in the right panel. Arrowheads indicate muscle cells with F310 signals in the slow muscle region. While a small number of slow muscle cells in WT sometimes showed immunoreactivity, the cell number was markedly increased in eKO. Scale bars, 100 μm. (H and I) Glycolytic muscle fibers were visualized by αGPD staining in WT (H) and eKO (I). Black broken lines indicate the boundary between slow and intermediate muscles, and the red broken line indicates the boundary between intermediate and fast muscles. Fast, intermediate, and slow muscle areas are labeled with F, I, and S, respectively. Note that the intermediate muscle region in eKO is hard to distinguish from the fast muscle region, blurring the boundary (I). Arrowheads in the right panel indicate muscle cells with αGPD signals in the slow muscle region. Scale bars, 100 μm. (J) Schematic illustration showing the rerouted innervation of pMNs. In eKO adults, synaptic silencing of fast muscles led to the innervation of fast muscle–specific pMNs on slow muscle. This reinnervation caused conversion of slow to fast muscles. The projections of sMNs that innervate fast muscles may not change.
the spinal cord. In larvae, motor neurons subsequently fire and cause trunk muscle contraction, leading to the C-bend behavior, whose maximum angle occurs within <12 ms after the initiation of muscle contraction (39). Adult sMNs that innervate slow muscles are disengaged by the Mauthner neuron excitation and remain suppressed for over 0.5 s (40). In light of these studies, the peak bending of the trunk in the eKO adults, which occurred in less than 20 ms, was unexpected (Fig. 5). We therefore hypothesized that slow muscles in eKOs were innervated by fast pMNs, which was corroborated by retrograde labeling and the conversion of muscle type from slow to fast (Fig. 6). Notably, the peak amplitude of C-bend behavior was smaller in adult eKOs than WTts (Fig. 5). This is presumably due to the reduction of muscle cell population responding to the motor neuron firing (Fig. 6).

eKO mice show muscle atrophy and die prematurely due to muscle weakness (24), in contrast to zebrafish eKOs, which survived to adulthood and displayed relatively normal swimming behavior. The difference between mice and zebrafish phenotypes may stem from several factors. First, it remains to be determined whether slow muscles in mammals express AChRs without γ or ε. eKO mice may therefore be unable to express any AChRs in muscles after the decrease of the γ subunit expression, in which case rewiring of motor neuron axons will be futile. Second, fish muscle did not show signs of atrophy, while eKO mice suffered from muscle weakness and atrophy. The lack of atrophy may result from the relatively young age of adult fish (3 to 5 months) used in the study. Many developmental factors are involved in the formation or growth of muscle fibers (41). It is possible that these developmental factors remain functional in fast muscles of eKO fish and prevent atrophy. Older eKO fish may present atrophy similar to mice and has yet to be explored and characterized. While the precise cause remains unknown, zebrafish eKOs also have a low survival rate (~25%), and it is possible that factors leading to premature death of eKO mice may have a role in zebrafish fatality. Thus, the difference between eKO zebrafish and mice phenotypes may actually be subtle.

The molecular and physiological mechanisms leading to the rewired innervation of pMNs, which is not genetically programmed and caused by synaptic silencing of fast muscles, are highly exciting. This phenomenon can be divided into three processes. First, pMN axons abandon nonresponsive fast muscles. Second, pMN axons explore, locate, and make synaptic contacts with active slow muscles. Third, pMNs compete with previously established sMNs and predominate innervation.

The third process presumably results from competition of axons innervating a single target: a well-established concept called synaptic elimination (42, 43). In mice diaphragm muscles, axons with more activity are favored over less active axons, resulting in a single innervation per muscle fiber.

The first and second processes are unique, lacking directly comparable experimental paradigms to the best of our knowledge. In developing eKOs, only slow muscles function after the termination of γ subunit expression. It is feasible that the higher activity of slow muscles relative to silenced fast muscles attracted motor neuron axons as the animal matured. The information of muscle activity can be transmitted over a distance. For example, injured neuron axons as the animal matured. The information of muscle activity can be transmitted over a distance. For example, injured peripheral nerves in mammals displayed activity-dependent enhancement of axon regeneration and functional recovery (44, 45). The molecular identity of this signal, which results in the attraction of pMN axons toward active muscle cells and the establishment of synaptic contacts against the genetic programming, will await further studies.

**MATERIALS AND METHODS**

**Fish lines and maintenance**

Zebrafish were maintained in the self-circulating systems at the National Institute on Alcohol Abuse and Alcoholism (NIAAA)/National Institutes of Health (NIH) and the Osaka Medical College. All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by NIAAA/NIH and Osaka Medical College.

**Genome editing**

A frameshift mutant of the AChR γ subunit gene (*chrng*) was generated by the CRISPR-Cas9 system. For generation of guide RNA (gRNA), oligo DNA for gRNA (Eurofins Genomics, Tokyo, Japan) was annealed and ligated with gRNA expression vector (DR274; Addgene), followed by digestion with Bsa I (New England Biolabs, Ipswich, MA) (46). After cloning and digestion by Dra I (New England Biolabs), gRNA was transcribed by T7 polymerase (Roche, Molecular Biochemicals GmbH, Mannheim, Germany). The Cas9 mRNA was transcribed using Pme I–digested Cas9 expression vector (MLM 3613; Addgene) by an mMESSAGE mMACHINE T7 ULTRA kit (Thermo Fisher Scientific, Waltham, MA). CRISPR-Cas9 solution containing gRNA (12.5 ng/μl) and Cas9 mRNA (300 ng/μl) was injected into the cytoplasm of fertilized one cell–stage eggs. Genomic DNA of F1 fish was extracted from the caudal fin. Amplification that includes the target region of each gene was generated by PCR. Amplions of the candidate fish were sequenced by a commercial company (Eurofins Genomics). F1 fish that had mutations were crossed. Homozygous KO F2 fish were selected by genome sequence (fig. S1 shows phenotypes of single KO lines). Frameshift mutants of the AChR ε subunit gene (*chrne*) were established similarly, except that TALEN was used instead of CRISPR-Cas9. Complementary DNA (cDNA) encoding TALEN was designed by Life Technologies Corporation (Carlsbad, CA). mRNAs were synthesized in vitro and injected at one cell stage into fertilized embryos. After checking somatic mutations, germline transmission was confirmed and established lines were used to generate homozygous fish.

**Swimming analysis**

High-speed image capturing of larval and adult zebrafish was performed with a Photron camera (Photron, Tokyo, Japan) at 1000 frames/s. Captured images were saved as JPEG and processed with ImageJ. For larvae (6 dpf), the trunk region was touched gently with a pipette to induce escape behavior (47). For adults, we dropped a 0.5-ml tube weighted with 1.5 g onto a point close to the animal’s head from 30 cm above the water (25). The head angle (C-bend) for each frame of the response was measured. In addition, swimming speed and total distance traveled during the escape were calculated. The frame at 0 ms was chosen before the movement was first detected. Measured angles were plotted against time.

**Histology**

Briefly, adult zebrafish (3 to 5 months old) were deeply anesthetized with tricaine (Tokyo Chemical Industry, Tokyo, Japan) before decapitation. Trunks were dissected into small segments and fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) at 4°C.
for 24 hours. The specimens were washed with PBS and immersed in 30% sucrose/PBS at 4°C overnight. The fixed specimens were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan) and frozen. Specimens were frontally cryosectioned at 20 μm using a cryostat (CM 3050S; Leica Microsystems, Wetzlar, Germany) and mounted onto MAS-GP type A–coated glass slides (Matsunami, Osaka, Japan). The sections were incubated with α-BTX and Alexa Fluor 488 conjugate (Thermo Fisher Scientific) [1:400; with PBS containing 0.3% Tween 20 (PBST)] for 2 hours and rinsed twice with PBS. The sections were coveredslipped with DAPI (4′,6-diamidino-2-phenylindole) Fluoromount-G (SouthernBiotech, Birmingham, AL) and observed under a TCS SP8 confocal microscope (Leica Microsystems).

For immunohistochemistry, adult zebrafish (3 to 5 months old) were fixed and mounted similarly. Specimens were frontally cryosectioned at 10 μm using a cryostat (CM 3050S; Leica Microsystems, Wetzlar, Germany) and mounted onto MAS-GP type A–coated glass slides (Matsunami, Osaka, Japan). The sections were incubated with F310 [1:50; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA] or synaptic vesicle glycoprotein 2A (SV2A) antibody (1:50; DSHB) overnight. After wash with PBST, the sections were incubated with secondary antibody, goat anti-mouse immunoglobulin G (H+L) Alexa Fluor 555 (Thermo Fisher Scientific) or 488 (1:500), for 2 hours. After a wash with PBST, sections were coverslipped with DAPI Fluoromount-G (SouthernBiotech) and observed under a confocal microscope (Leica TCS SP8, Leica Microsystems).

For αGPD staining, cryosectioned samples were incubated for 45 min at room temperature in 0.05 M tris buffer at pH 7.4 with 0.02% menadione, 0.05% nitroblue tetrazolium, and 0.01 mol disodium 2′-glycerophosphate. The sections were subsequently rinsed with PBS, coverslipped with DAPI Fluoromount-G (SouthernBiotech), and observed under a BZ-X700 microscope (KEYENCE, Osaka, Japan).

Histology for larval zebrafish was performed as previously described with some modifications (48). Larvae were anesthetized first in 10% Hank’s solution with 0.02% tricaine. The fish were kept in 100% Hank’s solution containing α-BTX and Alexa Fluor 488 conjugate (1:400) for 1 hour. Then, the fish were washed for 2 hours in toxin-free Hank’s solution to remove nonspecific binding of the toxin. Subsequently, larvae were embedded in 1% agarose and mounted on glass-bottom petri dish for observation under a TCS SP8 confocal microscope (Leica Microsystems).

Electrophysiological recordings
Recording of mEPC in the NMJ was performed as previously described with some modifications (47). Skinned larvae were pinned down to the recording chamber coated with Sylgard and immobilized by bathing in 10 μM nifedipine (Merck, Kenilworth, NJ). Patch clamp recordings were made by the whole-cell ruptured technique of muscle cells. The pipette solution used for the voltage-clamp recording was 120 mM KCl, 5 mM BAPTA, and 5 mM Hepes (pH 7.2). The extracellular solution contained 112 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 3 mM glucose, and 5 mM Hepes (pH 7.4). Membrane currents were recorded with an EPC10 amplifier and PatchMaster. For mEPC recordings, muscle cells were voltage-clamped at −90 mV, and 1 μM Tetrodotoxin (TTX) was added to the recording solution. The currents were sampled at 50 kHz and filtered at 3 to 5 kHz before analysis. Capacitive transients were compensated manually.

For puff application, a glass electrode [opening, ~30 μm; filled with bath solution containing 30 μM ACh and dextran–Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA)] was placed near the voltage-clamped muscle cell and positive pressure was applied using Picospritzer II (Parker Hannifin; 30 ms, 1 psi). ACh-induced currents were recorded by the whole-cell ruptured technique.

Ca²⁺ imaging
We amplified a zebrafish genomic DNA fragment containing the 5′-flanking region of the α-actin gene by PCR using PrimeSTAR (Takara, Shiga, Japan). The primers used had restriction sites (Sph I and Sal I), and the amplicon was inserted into the pT2RUASGCaMP7a plasmid (supplied by K. Kawakami’s laboratory), which swapped the upstream activation sequence with the promotor sequence of α-actin to drive the expression of GCaMP7a.

We injected the constructed plasmid into one cell–stage embryos from γ−/− e−/− pairs. The genotype of the injected embryos (at 6 dpf) was determined by responses to touch, and the embryos were anesthetized with 0.02% tricaine (Tokyo Chemical Industry) and pinned to a Sylgard dish. Tricaine was washed out, and embryos were bathed in Evans solution containing a muscle myosin inhibitor, N-benzyl-p-toluene sulfonamide at 5 mM (Merck), which does not affect the Ca²⁺ regulation (49). N-methyl-d-aspartate (50 μM) was applied to activate swimming (18). We performed line scanning at 26 Hz (38 ms/scan) of a fluorescent muscle cell. Fluorescence intensity was divided by an averaged (background) intensity. The resting intensity before [Ca²⁺] increase was chosen as the zero point. After recording, genotypes of the embryos (at 6 dpf) were determined by genomic DNA sequencing.

Digital droplet PCR
Total RNA was isolated from the whole body of WT or eKO larvae (n = 5 each) or from muscles of trunk regions of adult WT (n = 6 fish) or eKO fish (n = 5 fish) using NucleoSpin RNA XS (Takara). cDNA was synthesized using the PrimeScript RT Reagent Kit (Perfect Real Time, Takara). ddPCR was carried out using QX100 droplet digital PCR system following the manufacturer’s instructions (Bio-Rad, Pleasanton, CA). Briefly, for each 20-μl reaction, 0.5 μl of cDNA was mixed with 1 μl of target primers/probe (FAM) (20×), 10 μl of Bio-Rad’s ddPCR supermix for probes (2×), and 8.5 μl of nuclease-free water. We designed primers/probes in exon-exon boundary regions for each target gene; γ gene, e gene, and β-actin gene (Supplementary Materials). The entire reaction mixture was loaded onto a disposable plastic cartridge (Bio-Rad) together with 70 μl of droplet generation oil (Bio-Rad) and placed in the droplet generator (Bio-Rad). After processing, samples were transferred to a 96-well PCR plate. PCR amplification was carried out on a T100 thermal cycler (Bio-Rad) using a thermal profile: 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 60 s, 98°C for 10 min. After amplification, the plate was loaded on the droplet reader (Bio-Rad), and the droplets from each well of the plate were read automatically at a rate of 32 wells per hour. ddPCR data were analyzed with QuantaSoft analysis software (Bio-Rad), and the quantification of the target molecule was presented as the number of copies per microliter of PCR mixture after normalization with the β-actin control.

Retrograde labeling of motor neuron
Retrograde labeling of motor neurons was performed as previously described with some modifications (26). After anesthesia by 0.02% tricaine (Tokyo Chemical Industry), crystals of dextran–Alexa Fluor 488 (molecular weight, 10,000; Thermo Fisher Scientific) were dissolved
in distilled water and injected into fast or slow muscles using glass pipettes. Five to 6 hours after recovery from anesthesia, the animals were again deeply anesthetized and decapitated. For experiments using lipophilic fluorescent tracer DiI [or DiIC18(3)] (Biotium, Fremont, CA), crystals of DiI were inserted gently into the slow muscle area of eKO adults using a glass pipette. After placing the fish back in water for 24 hours, fish were deeply anesthetized and decapitated. Trunks were dissected and fixed with 4% paraformaldehyde/PBS at 4°C overnight. The specimens were washed with PBS and immersed in 30% sucrose/PBS at 4°C overnight. The specimens were embedded in O.C.T. compound (Sakura Finetek Japan) and frozen. Specimens were frontally cryosectioned at 20 µm using a cryostat (CM 3050S; Leica Microsystems) and mounted onto MAS-GP type A–coated glass slides (Matsunami) and coverslipped with DAPI Fluoromount-G (SouthernBiotech) for observation under a Leica fluorescence microscope (Leica Microsystems).

Statistics

Unpaired t test (two-tailed) was performed for statistical analysis. For multiple comparisons, t tests were performed with Bonferroni correction. Averages and SEM are displayed.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/15/eaax382/DC1

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES

1. A. Hess, Vertebrate slow muscle fibers. Physiol. Rev. 50, 40–62 (1970).
2. S. Schaffrin, C. Raggianni, Fibre types in mammalian skeletal muscles. Physiol. Rev. 91, 1447–1531 (2011).
3. F. Mascalzone, M. G. Romanello, P. A. Scapol, Histochemical and immunohistochemical profile of pink muscle fibres in some teleosts. Histochemistry 84, 251–255 (1986).
4. L. C. Rume, R. P. Funke, R. M. Alexander, G. Lutz, H. Aldridge, F. Scott, M. Friedman, Why animals have different muscle fibre types. Nature 335, 824–827 (1988).
5. S. Baxendale, C. Davison, C. Muxworthy, L. Saint-Amant, J. Y. Kuwada, K. Kang, R. M. Evans, Regulation of muscle fiber type and running endurance by PPAR-δ, subunit deletion causes muscle weakness and atrophy in juvenile and adult mice. Proc. Natl. Acad. Sci. U.S.A. 93, 13286–13291 (1996).
6. M. R. Brann, J. Ellis, H. Jørgensen, D. Hill-Eubanks, S. V. Jones, Muscarinic acetylcholine receptor subtypes: Localization and structure/function. J. Comp. Physiol. B 182, 10211–10218 (2014).
7. A. Muto, M. Ohkura, G. Abe, J. Nakai, K. Kawakami, Real-time visualization of neuronal activity during perception. Curr. Biol. 23, 307–311 (2013).
8. W. W. Cui, L. Saint-Amant, J. Y. Kuwada, Shocked Gene is required for the function of a premotor network in the zebrafish CNS. J. Neurophysiol. 92, 2898–2904 (2004).
9. S. Elwroth, M. Haldane, A. Knight, K. Mebus, P. W. Ingham, Expression of multiple slow myosin heavy chain genes reveals a diversity of zebrafish slow twitch muscle fibres with differing requirements for Hedgehog and Pdm1 activity. Development 135, 2115–2126 (2008).
10. V. Witzemann, H. Schwarz, M. Koenen, C. Berberich, A. Villarroel, A. Wernig, H. R. Brenner, B. Sakmann, Acetylcholine receptor γ-subunit deletion causes muscle weakness and atrophy in juvenile and adult mice. Proc. Natl. Acad. Sci. U.S.A. 93, 13286–13291 (1996).
11. N. Danos, G. V. Lauder, Challenging zebrafish escape responses by increasing water viscosity. J. Exp. Biol. 215, 1854–1862 (2012).
12. K. A. B)ratt, J. Song, A. El Manira, Pattern of innervation and recruitment of different classes of motoneurons in adult zebrafish. J. Neurosci. 33, 10875–10886 (2013).
13. F. de Graaf, W. van Raamsdonk, E. van Asselt, P. C. Diegenbach, Identification of motoneurons in the spinal cord of the zebrafish (Brachydanio rerio), with special reference to motoneurons that innervate intermediate muscle fibers. Anat. Embryol. 182, 93–102 (1990).
14. E. van Asselt, F. de Graaf, W. van Raamsdonk, Ultrastructural characteristics of zebrafish spinal motoneurons innervating glycolytic white, and oxidative red and intermediate muscle fibers. Acta Histochem. 95, 31–44 (1993).
15. W. van Raamsdonk, W. Mos, M. J. Smith-Ornel, W. J. van der Laarse, R. Fehres, The development of the spinal motor column in relation to the myotomal muscle fibers in the zebrafish (Brachydanio rerio). I. Posthatching development. Anat. Embryol. 167, 125–139 (1983).
16. T. J. Pucadyil, A. Chattopadhyay, Effect of cholesterol on lateral diffusion of fluorescent lipid probes in native hippocampal membranes. Chem. Phys. Lipids 143, 11–21 (2006).
17. K. D. Bergmeister, M. Aman, S. Muceli, I. Vujaklija, K. Manzano-Szalai, E. Unger, R. A. Byrne, C. Scheinecker, O. Riedl, S. Salminger, F. Onemli, G. H. Borschel, S. Farina, O. C. Amszmann, Peripheral nerve transfers change target muscle structure and function. Sci. Adv. 5, eaau2956 (2019).
18. H. Hirata, T. Watanabe, J. Hatakeyama, S. M. Sprague, L. Saint-Amant, A. Nagashima, W. W. Cui, W. Zhou, J. Y. Kuwada, Zebrafish relatively relaxed mutants have a ryanodine receptor defect, show slow swimming and provide a model of multi-minicore disease. Development 134, 2771–2781 (2007).
19. Y. Naganawa, H. Hirata, Developmental transition of touch response from slow muscle-mediated coolings to fast muscle-mediated burst swimming in zebrafish. Dev. Biol. 355, 194–204 (2011).
20. E. Daikoku, M. Saito, F. Ono, Zebrafish mutants of the neuromuscular junction: Swimming in the gene pool. J. Physiol. Sci. 65, 217–221 (2015).
21. Y. Egashira, B. Zempo, S. Sakata, F. Ono, Recent advances in neuromuscular junction research prompted by the zebrafish model. Curr. Opin. Physiol. 4, 70–75 (2018).
22. V. M. Luna, E. Daikoku, F. Ono, "Slow" skeletal muscles across vertebrate species. Cell. Biosci. 5, 62 (2015).
23. S. H. Devoto, E. Melancon, J. S. Eisen, M. Westerfield, Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development 122, 3371–3380 (1996).
24. R. Mongeon, M. Walogorsky, J. Urban, G. Mandel, F. Ono, P. Brehm, An acetylcholine receptor lacking both y and ε subunits mediates transsynaptic in zebrafish slow muscle synapses. J. Gen. Physiol. 138, 353–366 (2011).
25. M. Mishina, T. Takai, K. Imoto, M. Noda, T. Takahashi, S. Numa, C. M. Fischbasser, B. Sakmann, Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321, 406–411 (1986).
26. M. Walogorsky, R. Mongeon, H. Wen, R. N. Nelson, J. M. Urban, F. Ono, G. Mandel, P. Brehm. Zebrafish model for congenital myasthenic syndrome reveals mechanisms causal to developmental recovery. Proc. Natl. Acad. Sci. U.S.A. 109, 17771–17776 (2012).
27. J. Y. Park, M. Mott, T. Williams, H. Ikeda, H. Wen, M. Linhoff, F. Ono, A single mutation in the acetylcholine receptor β-subunit causes distinct effects in two types of neuromuscular synapses. J. Neurosci. 34, 10211–10218 (2014).
41. H. Ochi, M. Westerfield, Signaling networks that regulate muscle development: Lessons from zebrafish. Dev. Growth Differ. 49, 1–11 (2007).
42. M. Buffelli, R. W. Burgess, G. Feng, C. G. Lobe, J. W. Lichtman, J. R. Sanes, Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. Nature 424, 430–434 (2003).
43. M. A. Lanuza, J. Tomás, N. Garcia, V. Cilleros-Mañé, L. Just-Borràs, M. Tomás, Axonal competition and synapse elimination during neuromuscular junction development. Curr. Opin. Physiol. 4, 25–31 (2018).
44. E. Asensio-Pinilla, E. Udina, J. Jaramillo, X. Navarro, Electrical stimulation combined with exercise increase axonal regeneration after peripheral nerve injury. Exp. Neurol. 219, 258–265 (2009).
45. J. S. Park, K. H. Park, Treadmill exercise induced functional recovery after peripheral nerve repair is associated with increased levels of neurotrophic factors. PLOS ONE 9, e90245 (2014).
46. W. Y. Hwang, Y. Fu, D. Reyon, M. L. Maeder, S. Q. Tsai, J. D. Sander, J.-R. Yeh, J. K. Joung, Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. 31, 227–229 (2013).
47. M. Mott, V. M. Luna, J.-Y. Park, G. B. Downes, K. Epley, F. Ono, Expressing acetylcholine receptors after innervation suppresses spontaneous vesicle release and causes muscle fatigue. Sci. Rep. 7, 1674 (2017).
48. F. Ono, S. Higashijima, A. Shcherbatko, J. R. Fetcho, P. Brehm, Paralytic zebrafish lacking acetylcholine receptors fail to localize rapsyn clusters to the synapse. J. Neurosci. 21, 5439–5448 (2001).
49. A. Cheung, J. A. Dantzig, S. Hollingworth, S. M. Baylor, Y. E. Goldman, T. J. Mitchison, A. F. Straight, A small-molecule inhibitor of skeletal muscle myosin II. Nat. Cell Biol. 4, 83–88 (2002).

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