Supporting Information

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SI Methods

Animals. Zebrafish (*Danio rerio*) were maintained in a laboratory breeding colony on a 14-h light/10-h dark cycle. Embryos wereobtained from natural spawning of AB WT and transgenic fish lines and bred according to guidelines in *The Zebrafish Book* (1). The following transgenic lines were used for experiments (naming according to official zebrafish nomenclature): Et(20.6hsp70l:Gal4-VP16)s1020t (also known as Gal4s1020t) (2), UAS:GCaMP3 (3), and Tg(mnx1:mGFP)ml3 (also known as Hb9:mGFP) (4). Embryos were maintained at 28.5 °C in E3 embryo media (130 mM NaCl, 0.5 mM KCl, 0.02 mM Na_2HPO_4, 0.04 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 0.4 mM NaH_2CO_3) and staged according to external morphology (5). In all cases, spinal neurons from the medial segments (somites 6–9) were studied. Animal experiments were performed with oversight by the University of California at San Diego Institutional Animal Care and Use Committee.

Mosaic Labeling of Primary Motor Neurons. To generate mosaic expression of transgenes in primary motor neurons (PMN), plasmid DNA was prepared using Qiagen miniprep kits and injected at a concentration of 25 ng/μL in 0.1 M KCl into one to four cell-stage zebrafish embryos. An Hb9:eGFP construct was injected alone. The zebrafish Hb9 promoter was used to drive the expression of GAL4 on an activator plasmid (6). The following effector plasmids were coinjected with the Hb9:Gal4 construct: UAS:DsRed, UAS:DsRed:: UAS:human inward rectifier K^+ channel (hKir2.1), UAS:DsRed:: UAS:hKir2.1 mutant (7), or UAS:tetanus toxin light chain fused to eGFP (8). The basis for mosaic expression from DNA constructs remains to be determined. Injected embryos were raised at 28.5 °C in E3 medium containing phenylthiourea to block pigment formation.

Imaging Axon Outgrowth. Embryos stochastically expressing the Hb9:eGFP construct (4) were paralyzed with 20 μM α-bungarotoxin (Tocris Bioscience), embedded in 1% low-melting agarose at 16 h postfertilization (hpf; beginning of midtrunk PMN axon extension), oriented horizontally to obtain a side view for imaging, and kept at 28.5 °C in a heated chamber for time-lapse experiments. Live embryos from 16 to 26 hpf were imaged with a Leica SP5 confocal microscope capturing z-stack images at 30-min intervals to investigate axon outgrowth to their targets. Images were analyzed using ImageJ to project the z stacks and analyze axon trajectories.

Calcium Imaging. Calcium green-1 dextran (10 mM, 10 kDa; Molecular Probes) was injected into one cell-stage Hb9:mGFP embryos, or progeny of Gal4s1020t/UAS:GCaMP3 double-transgenic fish were identified for GCaMP3 expression (green fluorescence) using a fluorescent stereoscope. Embryos were raised in E3 medium at 28.5 °C, immobilized with 20 μM α-bungarotoxin, mounted as for imaging axon outgrowth, and kept at 28.5 °C in a heated chamber.

For studies of Ca^{2+} waves, live embryos were imaged at 0.2 Hz for 30 min with a Leica SP5 confocal microscope equipped with a 40× (N.A. 0.8) water immersion objective. For studies of Ca^{2+} spiking activity, images were collected at 4 Hz for 5 min or 7.6 Hz for 2 min. Images were analyzed with ImageJ. Event duration was calculated as the width at half-maximum. The number of Ca^{2+} transients was scored using a threshold of 20% (for waves) and 10% (for spikes) of $(F - F_0)/F_0$, where F_0 is the fluorescence at the beginning of the imaging period. These limits exceed two times the SD of baseline noise for all recordings and were used as a common threshold for all traces.

Morpholino Oligonucleotide Injection. Morpholino oligonucleotides (MOs) were obtained from Gene Tools. The sequences were as follows:

PlexinA3 MO 5′-ATACCAGCAGCCACAAGGACCTCAT-3′;
PlexinA3 control MO 5′-ATACCA*C*CA*C*CCA*G*AA*C*GACCT-*G*AT-3′.

The control MO sequence had a five-base mismatch. The effectiveness of these PlexinA3 and control MOs has been reported (9). MOs were solubilized in water and injected into recently fertilized eggs. For subthreshold coinjections with UAS:Kir:: UAS:DsRed cDNA, 0.3 mM PlexinA3 MO was used. This dose was the highest that resulted in minimal phenotypic defects that were not statistically different from control MO phenotypes.

In Situ Hybridization. PlexinA3 probe was labeled with digoxigenin using the Megascript kit (Ambion) and used on 24-hpf whole-mounted embryos according to standard protocols. Briefly, embryos were fixed in 4% (wt/vol) paraformaldehyde at 4 °C overnight, incubated with the probe at 70 °C overnight, and washed extensively at 70 °C. The hybridized probe was detected using an alkaline phosphatase-coupled antidigoxigenin antibody (Roche). The signal was developed using SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma-Aldrich). Control, tricaine-treated, and Kir-expressing embryos were processed simultaneously.

Immunohistochemistry. DsRed was immunodetected after in situ hybridization with a polyclonal antibody (Clontech Laboratories). Briefly, fish were washed in PBS containing 0.1% Tween (PBST), blocked in antibody solution [PBST with 10% (vol/vol) sheep serum; 2 mg/mL BSA] for 1 h, and then incubated with 1:1,000 Living Colors anti-DsRed polyclonal antibody (Clontech Laboratories) overnight at 4 °C in antibody solution as above. After rinsing four times for 15 min each in PBST/0.1% DMSO, embryos were incubated for 2 h at room temperature in the dark with 1:200 AlexaFluor 568 goat anti-rabbit IgG(HþL) secondary antibody (Molecular Probes) in antibody solution as before.

Statistics. Statistical analysis was performed with GraphPad Prism (GraphPad Software). Results are presented as mean ± SEM. Significant differences between two groups were determined by paired Student *t* test (Ca^{2+} imaging) or Fisher exact test (PMN axon trajectories).

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4. Flanagan-Steet H, Fox MA, Meyer D, Sanes JR (2005) Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. *Development* 132(20):4471–4481.
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9. Feldner J, et al. (2007) PlexinA3 restricts spinal exit points and branching of trunk motor nerves in embryonic zebrafish. *J Neurosci* 27(18):4978–4983.

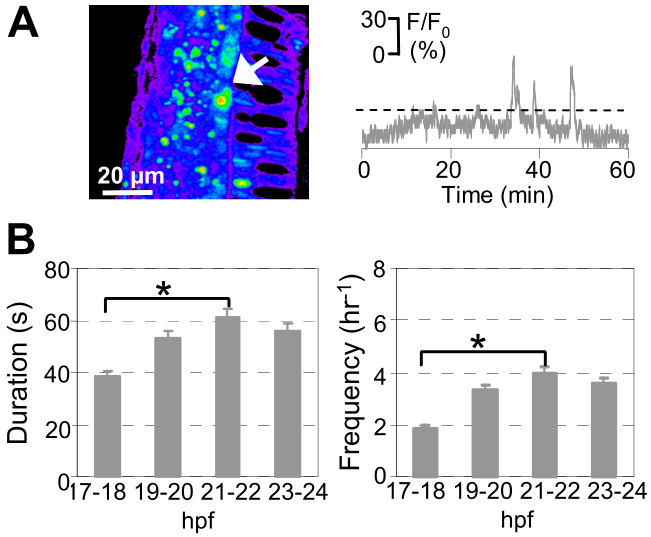


Fig. S1. Developing spinal secondary motor neurons (SMN) generate spontaneous intracellular Ca^{2+} waves. (*A*) A SMN (arrow) loaded with calcium green-1 dextran in the spinal cord of a zebrafish embryo at 20–21 hpf. Dorsal is to the left and caudal is at the bottom. Images were captured at 0.2 Hz for 30 min, and fluorescence intensity was normalized to baseline. Fluorescence is displayed on a pseudocolor scale, where purple represents the lowest intensity and red represents the highest intensity. Changes in fluorescence intensity in a SMN are plotted as a function of time. Ca^{2+} waves were identified as fluorescence transients greater than 20% of $\Delta F/F_0$ (dashed lines), more than two times the SD of the baseline, and >20 s in duration, calculated as the width at half-maximum. (*B*) Duration and frequency of Ca^{2+} waves in SMN. n = 15–20 neurons from seven to nine embryos for each time period; values are means ± SEM. *$P < 0.05$.

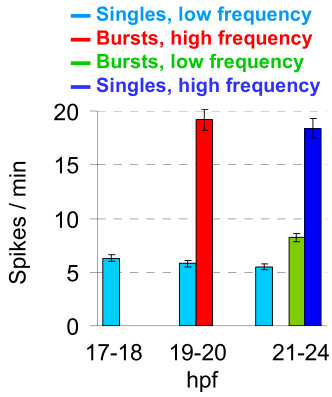
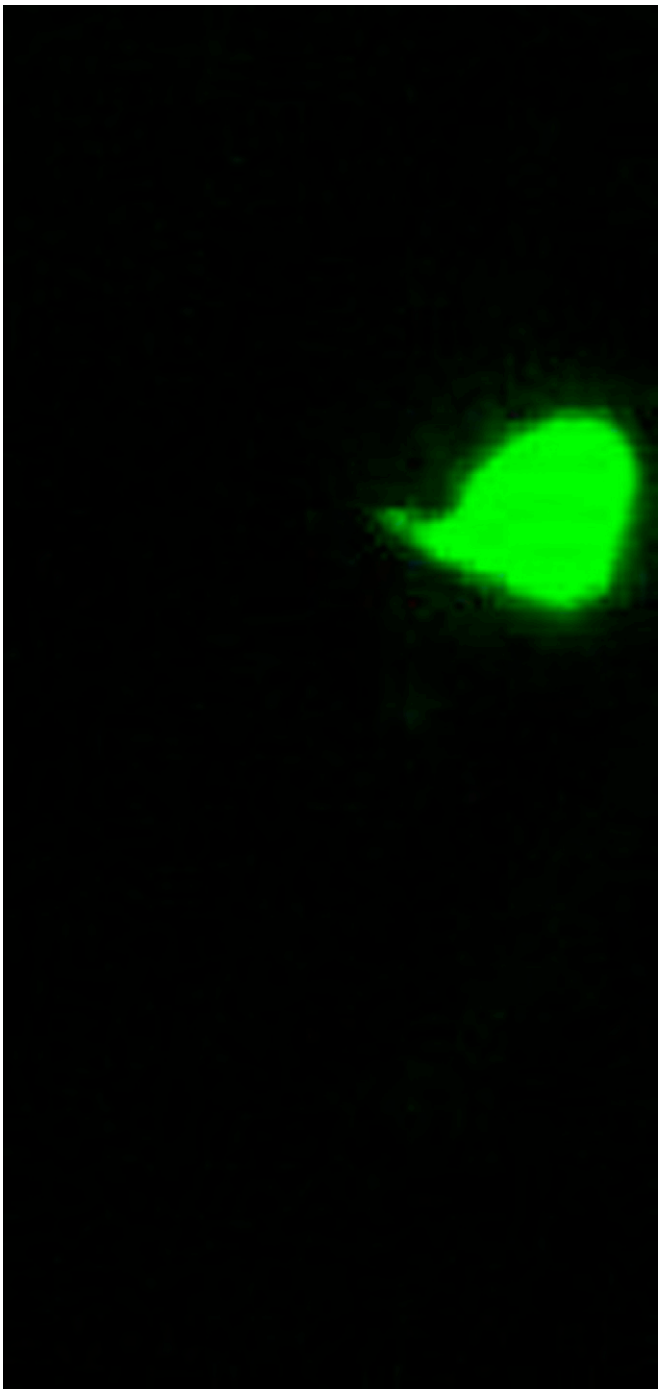
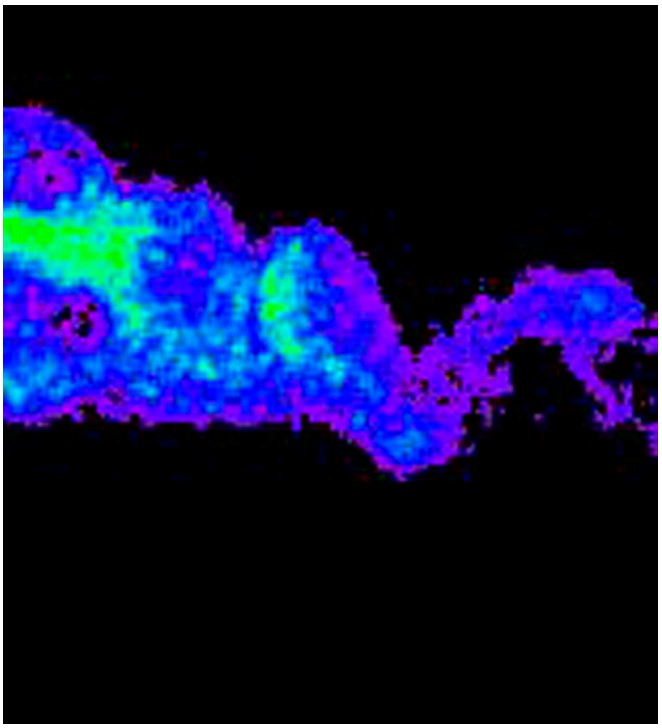


Fig. S2. PlexinA3 does not modulate PMN spontaneous Ca^{2+} spike frequency. Frequency of Ca^{2+} spikes in PMN of double-transgenic Hb9:Gal4/UAS:GCaMP3 embryos injected with PlexinA3 MO (0.5 mM) at the one-cell stage. n = 10 neurons from six to nine embryos for each period; values are means ± SEM.



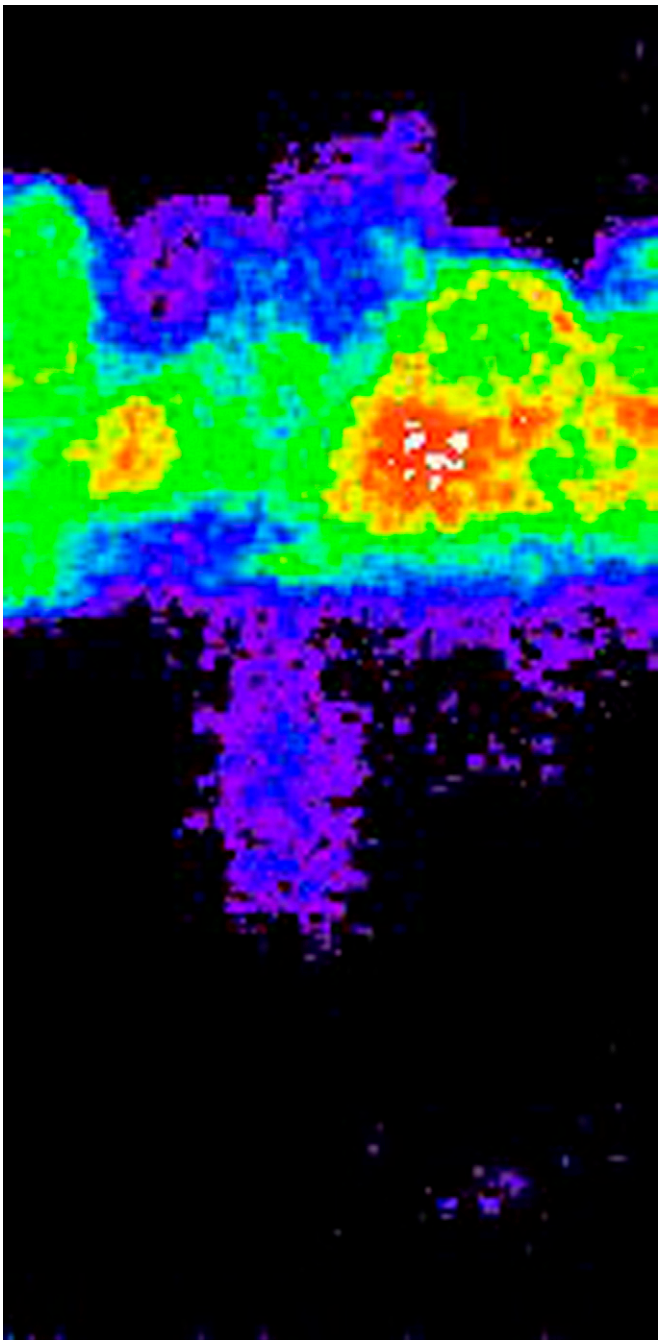
Movie S1. Time-lapse movie of the outgrowth of a PMN axon in a spinal medial segment of a WT embryo expressing eGFP in a single caudal primary motor neuron (CaP). The cell was imaged at 20-min intervals for 10 h. Lateral view; dorsal is up and rostral is to the left.

Movie S1



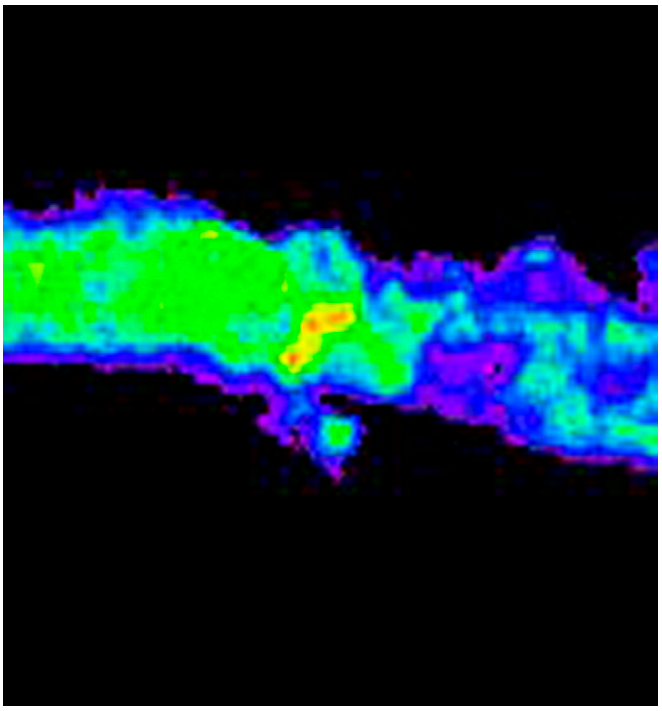
Movie S2. GCaMP3 activity acquired at 7.6 Hz for 37 s in a single CaP of a double-transgenic Hb9:Gal4/UAS:GCaMP3 embryo at 18 hpf. The Ca^{2+} signal is expressed first in the axon and propagates to the soma. Lateral view; dorsal is up and rostral is to the left. Fluorescence intensity is displayed on a pseudocolor scale from purple (low calcium) to red (high calcium).

Movie S2



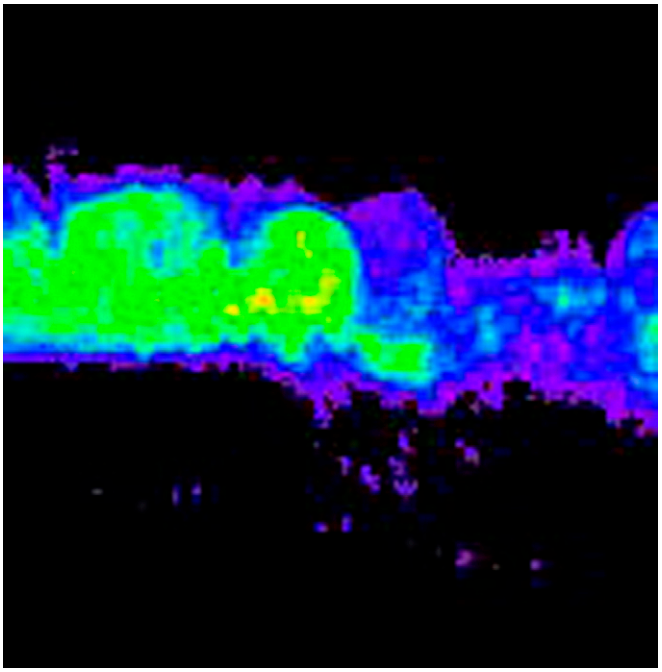
Movie S3. GCaMP3 activity acquired at 7.6 Hz for 38 s in the same CaP as in Movie S2, at 24 hpf. Display is the same as in Movie S2. The Ca^{2+} signal is expressed first in the distal axon and propagates to the soma more rapidly than at 18 hpf.

Movie S3



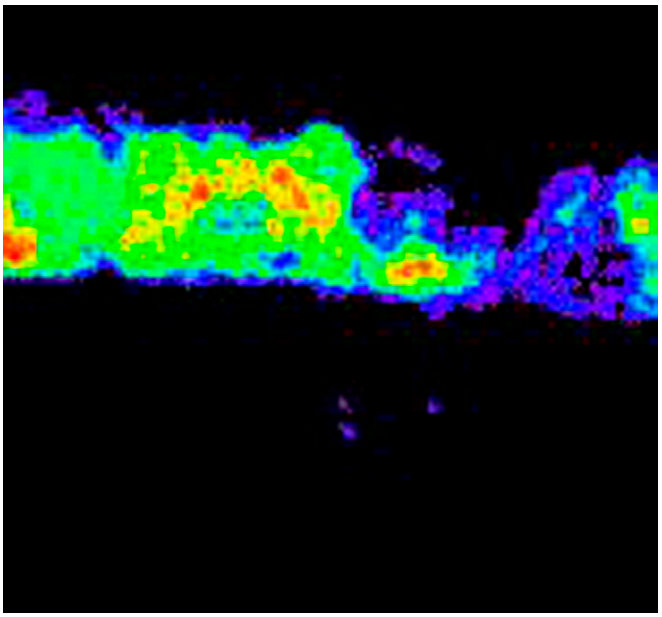
Movie S4. GCaMP3 activity acquired at 4 Hz for 26 s in a double-transgenic Hb9:Gal4/UAS:GCaMP3 embryo at 17 hpf. A CaP extending a process out of the neural tube starts generating single spikes. Display is the same as in Movie S2.

Movie S4



Movie S5. GCaMP3 activity acquired at 4 Hz for 22 s in a double-transgenic Hb9:Gal4/UAS:GCaMP3 embryo at 18 hpf. Spiking is observed in the same CaP as in Movie S4 and in a second cell located rostrally to the CaP that was later identified as a middle primary motor neuron (MiP). No correlation between CaP and MiP spike activity was observed at this stage. Display is the same as in Movie S2.

Movie S5



Movie S6. GCaMP3 activity acquired at 4 Hz for 20 s in a double-transgenic Hb9:Gal4/UAS:GCaMP3 embryo at 21 hpf. Correlated spike activity is observed in the same CaP and MiP as in Movie S5 and in a third cell located rostrally to the MiP that was later identified as a rostral primary motor neuron. Display is the same as in Movie S2.

Movie S6