Supporting Information

Kinkhabwala et al. 10.1073/pnas.1012185108

SI Experimental Procedures

Fish Care. All experiments were performed on zebrafish (*Danio rerio*) between 1 and 8 dpf obtained from a laboratory stock of wild-type and transgenic adults. Embryos at 2 dpf were selected while still in their egg casing (between 48 and 54 hpf). Larvae selected at 4 dpf were spontaneously swimming (between 96 and 106 hpf). Embryos and larvae were raised at 28.5 °C in the same system as adults (Aquatic Ecosystems), but experiments were performed at room temperature (~22 °C). At these early ages, embryonic and larval fish are still nourished by the remnants of their yolk sac. All procedures conform to the National Institutes of Health guidelines regarding animal experimentation and were approved by Cornell University's Institutional Animal Care and Use Committee.

Immunostaining. Standard whole-mount antibody staining procedures were used as described previously (1, 2). After fixation with 4% paraformaldehyde, the whole brain was carefully excised to allow for better penetration of the antibodies. The engrailed1 staining was performed with a rabbit anti-En1 antibody (a gift from A. Joyner, Sloan-Kettering Institute, New York; 1:250–1:500), and signal was detected with goat anti-rabbit antibody conjugated with HRP (Invitrogen; 1:200) and the Alexa Fluor 647-Tyramide system (Invitrogen). The chx10 staining was performed with a guinea pig anti-chx10 antibody (1:2,000) that was generated using bacterially expressed proteins (3), followed by a goat anti-guinea pig antibody conjugated with Alexa Fluor 633 (Invitrogen; 1:500). Both of the antibodies were shown to label neuronal populations in the zebrafish spinal cord identical to those detected by in situ hybridization for *eng1b* (1) and alx (*chx10*) (3), respectively.

Stochastic Labeling. Stochastic labeling was performed as described previously (4). Briefly, single-cell embryos were positioned in wells within an agar plate (1.5% DNA-grade agarose in 10% HBSS with methylene blue). To label early born neurons, injections of small volumes of constructs (30 ng/uL for each construct when injecting two simultaneously) were performed into single cells within 45 min of fertilization, before the first cell division. To label later differentiating neurons, embryos were injected into the yolk at a multiple-cell stage at or before the 1,000 -cell stage. Embryos were screened at either 3 or 4 dpf by using a Leica fluorescent dissecting scope to find embryos with only 1–3 cells labeled in the hindbrain.

Injected constructs included promoters for the glycine transporter 2, the vesicular glutamate transporter, and alx, described in prior studies (3–6). These were used to drive expression of fluorescent proteins as described in the text. For Brainbow labeling, three DNA constructs containing the following genes were mixed to a final concentration of 30 ng/uL each: UAS:Brainbow-1.1m (flanked by Tol-2 sites) (7), alx:Gal-4 (BAC), and CMV:CRE. Two microliters of the DNA mixture was added to 1.5 μL Tol-2 mRNA and subsequently injected into alx:GFP nacre (8) embryos at the single-cell stage. Fish positive for Brainbow-1.1m were imaged on the confocal, from hindbrain down to the spinal cord at 6 dpf to reconstruct the entire labeled neurons.

Transgenic Lines. The transgenic lines we used included ones described in prior studies (3, 5, 6), as well as two new ones with the promoters dbx1b and barhl2. These new lines, Tg(dbx1b:GFP) and Tg(barhl2:GFP), were constructed with the BACs zK17G17 and zC15L16, using a previously described method (3). The detailed approaches for generating these lines will be described elsewhere.

Confocal Imaging. Confocal imaging was performed as described previously (9, 10). Transgenic zebrafish were anesthetized in 0.02% MS-222 and embedded in 1.4% low-melting-point agar, typically with the dorsal side down and resting against a glass coverslip floor of a small Petri dish. The agar was covered with 0.02% MS-222 solution in 10% HBSS to prevent desiccation. Images were collected using an inverted Zeiss LSM confocal microscope with a Zeiss C-Apochromat 40× water lens. Green fluorescence was excited using a 488-nm laser light and red fluorescence using a 543-nm laser. Green fluorescence emission was typically collected with band-pass emission filters (505-530 nm or 505–550 nm), and red fluorescence emission was collected using a long-pass 560-nm filter. Single-image stacks were collected throughout the dorsoventral extent of hindbrain or spinal cord, and collection typically lasted 40 min to 1.5 h. To prevent photo damage, images were collected with high-gain settings and averaged. Fish remained healthy and anesthetized during this time.

Colocalization. Confocal image stacks containing green and red channels were observed using Imaris software (Bitplane). Using the colocalization add-on, minimum thresholds were determined for red and green channels slightly above noise levels for each channel. We ensured that the dimmest cells were not thresholded out. A new channel was generated that represented colabeled signal. For image stacks with low noise levels in both channels, the automated colocalization feature in Imaris was adequate for our purposes, but in noisier datasets, thresholds were adjusted by hand to be sure that the thresholds were above the background noise.

Neuronal Tracing and Location Measurements. Neurons were traced using the filament reconstruction feature provided in the Imaris software package (Bitplane). For image stacks with low background noise levels, automated reconstructions were performed choosing a diameter for a starting point (cell body) and a minimum diameter for end points along projections from the starting point. A threshold was used to adjust the number of start and end points for optimal tracing of single neurons. In cases where background noise levels were high, the autodepth feature of the Imaris filament software was used to trace neurons by hand along various 2D planes transecting the 3D volume, while allowing the program to determine the depth of the tracing. Reconstructions were constantly verified to be accurate representations of the labeled cell, and were used for illustration in this study because fine processes were hard to depict in 2D projections of confocal image stacks. The dorsoventral position of a particular cell within a stripe was determined by dividing the distance of the middle of the cell body to the bottom of the stripe by the length of the entire stripe.

Photoconversion and Analysis. Huc:Kaede and alx:Kaede transgenic embryos (3, 11) were illuminated with UV light using a mercury bulb source for 10–40 s within their chorion. Immediately afterward, photoconversion was confirmed by observation of the presence of red expression and an absence of green expression using green and red filters. Photoconverted fish were then kept at 28.5 °C in a light-tight container until the day of imaging.

To determine which cells were exclusively green, and thus not expressing Kaede at the time of photoconversion, a colocalization was performed between the red and green channels (see coloc-

alization section of *Experimental Procedures*) and a new channel was constructed. A mask of this new channel was used to remove all of the green expression from colocalized regions to produce the images shown in Fig. 4*D*.

For quantification of intensities in the neuropil, analysis was performed in MATLAB. Horizontal confocal image stacks were rotated by 90° using Imaris software to obtain cross-sections. Neuropil regions were cropped in cross-section, and a series of tiffs within a local region, including the dorsoventral and mediolateral extent of the labeled neuropil, was generated in Imaris and analyzed using MATLAB (generally 15–100 slices). To generate a single image of this region, tiffs were summed and divided by the number of sections to show an averaged image plane of red and green intensities. To quantify the dorsoventral extent of red and green expression, voxels at the same dorsoventral position were summed across the mediolateral extent to obtain the intensity at a given dorsoventral location. Each value was divided by the maximum value of the distribution to generate a normalized distribution, because the absolute intensity differences between red and green expression might be related to imaging conditions and not differences in the distribution of red and green.

Targeted Whole-Cell Patching of alx Neurons in Exposed Brain Preparation for Input Resistance Measurements. Larvae were anesthetized in 3-aminobenzoic acid ethyl ester (0.02% in HBSS) and then immobilized using α-bungarotoxin (Sigma-Aldrich; 0.1% in HBSS). To measure input resistance of alx neurons in hindbrain, we initially used a dissection procedure similar to one described previously (12). We later switched to a less-extensive exposure of the brain, leaving more of the head, including the eyes, intact to minimize potential damage to the neurons. Procedures for whole-cell recordings are described in the following section. After the electrophysiological measurements, the preparation was fixed with 4% formaldehyde with the pipette in place to avoid the movement of the cell body during the retraction of pipette. Then a z stack was acquired with a confocal microscope (LSM 510 META; Zeiss) for the measurement of the soma position relative to the alx stripe.

In Vivo Whole-Cell Recordings in the Hindbrain. Whole-cell recordings were done in current clamp mode in 5-dpf alx:GFP nacre transgenic larvae using modifications of methods described previously (5, 12–14). Larvae were anesthetized using 3-aminobenzoic acid ethyl ester (MS-222, 0.02% in HBSS), paralyzed with α-bungarotoxin (1 mg/mL in extracellular solution) and pinned twice through the notocord with tungsten pins to Sylgard in a small Petri dish. The skin was removed from axial muscle between the pins for later ventral root recordings. The head was then rotated 90° and pinned through the mouth. A small incision was made in the skin along a dorsal portion of the head for ease of patch electrode insertion. The dish, containing extracellular saline solution (134 mmol/L, NaCl, 2.9 mmol/L KCl, 1.2 mmol/L MgCl2, 10 mmol/L Hepes, 10 mmol/L glucose, and 2.1 mmol/L CaCl2; adjusted to pH 7.8 with NaOH), was placed on a compound microscope (BX51WI; Olympus) and a glass microelectrode filled with extracellular solution was placed over an intermyotomal cleft where the axial skin was removed. A MultiClamp 700A amplifier (Molecular Devices) was used to monitor extracellular signals in current clamp mode at a gain of 1,000, with the low- and high-frequency cutoff set at 300 and 5,000 Hz, respectively.

Patch-clamp electrodes were pulled from thin-walled glass capillaries to 10–20 MΩ resistances. To record from hindbrain neurons, the electrodes were filled with intracellular saline solution (ionic composition in millimoles per liter: 125 K-gluconate, 2.5 MgCl2, 10 EGTA, 10 Hepes, 4 Na2ATP; 6.25 mg sulfur rhodamine-B, adjusted to pH 7.3 with KOH) and then gently advanced into the brain using motorized micromanipulators (Sutter Instrument Co. or Luigs–Neumann). Constant positive pressure (~20 mmHg) was applied to the micropipette using a pneumatic transducer (Bio-Tek Instruments Inc.) until the tip of the electrode was brought within close proximity to a cell body. Cell bodies were visualized using differential interference contrast (DIC) optics, and the alx-positive identity of a cell was confirmed by briefly switching to an attenuated epifluorescent light source. A GΩ seal was then obtained either by equilibrating the micropipette to atmosphere or by applying gentle suction. A holding potential of −65 mV was applied once the micropipette had become cell attached and the membrane was penetrated with suction pulses. Whole-cell current clamp recordings were made with a MultiClamp 700A amplifier at a gain of 20 (rf = 500 mΩ) filtered at 30 kHz and digitized at 63 kHz.

Calcium Imaging. Transgenic 4-dpf alx:DsRed Casper fish (15) were first anesthetized using 3-aminobenzoic acid ethyl ester (MS-222, 0.02% in HBSS) and embedded in low-melting-point agarose (1.6% in HBSS; Sigma). A patch electrode (5–10 MΩ resistance) was filled with 20% Oregon Green BAPTA-1 (10,000 M_r; Invitrogen/Molecular Probes), and the indicator was electroporated along the dorsoventral axis of the alx stripe in caudal hindbrain by using a single-cell Axoporator (−4 V, 20-ms duration square pulse; Molecular Devices). Larvae were then removed from the agarose, placed in a Petri dish containing HBSS, and stored in an incubator (28.5 °C) overnight.

The following day, we imaged the calcium indicator in hindbrain on a Zeiss LSM 510 inverted confocal microscope while recording from a ventral root using methods similar to those applied previously (5, 16). The larva was oriented on its side, with the head at 90° relative to the tail, and pinned in place. Once a stable root recording was in place, the hindbrain was imaged. A time series capturing OGB-1 and alx:DsRed transgenic fluorescence (typically at about three images per second) was acquired while simultaneously monitoring ventral root activity. Swimming often occurred spontaneously, but was also elicited by either flashes of blue light or a brief electrical stimulus applied to the end of the tail. Following each experiment, we acquired high-quality image stacks of the hindbrain for later reconstruction and cell positioning.

Analysis of calcium imaging and ventral root recordings was performed using custom written MATLAB software. ROIs were selected to include single cells for measuring fluorescence intensity as an indicator of neuronal activity. The ventral root bursts were used to estimate swimming frequency during this activity, measured as the reciprocal of the period between the start of each successive burst. Due to the coarse temporal resolution of calcium imaging, we took a conservative approach to the measure of minimum swimming frequency at which a neuron was recruited, similar to McLean et al. (5). For each swimming episode in which a neuron showed a calcium response (defined as >9% fluorescence increase over baseline), the fastest swimming frequency during that episode was recorded. We then averaged the lowest three of these values to estimate the minimum speed of recruitment, being sure to bracket the lowest frequency of recruitment by having episodes in which the neurons did not respond. The number of trials collect per fish was about 100, but ranged from 50 to >200. The images were typically sampled every 245 ms.

Image stacks were then reconstructed in 3D using Imaris software (Bitplane). Neurons that were active during the experiment were identified and isosurfaces were generated for each. The length of the left and right stripes (at the position of the cell body) were each measured three times and averaged. The distance from the top of the stripe to the center of the cell body was then measured three times and averaged. This distance along the dorsoventral axis of the stripe was then normalized for total stripe length at the position of the cell body.

1. Higashijima S, Masino MA, Mandel G, Fetcho JR (2004) Engrailed-1 expression marks a primitive class of inhibitory spinal interneuron. *J Neurosci* 24:5827–5839.
2. McLean DL, Fetcho JR (2004) Ontogeny and innervation patterns of dopaminergic, noradrenergic, and serotonergic neurons in larval zebrafish. *J Comp Neurol* 480: 38–56.
3. Kimura Y, Okamura Y, Higashijima S (2006) alx, a zebrafish homolog of Chx10, marks ipsilateral descending excitatory interneurons that participate in the regulation of spinal locomotor circuits. *J Neurosci* 26:5684–5697.
4. Higashijima S, Mandel G, Fetcho JR (2004) Distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in embryonic and larval zebrafish. *J Comp Neurol* 480:1–18.
5. McLean DL, Fan J, Higashijima S, Hale ME, Fetcho JR (2007) A topographic map of recruitment in spinal cord. *Nature* 446:71–75.
6. Bae YK, et al. (2009) Anatomy of zebrafish cerebellum and screen for mutations affecting its development. *Dev Biol* 330:406–426.
7. Livet J, et al. (2007) Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* 450:56–62.
8. Lister JA, Robertson CP, Lepage T, Johnson SL, Raible DW (1999) nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* 126:3757–3767.
9. Hale ME, Ritter DA, Fetcho JR (2001) A confocal study of spinal interneurons in living larval zebrafish. *J Comp Neurol* 437:1–16.
10. McLean DL, Masino MA, Koh IY, Lindquist WB, Fetcho JR (2008) Continuous shifts in the active set of spinal interneurons during changes in locomotor speed. *Nat Neurosci* 11:1419–1429.
11. Sato T, Takahoko M, Okamoto H (2006) HuC:Kaede, a useful tool to label neural morphologies in networks in vivo. *Genesis* 44:136–142.
12. Drapeau P, Ali DW, Buss RR, Saint-Amant L (1999) In vivo recording from identifiable neurons of the locomotor network in the developing zebrafish. *J Neurosci Methods* 88:1–13.
13. Masino MA, Fetcho JR (2005) Fictive swimming motor patterns in wild type and mutant larval zebrafish. *J Neurophysiol* 93:3177–3188.
14. Bhatt DH, McLean DL, Hale ME, Fetcho JR (2007) Grading movement strength by changes in firing intensity versus recruitment of spinal interneurons. *Neuron* 53: 91–102.
15. White RM, et al. (2008) Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* 2:183–189.
16. McLean DL, Fetcho JR (2009) Spinal interneurons differentiate sequentially from those driving the fastest swimming movements in larval zebrafish to those driving the slowest ones. *J Neurosci* 29:13566–13577.