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Supplementary Materials for

Orderly Compartmental Mapping of Premotor Inhibitory Inputs in Developing Spinal Cord

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Materials and Methods

Animals

Adult wildtype and transgenic zebrafish were maintained at 28.5ºC on a 14/10-hr light/dark cycle in a custom-built facility (Aquatic Habitats). Daily crosses of adults provided zebrafish embryos that were raised at 28.5ºC. Experiments were performed in zebrafish up to 5 days post fertilization, at which point they have a fully inflated swim bladder and are free-swimming. At these developmental stages, zebrafish have not yet sexually differentiated and can survive on embryonic yolk stores. All procedures were performed in accordance with NIH guidelines for animal experimentation and were approved by the Northwestern University Institutional Animal Care and Use Committee (#IS00002671).

Plasmids and BACs

The *dmrt3a:Cre* and *dmrt3a:hsGFP* bacterial artificial chromosomes (BAC) were generously provided by Shin-ichi Higashijima prior to publication. Unpublished plasmids listed in Table S1 were generated using the Tol2kit (*31*) and Gateway cloning (Invitrogen) using primers listed in Table S2. To generate *mnx1*:mCerulean, we first amplified mCerulean from CMV-Brainbow1.0H (Addgene 18720; (*32*)) with primers that were flanked by gateway cloning sites. The mCerulean PCR product was first subcloned into a middle entry vector and subsequently subcloned into a Tol2 destination vector with Tol2 transposable elements flanking three copies of the 125 basepair (bp) *mnx1* enhancer (*33*) and a SV40 polyadenylation (pA) signal. Constructs for GRASP were subcloned into middle entry vectors from plasmids containing CD4-spGFP1-10 and CD4-spGFP11 (gift from Marco Gallio; (*12*)) by amplifying the CD4-spGFP1-10 and CD4-spGFP11 fragments with primers that were flanked by gateway cloning sites. The CD4-spGFP1-10 middle entry vector was subcloned under a 3x125 bp element *mnx1* enhancer, and the CD4-spGFP11 middle entry vector was subcloned under a 10X element UAS promoter to generate UAS:CD4-spGFP11. Both the *mnx1*:CD4-spGFP-1-10 and UAS:CD4-spGFP11 plasmids contained Tol2 transposable elements and a SV40 polyadenylation (pA) signal. A construct driving cytosolic Dendra expression, UAS:Dendra, was generated by amplifying Dendra from UAS:Dendra-kras (*34*) flanked by gateway cloning sites. The Dendra PCR product was subcloned into a middle entry vector, and then subcloned into a Tol2 destination vector under a 10X element UAS promoter to generate UAS:Dendra.

Transgenic lines

The Tg[dmrt3a:hsGFP] line was generously provided by Shin-ichi Higashijima prior to publication. A full list of transgenic lines is provided in Table S3. Transgenic fish were generated using Tol2 based transgenesis (*35*) where plasmids were co-injected into embryos at the single cell stage along with in vitro transcribed transposase mRNA (*36*). Embryos were secured in an agar plate (1.4% w/v agar), and injections were performed using microinjection needles with a tip diameter of 3-5 µm made from thin wall glass capillaries (Warner Instruments, GT100TF-3). The concentration of injected plasmids was 15-25 ng/µl and the concentration of transposase mRNA was 50 ng/µl. Messenger RNA was synthesized using the mMessage mMachine kit (Ambion). Putative founder embryos (F_0) from injected fish were raised to sexual maturity. These were then crossed with wild-type adults to generate transgenic F1 embryos that were screened for fluorescence at 36-hour-post-fertilization (hpf) using an epifluorescent microscope (Zeiss SteREO Discovery.V20).

To generate stable Tg[*dmrt3a*:Gal4; UAS:Dendra] fish expressing the photo-convertible protein Dendra in dI6 neurons, we injected a UAS:Dendra plasmid into embryos from Tg[*dmrt3a*:Gal4] fish (*37*). Tg[*dmrt3a*:Gal4; UAS:pTagRFP] fish expressing red fluorescent protein in dI6 neurons, were generated by crossing two existing transgenic lines, Tg[*dmrt3a*:Gal4; UAS:EGFP] and Tg[*mnx1*:Gal4; UAS:pTagRFP] (*38*) and raising embryos expressing red fluorescent protein only in dI6 neurons to adulthood. To generate stable Tg[*mnx1*:mCerulean] fish expressing membrane-tagged cyan fluorescent protein in motor neurons, we injected *mnx1*:mCerulean plasmid with transposase mRNA in wild-type embryos. Double transgenic Tg[*glyt2*:lRl:Gal4; *mnx1*:mCerulean] fish were generated by crossing Tg[*glyt2*:lRl:Gal4] (*8*) and Tg[*mnx1*:mCerulean] lines and raising embryos positive for motor neuron cyan fluorescence, and red fluorescence in glycinergic neurons. For GRASP experiments relying on transient expression (see below), we made a Tg[*dmrt3a*:Gal4; UAS:pTagRFP; UAS:CD4-spGFP11] to drive the expression of spGFP11 in all dI6 positive neurons, by injecting UAS:CD4-spGFP11 into Tg[*dmrt3a*:Gal4; UAS:pTagRFP]. For more stable expression of GRASP, we also made a transgenic line with 5 transgenes – Tg[*dmrt3a*:Gal4; UAS:pTagRFP; UAS:CD4-spGFP11; *mnx1*:mCerulean; *mnx1*:CD4-spGFP1-10] – by injecting a combination of UAS:CD4-spGFP11, *mnx1*:mCerulean, and *mnx1*:CD4-spGFP1-10 plasmids into Tg[*dmrt3a*:Gal4; UAS:pTagRFP] embryos. In order to detect smaller punctate GRASP signals, fish were screened on the confocal microscope.

Transient expression

For transient labeling of neuron morphology and putative synapses, we used the Gal4-UAS system to drive sparse, mosaic expression of reporter constructs selectively in motor neurons or dI6 neurons (*39, 40*). Motor neuron Gal4 expression was driven by the zebrafish mnx1 promoter (*mnx1*:gal4) or the bacterial artificial chromosome for vesicle acetylcholine transporter (*vachta*:Gal4) (*18*). Two reporter constructs containing upstream activating sequences (UAS) were co-injected – UAS:pTagRFP was used to visualize the morphology of motor neurons (*41*), and UAS:eGFP-GlyRα1 labeled putative glycinergic synapses (*8*). Transient labeling of dI6 neurons was achieved in one of three ways. First, *dmrt3a*:Gal4 was co-injected with UAS:pTagRFP and UAS:Syp-GFP (*42*) in wild-type fish or transgenic fish that express cyan fluorescent protein in motor neurons (Tg[*mnx1*:mCerulean]). Second, *dmrt3a*:Cre was co-injected with UAS:pTagRFP and UAS:Syp-GFP in double transgenic fish (Tg[*glyt2*:lRl:Gal4; *mnx1*:mCerulean]) generated by crossing Tg[*glyt2*:lRl:Gal4] and Tg[*mnx1*:mCerulean] fish. Third, UAS:Syp-GFP and UAS:mCerulean were co-injected in transgenic fish that express red fluorescent protein in dI6 neurons (Tg[*dmrt3a*:Gal4; UAS:pTagRFP]). For transient labeling in GRASP experiments, Tg[*dmrt3a*:Gal4; UAS:pTagRFP; UAS:CD4-spGFP11] embryos were injected with two plasmids – *mnx1*:mCerulean and *mnx1*:CD4-spGFP1-10 – to unambiguously assign GRASP signal to individual motor neurons.

Electrophysiology

For electrophysiological recordings, larvae were first anesthetized in ethyl 3-amino-benzoate methanesulfonic acid (MS-222; 0.02% w/v, Sigma-Aldrich) and then immobilized in α-bungarotoxin (0.1% w/v, Sigma-Aldrich), both of which were dissolved in extracellular solution (1 mg/ml). The composition of the extracellular solution was as follows (in mM): 134 NaCl, 2.9 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 glucose and 2.1 $CaCl_2$, adjusted to pH 7.8 with NaOH. After 3-5 min, fish were removed from the α-bungarotoxin solution and secured on their right side to the bottom of a Sylgard-coated, glass-bottomed dish filled with extracellular solution, using custom-

etched tungsten pins inserted through the notochord. In a subset of experiments (n = 21), the skin just behind the swim bladder past the anus was removed using fine forceps, to enable suction electrode recordings of motor neuron axons (*43*). To access spinal neurons, 1-2 muscle segments were dissected away carefully under a dissecting microscope with a sharpened tungsten dissecting pin.

After the dissection, spinal neurons were targeted using a 40x/1.0 NA water-immersion objective (W Plan-Apochromat; Zeiss) on a compound microscope (AxioExaminer; Zeiss) equipped with an epifluorescent light source, differential interference contrast (DIC) optics and a CCD camera (Rolera-XR; Q-Imaging). dI6 neurons were targeted on the left (upper) side based on fluorescence in either the Tg[*dmrt3a*:hsGFP],Tg[*dmrt3a*:Gal4; UAS:GFP], or Tg[*dmrt3a*:Gal4; UAS:Dendra] lines, while primary motor neurons were targeted 1-2 segments (~100-200μm) rostral or caudal to the dI6 neurons on the opposite (right) side based on their size and position in DIC. For whole-cell recordings, standard wall glass capillaries (Warner Instruments, G100F-3) were pulled to make recording pipettes between 5-15 MΩ, which were then backfilled with patch solution containing either Alexa Fluor 488 or 568 hydrazide (50 μM; ThermoFisher) or sulforhodamine (0.025% w/v; Sigma-Aldrich) to visualize cell morphology at the end of the experiment. Post-hoc images were captured using QCapture software (Q-imaging) or on a confocal microscope (LSM710; Zeiss). To get clearer images of the contralateral axon of dI6 neurons and the contralateral motor neuron for analysis, fish were flipped and secured on their left side. For extracellular recordings of motor neuron axons, suction electrodes fashioned from patch electrodes were broken at the tip (20-50 μm diameter), fire polished (Narishigi), filled with extracellular solution and placed over the intermyotomal clefts. Recording pipettes were positioned using motorized micromanipulators (MP-225; Sutter Instruments). 'Fictive' swimming was evoked using a tungsten concentric bipolar electrode placed under the tip of the tail using a manual micromanipulator (Y-31CF; Narishige). A brief electrical stimulus (5-15 V; 0.2-0.4 ms) was delivered via an isolated stimulator (DS2a-Mk.II; Digitimer).

For dI6 neurons recorded in current-clamp mode, the composition of patch solution was as follows (in mM): 130 K-Gluconate, $2MgCl_2$, 0.2 EGTA, 10 HEPES, $4Na_2ATP$, adjusted to pH7.3 with KOH. For primary motor neurons recorded in voltage-clamp mode, the composition of the patch solution was (in mM): 122 $CsMeSO_3$, 0.1-1 QX314-Cl, 1 TEA-Cl, 3 $MgCl_2$, 10 HEPES, 1 EGTA, 4 mM Na_2-ATP, adjusted to pH7.3 with KOH. All electrophysiological recordings were acquired using a Multiclamp 700B amplifier, a Digitdata series 1322 A digitizer, and pClamp software (Molecular Devices). Bridge balance and electrode capacitance were corrected for in current-clamp recordings. Voltage clamp recordings were performed with 60% series compensation at a 0 mV holding potential (taking into account a –11 mV liquid junction potential). In all voltage-clamp recordings, compensated access resistance was below 50 MΩ. Electrical signals from whole-cell recordings were filtered at 30 kHz and digitized at 63 kHz at a gain of 10 (feedback resistor, 500 MΩ). Electrical signals from motor neuron axons were recorded at a gain of 1000 with low and high-frequency cutoffs set at 100 and 4000 Hz, respectively. Synaptic connections were assessed by injecting 5-10 ms current steps at 1Hz to trigger a single spike in the presynaptic dI6 neuron while recording inhibitory postsynaptic currents (IPSCs) in the postsynaptic motor neuron.

Confocal imaging and staged photoconversions

For confocal imaging, zebrafish larvae were anesthetized in 0.02% w/v MS-222 and embedded on their side in low-melting-point agar (1.4% in system water) in a glass-bottomed dish. Once

solidified, the agar was covered with more anesthetic solution to prevent agar desiccation and fish movement. Fish were imaged and photoconverted on a confocal microscope equipped with an argon laser (488, 458 nm) and a helium/neon laser (543 nm), using a 20x/1.0-NA water-immersion objective (Zeiss). For photoconversion experiments, fish were additionally illuminated for 1-2 min using a DAPI epifluorescence filter set. Conversion from green to red was confirmed using FITC and rhodamine settings. Photoconverted fish were then raised at 28.5ºC in the dark to avoid background photoconversion. Motor neurons, dI6 neurons, and GRASP data were sampled between muscle segments 7-22. DIC images of the spinal cord were acquired simultaneously to identify anatomical landmarks for purposes of analysis.

Analysis

Three-dimensional (3D) imaging data were analyzed using Imaris (Bitplane). Motor neuron and dI6 neuron morphology were reconstructed in 3D using the Filament function. Axon lengths of dI6 neurons were quantified in a 100 µm window rostral and caudal to the cell body (approximating 1-2 body segments). Putative GlyRα1, synaptophysin and GRASP puncta were detected using the Spots function with a threshold value of 1 µm. Cell bodies of dI6 neurons were digitized using the Spots function with a threshold value of 5 µm. Putative GlyRα1 puncta were classified into somatic, axonic or dendritic puncta based on their location on motor neurons. To quantify spatial distribution of puncta, dI6 axons and cell bodies across fish, variations in spinal cord height and height were normalized relative to the dorsal and ventral aspects of the spinal cord as well as along mediolateral landmarks marked the central canal and the lateral-most boundary between the spinal cord and the axial musculature. Landmarks in each image were marked using the Measurements function. Coordinates and landmarks were then exported from Imaris and plotted using previously published MATLAB scripts (*18*) interfaced with Imaris. Distribution contours of GlyRα1, synaptophysin and GRASP puncta as well as dI6 neuron cell bodies and axons were made in MATLAB using the kde2d function (MATLAB File Exchange). This function estimates a bivariate kernel density over the set of normalized coordinates using a 32 by 32 grid (*44, 45*). Based on the calculated densities, contour plots were then generated using the "contour" function in MATLAB. Contour plots are displayed as contour lines in increasing increments of one sixth of the peak density where innermost contours represent the highest density of coordinates.

Electrophysiology data were analyzed off-line using DataView (*46*), MATLAB (MathWorks), and Excel (Microsoft). DataView was used to identify spikes in current-clamp recordings of dI6 neurons, the tail stimulus artifact, as well as the timing of the first inhibitory post-synaptic current in motor neurons after the stimulus. MATLAB was used to analyze the timing of spikes and inhibitory events relative to the stimulus. A combination of DataView and MATLAB was used to analyze synaptic connectivity between dI6 neurons and motor neurons. Presynaptic action potentials were aligned in DataView to the peak of the action potential (10-50 sweeps), and time-locked IPSCs were marked for subsequent amplitude analysis in MATLAB. Evoked IPSC amplitudes were calculated from successful events and normalized to baseline values measured in a 2 ms window preceding the spike. Failure rates were calculated as the percentage of presynaptic spikes that did not result in time-locked evoked IPSCs in the postsynaptic motor neuron. Unconnected pairs were assigned 0 mV amplitudes and 100% failure rates for purposes of analysis, since we could not rule out failure rates that exceeded our sample size (10-50 spikes). Cyclical motor bursts from suction electrode recordings during 'fictive' swimming were identified in DataView and subsequently exported to MATLAB. 'Fictive' swimming frequency

was calculated as the inverse interval between the onset of motor bursts. Statistical analysis was performed using the StatPlus plugin to Excel (AnalysSoft). Data were first tested for normality and then the appropriate parametric or nonparametric test was selected. Degrees of freedom are reported parenthetically with the respective t, U, F, and H statistics, per convention. Significance was set at $p < 0.05$.

Table S1. List of plasmids

Name	Plasmid/BAC	Source
dmrt3a:Gal4	BAC	(Satou et al., 2013)
dmrt3a:Cre	BAC	Gift from Shin-ichi Higashijima
dmrt3a:hsGFP	BAC	Gift from Shin-ichi Higashijima
mnx1:Gal4	Plasmid	(Zelenchuk & Brusés, 2011)
mnx1:mCerulean	Plasmid	This paper
mnx1:CD4-spGFP1-10	Plasmid	This paper
UAS-CD4-spGFP11	Plasmid	This paper
UAS:Dendra	Plasmid	This paper
UAS:pTagRFP	Plasmid	(Menelaou et al., 2014)
UAS:Syp-GFP	Plasmid	(Niell, Meyer, & Smith, 2004)
vachta:Gal4	BAC	(Kishore & Fetcho, 2013)

Table S2: List of primers for cloning

5' pME-mCerulean	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACGGAATTAATTCACAGCCACCA
3' pME-mCerulean	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGTCGCGGTGATCTAGAGTC
5' pME-Dendra	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGAACACCCCGGGAATTAAC
3' pME-Dendra	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAAGATCTCTTGTACACGCCGCTGTC
5' pME-CD4-spGFP 1-10	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGCCACCTTCAACATCATTGCTGCTCCTCGCAG
3' pME-CD4-spGFP 1-10	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAGCGCCTTCGGTGCCGGCACCTGACACAGAA
5' pME-CD4-spGFP 11	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGCCACCTTCAACATCATTGCTGCTCCTCGCAG
3' pME-CD4-spGFP 11	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAGCGCCTTCGGTGCCGGCACCTGACACAGAA

Table S3: List of transgenic lines

Name	Source
dmrt3a:hsGFP	Gift from Shin-ichi Higashijima
dmrt3a:Gal4; UAS:GFP	(Satou et al., 2013)
dmrt3a:Gal4; UAS:Dendra	This paper
dmrt3a:Gal4; UAS:pTagRFP	This paper
dmrt3a:Gal4; UAS:pTagRFP; UAS:CD4-spGFP11	This paper
*dmrt*3a:Gal4; UAS:pTagRFP; UAS:CD4-spGFP11; *mnx1*:CD4-spGFP1-10; *mnx1*:mCerulean	This paper
mnx1:mCerulean	This paper
*glyt2:*lRl:Gal4	(Satou et al., 2013)
*glyt2:*lRl:Gal4; *mnx1:*mCerulean	This paper