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Modular organization of axial microcircuits in zebrafish

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Abstract

Locomotion requires precise control of spinal networks. In tetrapods and bipeds, dynamic regulation of locomotion is simplified by the modular organization of spinal limb circuits, but it is not known whether their predecessors, fish axial circuits, are similarly organized. Here, we demonstrate that the larval zebrafish spinal cord contains distinct, parallel microcircuits for independent control of dorsal and ventral musculature on each side of the body. During normal swimming, dorsal and ventral microcircuits are equally active; but during postural correction, fish differentially engage these microcircuits to generate torque for self-righting. These findings reveal greater complexity in the axial spinal networks responsible for swimming than previously recognized and suggest an early template of modular organization for more complex locomotor circuits in later vertebrates.

Animals make effective progress through unpredictable environments by rapidly adjusting ongoing locomotor movements (1, 2). In limbed vertebrates, spinal microcircuits are organized into modules for efficient coordination of joints, limbs and trunk during locomotion (3, 4). However, it is unknown how the modular organization of complex spinal circuitry in tetrapods and bipeds could have arisen from the simpler, ancestral axial network of fish (5). Current models of axially-based locomotion in fish engage left-right alternation circuits, but do not include equivalent ipsilaterally organized microcircuits akin to those seen in higher vertebrates (6). Instead, it is thought that differential activation of dorsal and ventral musculature for behaviors such as postural control is achieved by supraspinal descending commands (7). To address this evolutionary gap, we explored whether axial circuitry in fish contains more complexity than previously recognized.

In larval zebrafish, as with adults, swimming is produced by the left-right alternation of axial muscles activated in waves by segmentally iterated pools of motor neurons (MNs). In each spinal hemi-segment is a cohort of four early-developing “primary” MNs, whose axon arborizations collectively tile the dorsal and ventral musculature (Fig. 1H) (8, 9). Because all primary MNs exhibit identical recruitment patterns during swimming (9, 10), there should be no confounding effects of speed-dependent differences on the configuration of their premotor circuits (11). Thus, current models would predict that they are embedded in a shared premotor network.

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To test this prediction, we performed voltage clamp recordings from pairs of MNs during fictive swimming. As expected (12, 13), all MNs received barrages of excitatory post-synaptic currents (EPSCs) in phase with ipsilateral motor activity (Fig. 1A). To evaluate whether these barrages, which are synchronous on a large temporal scale (tens of ms), were derived from a shared premotor network, we assessed whether they were also synchronous at a fine temporal scale (tens of μ s) (14).

In pairs of MNs innervating the same muscle quadrant, EPSCs during fictive swimming were indeed highly synchronous (Fig. 1B), as exemplified by an overlay of 100 consecutive EPSCs from one neuron and the associated EPSC-triggered average in the other (Fig. 1C). Unexpectedly, excitatory inputs to MN pairs innervating different muscle quadrants lacked synchrony at fine time scales (Fig. 1D), with accordingly small EPSC-triggered average responses (Fig. 1E). Analysis of all data revealed significantly larger EPSC-triggered averages for “in quadrant” (i.e., either dorsal/mid-dorsal or ventral/mid-ventral pairs), as opposed to “out of quadrant” pairs (Fig. 1F). Similarly, a measure of event cross-correlation scores for the pooled data revealed a significant peak at 0 ms for in quadrant versus out of quadrant pairs (Fig. 1G) (see also Methods and Figs. S1 and S2). The low synchrony for out of quadrant pairs is incompatible with the assumption that dorsal- and ventral-projecting MNs are embedded in a shared presynaptic network. Instead, premotor excitatory inputs are segregated into two largely distinct microcircuits, governing dorsal and ventral musculature (Fig. 1H).

Are inhibitory premotor pathways similarly segregated? On a longer time scale, barrages of inhibitory postsynaptic currents (IPSCs) recorded in two MNs during fictive swimming occurred synchronously, predominantly out of phase with the motor output (Fig. 2A). Similar to EPSCs, however, at fine temporal scales in quadrant pairs exhibited strong synchrony of IPSCs and a large IPSC-triggered average (Figs. 2B, C), whereas out of quadrant MN pairs revealed weaker evidence for IPSC correlation and smaller IPSC-triggered averages (Figs. 2D, E). These observations were borne out by analysis of group data, showing that in quadrant pairs exhibited a ~2.3-fold larger IPSC-triggered average (Fig. 2F) and twice the magnitude of cross-correlation at 0 ms (Fig. 2G; see also Fig. S2) as out of quadrant pairs. Thus, similar to premotor excitatory networks, premotor inhibitory networks are also segregated into two distinct microcircuits for the differential control of dorsal and ventral musculature, although the smaller magnitude difference between in quadrant and out of quadrant cross correlograms (cf. Figs. 1G, 2G) suggests, as a conservative estimate, less stringent specificity for inhibitory connectivity (Fig. 2H).

In mammalian cortex, pyramidal neurons that receive shared excitatory inputs are also more likely to be connected to each other (14). Similarly, there is preferential gap junctional coupling between mammalian limb MNs of shared modular function, e.g. ankle flexors (15). We found that electrical coupling between MNs was two-fold higher for in quadrant as compared to out of quadrant pairs (Fig. S3). Thus the segregated microcircuits at the premotor level are supported by increased connectivity among MNs themselves.

To assess whether excitatory segregation derives from spinal sources, and to identify specific premotor populations of relevance, we used an optogenetic approach to selectively

drive firing in the *V2a/Chx10+* population (Fig. 3A; Fig. S4). *V2a/Chx10+* neurons are the largest class of ipsilaterally projecting glutamatergic premotor neurons in the spinal cord and have a conserved function during locomotion in both zebrafish and mice (16, 17). Subsequent recording from MN pairs revealed that the EPSCs elicited by pulses of illumination were highly synchronous between in quadrant pairs (Fig. 3B), but asynchronous between out of quadrant pairs (Fig. 3C). Analysis of group data showed that EPSC-triggered averages were 15-fold larger for in quadrant than out of quadrant MN pairs (Fig. 3D). Thus, *V2a/Chx10+* neurons in the spinal cord are segregated into two subtypes, yielding distinct excitatory control over dorsal and ventral musculature.

Premotor inhibition can derive from both ipsilateral and contralateral spinal sources. Are both sources of spinal inhibition similarly segregated? We took advantage of the fact that inhibition arriving in phase with the locomotor signal derives exclusively from ipsilateral spinal sources, while inhibition arriving out of phase derives from commissural spinal sources (18). Both IPSCs occurring in phase and those occurring out of phase with ipsilateral activity exhibited ~2.5-fold larger IPSC-triggered average responses for in quadrant than for out of quadrant pairs (Fig. 3E, F). We conclude that both ipsilaterally- and contralaterally-derived spinal inhibitory drive is also segregated into two microcircuits.

To assess the utility of these parallel axial microcircuits for ethologically relevant behaviors, we focused on their recruitment during postural correction, which is known to rely on differential dorsal and ventral muscle activity (7, 19). Normally, a side-lying larval zebrafish will right itself at the beginning of a swim bout (Fig. 4A) (20). We quantified the motor activity underlying this behavior using calcium imaging, which allowed visualization of the activity of the dorsal- and ventral-projecting primary MNs on both sides of the fish (Fig. S5A). At the onset of evoked swim bouts, bursts of activity are visible as Ca^{2+} transients (Fig. 4B). In side-lying fish, the amplitude of the Ca^{2+} transient was larger in ventral-projecting than in dorsal-projecting MNs on the ear-up side; but this pattern was inverted on the ear-down side of the fish (Fig. 4C). This asymmetric motor drive thus produces self-righting torque to roll the fish dorsal side up (Fig. 4D) (7), and is likely to reflect the overall motor activation pattern in more modest postural correction as well. In contrast, when fish were embedded dorsal side up in a normal orientation, no significant differences were seen in the pattern of activation (Fig. S5B).

Is this bias in activity due to differential excitation or inhibition? Paired physiological recordings from MNs on the ear-up side of side-lying fish revealed that, during swimming, EPSCs were significantly more frequent in ventral-projecting than dorsal-projecting MNs. In addition, IPSCs occurred more frequently in MNs controlling dorsal than ventral musculature (Fig. 4F). Average EPSC and IPSC amplitude did not differ between the MN types, however (Fig. S6).

To test whether vestibular signals were responsible for these asymmetries in behavioral and physiological activity, we evaluated the *rock solo* mutant fish line, in which the anterior otolith (the sole gravity sensor at this stage (21)) is absent (22). *Rock solo* mutant fish failed to develop self-righting during the first week post fertilization, in contrast with their heterozygous and wild-type siblings (WT/Het: 82% successful righting, 49 trials, 13 fish;

rock solo, 48% righting, 50 trials, 20 fish; $p < 0.0001$, Fisher exact test). MN imaging in side-lying *rock solo* mutant fish revealed no reliable asymmetry of activity (Fig. 4E), consistent with the absence of self-righting behavior. Similarly, physiological recordings from *rock solo* $-/-$ fish, or from fish in which the otoliths were surgically removed immediately prior to the experiment to avoid developmental confounds (23), revealed no consistent bias for synaptic events in the MNs controlling dorsal and ventral musculature (Fig. 4G).

Critically, however, the differences in EPSC and IPSC synchrony for in quadrant versus out of quadrant pairs persisted in otolith-deficient animals (Fig. 4H). This observation confirms that spinal axial microcircuitry exhibits segregated, modular connectivity to different motor pools even in the absence of structured descending vestibular signals. Collectively, our findings challenge the traditional view of axial network organization (12, 13, 24) and shift the locus of distinct dorsal/ventral control in fish from supraspinal regions to modular, intrinsic spinal circuits.

The evolution of vertebrates from fishes to mammals involved fundamental changes in body plan, muscle distribution and posture (25). These changes not only involved reorganization of the trunk musculature, but also an increasing reliance on the limbs to locomote (26). Commensurate with these changes, locomotor networks in the spinal cord must have adapted. Studies of the molecular mechanisms responsible for generating spinal locomotor circuitry have identified conserved programs regulating the differentiation of axial and limb spinal microcircuitry (27, 28).

While limb microcircuits must have originated from more primitive axial ones, it is unclear how the modular organization of limb networks could have arisen from a single, homogeneous axial network (5). Our work now demonstrates that axial networks, like limb networks, are organized in a modular fashion to facilitate differential control of muscles during locomotor-related behaviors such as postural correction (29). Thus, the modular organization of axial locomotor control systems may have served as a functional template for the evolution of limb control systems (Fig. 4I). We suggest that refinement and reweighting of inhibition between these modules is a plausible candidate mechanism for generating new locomotor patterns from ancient axial circuits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Distinct excitatory microcircuits govern dorsal and ventral musculature

A) Top, recording set-up with schematic of example primary motor neuron (MN) axon arbores in the musculature. Bottom, dual whole-cell recording of EPSCs during swimming from MNs projecting to mid-dorsal and ventral territory. Traces vertically offset for clarity. Simultaneous ventral root recording (grey) shows that excitatory barrages occur in phase with the ipsilateral fictive bend.

B) Expanded time scale, EPSCs recorded during swimming from example in-quadrant (mid-ventral and ventral) MNs (Fig. 1H). Asterisks indicate synchronous EPSCs in both neurons ($t < 150 \mu s$). Scale as in **(D)**.

C) 100 consecutive EPSCs recorded in the mid-ventral MN (grey, top; average in light brown) and simultaneously recorded signal in the ventral MN (grey, bottom) reveals a significant EPSC-triggered average (dark brown).

D – E) As for B – C, for an example out of quadrant (mid-ventral and dorsal) MN pair, revealing few synchronous EPSCs and small EPSC-triggered average (dark blue).

F) Summary EPSC-triggered averages, means $\pm$ SEM, for in quadrant ($n = 15$) and out of quadrant ($n = 19$) MN pairs. ***, $p < 0.001$, unpaired t-test of peak EPSC amplitude. Gray bar indicates period of baseline normalization.

G) Cross-correlogram of EPSC timing, averages from all MN pairs. Means $\pm$ SEM; bin = 50 μs , N as in **(F)**. ***, $p < 0.001$, unpaired t-test at 0 ms.

H) Schematic of proposed premotor excitatory circuitry. An individual excitatory premotor neuron (open circles) preferentially synapses either on dorsal- or ventral-projecting MNs (solid lines), but not both.

Fig. 2. Distinct inhibitory microcircuits govern dorsal and ventral musculature

All panels as in Fig. 1, for IPSCs.

A) Example dual recording of IPSCs from MNs projecting to mid-dorsal and ventral territory.

B) Expanded time scale, IPSCs recorded during swimming from example in-quadrant (mid-dorsal and dorsal) MN pair. Scale as in (D).

C) 100 consecutive IPSCs recorded in mid-dorsal MN (top) and simultaneously recorded signal in the ventral MN (bottom) reveals a significant IPSC-triggered average (dark blue). Scale as in **(E)**.

D – E) As for **B – C**, for an example out of quadrant (mid-ventral and dorsal) MN pair, revealing few synchronous IPSCs and small IPSC-triggered average (dark blue).

F) Summary IPSC-triggered averages, means $\pm$ SEM, for in quadrant ($n = 13$) and out of quadrant ($n = 17$) MN pairs. ***, $p < 0.001$, unpaired t-test of peak IPSC amplitude.

G) Cross-correlogram of IPSC timing, averages from all MN pairs. Means $\pm$ SEM; bin = 50 μ s, N as in **(F)**. **, $p < 0.01$, unpaired t-test at 0 ms.

H) Schematic of proposed premotor inhibitory circuitry. An individual inhibitory premotor MN preferentially synapses either on D or V MNs (solid lines), but rarely on both (dotted lines).

Fig. 3. Intraspinal premotor inputs are segregated into dorsal and ventral microcircuits

A) Sagittal view of 4 dpf larva expressing *alx:Gal4::UAS:CatCh* in two premotor neurons with typical V2a morphology (ipsilateral axon, primarily descending). Rostral is to the left; white bars mark muscle segments.

B) Example of synchronous EPSCs evoked optogenetically in a dorsal/mid-dorsal MN pair. Green bar indicates light exposure.

C) Asynchronous EPSCs evoked in a dorsal/mid-ventral MN pair in another fish.

D) Summary of EPSC-triggered averages for in quadrant and out of quadrant MN pairs (n = 5 pairs each). *, $p < 0.05$, unpaired t-test of peak amplitudes. Varying baseline period is due to frequent barrages of light-elicited EPSCs (as in B).

E) IPSC-triggered average responses in phase with ipsilateral motor activity, as measured by ventral root recordings (schematic, bottom), from in quadrant (n = 7) and out of quadrant (n = 14) pairs. ***, $p < 0.001$, unpaired t-test of peak amplitudes.

F) As in (E), but for recordings during mid-cycle, out of phase with ipsilateral motor activity.

Fig. 4. Descending vestibular information differentially recruits dorsal and ventral microcircuits

A) Time-lapse images of a 3 dpf larva engaging in self-righting from right ear up to dorsal-up orientation. Pictures were taken under normal illumination for display purposes only; behavioral data were gathered with infrared illumination. Scale, 1 mm.

B) Example Ca^{2+} imaging data from primary MNs in one segment of a side-lying fish. Arrow indicates time of brief electrical stimulus applied to tail to induce swimming. Scale bars: 20% $\Delta\text{F}/\text{F}$, 5 s.

C) Ventral-projecting MNs exhibit larger Ca^{2+} signals than dorsal-projecting MNs on ear-up side (left; $n = 13$ MN pairs from 9 fish); pattern is reversed on ear-down flank (right; 7 pairs from 7 fish). *, $p < 0.05$, paired t-test.

D) Schematic of asymmetric drive to the four quadrants of musculature, producing torque for self-righting.

E) Ca^{2+} imaging summary as in (D), for rock solo mutant animals (ear-up: $n = 7$ pairs from 7 fish; ear-down: $n = 8$ pairs from 8 fish). n.s., not significant, paired t-test.

F) Physiological recordings from MN out of quadrant pairs on the ear-up side of WT animals during fictive swimming reveals that EPSCs are more frequent in ventral-projecting MNs than dorsal ($p < 0.001$, paired t-test, $n = 19$ pairs), whereas IPSCs are more frequent in dorsal-projecting MNs than ventral ($p < 0.001$, 17 pairs). Each dot represents either EPSC or IPSC data from a single out of quadrant pair. Unity line is shown in dashed grey.

G) As in (F), for vestibular-deficient animals. No differences are seen in the number of EPSCs ($p = 0.11$, $n = 10$ pairs) or IPSCs ($p = 0.50$, $n = 9$ pairs) between dorsal- and ventral-projecting MNs.

H) Higher in quadrant than out of quadrant synchrony persists in rock solo mutants and otolith ablated animals for both EPSCs and IPSCs. **, $p < 0.01$, unpaired t-test of peak amplitude (EPSCs: $n = 6$ in quadrant, 10 out of quadrant pairs; IPSCs: $n = 6$ in quadrant, 9 out of quadrant pairs). Scale bars: 4 pA, 0.5 ms; 10 pA, 1.5 ms.

I) Schematic summary of results: prior models of a single premotor circuit for dorsal and ventral control in fish (left); current model supporting parallel, modular microcircuits (middle); and proposed homology to limb control in tetrapods (right).