Developmental switch in the function of inhibitory commissural V0d interneurons in zebrafish

Graphical abstract

Highlights
- V0d interneurons in larval zebrafish participate in high-speed locomotion
- V0d interneurons undergo a functional switch during development
- In adult zebrafish, V0d interneurons participate in slow-speed swimming
- Ablation of V0d diminishes mid-cycle inhibition during swimming in adult zebrafish

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In brief
Picton, Björnfors et al. show that V0d interneurons undergo a functional switch within the locomotor circuit during development. In zebrafish larvae, V0d interneurons participate in high-speed locomotion. During development, the V0d interneurons diversify and switch their primary function to provide mid-cycle inhibition at slow swimming speeds.
Developmental switch in the function of inhibitory commissural V0d interneurons in zebrafish

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SUMMARY

During development, all animals undergo major adaptations to accommodate behavioral flexibility and diversity. How these adaptations are reflected in the changes in the motor circuits controlling our behaviors remains poorly understood. Here, we show, using a combination of techniques applied at larval and adult zebrafish stages, that the pattern-generating V0d inhibitory interneurons within the locomotor circuit undergo a developmental switch in their role. In larvae, we show that V0d interneurons have a primary function in high-speed motor behavior yet are redundant for explorative swimming. By contrast, adult V0d interneurons have diversified into speed-dependent subclasses, with an overrepresentation of those active at the slowest speeds. The ablation of V0d interneurons in adults disrupts slow explorative swimming, which is associated with a loss of mid-cycle inhibition onto target motoneurons. Thus, we reveal a developmental switch in V0d interneuron function from a role in high-speed motor behavior to a function in timing and thus coordinating slow explorative locomotion. Our study suggests that early motor circuit composition is not predictive of the adult system but instead undergoes major functional transformations during development.

INTRODUCTION

The coordination of movement is key to the smooth execution of motor actions and requires the control of muscle activity around different joints and across the body axis.¹⁴ Coordinated movements are already expressed at birth.⁵⁻⁸ However, studies across animals have shown that these early motor circuits undergo major changes during development and body growth to accommodate behavioral flexibility.⁸⁻¹² The extent of these functional transformations of early motor circuits, and how these changes are implemented at the level of specific interneuron subtypes, has remained a challenge to decipher.

Locomotion underpins a large component of the behaviors displayed by animals.²⁻¹³⁻¹⁷ The core vertebrate locomotor network (central pattern generator, CPG) within the spinal cord is composed of motoneurons (MNs) and several major interneuron classes, whose development and properties are dictated by distinct molecular pathways.¹⁸,¹⁹ Once the locomotor circuit is established, each of the cardinal interneuron classes is considered to perform specific roles in rhythm generation and pattern formation across vertebrate species.¹³⁻¹⁵,¹⁷⁻¹⁹

A key CPG class are the commissural V0 interneurons, which have a conserved role in the control of the coordination of the left-right motor pattern during locomotion in vertebrates.²²⁻²⁴ The V0 population expresses the transcription factor dbx1 and is composed of two major subclasses: the excitatory V0v and the inhibitory V0d interneurons,⁵⁻⁷ in addition to a smaller subclass of cholinergic V0c interneurons.²⁸ The genetic deletion of dbx1 (all V0 interneurons) leads to an intermittent loss of left-right alternation during fictive locomotion in mice.²⁵ How when selective ablation of the V0 subclasses (V0d or V0v) has shown that they preferentially contribute to locomotor coordination in mice in a speed-dependent manner, with V0d interneurons controlling left-right limb alternation at slow walking speeds, whereas V0v interneurons perform this role at higher speeds during trotting.²⁴,²⁶ In contrast to mice, V0d interneurons in larval zebrafish are active reliably only at the highest swimming speeds,²³ whereas MCoDs, a specific V0v interneuron type, are only active during slow swimming.³⁰⁻³¹ In adult zebrafish, V0v interneurons are largely recruited at higher speeds, as proposed in mice.³² The apparently inverted pattern of recruitment of respective V0 interneuron classes may represent a species difference related to the distinct locomotor strategies of mammals and fish. Alternatively, this difference could instead be associated with functional changes in the properties of V0 interneuron populations during development from larvae to adult. Whether a diversification and functional switch occurs in the V0d class has not yet been addressed.

In this study, we assess the morphological properties and activity profiles of V0d interneurons from early larval stages through development to adult stages. We show that, early in development, V0d interneurons participate and contribute to locomotion only at the highest speeds and their ablation has no apparent effects on slow, spontaneous, explorative swimming. Later in development, the ablation of V0d interneurons has a range of effects on locomotor behavior. Consistent with
this finding, we show a diversification within the V0d population at adult stages, including morphological heterogeneity and the formation of modular subclasses of V0d interneurons involving differences in intrinsic properties and speed-dependent recruitment at slow, intermediate, or fast speed. At the population level, however, the majority of recorded V0d interneurons were recruited at slower speeds of locomotion, and we show that the inhibitory input from these V0d interneurons to contralateral MN pools is essential for mid-cycle inhibition during locomotion. Together, this study reveals a functional switch during development in which V0d interneurons transition from a role in the fastest locomotor speeds to a core function across the locomotor speed range. Thus, our results highlight that the functional composition of motor circuits early in development is not predictive of the adult locomotor network, but rather major transformations are associated with the maturation of motor behavior.

RESULTS

A developmental shift in the distribution and function of V0d interneurons

The distribution and position of V0d interneurons were examined at three developmental stages (5 days post-fertilization [dpf], 4 weeks post-fertilization [wpf], and 12 wpf) in a zebrafish line in which GFP expression was driven by $dbx1b$ in glycinergic neurons ($Tg[glyt2:loxP-RFP-loxP-GFP] 	imes Tg[dbx1b:Cre]$). At 5 dpf, the V0d interneurons were restricted to a dorsomedial position in the spinal cord. During development, there was a gradual ventral and lateral shift in the position of these interneurons before settling along a medio to ventrolateral band in the gray matter at 12 wpf (Figure 1A). There was no overall significant difference in the number of V0d interneurons in larval and adult zebrafish ($39.17 ± 2.4$ V0d per segment in larvae).
versus $33.8 \pm 1.4 \text{ V0d per segment in adult, } p > 0.05$). To assess whether the V0d interneurons in larvae and adult represent the same neuronal population, we performed a series of BrdU incubation experiments (Figure S1A). In zebrafish treated with BrdU at 3–29 h post-fertilization (hpf), all GFP-labeled V0d interneurons at 5 weeks old were double labeled with BrdU ($n = 279$ V0d interneurons in 5 fish). On the other hand, no V0d interneurons were double labeled with BrdU when treated at 5–7 dpf ($n = 4$ fish) or 12–14 dpf ($n = 5$ fish) (Figure S1A). These results indicate that V0d interneurons are born during embryonic development and that no new V0d interneurons are added at later development stages.

At 5 dpf, V0d interneurons also displayed relatively homogeneous morphological features, as previously described. Their dorsal somata were largely devoid of dendrites and had a primary axon that projected ventrally before crossing to the opposite side of the spinal cord where it bifurcated into ascending and descending axonal branches (Figure 1B). There were collaterals originating from the main axon both ipsilaterally and contralaterally (Figure 1B). In zebrafish larvae (4–6 dpf), these V0d interneurons were largely unrecruited during fictive swimming and displayed only subthreshold oscillations at lower frequencies (<3 Hz; Figures 1C and 1E; $n = 12$ cells from 12 fish) but became active at the highest fictive swimming frequencies (>40 Hz; Figures 1D and 1E), consistent with a previous study. Collectively, these data suggest that at larval stages the V0d interneuron class is a relatively morphologically and functionally homogeneous population that may only contribute to locomotion at the highest speeds.

The contribution of V0d interneurons to larval zebrafish swimming activity in vivo was assessed using behavioral analysis combined with two-photon ablation. The V0d interneurons were selectively targeted for two-photon ablation across approximately 20 full spinal segments (Figures 2A and S1B). The ablation of V0d interneurons did not affect slow explorative swimming (Figure 2B), consistent with their lack of recruitment at lower frequencies. None of the analyzed parameters of slow explorative swimming were affected, including the number of spontaneous swim episodes (Figure 2C), overall swim distance (Figure 2D), and swim velocity (Figure 2E).

We next assessed the effect of ablation of V0d interneurons on sound-induced escape and the subsequent sequence of high-speed swimming in larval zebrafish (Figure 2F). Both control and ablated fish performed C-start escape behavior followed by fast swimming (Figure 2F). There was no difference in the overall swim distance (Figure 2G); however, there was a small but significant overall decrease in the velocity of the high-speed swimming sequence (Figure 2H). These results show that early in zebrafish development, V0d interneurons do not contribute to slow explorative swimming and suggest a primary involvement in evoked, high-speed motor behavior, such as escape.

V0d interneurons have been proposed to play a key role in controlling left-right alternation during locomotion in vertebrates, particularly at slower speeds. However, our results do not support such a role for these interneurons in larval zebrafish, given the negligible effect of their ablation on swimming behavior at these early stages, suggesting the involvement of other inhibitory commissural interneuron classes for slow swimming in zebrafish larvae (see discussion). To test whether V0d interneurons continue to play a role only in high-speed motor behavior in zebrafish throughout development, we carried out a similar behavioral analysis combined with two-photon ablation at juvenile/adult stages (4–7 wpf; Figure 3A). In contrast to the larvae, ablation of V0d interneurons had clear effects on slow explorative swimming (Figure 3B). There was a significant reduction in the overall number of spontaneous swim episodes (Figure 3C) that was associated with a decrease in swim distance (Figure 3D) and a reduction in the velocity (Figure 3E). The ablation of V0d interneurons had no effect on the sound-induced C-start escape (Figure 3F) but resulted in a significant decrease in the distance of the subsequent swim sequence (Figure 3G), without affecting the overall mean velocity of the escape-swim sequence (Figure 3H). Together, these data show that in juvenile/adult zebrafish, V0d interneurons participate in a range of locomotor speeds, including slow explorative swimming, and they suggest a functional switch in the role of V0d interneurons between larval and juvenile/adult stages.

### The pattern of activity of V0d interneurons in adult stages

The swimming CPG in adult zebrafish is composed of three speed modules (slow, intermediate, and fast) recruited sequentially with increased swim frequency. To examine the activity pattern of the V0d population in juvenile/adult zebrafish, V0d interneurons were targeted for whole-cell patch-clamp recordings using an ex vivo brainstem–spinal-cord preparation, and their activity was analyzed during fictive swimming. In contrast to the larvae, subsets of V0d interneurons were recruited across the full range of swimming speeds, including recruitment of slow V0d interneurons at the absolute slowest swim frequencies (Figure 4). Consistent with a modular organization, we identified three types of V0d interneuron matching the recruitment speed of the slow, intermediate, and fast circuit modules, respectively (Figure 4). The slow V0d interneuron type was reliably recruited at the onset of swimming (<3 Hz), fired single or bursts of action potentials within each cycle, and maintained its activity across the full-speed range (Figure 4A; $n = 52$ cells). These slow V0d interneurons displayed swim-related oscillations with a relatively constant high amplitude across the whole speed range (Figures 4B and 4C). The intermediate type was not active at the slowest speeds (1–3 Hz), where they showed only low amplitude, subthreshold oscillations, but became recruited once the swimming frequency reached the intermediate range (4–6 Hz) at which they displayed the highest amplitude of oscillations (Figures 4D–4F; $n = 37$ cells). The fast type was not recruited at slow and intermediate speeds and displayed only very small subthreshold oscillations (Figures 4G–4I; $n = 27$ cells). This type only occasionally became active at the very fastest swim speeds (>8 Hz; Figure S2). The somata of the three types of V0d interneurons showed an overlapping distribution with no topographic preference in the spinal cord (Figure 5A; $n = 116$ cells). However, among the whole set of recordings, the slow subtype was most represented (45%), followed by the intermediate (32%), with only relatively few fast V0d interneurons (23%) (Figure 5B). These results show that in adult zebrafish, V0d interneurons are active across the full...
Figure 2. Ablation of V0d interneurons in young larval zebrafish affects escape behavior but not spontaneous swimming

(A) Confocal images of the spinal cord of a larval zebrafish (5 dpf) showing V0d interneurons before (“pre-ablation”) and after two-photon laser ablation (“post-ablation”). The panel on the right shows the expansion of the area indicated by a dashed line. V0d interneurons are shown in green and non-V0d glycineric interneurons in orange.

(B) Tracking of spontaneous explorative swimming in vivo in a control larval zebrafish and following the ablation of V0d interneurons.

(C) Ablation of V0d interneurons had no effect on the number of spontaneous swim episodes (n = 8 control animals; n = 8 ablated animals; two-tailed Student’s t test).

(D) Ablation of V0d interneurons had no effect on the overall spontaneous swim distance (n = 8 control animals; n = 8 ablated animals; Mann-Whitney test).

(E) Ablation of V0d interneurons had no effect on swim velocity (n = 8 control animals; n = 8 ablated animals; two-tailed Student’s t test).

(F) A sequence of escape and fast swimming evoked by a sound stimulus in control and in a V0d-ablated larval zebrafish.

(G) Ablation of V0d interneurons had no effect on escape distance (n = 8 control animals; n = 7 ablated animals; two-tailed Student’s t test).

(H) Ablation of V0d interneurons significantly reduced the overall velocity of the evoked escape sequence (n = 8 control animals; n = 7 ablated animals; *p < 0.05; two-tailed Student’s t test).

See also Figure S1.
spectrum of speeds, but with the majority active at speeds involved in slow, explorative swimming. This highlights a developmental shift in the pattern of activity within the V0d class of commissural inhibitory interneurons.

The intrinsic properties and morphology of adult V0d interneurons
The three types of V0d interneurons displayed differences in their firing patterns and excitability. Slow and intermediate
V0d interneurons showed a bursting or tonic firing pattern in response to depolarization steps, with the highest proportion of bursting neurons found among slow V0d interneurons (Figures 5G and 5H). Fast V0d interneurons instead showed either a tonic or adapting firing pattern (Figure 5I). In addition, there was scaling of the excitability to match the order of recruitment of the three V0d interneuron types. The slow type had the lowest spike threshold, the most depolarized resting membrane potential, and the highest input resistance (Figures 5C–5F). The fast type, on the other hand, had the highest spike threshold, the most hyperpolarized resting membrane potential, and the lowest input resistance (Figures 5C–5F).

In contrast to the larvae in which V0d interneurons lack dendritic arborizations and show bidirectional axonal projections, the three V0d interneuron types in adult zebrafish displayed variable morphologies in terms of the extent of their dendritic arborizations and directionality of axonal projections (Figures 5J–5M). Each of the V0d types showed either a bidirectional or unidirectional descending axonal projection, with collaterals reaching distinct regions of the gray matter (Figures 5J–5M). None of the V0d interneurons analyzed showed purely ascending axon projections, which is only observed in this transgenic line in the recently described intraspinal lateral proprioceptors.38

These results show that the three V0d interneuron types involved in adult swimming had distinct combinations of intrinsic properties and firing patterns related to their order of recruitment as well as extensive dendrites and variable axon projection direction and distance.
Figure 5. Intrinsic properties and morphologies of V0d interneuron subtypes in juvenile zebrafish
(A) Plot of the soma position of recorded V0d interneurons showing no topographical organization of V0d interneuron subtypes.
(B) Proportion of each V0d interneuron subtype identified among recorded neurons showing that the majority were slow or intermediate.

(legend continued on next page)
A dorsally located non-swimming-related V0d interneuron type
In addition to the three major V0d classes involved in swimming, we also identified a distinct, infrequent type of V0d interneuron. The somata of these interneurons were located dorsally in close association with primary MNs (pMNs) (Figures 6A and 6B). This subtype had a large descending axon that projected over many segments on the contralateral side to the soma, often with a thin ascending collateral that extended between 0.5 and 2 segments (Figures 6B and 6C). The descending axon drifted dorsally with many collaterals directed toward pMNs and dorsal V0d counterparts on the opposite side of the spinal cord (Figure 6C). These interneurons displayed a strongly adapting firing pattern (Figure 6D) similar to that of pMNs as well as a high spike threshold and low input resistance (Figures 6D and 6E; n = 10 cells). Furthermore, this V0d interneuron type was not active during fictive swimming and displayed very weak membrane potential oscillations (Figure 6F) akin to those seen in pMNs.

These findings suggest that these V0d interneurons represent a specialized subtype that is primarily connected with the fast pMNs, do not seem to be involved directly in swimming, and may contribute to other fast behaviors, such as escape or steering.

V0d interneuron ablation disrupts mid-cycle inhibition and rhythmic firing in contralateral motoneurons
The ablation of V0d interneurons in adult zebrafish affected slow explorative swimming, and at these stages, they are reliably recruited across locomotor speeds. We next examined how the ablation of V0d interneurons may affect mid-cycle inhibition in contralateral MNs and the swimming pattern (Figure 7). Ablation was restricted to a subset of V0d interneurons on one side of the spinal cord (20–40 V0d interneurons ablated across approximately 5 segments), whereas mid-cycle inhibition and the swimming pattern were monitored in back-labeled slow MNs on the opposite side during electrically evoked fictive swimming (Figures 7A and 7B). In control fish, MNs displayed alternating on-cycle depolarization and mid-cycle inhibition (Figure 7C). In control fish, the trough value of this inhibition was relatively stable throughout the whole swimming episode, consistently reaching a value close to the resting membrane potential that effectively silenced MN firing during each mid-cycle period (Figures 7C and 7E). By contrast, the ablation of V0d interneurons on one side resulted in a pronounced disruption of mid-cycle inhibition in contralateral MNs (Figures 7D and 7E). The absolute trough value of mid-cycle inhibition was significantly more depolarized, an effect that became progressively more pronounced as the swimming frequency declined during the swimming episode. As a result, toward the end of the swimming episode, MNs switched to a more continuous arrhythmic firing pattern driven by a tonic background depolarization in the absence of mid-cycle inhibition (Figures 7D–7F). The analysis of pooled data shows that the trough value of MN mid-cycle inhibition was significantly more depolarized following V0d ablation compared with the control (Figure 7F).

These results demonstrate that in adult zebrafish, V0d interneurons provide important rhythmic inhibitory input to contralateral MNs across locomotor speeds, especially during slow swimming, which ensures the proper timing of MN activity during locomotion.

DISCUSSION
Early studies in lamprey and Xenopus tadpoles provided the first blueprint for the spinal network controlling locomotion in vertebrates. The coordination of left-right alternation along the body axis has been shown to be mediated by commissural interneurons, which was revealed using pharmacological, surgical, and genetic perturbations. Furthermore, studies in invertebrates and vertebrates have highlighted the intrinsic plasticity of rhythmic motor networks, in terms of both neuromodulation and developmental remodeling. Understanding how developmental network changes are reflected in the functional properties of defined interneuron classes in the vertebrate spinal cord remains an ongoing challenge. The development of molecular tools to label interneuron subgroups, especially in mice and zebrafish, has now allowed for a detailed characterization of each of the genetically defined neural classes within the locomotor CPG, including those controlling locomotor coordination. For example, through a selective genetic ablation of V0 subclasses in mice, it was shown that V0d interneurons mediate left-right limb alternation at slow speeds (walk), whereas V0v interneurons perform this role at high speeds (trot). However, accumulating evidence points toward a large degree of diversity within each major CPG neuron class. Furthermore, functional and morphological changes in CPG neuron populations are known to occur during
Figure 6. A specialized V0d interneuron likely involved in escape

(A) Soma position of dorsal specialized V0d interneurons in the spinal cord. Dorsal V0d interneurons were located dorso-medial close to the position of primary motoneurons.

(B) Top: example morphological reconstruction of a dorsal V0d interneuron (side view). Bottom: the axon projection direction and distances of dorsal V0d interneurons.

(C) Top: morphological reconstruction of a dorsal V0d interneuron (top down) showing the close proximity of the soma and contralateral axon with primary motoneurons (pMNs: indicated by gray-filled circles on the ipsilateral side and empty circles on the contralateral side). The dashed line indicates the spinal cord midline. Bottom: a side-view reconstruction of a dorsal V0d interneuron. Images below show the soma and the contralateral axon close to primary motoneurons in the regions indicated by red boxes. The white arrow indicates another dorsal V0d interneuron close to the axon of the contralateral recorded dorsal V0d interneuron.

(D) Firing pattern of a dorsal V0d interneuron that is strongly adapting.

(E) Dorsal V0d interneurons have a depolarized spike threshold and low input resistance relative to other V0d interneuron subtypes (n = 10 dorsal V0d interneurons).

(F) Example recording of dorsal V0d interneuron during electrically evoked fictive swimming, which shows only small, subthreshold rhythmic oscillations of the membrane potential.
Figure 7. Ablation of V0d interneurons in juvenile zebrafish disrupts mid-cycle inhibition in motoneurons during slow fictive swimming
(A) Experimental setup. Top: V0d interneurons were ablated along several segments on one side of the spinal cord. Bottom: recordings were then made from motoneurons on the opposite side of the spinal cord during swimming evoked by electrical stimulation.
(B) Image of the spinal cord before (“pre-ablation”) and after (“post-ablation”) two-photon ablation of 20–40 V0d interneurons across approximately 5 spinal cord segments.
(C) Recording of a control slow motoneuron during evoked fictive swimming in which mid-cycle inhibition silences motoneuron firing at each swim cycle.
(D) Recording of a slow motoneuron during fictive swimming following the ablation of V0d interneurons. The mid-cycle inhibition becomes weaker and motoneuron firing becomes tonic as the swim episode progresses (red dashes indicate the peak of mid-cycle inhibition).
(E) Pooled data showing the voltage difference between the trough value of mid-cycle inhibition and the resting membrane potential (mean and SEM; n = 9 control motoneurons from 7 separate animals; n = 8 motoneurons from 7 separate V0d-ablated animals).
(F) Following the ablation of V0d interneurons, the value of mid-cycle inhibition in motoneurons became significantly more depolarized compared with control motoneurons (n = 9 control motoneurons from 7 separate animals; n = 8 motoneurons from 7 separate V0d-ablated animals; **p < 0.01; ***p < 0.001; unpaired two-tailed Student’s t test).
development as locomotor circuits mature. Ultimately, addressing the question of diversity within interneuron types and across development requires a characterization of CPG neuron classes at the single-cell level as well as at different stages of spinal cord circuit maturation.

In this study, we have used the zebrafish model system to assess the functional properties of the V0d interneuron population at different stages of development and identify age-dependent roles for V0d interneurons. We show that larval V0d interneurons form a relatively homogeneous, dorsally located population in the spinal cord that fires reliably only during the highest locomotor speeds, in line with a recent study. Furthermore, we show that V0d interneuron ablation had no clear effects on slow explorative swimming but decreased the velocity of fast escape behavior. As development progresses, the morphologies, soma position, and recruitment patterns diversify. V0d interneurons at later stages form distinct subtypes, consistent with the speed-module organization of MNs and the excitatory V2a and V0v interneurons in adult zebrafish. The majority of V0d interneurons were, however, reliably recruited at the slow and intermediate speeds of locomotion, and the ablation of V0d interneurons at juvenile/adult stages disrupted spontaneous swimming in vivo. This was associated with a loss of mid-cycle inhibition in contralateral slow MNs that disrupts their rhythmic firing during swimming. Given that the population of small, slow MNs lacks synaptic input in early larval zebrafish stages, it is possible that this integration of V0d interneurons into the circuit for slow swimming involves a synaptic wiring between V0d interneurons and late-born slow MNs during development.

V0d interneurons have been proposed to act by providing rhythmic, monosynaptic inhibition to MNs and interneurons on the contralateral side of the spinal cord during locomotion. However, no previous studies have directly assessed how a loss of inhibitory inputs from V0d interneurons influences the rhythmic activity of contralateral interneurons during ongoing locomotion. Our results with respect to the adult zebrafish now show that mid-cycle inhibition in MNs is diminished by the ablation of contralateral V0d interneurons, which in turn affects the firing pattern of MNs during fictive swimming. Interestingly, this effect was least pronounced at the start of swim episodes, when swim frequency is highest, suggesting the contribution of additional sources of crossed inhibition at higher speeds. One source of this inhibition could be via a polysynaptic pathway mediated by excitatory commissural V0v interneurons promoting the activity of local, ipsilateral inhibitory interneurons. Indeed, V0v interneurons in the adult zebrafish locomotor network have been shown to be primarily of the fast type.

An alternative source of rhythmic inhibition could be from dmrt3-expressing interneurons, a commissural inhibitory class of dI6 interneurons, which have also been shown to contribute to quardrupedal gait control in mammals and to swimming in larval zebrafish. In larval zebrafish, two distinct subtypes of dmrt3+ interneurons have been described and shown to be responsible for coordinating slow locomotion at these stages. The genetic ablation of dmrt3 interneurons in larval zebrafish leads to a loss of locomotor coordination during fictive swimming at all speeds except for the highest frequencies. In a separate study, the mutation of dmrt3 in larval zebrafish (4–6 dpf) similarly led to deficits in slow spontaneous swimming, acceleration, and tail coordination in vivo. However, these effects faded during development, and juvenile fish (6 wpf) showed only a subtle decrease in their maximum swim speed, further supporting a primary role of V0d interneurons over dmrt3 interneurons for slower swimming in juveniles and adults. The picture that emerges suggests that dmrt3 interneurons are the major inhibitory commissural neuron class involved in the coordination of slow swimming in larval zebrafish. As the swim circuit matures, the role of V0d interneurons in slow swimming becomes more prominent, whereas the contribution of dmrt3 interneurons may shift toward a more selective role at higher speeds.

During real swimming in adult zebrafish, however, the mid-cycle inhibition provided by V0d interneurons will also be complemented by sensory-mediated inhibition from intraspinal proprioceptor neurons. These proprioceptive neurons are only present in adults and provide exclusively ascending commissural inhibitory feedback to excitatory V2a interneurons based on the curvature of the spinal cord during actual movements. Given the primarily descending axon trajectories of V0d interneurons (this study), future studies should aim to reconcile the roles of sensory-based ascending and network-generated descending inhibition in the context of freely moving adult zebrafish—a complex task that will likely require computational modeling of the swim network.

Overall, this study shows, through a detailed developmental and functional analysis at the single-cell level, how a key subclass of commissural interneurons shift their profiles during locomotor circuit maturation in a vertebrate. Our results demonstrate a developmental differentiation of V0d interneurons from a dorsal population primarily involved in strong, high-speed movements to a ventrolateral population composed of speed-dependent modular subtypes that coordinate locomotion at slower speeds. Thus, our study provides detailed insights into how motor circuits can transform during the developmental process to allow for the expansion and adjustment of behavior during the maturation of an animal.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

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AUTHOR CONTRIBUTIONS

L.D.P., E.R.B., and A.E.M. conceived the project and designed the experiments. E.R.B. and L.D.P. performed most of the experiments and analyses. P.F. performed the analysis of the behavioral experiments. I.P. performed the ex vivo ablation and recordings. M.B. performed the anatomical experiments and analysis. E.R.B., L.D.P., and A.E.M. prepared the figures and wrote the manuscript. All the authors contributed to the preparation of the figures and manuscript writing. L.D.P. and E.R.B. contributed equally, and each can list themselves as the first author.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Chicken polyclonal anti-GFP | Abcam | Cat# ab13970; RRID: AB_300798 |
| Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 488 conjugate | Invitrogen | Cat# A11039; RRID: AB 142924 |
| Streptavidin, Alexa Fluor 647 conjugate | Thermo Fisher Scientific | Cat# S32357 |
| Streptavidin, Alexa Fluor 488 conjugate | Thermo Fisher Scientific | Cat# S11223 |
| Streptavidin, Alexa Fluor 555 conjugate | Thermo Fisher Scientific | Cat# S32355 |
| Mouse anti-BrdU | Becton Dickinson | Cat# 347580; RRID: AB 10015219 |
| Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 | Thermo Fisher Scientific | Catalog # A10037; RRID: AB_2534013 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Ethyl 3-aminobenzoate methanesulfonate (MS-222) | Sigma Aldrich | Cat# E10521 |
| PBS – Phosphate-Buffered Saline (10X) pH 7.4 | Invitrogen | Cat# AM9625 |
| Neurobiotin | Vector Laboratories | Cat# SP-1120 |
| Alexa Fluor 647-dextran | Thermo Fisher | Cat# D22914 |
| Vectashield Hard Set Antifade Mounting Medium | Vector Laboratories | Cat# H1400 |
| Triton X-100 | Sigma Aldrich | Cat# T9284 |
| Bovine Serum Albumine | Sigma Aldrich | Cat# A2153 |
| 5-bromo-2’-deoxyuridine (BrdU) | Sigma-Aldrich | Cat# B5002 |
| Experimental models: Organisms/strains |        |            |
| Danio rerio: Tg[dbx1b:Cre] | Satou et al.33 | Higashijima lab |
| Danio rerio: Tg[glyt2:LOXP-DsRed-LOXP-GFP] | Satou et al.23 | Higashijima lab |
| Software and algorithms |        |            |
| IMARIS | Oxford Instruments | RRID: SCR_007370; https://imaris.oxinst.com |
| R Core Team (2019). R: A language and environment for statistical computing. | R Foundation for Statistical Computing, Vienna, Austria. | RRID: SCR_001905; https://www.R-project.org |
| pClamp | Molecular Devices | RRID: SCR_011323; https://moleculardevices.com |
| Spike2 | Cambridge Electronic Design | RRID: SCR_000903; https://ced.co.uk |
| MATLAB | MATLAB and Statistics Toolbox Release 2012b, The MathWorks, Natick, Massachusetts, United States. | RRID: SCR_001622; https://www.mathworks.com |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests should be directed to and will be fulfilled by the Lead Contact, Abdel El Manira (abdel.elmanira@ki.se).

Materials Availability
This study did not generate new unique reagents.

Data and code availability
- All data is available in the main text or the supplementary materials and are available upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experiments were conducted using zebrafish (Danio rerio). Zebrafish were raised and maintained in a core facility at the Karolinska Institute according to established procedures. All experimental protocols were approved by the local Animal Research Ethical Committee, Stockholm and were performed in accordance with EU guidelines.

METHOD DETAILS

The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

Electrophysiology
Larval zebrafish (4-6 dpf) or adult zebrafish (8-10 weeks old) of either sex were used in all electrophysiological experiments. For larval patch-clamp recordings, fish were first anaesthetized in 0.01% MS-222 (buffered to pH 7.2) and then two pins were placed through the notochord to pin the fish in a Sylgard-coated dish. The skin overlaying the muscles was removed and 2-4 muscle segments overlying the spinal cord were etched away to allow access for patch-clamp recordings. Zebrafish were then transferred to extracellular solution containing 6.25 μM α-bungarotoxin for 10-20 minutes. For adult recordings, zebrafish were first anaesthetized using 0.015% MS-222 (pH 7.2) dissolved in frozen extracellular solution and then the brainstem, spinal cord and vertebral column were dissected out of the body. For larvae and adult zebrafish, the dissected preparation was then transferred to a recording chamber maintained at room temperature (20-22 °C) that was continuously perfused with extracellular solution containing (in mM): 134 NaCl, 2.9 KCl, 2.1 CaCl2, 1.2 MgCl2, 10 HEPES and 10 glucose with pH 7.8 adjusted with NaOH and an osmolarity of ~290 mOsm.

For targeted recordings, V0d interneurons were labeled in (Tg[glyt2:loxP-RFP-loxP-GFP] x Tg[dbx1b:Cre]) zebrafish, referred to as Tg[glyt2, dbx:GFP]. Motoneurons were back-labeled by injecting dextran dye into the muscles. All recordings were performed using the whole-cell patch-clamp technique. Patch-clamp electrodes were filled with a solution containing (in mM): 120 K-gluconate, 5 KCl, 10 HEPES, 4 Mg2ATP, 0.3 Na4GTP, 10 Na-phosphocreatine with pH 7.4 adjusted with KOH and an osmolarity of 275 mOsm. Neurons were visualized using a fluorescence microscope (Axioskop FS Plus, Zeiss) equipped with IR-differential interference contrast (DIC) optics and a CCD camera (Hamamatsu, Japan). Intracellular patch-clamp electrodes were advanced into the spinal cord through the meninges using a motorized micromanipulator (Luigs & Neumann, Germany) while applying constant
positive pressure. In larval zebrafish experiments both spontaneous fictive swimming and swimming initiated by electrical stimulation of the skin (1 ms pulse) was recorded. In adult experiments where swimming was initiated, several tail muscles were left intact during the dissection and an extracellular recording electrode was placed at an intermyotomal cleft to record ventral root output. An extracellular stimulation electrode was placed dorsally at the border between the brainstem and spinal cord to induce swimming-related activity (10 x 1 ms pulses at 1 Hz).13 Extracellular signals were amplified using a differential AC amplifier (A-M Systems) and filtered with low and high cut-off frequencies of 300 Hz and 1 kHz, respectively. Intracellular signals were amplified using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and low-pass filtered at 10 kHz. Electrophysiological data was digitized at 10 or 20 kHz using a Digidata 1322A A/D converter (Molecular Devices) and acquired using pClamp software (Molecular Devices). The analysis of electrophysiological data was performed using Spike2 (Cambridge Electronic Design), Clampfit (Molecular Devices) and MatLab software.

The action potential threshold of V0d interneurons was determined from the measured membrane potential at which dV/dt exceeded 10 mV/ms. For zebrafish larvae, fictive swimming frequency was calculated by measuring the time delay between membrane potential oscillations during fictive swimming using a custom Matlab script. V0d firing probability during swimming was calculated by scoring each swim cycle and assigning a value of 1 if there was at least one action potential and 0 if the neuron was not recruited. Swim cycles were binned into 5 Hz frequency windows and an average spike probability was calculated for each frequency window across recorded cells. For adult zebrafish, fictive swimming was analyzed using a custom Matlab script. The minimum recruitment frequency of V0d interneurons was defined as the lowest locomotor frequency of a swimming episode at which the neurons were firing action potentials.

Immunohistochemistry
In adult zebrafish, a subset of V0d interneurons were passively filled with 0.25% neurobiotin for post hoc analysis of their morphology. Spinal cords with neurobiotin-filled neurons were dissected out and transferred into 4% PFA solution overnight at 4 °C. The tissue was then washed extensively in PBS then incubated in 0.5% PBS-Triton for 2-4 hours. Non-specific protein binding sites were blocked with 0.15% normal goat serum and 1% Triton X-100 in PBS. Samples were then incubated in streptavidin conjugated to either Alexa Fluor 488 (1:500, Invitrogen), Alexa Fluor 555 (1:500, Invitrogen), or Alexa Fluor 647 (1:500, Invitrogen). Samples were mounted on coverslips using anti-fade fluorescent mounting medium (Vectashield Hard Set, Vector Labs). Whole-mount imaging of the spinal cords was acquired using a laser scanning confocal microscope (Zeiss LSM 800-airy or LSM 700). The neuronal processes (axons and dendrites) of labeled neurons were traced and reconstructed using Adobe illustrator or Coreldraw.

For analysis of GFP expression in Tg(glyt2,dbx:GFP) transgenic zebrafish, fish were first anesthetized in MS-222 and the spinal cords were dissected out and fixed in 4% PFA overnight at 4 °C. The spinal cords were washed five times for 5 min in phosphate buffer saline (0.01M PBS; pH = 7.4). Non-specific protein binding sites were blocked with 3% BSA and 2% normal donkey serum and 1% triton-100 at room temperature. Spinal cords were then incubated with chicken anti-GFP (Abcam, ab13970) in 1% Triton X-100 in PBS at 4 °C for 72 h. After washing in PBS, the tissue was incubated with anti-chicken Alexa Fluor 488 antibody (Life Technologies, A11039) at 1:500 dilution overnight at 4 °C and subsequently rinsed in PBS and mounted using anti-fade fluorescent mounting medium (Vectashield Hard Set, Vector Labs). The number and position of neurons was identified from images taken with a laser scanning confocal microscope (LSM800, Zeiss), using a 40x water immersion objective. Graphs were prepared with python using plotting libraries Matplotlib version 3.0.3 and Seaborn version 0.9.0 (https://github.com/mwaskom/seaborn). The position of neurons from approximately 15 segments from 4 fish was plotted for each transgenic line.

BrdU treatment and immunostaining
Animals were treated with 5-bromo-2’-deoxyuridine (BrdU; Sigma-Aldrich, B5002) at a concentration of 0.7% in fish water. Fish were kept in BrdU solution for the time indicated in Figure S1. Fish were housed until 5-6 weeks of age and then processed for BrdU immunodetection. To visualize the incorporated BrdU, DNA denaturation was performed by incubating the tissue in 2 N HCl for 60 min (whole mounts) at 37 °C, followed by thorough washing in PBS and incubation with BrdU antibody (1:100, Becton Dickinson, 347580; RRID: AB_10015219). The tissue was subsequently processed according to the standard immunohistochemistry procedure described above.

Behavioral analysis
Tg(glyt2,dbx:GFP) larval zebrafish (4-6 dpf) were first anesthetized in 0.01% MS-222, then embedded on their side in 1.5% low-melt agarose and covered with fish water containing MS-222. They were then placed under a 2-photon/confocal microscope (Zeiss LSM980) and all V0d interneurons were photoablated (wavelength 910 nm) along approximately 20 segments of spinal cord. Control animals were anesthetized and embedded in the same way as ablated animals but were not subjected to two-photon laser ablation. Zebrafish were then transferred to fish water to recover from anesthesia and acclimate in the recording chamber for 20 minutes before analyzing their swimming.

For behavioral analysis, fish were placed in a circular glass dish (5.5 cm diameter) containing 10 mL of fish water, illuminated from below by an LED lightbox. First, spontaneous swimming was recorded over 2 minutes using a high-speed camera at 60 frames per second (fps). Then, evoked escape swimming was assessed by sound stimulation while recording at 600 fps. Trials in which stimulation unsuccessfully elicited a C-start maneuver were excluded from analysis and at least 3 minutes were left between stimulations.
The procedure was adapted for juvenile zebrafish (4-7 week old). After embedding, fish were covered with fish water containing 0.015% MS-222 and agarose covering the gills and mouth was removed. V0d interneurons were photoablated along 20 segments of spinal cord. The large ventrolateral cells present in the juvenile zebrafish spinal cord, previously identified as intraspinal proprioceptors, were not ablated. For behavioral analysis, juvenile zebrafish were placed in a circular glass dish (10 cm diameter) containing 30 mL of fish water. Spontaneous swimming was recorded over 2 minutes at 60 fps and sound evoked escape swimming at 350 fps.

The tracking of the fish was obtained by extracting the coordinates of the centroid of the fish using an ImageJ plugin, ‘AnimalTracker75’, and analyzed using custom MATLAB scripts (MathWorks). The average velocity, distance travelled, and the number of spontaneous swim episodes was analyzed with velocity and distance values expressed as body length per second (bl/s).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Graphs and statistical analyses were made using GraphPad Prism software (https://www.graphpad.com/). A two-tailed Student’s t test (two groups) or a one-way ANOVA with Tukey’s post-hoc multiple comparisons (>two groups) were performed as appropriate. Data was tested for normality and a non-parametric alternative test was used where normality was not confirmed. Differences were considered to be significant if p < 0.05.