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Reconciling the functions of even-skipped interneurons during crawling, swimming, and walking

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In all bilaterally symmetric animals, movements across the body are coordinated by interneurons that traverse the midline. Recent work is beginning to tease apart the functional complexity of interneurons labeled by the homeodomain transcription factor even-skipped, which provide a phylogenetically conserved source of commissural excitation during locomotion in both vertebrates and invertebrates. Here, we review recent studies of the roles of even-skipped neurons during locomotion in flies (EL neurons), fishes, frogs, and mice (V0v neurons). Comparisons across species reveal commonalities, which include the functional organization of even-skipped circuits based on birth order, the link between increased muscular complexity and even-skipped neuron diversity, and the hierarchical organization of even-skipped circuits based on their control of escape versus exploratory movements. We discuss how stronger links between different species enable testable predictions to further the discovery of principles of locomotor network organization.

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Introduction

Different forms of animal locomotion necessitate different patterns of muscle activation and with different levels of urgency. Whether crawling, swimming, walking, or flying, commissural projection neurons that traverse the midline are required to coordinate movements across the body during escape and exploratory movements. The discovery of shared transcriptional networks regulating interneuron specification has allowed for better comparisons between locomotor-related interneurons in different species and different stages of development [1]. The hope for such comparative approaches is the identification of core operational principles that explain locomotor control in vertebrates and invertebrates alike. Here, we highlight several recent investigations that are beginning to hint at evolutionarily conserved functions of a genetically identified source of commissural excitation in the coordination of bilateral activity during various forms of locomotion, namely interneurons labeled by the homeodomain transcription factor even-skipped [2–4].

Even-skipped interneurons and crawling

During larval stages of the fly, peristaltic crawling movements using axial muscles are the primary form of exploratory locomotion [5]. Crawling is characterized by waves of bilateral muscle contractions that pass from tail-to-head (forward crawling) or head-to-tail (backward crawling). Muscles in the abdominal segments are responsible for generating these movements and neurons within isolated abdominal ganglia controlling these muscles can produce rhythmic motor patterns appropriate for crawling in the absence of sensory inputs [6]. In addition to exploratory movements, larvae also produce a forceful ‘corkscrew’ response that allows them to escape threats [5].

Relatively recent work from the Doe lab using thermogenetic and optogenetic perturbation strategies in fly larvae has revealed a key role for a subset of laterally located even-skipped (Eve) positive interneurons (known as ‘ELs’) in the coordination of bilaterally symmetric locomotor movements [7]. Each abdominal ganglion contains bilateral clusters of excitatory (acetylcholine) EL interneurons. While all ELs were commissural, their axonal trajectories were diverse. Commissural axons from cells within each cluster had either ascending (e.g. A08c, A08s) or locally bifurcating (e.g. A08e1–3) projections where they formed direct synaptic contacts with motoneurons as well as with other motor-related and sensory-related interneurons. Activation or ablation of the EL population slowed crawling movements and frequently led to unilateral bends. Interestingly, the effects were limited to the amplitude of muscle contractions rather than the timing, suggesting these neurons are not part of the circuits that produce the crawling rhythm, but rather contribute to the intensity of body bends. This conclusion was supported by calcium imaging experiments in isolated preparations demonstrating EL neurons are not rhythmically active during ‘fictive’ crawling [7].

A more recent study from the Heckscher lab has revealed a more nuanced behavioral role for ELs [8••]. By using a molecular birth-dating strategy, Wreden *et al.* [8••] first demonstrated that EL interneurons comprise two developmentally distinct cohorts of cells. The authors then used optogenetic techniques in freely behaving larvae to examine how these cohorts might contribute to locomotion. Optogenetic activation of late-born ELs (A08c, A08s, A08e1–3) reduced the speed of crawling and perturbed symmetric body contractions, consistent with previous findings [7]. However, stimulation of early born ELs (A08x, A08m [9]) increased the speed of locomotion and induced corkscrew escapes. How might these two populations of ELs contribute to different behaviors? Electron microscopy revealed putative synaptic inputs to early born ELs from mechanosensory primary and secondary afferents, whereas late-born ELs received inputs from proprioceptive primary afferents [7,8••], suggesting ELs integrate different sensory information to facilitate selection of appropriate motor patterns.

In sum, these results indicate that in the developing *Drosophila* larvae, temporally arrayed cohorts of even-skipped neurons form distinct circuits to modulate the intensity and mode of locomotor output — early born neurons are active during more forceful escape movements, while later born neurons are active during weaker exploratory ones.

Even-skipped interneurons and swimming

The idea that temporal sequencing contributes to even-skipped neuron functional diversity is consistent with studies in zebrafish. Axial muscle activity during early locomotor movements in zebrafish alternates from left–right [10]. By freely swimming exploratory larval stages, rhythmic axial movements are accompanied by rhythmic movements of bilaterally paired pectoral fins [11]. By juvenile/adult stages, zebrafish also use paired pelvic fins and medial dorsal, anal, and caudal fins for propulsion and stabilization [12]. As in invertebrates, even-skipped (Evx1/2) interneurons in the spinal cord are also commissural and excitatory (glutamate) but are referred to as V0v neurons due to their ventral origin from the spinal p0 progenitor domain in mice [13,14].

In zebrafish, V0v neurons with ascending axons (a.k.a. commissural secondary ascending interneurons or CoSAs) are the first to differentiate [15]. These neurons are integrated into zebrafish motor networks early during development and are recruited during spontaneous, whole-body bends [16], which resemble escape bends used in response to threats in older fish. Recent work from the Lewis lab has revealed that V0v neurons in frog embryos are also primarily ascending [17,18], which is a time of life when frogs engage axially based escape movements [19,20]. These results are consistent with the idea of a conserved function for early born Eve/Evx interneurons during powerful escape behaviors.

So do later born V0v neurons also participate in exploratory behaviors? In zebrafish, the next V0v neurons to differentiate have bifurcating axons that can project locally or across multiple body segments [15], followed by V0v neurons with primarily descending axon trajectories (a.k.a., multipolar commissural descending neurons or MCoDs and unipolar commissural descending neurons or UCoDs) [21]. Of these, only MCoDs have been functionally characterized in larvae and they participate exclusively in exploratory low frequency swimming [22], which is accompanied by rhythmic activity in the pectoral fins [23]. At higher frequency escape responses, the fins are adducted against the body and MCoDs are inhibited [23]. Laser ablation of MCoDs uncouples fin movements from axial ones during exploratory swimming, but does not impact escape swimming [24]. This suggests they are not a part of the rhythm generator, but instead play a role in regulating the intensity of muscle contractions in a mode-specific manner, as proposed in fly larvae.

Recent work from the El Manira lab in juvenile/adult zebrafish has begun to allow for comparisons of V0v function throughout development [25••]. As zebrafish age, there is a leftward shift in the distribution of axial bending frequencies in freely swimming fish, from 20 to 90 Hz in embryos/larvae [21] to 1–60 Hz in juvenile/adults [26]. Larval and juvenile/adult fish spend most of their time in exploratory mode swimming at lower frequencies, which can be reliably observed in fictive preparations [27,28]. However, generating high frequency swimming in fictive preparations is more challenging. With this in mind, Bjornfors and El Manira [25••] performed a series of electrophysiological recordings from V0v neurons in juvenile/adults during fictive swimming, examined their recruitment at frequencies between 1–8 Hz, and then assessed whether they had ascending, bifurcating, or descending axonal trajectories [25••].

As observed in larvae, a fraction of descending V0v neurons participate in low frequency exploratory swimming in juvenile/adults (< 8 Hz). There were also many ascending and bifurcating V0v neurons that were not recruited at these low frequencies, consistent with their proposed birth order. Clearly, however, morphology was not always a perfect predictor of recruitment order since a fraction of ascending and bifurcating V0v neurons were also recruited below 8 Hz, while many descending V0vs were still not. Since higher frequencies of motor activity were not generated in this study, it remains to be seen if non-recruited V0v neurons participate in faster exploratory and escape swimming or if slower V0v circuits in adults are inhibited during transitions in swimming mode as they appear to in larvae [22]. On the basis of previous work in adults, one would expect such switches to occur above 21 Hz, during what is referred to as 'escape' [26].

Regardless, the work on fish and frogs not only supports the temporal assembly of escape then exploratory even-skipped circuitry observed in flies, but it also suggests that increased morphological diversity within the Eve/Evx population during development supports increased complexity of muscular coordination during locomotion.

Even-skipped interneurons and walking

Intensity and mode-specific participation of morphologically diverse V0v neurons according to birth order are also supported by recent experimental and computational studies in mice. During ambulatory locomotion, increases in speed are accompanied by increases in the cyclical frequency of muscles within limbs and changes in the timing and amplitude of muscle contractions within and between them [29,30]. Earlier work from the Kiehn lab [31] genetically ablating V0v neurons during development revealed profound deficits in left–right alternation between hindlimbs at high frequencies of fictive locomotion in neonates and real locomotion in adults. Additionally, there were more subtle deficits in forelimb–hindlimb coordination at low frequencies.

Previous tracing studies in embryonic mice from the Goulding lab revealed glutamatergic Evx1-positive V0v neurons have primarily ascending axonal projections and a small number have bifurcating axons [14]. In addition, the Arber lab has recently shown that by postnatal stages, there is a further population of descending V0v neurons that link the cervical and lumbar regions of the spinal cord that control the forelimbs and hindlimbs, respectively [32•]. Ablation of cervico-lumbar propriospinal connections, which include V0v neurons, disrupts low intensity exploratory walking in freely behaving animals and interlimb coordination at higher speeds. In addition, recent work using c-fos expression from the Gosgnach lab has demonstrated that V0v neurons in lumbar spinal cord are activated from low (0.18 Hz) to moderately high (0.48 Hz) frequencies of fictive locomotion [33], although links between morphology and recruitment could not be performed. Nonetheless, these observations suggest that ascending and bifurcating V0v neurons are among the earliest detectable and that V0v neurons play critical roles in coordinating activity along and across the body during both exploratory and escape movements, as reported in flies and zebrafish.

The idea that V0v neurons in mice represent a morphologically diverse population with speed-dependent patterns of recruitment was recently explored computationally by Danner *et al.* [34•]. One of the key predictions from this work is that locally and long-range projecting V0vs may be central to speed-dependent transitions between gaits, but are not part of the rhythm generating circuits. Under the proposed paradigm, locally, but not long-range projecting V0vs (i.e. cervical–cervical, lumbar–lumbar) receive supraspinal inhibitory drive that increases with locomotor frequency. This means transitions to the fastest gaits require inhibition of V0v neurons associated with slower gaits. Although gait-dependent inhibition needs to be confirmed physiologically, this pattern mirrors the one observed in zebrafish larvae, where V0v neurons engaged during slower exploratory swimming involving the pectoral fins are inhibited as fish transition to faster escape swimming with the fins adducted. The clear discontinuities in movements of the pectoral fins during transitions between exploratory and escape locomotion arguably reflect changes in gait despite relatively continuous variations of tail-beat frequency.

Collectively, these observations are consistent with the idea that V0v neuron differentiation in mammals follows the logic described in flies, fishes, and frogs and also provides evidence that V0v neurons operating during different modes of locomotion are functionally incompatible, since switching to a faster escape mode of locomotion involves inhibition of slower exploratory circuits.

Conclusions and predictions

Despite differences in body plans and forms of locomotion, consistent patterns are emerging related to the functional organization of even-skipped interneurons for escape and exploration. From flies and fishes, there is evidence for a temporal logic to the assembly of even-skipped circuitry, where early born neurons participate in faster escape movements and later-born neurons in slower exploratory ones. While this has yet to be tested in frogs and mice, one prediction is that V0v neurons engaged in the most powerful modes of locomotion are among the earliest born.

From flies, frogs, fishes, and mice, there is evidence that morphological diversity within the even-skipped population is linked to increases in muscular and behavioral complexity. Recent work from the Dasen lab has revealed remarkably conserved transcription-factor programs underlying the specification of fin motor pools and limb motor pools [35••]. This likely extends to the interneuronal networks controlling their activity and so future studies of V0v neurons providing fin-control should yield insights into the evolution of limb-control circuits. One prediction is that fin/limb V0v neurons represent a more temporally delayed cohort relative to axial V0v neurons. This cohort could be equally diverse and thus provide fin/limb–axis coordination along and across the body during different modes of locomotion.

From fishes and simulations of mouse locomotion, there is evidence for a tiered organization of even-skipped circuits, where V0v neurons active at slower speeds are inhibited as V0v neurons recruited at faster speeds are engaged. Why might there be differential control of V0v populations? Even-skipped neurons do not appear to be critical for rhythm generation, but rather help establish appropriate patterns of muscle activity across and along the body. It therefore makes some sense that interneurons responsible for producing one motor pattern are inhibited as those responsible for producing another are engaged. Future work identifying the source of this inhibition, whether from the brain or the spinal cord, is now required. It would also be worth seeing whether a similar tiered organization exists in the abdominal ganglion of flies and how EL diversity may increase commensurate with changes in body plan during fly development. For example, even-skipped interneurons are found in both larval and adult locusts [36].

In flies, EL interneurons receive distinct sources of afferent input related to their participation in escapes versus crawling. In zebrafish, it is conceivable that early born ascending V0v neurons with their more dorsally positioned somata are targeted by primary or secondary sensory afferents, which could evoke more forceful movements. Recent work from the Wyart lab has revealed that zebrafish cerebrospinal fluid-contacting neurons are mechanosensitive [37] and synapse onto V0v-MCoD neurons [38••]. Thus, it is possible that distinct types of V0v neurons receive input from different sensory modalities to select different modes of locomotion in fish as in fly larvae. V0v neurons activated during fictive locomotion in mice are distributed close to the central canal [33], so this form of afferent modulation could also apply more broadly.

A more recent study in mice from the Kiehn lab has demonstrated that descending midbrain control systems are also organized according to their role in evasion versus exploration [39•]. In addition, a very recent study in zebrafish from the Koyama lab has revealed a developmental logic to the wiring of descending control systems to spinal circuits, where hindbrain neurons active during slower swimming wire up to V0v-MCoDs after hindbrain-spinal networks for fast swimming [40••]. Consequently, the staggered assembly of even-skipped networks according to function and behavioral complexity could also apply to the descending control systems responsible for producing behaviors of differing urgency. While it remains to be seen how other features of organization of Eve/Evx circuitry are either shared or specialized between flies, fishes, frogs and mice, thanks to continuing work in these animals there should be plenty of predictions to test.

Conflict of interest statement

Nothing declared.

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