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Development of vestibular behaviors in zebrafish

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Most animals orient their bodies with respect to gravity to facilitate locomotion and perception. The neural circuits responsible for these orienting movements have long served as a model to address fundamental questions in systems neuroscience. Though postural control is vital, we know little about development of either balance reflexes or the neural circuitry that produces them. Recent work in a genetically and optically accessible vertebrate, the larval zebrafish, has begun to reveal the mechanisms by which such vestibular behaviors and circuits come to function. Here we highlight recent work that leverages the particular advantages of the larval zebrafish to illuminate mechanisms of postural development, the role of sensation for balance circuit development, and the organization of developing vestibular circuits. Further, we frame open questions regarding the developmental mechanisms for functional circuit assembly and maturation where studying the zebrafish vestibular system is likely to open new frontiers.

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Introduction

Gravity is a pervasive force across Earth. Most animals learn to control their orientation with respect to gravity, engaging reflexive movements that correct and maintain posture and that also serve as organizing principles of locomotor behavior. The normal posture that results from these movements is vital for locomotion and facilitates perception by stabilizing gaze. Therefore, the development of gravity-related behaviors and their underlying neural circuitry is a general problem of vital importance. Rigorous dissection of neural circuit function is only possible in the context of well-described behaviors, which dictate the constraints on the output of neural computations [1,2]. This poses a challenge for studies of posture in tetrapods, where a large number of muscles governing both limbs and trunk are engaged to maintain the animal's orientation, defying experimental analysis of the motor output. Furthermore, behavioral capacity is constrained by the composition of the underlying neural circuits, which are built during early development and refined as animals mature. Here we focus on vestibular function in an animal with a simpler body plan, the larval zebrafish, that serves as a powerful proving ground for hypotheses about the functional development of neural circuits.

As a small model vertebrate, zebrafish have four features that uniquely facilitate the work on gravity-related behaviors and the responsible neural circuits. First, their simpler body plan facilitates decoding both destabilizing physical forces and the development of compensatory behaviors. Second, zebrafish are genetically accessible, with established mutant lines that disrupt balance and posture [3]. Third, the external development of zebrafish embryos permits continuous access, whereas in amniotes much of the development takes place *in ovo* or *in utero* [4•,5–7]. Finally, the mostly transparent bodies of zebrafish larvae permit anatomical, electrophysiological, and optical [8•,9] approaches during external development.

Vestibular circuits serve to transform sensed instability into corrective motor output. In order to produce rapid sensory-motor transformations for both posture and gaze stabilization, the nervous system relies predominantly on a short reflex arc [10]. In the inner ear, an otolith (or mass of otoconia, in later vertebrates) sits atop of hair cells. As the head tilts or translates, the otolith slides relative to the hair cells, which transduce this mechanical stimulus into electrical signals, releasing glutamate onto vestibular afferents of the eighth cranial nerve. Afferents relay this activity to central vestibular neurons, which encode head tilt in a variety of directions and project directly to motor centers, including cranial motor nuclei and the spinal cord. These feed-forward systems are remarkably well-conserved across vertebrates [11,12], reinforcing the generality of findings in the larval zebrafish.

How zebrafish learn to stabilize posture

What physical challenges destabilize larval zebrafish ($\leq$30 days old) and how do they learn to respond? Simliar to other highly maneuverable animals, zebrafish bodies are inherently unstable [13]. Vestibular-deficient animals

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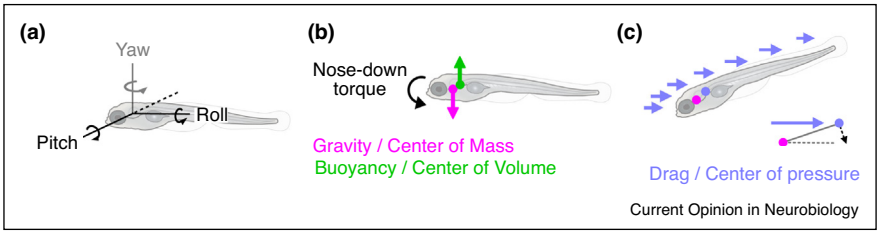
Figure 1



A diagram of forces that stabilize and destabilize larval zebrafish posture. **(a)** Three axes of rotation: Roll, Pitch (nose-up/nose-down) and Yaw (left/right turns). **(b)** Forces in the pitch axis: the buoyant force acts at the center of volume (green circle) to elevate the fish; gravity acts at the center of mass (magenta circle) to pull the fish down. The center of mass sits forward of the center of gravity which leads to a nose-down torque that will rotate a passive fish nose-down. **(c)** During forward translation, or in flow, the fish will rotate (shown here in the pitch axis) to align with the direction of drag (blue lines). The center of pressure (blue circle) is displaced caudally to the center of mass (pink circle) about which the fish rotates. This displacement acts as a moment arm, schematized in the corner, that generates stabilizing torque (black arrow) to align the body.

often swim in corkscrew fashion or upside-down, suggesting instability in the roll axis (Figure 1a) [14]. Anesthetized larvae are also unstable in the pitch axis, tipping headfirst (Figure 1a). Recent work offers a physical explanation for the nose-down torques that destabilize pitch [15••]. In still water, the two main forces acting on zebrafish are gravity and buoyancy (Figure 1b). Gravity acts at a zebrafish's center of mass to pull the fish down. Meanwhile, the buoyant force acts at the center of volume to push the zebrafish up. Because the center of mass for larval zebrafish sits rostral to the center of volume, fish are subject to a constant nose-down torque. Therefore, to maintain stability, fish must govern orientation in both the pitch and roll axes.

Zebrafish exhibit both active and passive mechanisms that govern orientation, with passive contributions mitigating some external destabilization and active components providing the remaining postural control. Larval zebrafish can actively rotate their bodies by selective contractions of the dorsal (epaxial) and ventral (hypaxial) trunk musculature. Differential contraction of the dorsal and ventral muscles on the right and left sides of the body subserves roll (Figure 2a) [16••]. Conversely, contraction of dorsal or ventral muscles on the left and right sides in concert serves to rotate fish in the pitch axis, though the precise organization of this activity has not been described [17]. Importantly, asymmetries in zebrafish morphology provide directional stability in both roll and pitch axes. Just as a weathervane rotates to adopt a stable orientation relative to the wind, fish orient in roll/pitch during forward translation (Figure 1c). Thus swimming and/or facing into flowing water passively stabilizes orientation.

Separating active and passive means of orientation enabled recent work showing that larval zebrafish learn to time their swimming to stabilize posture [15••]. Larval zebrafish locomote in discrete swim bouts. Their bodies are so small that translation is constrained by viscous forces that minimize glide [18–20]. During the pause between swim bouts, larvae lose the stabilizing effects of flow and are instead pitched nose downwards by gravity. Subsequent swim bouts, made on average once a second, translate fish and correct this destabilization in concert with active reorienting movements (Figure 1c). At 4 days old, larvae time their swim bouts with comparable frequency at most postures. As they develop, the probability of making a bout becomes correlated with instability. As zebrafish rotate away too far or too quickly from their preferred posture, the probability of initiating a bout increases. Consequently, unlike younger larvae, older larvae specifically time bursts of swim bouts to compensate for instability. Emergent control of locomotor initiation thus underlies developmental improvements to postural stability.

Larval zebrafish reduce their density by inflating a gas-filled organ called the swim bladder; failure to do so is fatal. This early behavior requires larvae to reach the surface and gulp air [21]. Unlike visual cues, vestibular cues are sufficient to orient and locomote to the surface. Normal larvae raised in the dark inflate their swim bladders properly, whereas fish missing their vestibular otoliths fail to inflate their swim bladder at a normal time, even when raised in the light [22•]. Therefore to swim properly, larvae must use their vestibular system to orient and surface [23].

Vestibular input is not required for spinal circuit development

The importance of locomotion for postural stability underscores the need for rapid functional development of the spinal circuits that generate axial swimming in response to vestibular input. In larval zebrafish, the spinal circuitry responsible undergoes a change in functional configuration from 1 to 3 days old, with a shift from a spontaneous coiling behavior to the beat-and-glide swim pattern. The spinal cord of the early embryo exhibits high levels of gap junctional coupling, which is thought to

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underlie coiling movements [24,25]. During the transition from coiling to beat-and-glide swimming, there is a shift from reliance on electrical to chemical synapses [26] and a concurrent transition in the ionic currents expressed in spinal cord premotor neurons [27]. Additionally, the elements of spinal circuitry that produce fast swimming are largely in place by 2 days old, whereas the motor and premotor components of slower muscle control, which are thought to underlie most postural movements [28], develop around 2–4 days old [29,30]. Over the same time period, reticular circuits acquire sensitivity to vestibular and auditory input [31]. Therefore, spinal cord maturation, encompassing both neuronal differentiation and intrinsic and synaptic changes, prepares the circuit for appropriate motor outputs in response to newly functional vestibular signals around 3 days old.

Does patterned sensory activity play an instructive role in vestibular circuit development? Recent work in zebrafish has found that gravitational sensation is likely dispensable for this process. Both the utricular otolith and associated hair cells develop by 1 day old, well before postural behaviors emerge [32,33]. The utricle is the only functional vestibular sensor in larval zebrafish [22•], due to the small size of the larval semicircular canals [34,35••]. Mutant zebrafish lacking a functional otogelin allele exhibit delayed development of the utricular otolith to ~2 weeks old, well after postural function normally develops. Intriguingly, once the utricular otolith is in place otogelin −/− larvae develop normal orientation to gravity, supporting the hypothesis that the underlying vestibulospinal circuit develops accurately in the absence of patterned sensory information [36]. Notably, the organization of the spinal circuit into distinct modules for differential control of dorsal and ventral musculature necessary for proper postural orientation persists in otogelin −/− larvae, even before the arrival of the otolith [16••]. Therefore, spinal motor circuit assembly does not rely on patterned descending vestibular signals.

Organization of developing vestibular circuits that stabilize gaze and posture

Vestibular circuits rely on specific connectivity to route gravitational signals to appropriate motor outputs. Research on vestibular-driven postural circuits, which operate through the rich complexity of the spinal cord, has lagged behind that of vestibulo-ocular gaze-stabilizing circuits. There, eye movements are controlled through a highly conserved set of six muscles, and gaze-stabilizing behavior can be readily measured. In the gaze-stabilizing circuit, two major classes of central vestibular neurons are sufficient to elicit compensatory eye movements during pitch and roll [37,38]. One class is sensitive to nose-up body rotations, the other to nose-down, and both can respond during roll towards the ipsilateral ear (ipsiversive roll). During ipsiversive roll, both classes are active and the eyes rotate appropriately. To compensate eye position during pitch, the two classes have distinct effects on motor neurons: one class evokes torsional eye rotations that compensate nose-up pitch, whereas the other rotates the eyes to compensate nose-down pitch (Figure 2b). Recent work used the zebrafish to confirm an anatomical asymmetry among gaze-stabilizing neurons previously suggested in mammals [39]. Using single-cell tracing, lesions, and selective activation, it was demonstrated that vestibular neurons were distributed in a 6:1 nose-up to nose-down ratio [40••].

Gaze stabilization in pitch and roll via the vestibulo-ocular reflex develops contemporaneously with consistent postural orientation [43]. At 3 days old, larval zebrafish show no measurable eye movements in response to body tilts in pitch or roll. 24 hours later, the responses are partially compensatory. By 10 days old, larval zebrafish can entirely compensate for ethologically relevant body tilts by counter-rotating their eyes.

Gravity guides both postural and gaze stabilizing reflexes. Do neural circuits coordinate the two? Recent anatomical and lesion work supports a role for central vestibular neurons that perform the vertical/torsional vestibulo-ocular reflex. In addition to projections to the extraocular motoneurons that stabilize gaze, these vestibular neurons project to a set of reticulospinal neurons in the nucleus of the medial longitudinal fasciculus [43]. These midbrain reticulospinal neurons respond to visual cues, including moving gratings, and affect the speed and direction of locomotion [44–46]. Intriguingly, following early photoablation of these vestibular neurons larvae fail to inflate their swim bladders, indicating malfunction of righting behaviors [40••]. This work suggests a postural role for gaze-stabilizing vestibular neurons, evidence of coordinated processing of vestibular sensation for behavior.

Open questions well-suited to the zebrafish model

How do molecular cues coordinate sensorimotor circuit assembly? Proper vestibular circuit organization requires central vestibular neurons to coordinate information flow across two synapses: for example, peripheral afferents sensitive to nose-up pitch tilts must selectively innervate central vestibular neurons that project to nose-down effectors to stabilize gaze (Figure 2b). There are four possible substrates for the origin of developmental cues that govern this process. Utricular hair cells with opposite polarity are genetically differentiable [47••], raising the possibility that they express cell surface signaling molecules that trigger identity formation in afferents. Alternatively, central vestibular neurons may use a process such as Notch-mediated lateral inhibition to instruct fate, similar to the spinal cord [48]. Thirdly, neuronal connectivity could be conferred retrogradely from the

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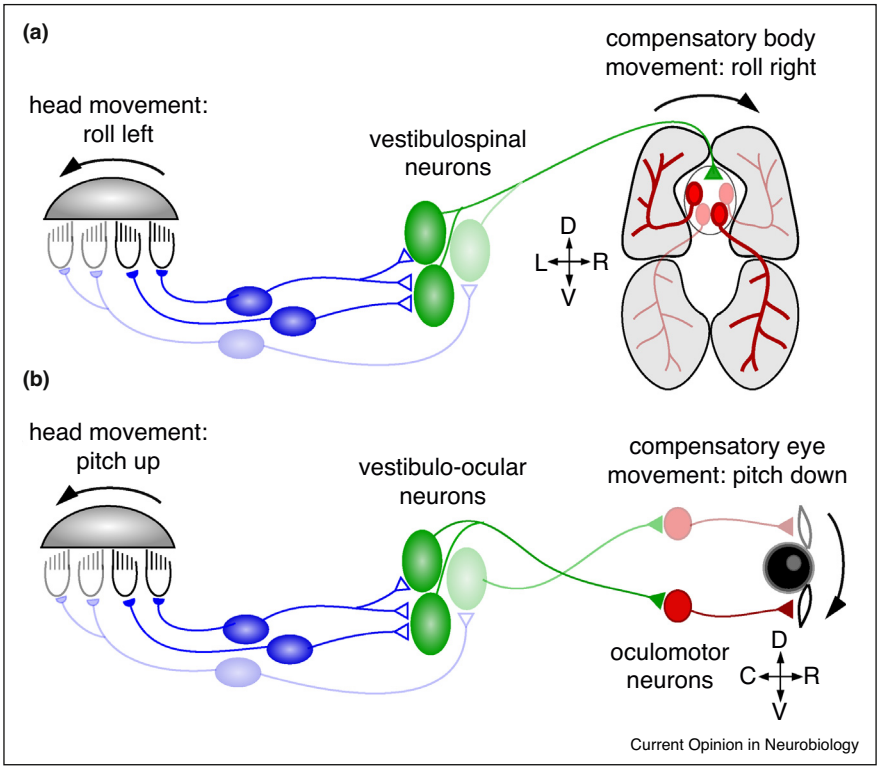
Figure 2



Schematic of vestibular circuits subserving posture and gaze stabilization. **(a)** At the onset of movement, the utricular otolith slides relative to the hair cells underneath, depolarizing some (black) and hyperpolarizing others (gray), depending on their ciliary orientation. Vestibular afferents (blue) relay hair cell signals to vestibulospinal neurons in the hindbrain (green). This vestibular drive sets up asymmetric activation of trunk musculature through as-yet unclear connectivity that is thought to rely both on direct synapses with motor neurons as well as indirectly via spinal premotor populations [41•,42]. In this example, as the fish rolls to the left, stronger motor drive to ventral muscle on the right and dorsal muscle on the left (gray shaded regions) produces a self-righting torque [16••]. **(b)** Equivalent schematic for vestibular-driven gaze stabilization. Here the brainstem vestibular nuclei are known to make direct connections to oculomotor neurons. Recent work has revealed that in pitch-related circuits, vestibulo-ocular neurons driving eye rotation downwards (active pathway, dark green) outnumber those driving upwards eye movements (inhibited pathway, pale green) by ~6:1 [40••].

oculomotor neuron pools, which are segregated spatially during development [49], as has been suggested for the chick [50] and shown for spinal interneurons [51]. Finally, temporal cues could guide fate decisions: recent *in vivo* timelapse imaging demonstrated how peripheral afferents develop systematically in time [52,53]. Similar organization by birthdate has been demonstrated in extraocular motoneurons [49] and spinal motor circuits [30] using genetically encoded photoconvertible markers of post-mitotic neurons. Disentangling these competing hypotheses will require observing and perturbing developing circuits, experiments facilitated by the accessibility of the zebrafish preparation.

The cerebellum is well-known for its capacity to implement multisensory integration [54] and drive motor learning [55]. Does it also serve to guide vestibular development? In zebrafish, Purkinje cells are functional by 5 days [56•,57•], and *in vivo* imaging has revealed that a cerebello-vestibular projection develops around 5–7 days old [58•,59]. This occurs after the early development of posture and gaze stabilization, but before refinements in bout timing and integration of sensory information from the semicircular canals. Thus, it should be possible to examine cerebellar contributions to vestibular circuit function by analysis of the ontogeny of postural behaviors relative to functional development of the Purkinje - vestibular synapse. Cerebellar signals could also direct the timing of swim bouts specifically to counter postural instability [15••], consistent with the known role of cerebellum in temporal precision [60,61].

Similarly, as larval zebrafish transition to juveniles and the semicircular canals begin to function, vestibular circuits must integrate these new signals without compromising behavior. The developmental processes that guide this integration are wholly unclear, but intriguing results in *Xenopus* point to a role for the semicircular canals in

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functional refinement of tuning [62••]. The cerebellum is implicated in integration of canal and otolith signals [63], and might also govern their developmental alignment. Finally, the mature cerebellum uses visual feedback to tune the strength of the vestibulo-ocular reflex. In larval zebrafish, visual information can be appropriately integrated to augment gaze-stabilization [43]. Whether the cerebellum uses visual information to drive refinement of gaze-stabilizing circuits is an open research question well-suited to the zebrafish due to its external development.

Conclusions

Thanks to a set of unique advantages, the larval zebrafish model has shed light on a number of long-standing questions regarding vestibular circuit assembly, organization, and behavioral development. Recent work has shown that basic vestibular reflexes develop quite early, followed by later refinements that improve stability of posture and gaze. Mutant zebrafish with delayed peripheral development show normal circuitry, suggesting that gravity sensation may be dispensible. The relative simplicity of orienting underwater revealed that larval zebrafish learn to time their movements to control posture. Finally, recent work used the optical access to define the anatomy and function of a set of vestibular neurons responsible for both gaze stabilization and early postural behaviors. These findings demonstrate the burgeoning impact of larval zebrafish model for understanding vestibular development.

Excitingly, the developing zebrafish vestibular system permits investigation of outstanding questions regarding the roles of genetic signals, activity, and cerebellar feedback in directing neural circuit development. Does the sensory or motor periphery provide vital instructive cues governing central neuron identity/circuit connectivity? How are cerebellar and peripheral inputs — the hallmarks of mature vestibular circuits — incorporated during development without disrupting behavior? Studies of the mature vestibular system have informed our understanding of nearly every aspect of systems-level neuroscience [64]. Studying vestibular circuit assembly and attendant behavior in the larval zebrafish stands to continue this trend, opening a new frontier to deepen understanding of functional neural development.

Conflict of interest statement

Nothing declared.

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