

Development of Sensory Systems in Zebrafish (*Danio rerio*)

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Abstract

Zebrafish possess all of the classic sensory modalities: taste, tactile, smell, balance, vision, and hearing. For each sensory system, this article provides a brief overview of the system in the adult zebrafish followed by a more detailed overview of the development of the system. By far the majority of studies performed in each of the sensory systems of the zebrafish have involved some aspect of molecular biology or genetics. Although molecular biology and genetics are not major foci of the paper, brief discussions of some of the mutant strains of zebrafish that have developmental defects in each specific sensory system are included. The development of the sensory systems is only a small sampling of the work being done using zebrafish and provides a mere glimpse of the potential of this model for the study of vertebrate development, physiology, and human disease.

Key Words: dorsal root ganglion; inner ear; lateral line; olfactory system; vestibular system; visual system

Introduction

The zebrafish (*Danio rerio*) is a powerful model organism for the study of vertebrate biology in that it is well suited to both developmental and genetic analysis. Because of its external fertilization, optically clear chorion, translucent embryo, and rapid development, as well as the accessibility of early developmental stages, the zebrafish is being used to analyze a myriad of developmental events. The development of the sensory systems exemplifies the work utilizing zebrafish. Zebrafish possess all of the classic sensory modalities: taste, tactile, smell, balance, vision, and hearing. Of these, only taste has not been studied in the zebrafish. Although it is clear that fish can hear (Fay and Edds-Walton 2000), hearing is not discussed in depth in this article. Zebrafish do not possess any overt specialization of the inner ear (e.g., a cochlea) for hearing although they do have Weberian ossicles that are capable of transmitting vibration from the gas-filled swim bladder to the inner ear. Both the lateral line and the inner ear are used to detect vibrational stimuli in a manner that could be interpreted as “hearing.”

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By far the majority of studies performed in each of the sensory systems of the zebrafish have involved some aspect of molecular biology or genetics. This involvement is the result of several large-scale mutagenesis projects, which were undertaken to identify genes that play essential roles during zebrafish development from fertilization to the embryo-larva transition at 72 hr after fertilization (all times after fertilization are for embryos maintained at 28.5°C) (Driever et al. 1996; Haffter et al. 1996). Interpretation of the results from those screens was made easier by the now-classic description of the pre-embryonic and embryonic stages of zebrafish development (Kimmel et al. 1995).

Each section of this article provides a brief overview of a specific sensory system in the adult zebrafish, followed by a more detailed overview of the development of the specific sensory system. Each section ends with a brief discussion of some of the mutant strains of zebrafish that have developmental defects in the specific sensory system. (A more complete description of all of the mutants can be found at <<http://zfin.org/>>.)

Dorsal Root Ganglia

As in all vertebrates, the zebrafish dorsal root ganglia are organized segmentally. Each neuron of a dorsal root ganglion has an axon that projects into the spinal cord via a dorsal root and projects peripherally via a spinal nerve. The sensory neurons of the dorsal root ganglion are derived from neural crest cells. Neural crest cells are transient, embryonic, migratory cells that originate at the lateral edges of the neural plate. Although as a group neural crest cells have been quite well studied in the zebrafish, dorsal root ganglia cells, specifically, have not. Before dorsal root ganglia formation, zebrafish skin is innervated by Rohon-Beard spinal sensory neurons. The Rohon-Beard cells die as dorsal root ganglia neurons become established (Bernhardt et al. 1990). Although this timing suggests that the dorsal root ganglion neurons might participate in Rohon-Beard cell death, results of surgical or genetic dorsal root ganglia removal do not support this hypothesis. Rohon-Beard cells and neural crest cells are intermingled in the lateral neural plate. Mutants defective in Delta-Notch signaling have more Rohon-Beard cells and fewer trunk neural crest cells, providing evidence that Delta-Notch-mediated lateral inhibition segregates Rohon-Beard cell and trunk neural crest cell fates (Cornell and Eisen 2000). Only the earliest migrating trunk neural

crest cells generate dorsal root ganglia neurons. Neural crest cells that generate dorsal root ganglia neurons show specific early migratory behaviors that predict their fate (Jesuthasan 1996). These behaviors provide evidence that they are specified before they reach the dorsal root ganglia and that neural crest cells thus become heterogeneous quite early in development. Each dorsal root ganglion initially contains only one to three cells in the zebrafish; by adulthood, each dorsal root ganglia contains approximately 100 neurons. The source of the additional cells is unknown; however, bromodeoxyuridine labeling studies suggest that some neurons may be capable of dividing.

It is possible to identify premigratory neural crest cells by *snail2* (Thisse et al. 1995) and *forkhead6* (Kelsh et al. 2000) in situ hybridization staining. The *alyron* mutant has a significantly reduced number of neural crest cells and at later stages lacks dorsal root ganglia (Cretokos and Grunwald 1999). The *sdp*^{w15} (*sensory deprived*) mutant has no dorsal root ganglia neurons. The *narrowminded* mutant lacks both dorsal root ganglia and Rohon-Beard cells, suggesting a genetic link between formation of neural crest and primary sensory neurons (Artinger et al. 1999).

Olfactory System

Vertebrates can recognize and discriminate among a large number of odorants of diverse molecular structure. Humans, for example, are capable of distinguishing among thousands of distinct odors. In the vertebrate olfactory system, zebrafish accomplish the tasks of molecular recognition and neural coding because they possess a well-developed sense of smell that governs a variety of behaviors. Both the number of odorant receptor genes and the number of glomeruli in the olfactory bulb are approximately one order of magnitude smaller than those of mammals. However, the spatial organization of functional properties within the sensory surface and the olfactory bulb are comparable to mammals (Korsching et al. 1997). The reduced olfactory system of zebrafish, together with the suitability of this species for developmental and genetic studies, make zebrafish an appealing model system to study olfactory development and the representation of olfactory information in the central nervous system (CNS¹).

The olfactory organ is a unique cellular population that arises outside the CNS from the ectodermally derived olfactory placode. Initially, the olfactory placode is an apparently homogeneous population of cells that first appears at 17 hr after fertilization as a thickening of the ectoderm and that later invaginates to form the nares by 32 hr. As the placode differentiates into the olfactory epithelium, stratified cell types characteristic of the adult epithelium appear and include basal cells, sensory neurons, and support cells. Throughout life, the basal cells generate olfactory sensory neurons that

migrate toward the apical surface of the epithelium while extending axons and dendrites (Byrd and Brunjes 1998). The cell bodies of the neurons remain in the epithelium, and their axons grow through the olfactory nerve into the differentiating CNS. As soon as the axons are within the CNS, they segregate to form glomeruli, or clustered grape-like structures in the olfactory bulb (Dynes and Ngai 1998). The glomerular organization of olfactory afferents is characteristic of both invertebrates and vertebrates. Within a species, the glomeruli are organized into a stereotyped pattern in the olfactory bulb. Dye labeling has shown that individual glomeruli in the olfactory bulb receive inputs from sensory neurons scattered throughout the olfactory epithelium (Hansen and Zeiske 1993). Recent studies indicate that the axons of sensory neurons expressing the same olfactory receptor converge on the same glomerulus, express receptor transcript in their axon terminals, and respond to the same small subset of odorants (Byrd et al. 1996). This finding led to the hypothesis that olfactory receptors play a role in sensory axon guidance.

The pathway that the sensory axons follow into the CNS is established by a precocious class of neurons, the pioneer neurons (Whitlock and Westerfield 1998). The appearance of the pioneer neurons precedes the olfactory neurons. The pioneer neurons establish initial contact with the telencephalon, are then used as a scaffold by the developing sensory afferents, and then die. Interestingly, these pioneer neurons are not known to express any of the individual odorant receptors (Whitlock and Westerfield 1998). This suggests that odorant receptors are not necessary for pathway recognition, but a role in target recognition has not been ruled out.

Individual odorants are thought to activate specific G-protein-coupled receptors encoded by a large multigene family. Each olfactory neuron expresses only one or a few odorant receptor genes. Thus, the problem of discerning which receptor has been activated (and concomitantly the molecular identity of the odorant stimulus) can be reduced by the nervous system to a problem of identifying which cell has been activated. Neurons with common odorant receptor specificities in turn converge to a small number of glomeruli in the olfactory bulb, suggesting that spatial patterns of innervation in the olfactory bulb are used to encode olfactory information. The logic underlying olfactory coding therefore is a direct consequence of the exquisite selectivity of odorant receptor gene regulation and the concomitant targeting of specific olfactory neurons in the olfactory bulb.

Relatively few, if any, mutants are known to have defects limited to the olfactory system. This condition might reflect the interests of the laboratories involved in the original mutagenesis projects and the techniques and criteria that were used to screen the mutants.

Lateral Line

The lateral line derives its name from the linear arrangement, from rostral to caudal, of mechanosensory neuromasts along

¹Abbreviation used in this article: CNS, central nervous system.

the lateral aspect of the body. Neuromasts are also distributed throughout the surface of the head and are considered part of the lateral line. Each neuromast consists of a group of support cells and hair cells that resemble the cupula of the semicircular canals of the inner ear. Each neuromast is a mechanosensory end-organ that is sensitive to low-frequency (1- to 200-Hz) vibrations. Mechanosensory information reaches the brain via the rostral and caudal lateral line nerves on each side. Input from these nerves is used primarily for prey localization, navigation, schooling behavior, and predator avoidance.

The rostral lateral line, neuromasts of the head and their associated sensory neurons, develops from a placode just rostral to the otic placode and from neural crest cells (Collazo et al. 1994). These cells comigrate to stereotypic positions on the head during the first 5 days after fertilization. The caudal lateral line of zebrafish develops from an embryonic primordium that migrates from its initial postotic position to the base of the tail. This migration takes place along a very specific pathway on the surface of the basal lamina of the skin immediately over the horizontal myoseptum. The horizontal myoseptum appears to be necessary for normal migration because under conditions in which the underlying myoseptum is disturbed during development, the primordium migrates abnormally. For instance, some zebrafish mutants, such as *no tail*, are characterized by an absence of the entire horizontal myoseptum. In these mutants, the primordium migrates erratically, sometimes over the yolk of the embryo. Local disruptions of the myoseptum can be induced by heat shock during somite formation. In heat shock-treated animals, the primordium migrates normally until it encounters the abnormal myotomes, at which point it migrates along abnormal pathways. In wild-type embryos, in the presence of basic fibroblast growth factor (but not acidic fibroblast growth factor), the primordial cells dissociate and migrate in all directions (Metcalf 2000). Neuromasts are first recognizable 2 days after fertilization. In the 5-day-old zebrafish larva, there are 10 to 11 neuromasts, 15 to 20 sensory neurons, and approximately 10 efferent neurons comprising the caudal lateral line on each side. As the fish grows, neuromasts and sensory neurons continue to develop to maintain an even spacing along the lateral aspect of the fish.

Interestingly, the lateral line projections appear to be organized in a somatotopic fashion (Alexander and Ghysen 1999). The sensory axons for individual neuromasts occupy stereotypic positions in the lateral line nerves. Thus, the positions of the neuromasts on the body of the fish appear to be represented in the central projections of the sensory neurons. This somatotopy is similar to the tonotopic projections of the cochlear hair cells in mammals. Other similarities, including a myosin isoform, which causes a zebrafish version of hereditary deafness when defective (Ernest et al. 2000), have suggested that the zebrafish lateral line might also be used as a model for mammalian hearing.

Two mutants with lateral line defects were identified in the original mutagenesis screens, *dog* and *hypersensitive* (Whitfield et al. 1996). The *dog* mutant has a significantly

reduced number of neuromasts on the trunk and tail, and the *hypersensitive* mutant has an increased number of neuromasts in the same regions. Both of these mutants have a normal number and normal distribution of neuromasts on the head. This characteristic suggests that either different cues are used to specify the fates of cells of the preotic and postotic placodes or that the migratory cues for these cells are different.

Vestibular System

The zebrafish inner ear consists of a vestibular end-organ that, almost certainly, also serves as an auditory organ. In fishes, as in other vertebrates, the vestibular end-organs are divided into a gravity receptor system, with three subdivisions and an angular acceleration receptor system (Platt 1993). The gravity receptor system on each side consists of utricular, saccular, and lagenar maculae, each covered by an otolith. In zebrafish, each otolith has a stereotypic shape, and the maculae have characteristic shapes, patterns of sensory hair cell arrangement, orientations of the ciliary bundles of the hair cells, and thus characteristic patterns of polarization (Platt 1993). The angular acceleration receptor system consists of three orthogonal semicircular ducts, each with an ampulla containing sensory cristae.

Little is known about the central projection of the primary afferent neurons of the zebrafish vestibular end-organs. If the adult zebrafish is similar to the adult goldfish, the primary afferent projections are likely to terminate in three of the five primary octaval nuclei (Wullmann et al. 1996). In addition, there probably are tonotopic auditory afferent projections from the inner ear to the hindbrain similar to those documented in the goldfish (Echteler 1985). Presumably, the sensory epithelia of the vestibular end-organ also receive efferent innervation in the zebrafish. Although these projections have not been documented in the adult zebrafish, they are probably similar to those seen in other fishes. In the goldfish, the cell bodies for the vestibular efferents are found in the tegmental motor nucleus and the ventral and medial vestibular nuclei (Strutz et al. 1980). In other animals, the axons of the vestibular efferents travel adjacent to the axons from cells of the facial motor nucleus (Bell 1981; Fritzscht and Nichols 1993). This relationship probably reflects a common developmental origin for both the vestibular efferents and the facial motor neurons (Fritzscht and Nichols 1993). A common developmental origin for these two types of fibers has also been proposed for the zebrafish (Higashijima et al. 2000).

In the zebrafish, inner ear development is probably initiated by release of fibroblast growth factor 3 from the hindbrain (probably the 4th rhombomere) (Adamska et al. 2000). The fibroblast growth factor 3 release plays a causal role (B. B. Riley, Texas A & M University, College Station, Texas, personal communication, 2001) in restricting *dlx-3* expression to the forming otic placode (Malicki et al. 1996) 14 hr after fertilization in zebrafish. The otic placode becomes visible approximately 16 hr after fertilization and forms a

vesicle with a lumen by cavitation at approximately 18 hr (Haddon and Lewis 1996). Otolith growth is initiated at 18 to 18.5 hr by localized accretion of free-moving precursor particles. This otolith “seeding” occurs as kinocilia of precociously forming hair cells (tether cells) bind seeding particles, thereby localizing otolith formation (Riley et al. 1997). Tether cells usually occur in pairs at the rostral and caudal ends of the ear (Riley et al. 1997). The utricular and saccular otoliths are visible in the lumen of the otic vesicle by 19.5 hr of development at 28°C (Haddon and Lewis 1996); however, the lagenar otolith does not appear until approximately 9 days after hatching (Riley and Moorman, 2000). Development of the first two otoliths on each side appears to precede the development of the epithelial swellings destined to become maculae (Haddon and Lewis 1996). Approximately 24 hr after fertilization, the first sensory hair cells are seen as grouped in two small patches, one beneath each otolith, corresponding to future maculae (Chapman and Fraser 1996; Haddon and Lewis 1996). This development coincides with the expression of *msx-D* in the macular epithelia (Ekker et al. 1992). By 36 hr of development, the utricular and saccular maculae are well formed. Zebrafish hatch after 72 hr at 28°C and are then referred to as larvae. Over the next several weeks of development, no major changes in the utricular and saccular maculae are seen.

Between 24 and 52 hr, the primary afferents from the maculae and the cristae project to the vestibular nuclei and innervate a region of the hindbrain that is longer and differently shaped than that seen in older animals (Chapman and Fraser 1995). Between 52 and 65 hr, the axonal arbors of the primary afferents are pruned to the specific, nonoverlapping regions seen in the larva (Chapman and Fraser 1995). Over the next several weeks, there are probably additional changes in the projection patterns for the saccular primary afferents. The zebrafish maculae might be specialized for both vestibular and auditory functions as in the goldfish (Popper and Fay 1993). Therefore, the projection patterns in the adult zebrafish might not be as clear-cut as those seen in the larva. Higashijima and colleagues (2000) have recently shown that the efferents that project to both the lateral line neuromasts and the vestibular end-organ pioneer the pathway from the brainstem to the periphery. In the zebrafish, they pioneer this pathway both for themselves and for the efferents of the facial nerve approximately 24 hr after fertilization. By 96 hr after fertilization, branches are seen innervating the sensory epithelia of the utricle and saccule, and innervating the cristae ampullaries of the semicircular canals. Thus, the timing of outgrowth of the efferents and the timing of ingrowth of the primary afferents appear to coincide. This apparent correspondence suggests that common mechanisms are involved in these two phenomena. To date, no genes have been identified as playing a role in guiding the vestibular primary afferent axons to their central targets. However, no one has screened any of the known zebrafish mutants for these types of defects.

Mutations giving rise to anatomical defects in the inner ear have been isolated in the large-scale screens for mutations

that cause visible abnormalities in the zebrafish embryo. Mutations have been identified that affect specification of the otic placode (Malicki et al. 1996), presence or size of the otoliths, size and shape of the otic vesicle, and formation of the semicircular canals (Malicki et al. 1996; Whitfield et al. 1996). Also identified have been mutants that have no visible defects of inner ear morphology but that display swimming behaviors, which suggest vestibular deficits (Granato et al. 1996). These “circling” mutants might have defects of the vestibular primary afferent projection patterns; however, these projection patterns have yet to be analyzed in any of the zebrafish mutants.

Visual System

The visual system is the best studied of all the sensory systems of the zebrafish. The zebrafish eye has the characteristic vertebrate retina consisting of a neural retina and the retinal pigmented epithelium. The neural retina has the characteristic three cell layers with seven major cell types including six types of neurons and the Muller glia.

In the embryonic zebrafish, the flat optic vesicle develops into a nearly spherical eye cup from 16 to 24 hr after fertilization, when cellular proliferation is very low (Li et al. 2000). The optic lobe of the diencephalon can first be distinguished at 12 hr after fertilization. The pigmented epithelium and the neural retina become distinct starting at 15 hr after fertilization. Invagination of the optic lobe is initiated at 16 hr and is accompanied by the formation of the lens rudiment. By the end of somitogenesis (24 hr), the lens is spherical and has detached from the epidermis. At this age, the optic cup consists of two distinct layers: a thick layer of columnar pseudostratified neuroepithelium, from which the neural retina will form, and a thin layer of flat pigmented epithelial cells. The first pigment granules appear in the pigmented epithelium at approximately 24 hr.

Approximately 27 hr after fertilization, proliferation in the retina accelerates, resulting from a series of inductive events initiated by the prechordal plate and progressing from the optic stalk. A cascade of genes involving *ath5* (the zebrafish homologue of *Drosophila atonal*) (Masai et al. 2000), *sonic hedgehog*, and *tiggy-winkle hedgehog* (Stenkamp et al. 2000) initiate and propagate a wave of differentiation beginning in the ventral pole of the optic cup. The first neurons are born in an orderly spatial-temporal sequence that begins in the ventral-nasal retina and sweeps around the retina like the hands of a clock to produce, by approximately 72 hr after fertilization, the central patch of early neurons. Thereafter, new neurons are added at the retinal margin. As in other vertebrates, the retinal ganglion cells are the first neurons to be born in the zebrafish retina. Birth-dating studies indicate that the first postmitotic neurons appear between 29 and 34 hr. At 36 hr, none of the neuronal cell layers are clearly distinguishable. By 60 hr, the vast majority of the neurons in the central retina have already been born and are organized into three nuclear layers sepa-

rated by two plexiform layers. The stratification of the retina becomes progressively more distinct at later stages of development. Interestingly, the stratification of the neural retina appears to be directed by cells of the pigmented epithelium via a pathway involving the *mosaic eyes* gene (Jensen et al. 2001).

The photoreceptor cell layer of the retina contains five photoreceptor types (distinguished on morphological criteria): rods, short single cones, long single cones, and long and short members of the double cone pair. Initially, different photoreceptor types are not distinguishable by morphological criteria. Short single cones can be first distinguished from other photoreceptor cells at approximately 4 days after fertilization. By 12 days, all photoreceptor types can be distinguished on the basis of morphological criteria.

Retinal ganglion cell axons leave the eye during the second day after fertilization. Ganglion cell axons first reach the brain approximately 42 hr after fertilization and complete the innervation of their several targets by approximately 72 hr after fertilization. Thus, a precise retinotectal map is established very early in development and is the result of a two-step process. First, after leaving the eye, the growth cones of the retinal ganglion cell axons navigate through the brain to find the contralateral tectal lobe. These axons follow a distinct pathway along the ventral and lateral surface of the diencephalons. Axons from the two eyes cross each other at the ventral midline of the diencephalons to form the chiasm. Retinal axons grow near, but not on, pre-existing axons of the tract of the postoptic commissure and the postoptic commissure itself (Burrill and Easter 1995) as they grow toward and cross the ventral midline. Second, once they have entered the tectum, retinal axons travel farther to their individual target sites within the tectal field. Here, the projection is topographically organized in that neighboring retinal cells connect to neighboring places in the tectum. Axons from retinal ganglion cells in the dorsal portion of the nasal retina project to the caudal portion of the ventral tectum. Axons from retinal ganglion cells in the ventral portion of the temporal retina project to the rostral portion of the dorsal tectum. Thus, the retinal image is doubly inverted and topologically mapped onto an area of the brain that is concerned with processing visual information. While tecta and retinae grow by addition of new cells, the map adapts accordingly, thus ensuring a stable representation of the retinal image for the rest of the animal's life.

Behavioral experiments have revealed that the fish first sees changes in light intensity at approximately 68 hr after fertilization and first makes eye movements that track the stripes on a rotating drum beginning approximately 72 hr after fertilization, providing evidence for pattern vision. This optokinetic response improves over the next day to achieve adult levels of performance by 96 hr after fertilization. Direct visualization of the retinal image reveals that the eye is initially far-sighted but gradually becomes emmetropic by approximately 72 hr after fertilization. Electron microscopic

and immunocytochemical data indicate that the extraocular muscles mature at approximately this same time, which is normally the beginning of the first day after hatching (Easter and Nicola 1996).

Mutants with defects in numerous aspects of retinal development have been identified and have included the following: specification of the eye anlage, growth rate of the optic cup, establishment of retinal stratification, specification or differentiation of retinal neurons, and formation of the dorsoventral axis in the developing eye (Malicki et al. 1996). Mutants with defects in retinotectal projections have also been identified. The mutations affect distinct steps in the retinotectal pathway, from pathfinding between eye and tectum (Karlstrom et al. 1996) to map formation along the dorsal-ventral and the rostral-caudal axes of the tectum (Trowe et al. 1996). Mutations that disturb axon pathfinding to the tectum for the most part do not disrupt retinotopic mapping, and vice versa. Analysis of the mutants has suggested important mechanisms of retinal pathfinding from eye to tectum. For instance, a series of sequential cues appears to guide retinal axons to the contralateral tectal lobe.

In addition to identifying these genes, many of the identified genes are now being cloned. For instance, a mutation in the gene *astray* leads to retinal ganglion cell axons innervating multiple abnormal targets throughout the brain in addition to innervating the normal target in the tectum. *Astray* has recently been cloned and is a novel zebrafish *roundabout* homologue (Fricke et al. 2001). *Roundabout* genes are members of the immunoglobulin superfamily that have been shown to play an important role in axon pathfinding as a receptor on neuronal growth cones in *Drosophila melanogaster*. Interestingly, *astray* mutants have high levels of *astray* mRNA in both the cell bodies of the retinal ganglion cells and the cell bodies of the vestibular primary afferents. This characteristic suggests that the same guidance molecules might be used in several different developing sensory systems.

Summary

Studies related to the development of the sensory systems comprise only a small sampling of the studies being performed using zebrafish. Large-scale genetic screens of zebrafish have identified hundreds of mutant phenotypes, many of which resemble human clinical disorders. At the time of this writing, more than 1100 genes have been identified and cloned in zebrafish, and more than half have been mapped to a specific linkage group. The creation of critical genetic reagents, including the ability to "knock-down" the function of specific genes (Nasevicius and Ekker 2000), coupled with the rapid progress of the zebrafish genome initiatives directed by the National Institutes of Health in the United States and The Sanger Center in the United Kingdom, are bringing this model system to its full potential for the study of vertebrate development, physiology, and human disease.

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