

# *The Zebrafish in Biomedical Research*

*Biology, Husbandry, Diseases, and Research Applications*



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American College of Laboratory  
Animal Medicine Series



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Research Applications

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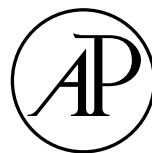
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An imprint of Elsevier

Academic Press is an imprint of Elsevier  
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525 B Street, Suite 1650, San Diego, CA 92101, United States  
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States  
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

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#### Library of Congress Cataloging-in-Publication Data

A catalog record for this book is available from the Library of Congress

#### British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library

ISBN: 978-0-12-812431-4

For information on all Academic Press publications visit our website at <https://www.elsevier.com/books-and-journals>

*Publisher:* Andre G. Wolff

*Acquisition Editor:* Andre G. Wolff

*Editorial Project Manager:* Barbara Makinster

*Production Project Manager:* Poulouse Joseph

*Cover Designer:* Greg Harris

Typeset by TNQ Technologies



# Zebrafish as a Model for Revealing the Neuronal Basis of Behavior\*

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## Introduction

While much of the original work using the zebrafish model in neuroscience was focused on developmental questions (Eaton, Farley et al., 1977; Eisen, 1991; Eisen, Pike, & Debu, 1989; Grunwald, Kimmel, Westerfield, Walker, & Streisinger, 1988; Kimmel, 1982; Kimmel, Sessions, & Kimmel, 1981; Myers, Eisen, & Westerfield, 1986; Streisinger, Coale, Taggart, Walker, & Grunwald, 1989), zebrafish offer major advantages for revealing how vertebrate brains produce behavior (Fetcho & Liu, 1998; Kimmel, Eaton, & Powell, 1980). This role for fish might not seem so obvious, but the advantages of small size, transparency, and genetic tools that lie at the heart of the power of the zebrafish model also catalyze its role in studies of brains and behavior. These studies are typically not directed toward understanding fish *per se*, but rather have as their goal the discovery of principles underlying brain function that apply to vertebrates broadly. This is possible because, to a first approximation, all vertebrate brains are the same (Butler & Hodos, 1996).

## Vertebrate Brains Have Much in Common Across Species

Nearly every region in the nervous system, with the notable exception of the multilayered cerebral cortex in mammals, is present in all vertebrates, including such important regions as the olfactory bulbs, retina,

telencephalon, hippocampus, amygdala, thalamus, hypothalamus, optic tectum, basal ganglia, hindbrain, cerebellum, spinal cord and the major dopaminergic, serotonergic, and noradrenergic neuromodulatory systems (Butler & Hodos, 1996). This is not too surprising because vertebrates evolved to do many of the same behaviors. They all use their senses (vision, hearing, smell, proprioception) to move about in coordinated ways to find food, water, shelter, and mates, and avoid becoming food. They learn about the external world through experience and store that information to produce adaptive behavior. While the execution (swimming vs. walking for example) might be different across animals, the brain regions and neural computations that process internal and external sensory information and produce appropriate outputs have much in common across species, allowing us to use zebrafish to inform us about how brains and spinal cords work across vertebrates generally.

## The Challenge of Understanding How Brains Generate Behavior

Our understanding of the generation of behavior by vertebrate brains is still in its infancy; it is one of the greatest remaining biological puzzles. To set the stage for the power of zebrafish and its place along the path to revealing brain function, a short account of what is needed to explain how any particular behavior is

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\*Supported in part by grants from the NIH (NINDS: F32-NS083099, F32-NS084654 and R01-NS026539).

produced by a brain will provide a useful context for discussion.

## Behavior

The challenge begins with a proper definition of the behavior of interest. It could be a relatively simple motor behavior, such as the escape behavior that fish use to avoid predators or a seemingly simple decision to turn to the left or right. On the other hand, the behavior might be much more complex, such as learned avoidance of dangerous places, navigation through a complex environment (3D for fish), or chasing down a prey item. In either case, the behavior must be studied rigorously to define how it is shaped by sensory information from the environment and prior experience, and the details of the movements that underlie it. These transformations from sensation to decision and action, and the influence of behavioral history (learning and memory) and internal state (hunger, fear, sexual, or general arousal, etc.), are what must be explained at the level of the neurons and circuits in the brain and spinal cord.

## Neurons

The next critical step is to reveal what neurons are involved by somehow monitoring their activity in the brain. Given that most brain regions are heterogeneous, with neurons of different function and connectivity, the ability to resolve the activity of individual cells is critical. Electrophysiological or imaging approaches are methods of choice. Electrophysiology, such as whole-cell patch recording from individual cells, can resolve the exact firing pattern of the neurons and even the synaptic inputs from other cell types. It also allows the detailed study of the electrical/ion channel properties of the cells, which influence how they respond to synaptic input from other neurons. The study of how these properties and circuit function can be modified by neuromodulators that can reshape activity without altering physical connections between neurons also demands electrophysiology (Lovett-Barron, Andalman et al., 2017). The limitations of electrophysiology are its invasiveness (surgical exposure and paralysis are typically needed) and an inability to record from more than a few neurons at once. Both of these limitations are overcome by using imaging, done most commonly with fluorescent calcium indicators, which change intensity when calcium levels in neurons rise (or fall) during activity. Here, the benefit of the transparency and small size of the larval fish is enormous, as large numbers of neurons, even deep in the brain, can be imaged with single-cell resolution to tie the activity of both individual neurons and neuronal populations to

behavior. The benefit, however, comes at the cost of temporal resolution, as the millisecond and submillisecond resolutions of electrophysiology are impossible to achieve with the slow kinetics of calcium sensors and their indirect ties to the actual electrical activity. Fortunately, zebrafish are accessible both to electrophysiological and imaging approaches (Fetcho & O'Malley, 1995; Legendre & Korn, 1995), so one can obtain both high temporal resolution and population-level information about the ties between active neurons and the behavior of interest.

## Wiring

The patterns of neuronal activity during a behavior are not sufficient to reveal how that behavior is produced by a brain because the brain uses connections/wiring between neurons to compute and transform external and internal information into appropriate action. The precise wiring is critical, although it sometimes does not get the attention it deserves. It is what defines the circuit! This is often missing from studies of zebrafish because it is one of the hardest bits of information to obtain. The most direct path to it is to record from pairs of neurons, then activate one and monitor the electrophysiological response in the other, to reveal the type (chemical, electrical, excitatory, inhibitory, neuromodulatory) of synapse, its strength, and the plasticity of the connection in response to different patterns of activation of the presynaptic cell. This pairwise (and even triple) recording can be done in zebrafish with optical targeting of electrodes to particular neurons (Koyama et al., 2016). It is hard, but feasible. Newer approaches to reveal connectivity combine optical activation of neurons with optical (or better, electrophysiological) monitoring of responses in potentially connected cells (Forster, Dal Maschio et al., 2017).

Another approach to connectivity that is just now reaching fruition in zebrafish is electron microscopic reconstruction of large regions of the brain, which reveals the pattern of connections of the neurons because both neuronal processes and their input and output synapses are visible at EM resolution. This does not provide direct functional information about connection strength or synaptic properties, but it is still critical information as it forms a ground plan for circuit wiring. Remarkably, there are still very few cases of any brain, in which, we know even how many output connections a single neuron has and how they are distributed because it is so difficult to obtain them with physiological methods. Still, this is critical for understanding circuit function and computations. EM provides this and can do so even for entire regions of the brain. In the small larval zebrafish, this opens access



to the baseline structural connectivity anywhere in the brain via whole-brain EM sectioning (Hildebrand et al., 2017; Wanner, Genoud, Masudi, Siksou, & Friedrich, 2016). The combination of light level imaging of structure, even at high resolution with expansion microscopy (Freifeld et al., 2017), and activity with EM level connectivity anywhere in the brain makes zebrafish unique among the vertebrates as a model for revealing how brains produce behavior.

## Models

The complexity of the wiring in brains, the varying electrical properties of different neurons and the multitude of interactions among cells makes understanding how the brain generates behavior a challenge, even with activity and wiring in hand. We typically cannot look at the data and intuit how computation works. This demands models, grounded in the data, that simulate the neuronal interactions, so that we can see whether the information we have captures what is needed to produce observed patterns of activity appropriate for driving behavior (Koyama et al., 2016; Naumann et al., 2016).

Importantly, such models should have predictive power, since understanding and explanation in science is synonymous with predictive power. Indeed, even brains themselves are mostly concerned with predicting what to do next. Tests of the predictions assess model quality. Zebrafish are especially amenable for testing predictions because the model predictions often take the form of what happens to the behavioral output (and activity of other cells in the circuit) when particular neurons are removed or activated or inactivated. The transparency of the zebrafish allows for very specific optical and genetic perturbations, including application of light-activated proteins for activating or silencing neurons, discussed in later sections (Douglass, Kraves, Deisseroth, Schier, & Engert, 2008; Kimura, Satou et al., 2013).

## The Goal

In the end, we can claim to understand the behavioral features under study when we have a model built upon known information about cell types, their electrical properties and activity, and their wiring that both produces the output seen during the behavior and that generates predictions that have been confirmed by empirical biological methods. The model need not include everything, and likely will not, as biological systems are complex at many levels. Predictions will eventually falter, but further biological studies will reveal critical features that are missing from the model

and can be added to expand its predictive power. The goal is the simplest model that explains what is known, has large predictive value, and hopefully reveals features of the computational algorithms used to transform internal and external sensation and stored information into adaptive actions.

## A History of Zebrafish as a Model for Understanding the Generation of Behavior

### The Beginning

All of the steps toward revealing the neuronal basis of behavior summarized in the previous sections become easier if one can simply look to the brain of an animal and see everything that is happening all at once. Larval zebrafish are now close to offering that, but reaching the required level of sophistication took several decades of effort starting with the genetic studies of George Streisinger in the late 1970s and early 1980s. Working nearly alone, so as not to risk the careers of his students and postdocs, Streisinger pioneered methods for the induction and study of mutations in the zebrafish genome using gamma rays (Chakrabarti, Streisinger, Singer, & Walker, 1983; Stahl, 1994). He also developed methods of inducing haploidy in the progeny of mutants, which facilitated the identification of recessive lethal mutations, as the haploid zygotes can survive for several days after fertilization (Kimmel, 1989; Patton & Zon, 2001; Streisinger et al., 1989; Streisinger, Walker, Dower, Knauber, & Singer, 1981). These pioneering genetic methods, along with *in vivo* imaging of zebrafish during development (Eisen, Myers, & Westerfield, 1986; Liu & Westerfield, 1988), initial studies of motor innervation and function by the Oregon group (Liu & Westerfield, 1988), and their generosity in helping others with the model, catalyzed large-scale screens for mutations affecting embryogenesis in zebrafish in Boston (USA) and Tübingen (Germany), which identified roughly 2000 genes necessary for normal development (Driever, Solnica-Krezel et al., 1996; Haffter, Granato et al., 1996).

### Mutagenesis and Behavior

Studies of genetic effects on behavior took a leap forward in zebrafish work because the big screens included behavior-based assays in the larvae that took advantage of the early development of motor behavior in order to identify mutations producing defects in motility and optomotor processing. In the screening by Granato et al (Granato, Van Eeden et al., 1996), mutagenized progeny were examined for motility defects in response to touch (which develops in normal embryos at around 24 h postfertilization). This screening

identified 166 mutations that resulted in motility defects and included mutations that affected muscle development, incapacitated proteins necessary for synaptic function at the neuromuscular junction or centrally, and genes necessary for correct wiring of the nervous system. Another interesting screen exploited the tendency of larval zebrafish to swim along and visually track moving gratings (termed the *optomotor response*). In an optomotor-based assay (Neuhauss, Biehlmaier et al., 1999), mutants from the Tübingen screen (450 lines) were exposed to a moving grating displayed on a monitor, and those that failed to accumulate at one end of a rectangular arena were examined for defects in visual anatomy and sensitivity. This screen identified 25 mutants, 13 of which exhibited retinal dystrophy (loss or degeneration of cell layers in the retina) and others that exhibited defects, such as lens degeneration, deficiency in the pigment melanin, miswiring of axons, and absence of retinal ganglion cells, among other defects.

### The Quantitative Study of Behavior in Larval Zebrafish

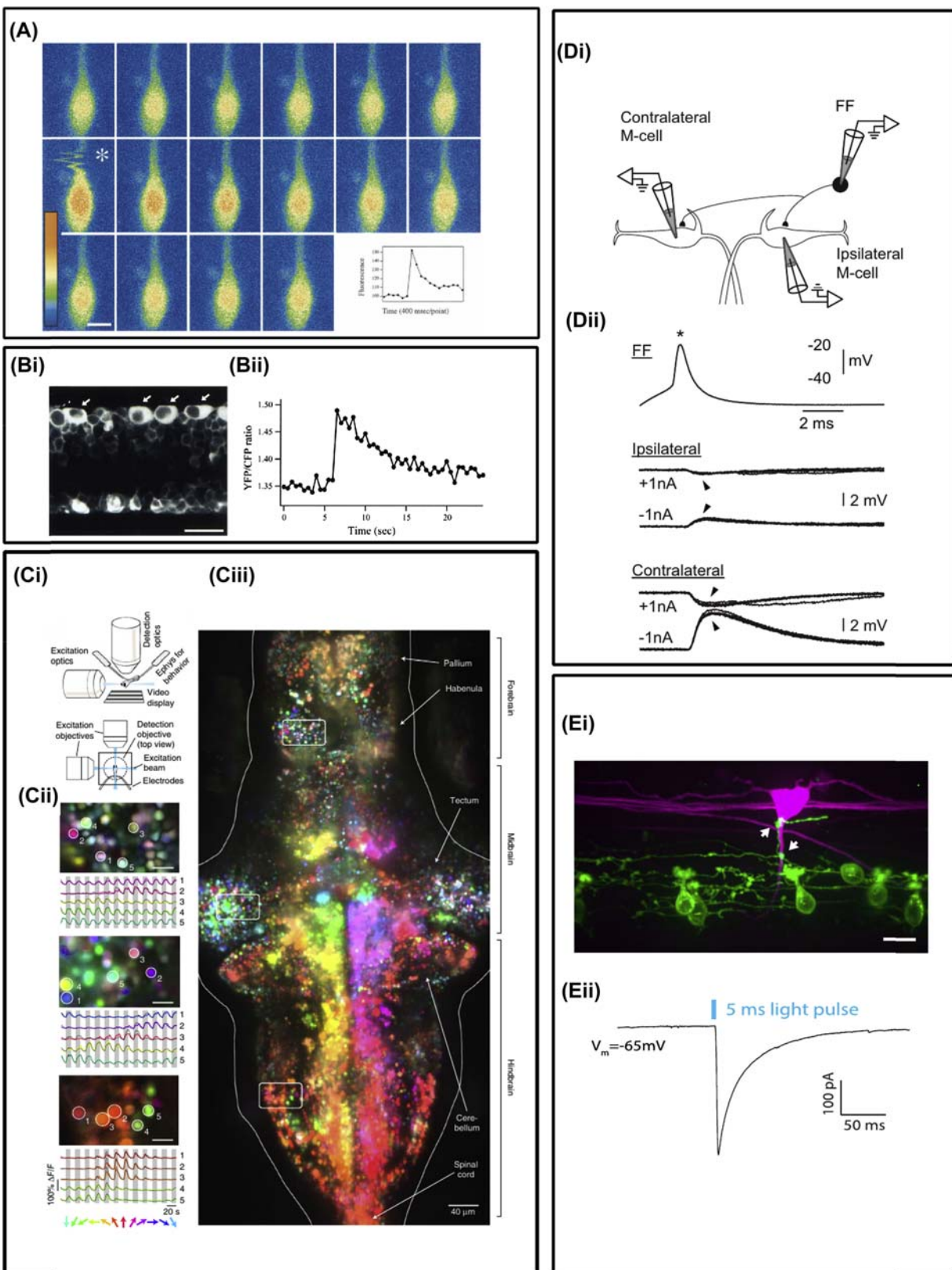
Investigations into the neuronal basis of behavior in normal and mutant zebrafish depend on a critical foundation in the careful quantitative analysis of the behaviors. Early on, scientists were concerned with the relationship between activity in the giant Mauthner cell and the escape behavior it was thought to initiate in teleost fish (including the zebrafish), and sought to relate the activity of this cell to the behavior (Eaton & Farley, 1975). Because the escape response (C-start) elicited by this cell is so fast, there were early technological limitations in being able to record the movement of the animal with sufficient temporal resolution. In a C-start, fish make a characteristic fast bend to one side (so that the animal resembles a C) before a weaker bend to the other side followed by lower amplitude bends as the animal swims away (Kimmel, Patterson, & Kimmel, 1974). Kimmel et al. (Kimmel et al., 1974) were able to reveal the latency of this rapid response by using a photoresistor to detect breaks in a beam of light. This method determined that a fast C-start response develops after 4 days postfertilization (dpf) in zebrafish, although slower responses were present after 3 dpf. The C-start has a latency of 15 ms in the larvae, with similar latencies (and general movement patterns) in adult animals. Advances in video technology allowed higher rates of image acquisition. Eaton et al. (Eaton, Farley et al., 1977) filmed larval escapes to a probe touch at 133 frames per second and were able to obtain more detailed information about the series of movements larvae produce during a C-start escape.

At 44–48 hpf and 88–95 hpf, analysis of these images revealed a response latency of 10.2 and 12.3 ms respectively. Video imaging of larvae at 1000 Hz with custom-written automated computer analysis (Liu & Fetcho, 1999), and more recently with software to analyze multiple animals simultaneously (Burgess & Granato, 2007), has refined this number even further; in response to an acoustic tone, 6 day old larvae produced either a short latency C-Start with a mean of 5.3 ms or a longer-latency C-start with similar movement kinematics as the fast one, but with a latency averaging 28.2 ms.

Although early behavioral studies focused on C-start escapes, the combination of high-speed imaging and detailed quantification they pioneered have provided the foundation for inquiries into circuits controlling other aspects of zebrafish behavior. Classification of slow and fast swimming behaviors, routine turns, and prey-capture behaviors by careful quantification of tail bend angles and frequencies, swim velocities, and other fin and eye movements, provided a valuable reference point for many studies of zebrafish neural circuits (Budick & O'Malley, 2000; Burgess & Granato, 2007).

### The Advent of *in vivo* Functional Imaging in Zebrafish

Taking advantage of the transparency of zebrafish, researchers took a key step toward understanding the behavioral function of neurons by performing the first imaging of neuronal activity of single cells in the brain and spinal cord of an intact vertebrate (Fetcho & O'Malley, 1995; O'Malley, Kao, & Fetcho, 1996) (Figure 46.1A). Neurons were filled by injection of dextran tagged synthetic calcium indicators that are picked up by neuronal processes into the muscle or spinal cord and the labeled neurons were imaged with confocal microscopy in restrained fish embedded in agar. These indicators increase their fluorescence as calcium levels rise in electrically active neurons. The early experiments revealed the potential to simply watch the activity of cells even deep in a vertebrate brain. However, it was important to develop methods for filling neurons with indicators that did not depend on damaging their processes to facilitate indicator uptake. This was done initially by injecting calcium indicator into single-cell embryos and raising them to larval stages, at which time, cells throughout the body, including brain and spinal cord, were filled with an indicator that could be used to detect neuronal activity (Cox & Fetcho, 1996). A better way was on the horizon, however, with the demonstration of the ability to make transgenic fish with the beautiful expression of fluorescent proteins (Higashijima, Hotta, & Okamoto, 2000; Higashijima, Okamoto, Ueno,



**FIGURE 46.1** (A) Calcium green-dextran backfilled Mauthner neuron exhibits a dramatic increase of fluorescence during an escape response (hotter colors represent higher image intensities, see color bar lower left). Each image is 400 ms apart, and the scale bar is 10 microns. Inset in bottom right indicates the level of fluorescence in each image. Adapted from (O'Malley et al., 1996). (B) Cameleon expressed throughout the nervous system, and in particular, Rohon-Beard (RB) sensory neurons in the spinal cord (left, arrows). The RB neurons exhibit an increase in the YFP/CFP ratio in response to an electrical stimulus applied at 5 s. Adapted from (Higashijima et al., 2003). (C) Whole-brain light-sheet imaging during visual adaptation to moving gratings. Larval fish was suspended over a video display (top left) while the motor responses were monitored from spinal ventral roots. Tuning of individual neurons was assessed by their response to gratings moving in a particular direction (traces one to five, see arrows on bottom) and each neuron was color coded by its preferred direction (see bottom arrows). Each box on the left is a magnified region from the image on the right, corresponding to habenula, tectum, and hindbrain. The preferred tuning of all imaged neurons during the course of the experiment is shown by the color map on the left. Adapted from (Freeman, Vladimirov et al., 2014). (D) A triple patch recording showing the activity of the ipsi and contralateral Mauthner neuron after depolarization of a feedforward interneuron. Adapted from (Koyama et al., 2016). (E) Chr2-mCherry (green) expressed in spinal cSF neurons and GFP expressed in a sensory-related CoPA interneuron (top). Targeting the cSF neurons with blue light (bottom) causes current to flow into the CoPA neuron due to synaptic contact between the cells (arrows top). Adapted from (Hubbard et al., 2016).



Hotta, & Eguchi, 1997; Long et al., 1997). In parallel, the first genetically encoded fluorescent calcium sensor was engineered by adding a calcium-binding domain to a fluorescent protein (Miyawaki, Llopis et al., 1997). Subsequently, zebrafish were the first vertebrates with a transgenic fluorescent indicator of neuronal activity expressed in all neurons (Higashijima, Masino, Mandel, & Fetcho, 2003) (Figure 46.1B).

The early genetically encoded calcium sensors worked in fish but had relatively small signals, so there was a lag in their adoption after an initial successful demonstration of their functionality (Higashijima et al., 2003). Most early works applied various synthetic indicators, although there was some application of the early genetic indicators by the Friedrich lab (Li, Mack et al., 2005), which also did important early imaging work of exposed portions of adult zebrafish brains (Friedrich & Korsching, 1997). This has changed recently with the optimization of indicators that can produce robust calcium transients, in the best situations detecting calcium transients from even single action potentials (Chen, Wardill et al., 2013; Nagai, Yamada, Tominaga, Ichikawa, & Miyawaki, 2004; Tian, Hires et al., 2009). Many of these are based on an approach, in which, a calcium-sensitive protein linker (calmodulin) is placed in a split fluorescent protein such that the conformation changes upon calcium binding in a way that partially reconstitutes the normal structure of the fluorescent protein to produce a fluorescence increase (Nagai, Sawano, Park, & Miyawaki, 2001). Optimization of these so-called GCaMPs over the last decade has generated a variety of indicators with varying time-constants, sensitivity to changes in calcium concentrations, and improved brightness (Chen, Wardill et al., 2013; Nagai et al., 2004; Tian, Hires et al., 2009). Improved indicators expressed under the original pan-neuronal promoter produce robust signals in neurons throughout the brain in larval fish (Ahrens, Li et al., 2012; Ahrens, Orger et al., 2013). These new indicators, along with other technological developments including very sensitive, fast cameras and fast laser-based imaging with sheets of light swept through the brain, has led to the recent imaging of activity of nearly every neuron in the larval brain at about two brains a second as a fish restrained in agar attempted to move (Ahrens, Orger et al., 2013) (Figure 46.1C). In this case, the movements themselves can be observed by freeing portions of the body, as done initially in early functional imaging experiments (Ritter, Bhatt, & Fetcho, 2001), or motor activity monitored by recording from motor nerves in paralyzed fish (Masino & Fetcho, 2005). Active neurons can then be mapped onto a zebrafish brain atlas (Randlett, Wee et al., 2015), or they can be monitored in fish with known neuronal subtypes

labeled in a different color than the calcium indicator to reveal more about the active neurons. Zebrafish is the only vertebrate in which simultaneous whole-brain imaging of neuronal activity with simultaneous behavioral monitoring is possible, after a history of innovations in the model.

Revealing circuits, however, depends on knowing more about the neurons than their activity. Fortunately, transgenic approaches have produced beautiful lines that label neurons with different neurotransmitter phenotypes and transcription factor identities (Bae, Kani et al., 2009; Higashijima, 2008; Kinkhabwala, Riley et al., 2011; Satou et al., 2013). Labeling fluorophores targeted to the membrane can also reveal the detailed projections of the cells (Forster, Arnold-Ammer et al., 2017; Pan, Freundlich et al., 2013). The combination of calcium imaging in one color with imaging of morphology or cell type markers in a different color provides the link between activity and some of the other key features that determine a neuron's role in a behavioral circuit.

This combination has been used in many ways, but one example is the combination of structural and functional imaging (Farrar, Kolkman, & Fetcho, 2018; Kimura, Satou et al., 2013; Kinkhabwala, Riley et al., 2011; Koyama, Kinkhabwala, Satou, Higashijima, & Fetcho, 2011) that revealed a striking pattern of alternating glycinergic and glutamatergic columns in the hindbrain, an organization that is less evident in adult vertebrates as neurons migrate (Higashijima, Schaefer, & Fetcho, 2004). This columnar patterning reflects a ground plan for circuit formation during hindbrain development. Mapping this ground plan in zebrafish revealed the transcription factors that define these columns, the morphologically different cell types localized to particular columns, the disposition of neurons for particular circuits within the columnar pattern, and the orderly recruitment of neurons during swimming by location/birth order in columns—patterns also evident in spinal cord (McLean, Fan, Higashijima, Hale, & Fetcho, 2007; McLean & Fetcho, 2009).

While much of the work with functional imaging has focused on calcium sensing, zebrafish offer the opportunity to look at fluorescently tagged synaptic markers *in vivo* (Chow, Zuchowski, & Fetcho, 2017; Niell, Meyer, & Smith, 2004), which has allowed studies of synapse formation during circuit construction in development (Niell et al., 2004), as well as the first *in vivo* imaging of the activity-dependent translocation of a kinase implicated in changes in synaptic strength to synaptic sites *in vivo* (Gleason et al., 2003). The possibilities for tying molecular dynamics to synaptic and circuit dynamics are enormous in zebrafish, but still largely untapped.

## Electrophysiology in Zebrafish

Electrophysiology provides high temporal resolution information about neuronal activity that is missed by the slower calcium imaging. The giant Mauthner cell and its role in the initiation of escape from predators in fishes and amphibians discussed earlier was the focus of early physiological studies in fish (Diamond, 1971; Faber & Korn, 1978). The escape response can be elicited experimentally by mechanical or acoustic stimuli, such as touch or water pressure waves (Eaton, Bombardieri et al., 1977; Eaton & Farley, 1975; Eaton, Nissanov, & Wieland, 1984; Kimmel et al., 1974). The M-cell is much larger than other neurons, and as such, produces a noticeable change in the electric field in and around the M-cell and, possibly, even outside of the fish when an action potential occurs. These can be recorded utilizing extracellular electrodes. Early on, such recordings, along with a high-speed video of escaping zebrafish larvae, revealed that the response latency in larvae was much the same as in adult animals (Eaton & Farley, 1975). The M-cell could be detected responding to stimuli as early as 40 hpf, but with increases in response amplitude and decreases in escape duration up to 100 hpf (Eaton, Farley et al., 1977).

While extracellular recordings are useful, they often do not allow identification of the neuron being monitored, can suffer from low signal-to-noise, and may not permit satisfactory discrimination between action potentials generated by nearby neurons. Other studies, initially in goldfish, used sharp electrodes inserted into individual cells (Faber, Fetcho, & Korn, 1989; Korn & Faber, 2005) to reveal circuit connections of identified neurons. Single-cell electrophysiology from identified neurons in larval zebrafish, however, reached fruition with the application of patch electrophysiology, which was already being widely used in other animals, especially mammals. This approach was critical because larval neurons are smaller than mammalian cells (many are 5–10  $\mu\text{m}$  in diameter) and difficult to record with sharp electrodes, but amenable to patch recording, in which the recording electrode (usually a hollow glass pipette tapered to a polished tip filled with a physiological solution, a few microns in diameter) seals onto a patch of membrane that is then ruptured to give electrical access to the inside of the neuron. The neuron can then also be filled with dye via the recording electrode to reveal its structure. The earliest patch physiology in zebrafish was performed by Legendre and Korn (1994, 1995) and was used to study the quantal nature of the synaptic release of glycine (an inhibitory neurotransmitter) onto M-cell, and the voltage-dependence of the glycine receptor channel conductance. Patch recording from spinal neurons followed later (Drapeau, Ali, Buss, & Saint-Amant, 1999). Extending physiological

work on the M-cell in zebrafish beyond studies of inhibitory conductances in larvae, Hatta and Korn used whole-cell patch recording to characterize the electrophysiological properties of the M-cell in adult zebrafish and compare its properties to the known ones of adult goldfish (Hatta & Korn, 1998). Physiological properties between the two species were very similar.

This early work in zebrafish larvae and adults, along with anatomical studies suggested that a class of interneurons called cranial relay neurons played a major role in Mauthner cell circuitry in zebrafish, as previously shown for goldfish (Faber et al., 1989; Korn & Faber, 2005). However, it would take simultaneous paired and triple neuron patch recordings a decade later to definitively show the circuit connectivity of cranial relay neurons and the M-cell in zebrafish larvae and to begin to reveal synaptic connectivity of neurons in the larval brain and spinal cord (Bhatt, McLean, Hale, & Fetcho, 2007; Koyama et al., 2011; Koyama et al., 2016; Satou, Kimura et al., 2009) (Figure 46.1D).

Whole-cell recordings allowed scientists to narrow down the defects present in some of the zebrafish mutants generated in the Tubingen screen. For example, a careful electrophysiological analysis of the *twitch once* mutant, whose body movements fatigue easily, revealed a previously undiscovered consequence of a mutation in the protein rapsyn, which was known to localize acetylcholine receptors on the surface of muscle fibers (Ono, Shcherbatko, Higashijima, Mandel, & Brehm, 2002). Such a mutation causes muscles to become less responsive to high-frequency stimulation, leading to quick fatigue in response to stimuli—an observation that presaged the identification of human patients with similar deficits. Similar physiology led to critical discoveries about synaptic transmission and the functional organization of nerve-muscle connections (Wang & Brehm, 2017; Wen et al., 2013; Wen, McGinley, Mandel, & Brehm, 2016).

Patch electrophysiology was also key to revealing the circuit properties of interneuron-motoneuron connections and network behavior in the spinal cord. Early work recording from single spinal motoneurons, red/white muscle fibers, and interneurons with characteristic morphology gave indications of the spinal motoneurons and interneurons active during locomotion in larvae (Drapeau et al., 1999; Saint-Amant & Drapeau, 2000) that complemented prior studies of motoneurons in adults (Liu & Westerfield, 1988). Paired recordings of a specific interneuron class (Chx10 CiDs) in the spinal cord and large primary motoneurons that innervate massive numbers of muscle fibers in a body segment revealed that the motoneurons receive excitatory input from this class (Bhatt et al., 2007). This circuit is active during fast swimming and escapes. However, during slow swimming, paired recordings (and functional

imaging of populations) revealed that a different class of commissural interneurons, the MCoDs, provide drive to the smaller secondary motoneurons that innervate fewer muscle fibers (McLean et al., 2007; McLean, Masino, Koh, Lindquist, & Fetcho, 2008; Ritter et al., 2001). Paired recordings between the CiDs and MCoDs reveal that when the CiD network is active, the MCoD network is inhibited (they are mutually exclusive in the activity), which revealed, as a general principle, that different interneuron networks are used in the spinal cord depending on locomotor speed and strength. The findings from these studies in larval zebrafish were later confirmed to operate across vertebrates moving at different speeds. Mutations of interneuron networks in both mice and horses walking, galloping or bounding revealed defects at certain speeds depending on which, interneurons were disrupted (Andersson, Larhammar et al., 2012; Crone, Zhong, Harris-Warrick, & Sharma, 2009; Kullander, Butt et al., 2003). Today, electrophysiological approaches continue to reveal with high temporal resolution the physiological activity of cells and the connectivity that underlies behavior.

### Circuit Perturbations

Zebrafish offer powerful ways to perturb neuronal activity to test ideas and formal models of the role of those neurons in behavior. Early perturbations in larvae used laser ablation of specific neurons, followed by behavioral testing. Calcium imaging of activity within the reticulospinal neurons in hindbrain suggested that several large reticulospinal neurons (a class of neurons in the hindbrain, which projects into the spinal cord), along with the Mauthner cells, contributed to the fast escape response (O'Malley et al., 1996). To demonstrate their respective contributions, Liu & Fetcho (Liu & Fetcho, 1999) used a focused excitation laser to kill calcium-dye loaded neurons via a phototoxicity effect. These experiments revealed that two non-Mauthner reticulospinal neurons were important, along with the Mauthner cell, in head-touch triggered escapes, whereas the Mauthner cell acted independently of them in tail-triggered escapes. The development of high-power long-wavelength lasers for multiphoton fluorescence imaging allowed for direct, specific ablation of neurons via heating effects, with the care necessary to avoid damaging the surrounding tissue. Even higher power lasers and multiphoton excitation can be used for ablation and cutting of processes *in vivo* with minimal heating (Koyama et al., 2016). Another method for cell ablations relies on introducing a nitroreductase enzyme into zebrafish neurons, which metabolizes the prodrug metronidazole into a cytotoxic compound (Curado et al., 2007). This method has the advantage

of being genetically targeted to specific cell types and can be used to ablate larger populations of neurons that would be more time-consuming to achieve using laser ablations. For example, nitroreductase mediated ablation of serotonergic dorsal raphe neurons was used to demonstrate their role in visual sensitivity (Yokogawa, Hannan, & Burgess, 2012).

Until relatively recently, stimulation during single-cell electrophysiology was the only method available for affecting the activity of a specific neuron without ablation or chemical reagents. This changed with the advent of the field of optogenetics, in which, light-sensitive ion channels (e.g., channelrhodopsin from the alga *Chlamydomonas reinhardtii*), genetically expressed by neurons, may be used to excite or inhibit cells when they are exposed to specific wavelengths of light (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005). This approach was easy to implement and rapidly adopted in neuroscience in many models. In the earliest published use of optogenetics in zebrafish, mechanosensory neurons in the trigeminal nucleus and spinal cord were transgenically modified to express channelrhodopsin, and exposure to blue light was sufficient to trigger an escape response in 24 hpf embryos (Douglass et al., 2008)—with optogenetic activation of mechanosensory neurons simulating a noxious touch stimulus. Understandably, optogenetics has revolutionized the field of neuroscience, with impact on zebrafish neuroscience as well. Since the earliest days, a wide variety of more effective optogenetic constructs have been engineered from the original ion channels, and new tools specifically for zebrafish are available (Forster, Dal Maschio et al., 2017).

Optogenetics may be used to reveal the behavioral consequences of circuit activation. For example, expression of ChR2 and halorhodopsin specifically in Chx10 (a transcription factor) positive hindbrain neurons was used to conclusively link their activity with the initiation of (and stopping, in the case of halorhodopsin) swimming in the animal (Kimura, Satou et al., 2013). In other work, activating subsets of a group of spinally projecting midbrain cells (the nucleus of the medial longitudinal fasciculus) elicited smooth tail bending in a direction-specific manner, implicating these neurons in the generation of body posture and steering (Thiele, Donovan, & Baier, 2014).

Another application of optogenetics in zebrafish is to fill or supplement a role traditionally occupied by paired-patch electrophysiology—demonstrating the connectedness of neurons in a neural circuit. A recent example comes from studies of the cerebrospinal fluid contacting neurons (CSF-cNs), which are now known to be a type of proprioceptive sensory neuron in the spinal cord that modulate bending movements



(Hubbard, Böhm, Prendergast, Tseng, Newman, Stokes, & et al., 2016). Hubbard and colleagues demonstrated that these neurons connect both to a specific type of primary motoneuron (the CaP, an individually identifiable cell present in each repeating segment of the tail) and a sensory interneuron by recording the motoneurons or interneurons with traditional electrophysiology while using patterned light to stimulate a subset of CSF-cNs expressing channelrhodopsin (Figure 46.1E). A definitive advantage of optogenetics over electrophysiology for neuronal activation studies is the ability to stimulate multiple individual neurons in a class at once, or in any sequence, in a non-invasive manner. This allowed the authors of this study to examine the convergence of input from the CSF-cNs to motoneurons.

Functional imaging and optogenetics applied in zebrafish have also included studies of eye movements, where imaging of activity revealed how a neuronal integrator was implemented at the population level (Miri et al., 2011)—a discovery that informed work on primates (Joshua & Lisberger, 2015). Subsequent work applied optogenetics to push the network dynamics of the brain from one regime to another in order to test mathematical models of network function (Gonçalves, Arrenberg, Hablitzel, Baier, & Machens, 2014). In that work, none of the tested models fully predicted the results, and the experiments led to the creation of a new model that had not been anticipated.

### Imaging and Perturbations in Freely Swimming Zebrafish

The vast majority of modern imaging and perturbation techniques only work well in restrained fish. However, there are a handful of methods developed for recording and perturbing neuronal activity in freely swimming zebrafish larvae. One system for recording the activity of unrestrained animals relies on bioluminescence using the jellyfish protein aequorin. Aequorin emits light upon a rise in calcium during neuronal activity in the absence of light stimulation, unlike fluorescent proteins (Naumann, Kampff, Prober, Schier, & Engert, 2010). Another approach conditionally converts a green fluorophore (CaMPARI) to red based on the activity level of neurons, when and only when they are in the presence of UV light. This allows scientists to reveal populations of neurons that were active specifically during a particular interval of a behavioral task at the time the UV light was applied (Fosque, Sun et al., 2015). Calcium responses can also be imaged in unrestrained fish at times when they are not moving (Muto & Kawakami, 2016), but imaging of cells in larvae while they are moving is a much greater challenge only now being attacked

(Kim et al., 2017). Finally, a number of perturbation approaches have been developed and used in animal models to genetically render neurons sensitive to chemical compounds to which they are normally insensitive. The compounds can then be applied to activate (or silence) specific sets of neurons. This methodology relies on introducing chemically activated ion channels under genetic control. For example, zebrafish neurons are not normally sensitive to capsaicin or to menthol but can be made sensitive by the introduction of ligand-gated ion channels from other species (Chen, Chiu, McArthur, Fetcho, & Prober, 2016). These approaches, along with recent technological developments to perform high-resolution fluorescence imaging in moving animals, provide a path to studies of neuronal function in freely behaving animals (Kim et al., 2017; Symvoulidis, Lauri et al., 2017).

### Advantages and Disadvantages of Zebrafish Compared to Other Model Organisms for the Study of the Neural Basis of Behavior

#### What is a Model Organism in the Context of Systems Neuroscience?

A model organism is one of a few species that have been chosen for extensive study by large communities of biologists. These few species typically: share a relatively short generation time, facilitating genetic approaches; share a wide range of available tools and techniques; and are amenable to genetic manipulation. The implicit assumption of studying model organisms is that the neurobiological principles gleaned from a small number of species will ultimately be widely applicable to many species (including humans) because of shared organizational features and shared behavioral problems that all species must solve. These are coupled with similar principles underlying the circuit level solutions of shared behavioral problems, such as finding food or mates, determining what is good or bad in the world, or making adaptive behavioral choices. Of course, much is also to be gleaned from studies of non-models with specialized behavioral abilities, ala Krogh's principle, which states "For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied," especially as genetic engineering technology moves toward powerful methods like CRISPR, which allows access to genetic manipulation and labeling in species more broadly (Albadri, Del Bene, & Revenu, 2017; Kimura, Hisano, Kawahara, & Higashijima, 2014; Krogh, 1929; Liu & Westerfield, 1988).



## Nematodes, Flies, and Mice Versus Zebrafish

The relative merits of zebrafish for behavioral work can best be appreciated through comparison with other model organisms used in neuroscience today. Here, we focus on nematode worms, fruit flies, zebrafish, and mice, due to their wide use and genetic accessibility.

The nematode roundworm *C. elegans* is quite small (about 1 mm in length) and exists in a male form and a hermaphrodite form. The development of the animal is highly stereotypical across individuals. As there are only 302 neurons in the adult animal, it was possible for developmental biologists to track the lineage of each cell from the single-cell stage (Sulston & Horvitz, 1977; Sulston, Schierenberg, White, & Thomson, 1983) and to identify specific cells across different individuals. Not only is every neuron individually identifiable, but the entire network of synaptic and electrical connectivity between neurons has been mapped with serial electron microscopy (White, Southgate, Thomson, & Brenner, 1986). *C. elegans* exhibits a number of interesting behaviors, such as movement toward sources of food, movement away from noxious stimuli, and mating behaviors (Bargmann, 1993). They are amenable to genetic manipulation, and all of the genetically expressed molecular tools known to work in zebrafish also work in *C. elegans*. Like the larval zebrafish, nematode worms are completely transparent, so it is possible to perform fluorescence microscopy anywhere in their bodies. However, the small scale of the animal and the internal pressurization needed to maintain its body integrity make electrophysiological recordings substantially more challenging than in fish (Goodman, Hall, Avery, & Lockery, 1998). Additionally, the neurons of the nematode worm typically function using graded potentials, unlike the action potentials utilized by most neurons in other animals (Lockery & Goodman, 2009). The main difference from other models, however, is that *C. elegans* manages to accomplish the challenges of survival and reproduction within its natural environment with many fewer neurons than more complex invertebrates and vertebrates. The distinction between nervous systems that solve behavioral problems with fewer neurons (as in *C. elegans*) versus many more neurons (as in zebrafish) gets to the heart of how neurons generate behavior.

In terms of nervous system complexity and behavioral repertoire, the fruit fly *Drosophila melanogaster* seems not wildly different from zebrafish. Larval zebrafish may exhibit less behavioral complexity than adult fruit flies because of the added requirements of flight and mating in the fly. Adult zebrafish, however, have additional behavioral complexity and cognitive abilities. Importantly, both free-swimming larval fish and adults must produce critical behaviors well enough for survival to

live to reproduce; in that sense, understanding the behavior in a larval animal is just as important as in adults, something that is also true for larval and adult flies. The adult fruit fly brain has roughly 135,000 neurons (Alivisatos et al., 2012), similar in amount to that of a larval zebrafish. Also, like zebrafish, fruit flies have sensory organs for vision, olfaction, taste, and touch/proprioception, although sensitivity to auditory stimuli may be more limited (Albert & Göpfert, 2015; Gillespie & Walker, 2001; Vosshall & Stocker, 2007). The wealth of genetic and molecular tools available to label these systems in *Drosophila* is better than those available for zebrafish.

Differences in the organization between vertebrate and invertebrate brains provide some unique advantages (and disadvantages) for *Drosophila*. Invertebrate neurons may rely less on using large numbers of neurons to drive behavior (via what is called population coding) than vertebrate ones (Pearson, 1993), and as a result, smaller subsets of neurons may have a similar genetic and functional identity in *Drosophila*. It has been possible to isolate small populations of interneurons—in some cases, single interneurons—genetically in the fly (Fischbach & Dittrich, 1989; Jenett, Rubin, Ngo, Shepherd, Murphy, Dionne, & et al., 2012). Single neurons with big behavioral effects are more common in insects, such as *Drosophila*, than in vertebrates, with the notable exception of the Mauthner cell. However, differences between the fly brain and the brain of vertebrates also lead to disadvantages in flies. The small size of the neurons and axonal and dendritic processes distant from the cell body make electrophysiological recording of the relevant activity of the neurons (which may occur in fine processes of tiny cells) more challenging in flies, although this is also an issue in many larval zebrafish neurons. Even calcium imaging data may have some unresolvable ambiguity, as active regeneration of voltage changes happening in neuronal processes may not be reflected in the calcium levels measured at the cell body. Furthermore, insights gained from studying neural circuits in zebrafish are more likely to apply to the more closely related humans than work in flies, if the goal is simply to understand humans rather than reveal principles of the biological organization more generally. The fruit fly is not as optically transparent as larval fish. Fruit flies also pupate between maggot stage and adults, so it is difficult to follow the development of neural circuits in the same individuals. In contrast, the development and formation of neural circuits are accessible at any stage in larval zebrafish because of its transparency.

Mice are the most popular model organism for systems neuroscience because of their close phylogenetic relationship with humans. Mice, unlike zebrafish, possess a neocortex, which is the subject of many studies in systems neuroscience because of its role in complex

behavioral tasks. Mice do exhibit cognitively sophisticated behaviors that do not exist in zebrafish, such as acoustic communication, social bonding, and caring for young (although there are fish that have some of these) (Bass & Baker, 1990). Despite relatively long generation times compared to flies and worms, there are sophisticated molecular tools available to mouse researchers that do not have close analogs in other model systems. For example, it is possible to introduce transgenic constructs into the brains of mice by applying viruses that infect particular types of neurons (Warden, Selimbeyoglu et al., 2012). Other synaptic crossing viruses, such as rabies virus, have been engineered to jump from neuron to neuron, tracing out the circuit of connected cells involved in a particular behavior (Callaway & Luo, 2015). Furthermore, mice can be trained to perform complex tasks and conditioned to fear certain stimuli, and in this way, have been fundamental in developing theories of memory formation and learning at a network level (Betley, Xu et al., 2015; Harvey, Collman, Dombeck, & Tank, 2009; Redondo et al., 2014). Because of their young age, larval zebrafish seem to have a lesser capacity for learning in some, probably less biologically relevant tasks. These differences may be mitigated by using adult zebrafish for studies of learning and memory, where appropriate. The large scale of the mouse brain also provides some advantages for optogenetics. Because it is so large, it is easier to excite neurons using light in freely moving mice because mini-microscopes can be mounted on their heads. For the most part, however, the large, opaque brain and the skull are a disadvantage for optical imaging.

In sum, each model, as one might expect, has strengths and weaknesses. The special advantages for studying the neuronal basis of behavior in zebrafish are optical and electrophysiological access to neurons anywhere (and even everywhere at once, using optical whole-brain imaging) in an intact vertebrate brain in an animal model with established tools to genetically target neurons.

### Major Avenues of Investigation in Zebrafish With a Couple of Case Studies

Because of its unique advantages, the zebrafish serves as a model system for the study of many neural circuits and behaviors. Zebrafish have been used to study a variety of visual behaviors (Helmbrecht, Dal Maschio, Donovan, Koutsouli, & Baier, 2018; Portugues & Engert, 2009), including the optokinetic (Beck, Gilland, Tank, & Baker, 2004; Chen, Bockisch et al., 2014; Emran et al., 2007; Kubo et al., 2014; Portugues, Feierstein, Engert, & Orger, 2014; Schoonheim, Arrenberg, Del Bene, & Baier, 2010) and optomotor (Ahrens, Huang et al., 2013;

Maaswinkel & Li, 2003; Naumann et al., 2016; Portugues, Haesemeyer, Blum, & Engert, 2015; Quirin et al., 2016) responses to visual motion. Indeed, these visual responses have been used as behavioral assays in mutagenesis screens to identify genes involved in visual system development and function (Brockerhoff et al., 1995; Muto, Orger et al., 2005; Neuhauss, 2003). Zebrafish also serve as a model of gaze (Beck et al., 2004; Bianco, Ma et al., 2012; Easter & Nicola, 1997; Greaney, Privorotskiy, D'Elia, & Schoppik, 2017; Mo, Chen et al., 2010) and postural stabilization (Ehrlich & Schoppik, 2017; Hubbard et al., 2016; Migault, van der Plas et al., 2018; Roberts, Elsner, & Bagnall, 2017; Semmelhack et al., 2014), as well as spatial navigation strategies, such as phototaxis (Ahrens, Huang et al., 2013; Burgess, Schoch, & Granato, 2010; Guggiana-Nilo & Engert, 2016; Horstick, Bayley, Sinclair, & Burgess, 2017; Lee, Ferrari, Vallortigara, & Sovrano, 2015). The olfactory system also plays critical behavioral roles in both larval and adult fish (Friedrich & Korsching, 1997; Li, Mack et al., 2005; Yaksi, von Saint Paul, Niessing, Bunschuh, & Friedrich, 2009). Considerable work has focused on motor behaviors and their control by the brain, including swimming (Ahrens, Li et al., 2012; Bagnall & McLean, 2014; Bhatt et al., 2007; Bianco, Kampff, & Engert, 2011; Borla, Palecek, Budick, & O'Malley, 2002; Budick & O'Malley, 2000; Burgess & Granato, 2007; Fidelin, Djenoune et al., 2015; Gahtan, Tanger, & Baier, 2005; Granato, Van Eeden et al., 1996; Hubbard et al., 2016; Kimura, Satou et al., 2013; Kinkhabwala, Riley et al., 2011; Liu & Westerfield, 1988; McLean & Fetcho, 2009; McLean et al., 2007; McLean et al., 2008; Menelaou, VanDunk, & McLean, 2014; Montgomery, Wiggin, Rivera-Perez, Lillesaar, & Masino, 2016; Mu, Li, Zhang, & Du, 2012; Patterson, Abraham, MacIver, & McLean, 2013; Portugues et al., 2015; Ritter et al., 2001; Sankrithi & O'Malley, 2010; Satou, Kimura et al., 2009; Thiele et al., 2014; Trivedi & Bollmann, 2013; Warp, Agarwal et al., 2012; Wiggin, Peck, & Masino, 2014; Wyart, Del Bene et al., 2009), escape movements (Burgess & Granato, 2007; Dunn, Gebhardt et al., 2016; Eaton & Emberley, 1991; Eaton & Farley, 1975; Eaton et al., 1984; Eaton, Bombardieri et al., 1977; Eaton, Lavender, & Wieland, 1982; Fetcho & O'Malley, 1995; Kimmel et al., 1980; Kinkhabwala, Riley et al., 2011; Korn & Faber, 2005; Koyama et al., 2011; Koyama et al., 2016; Lacoste, Schoppik et al., 2015; Lambert, Bonkowsky, & Masino, 2012; Liu & Fetcho, 1999; Liu, Bailey, & Hale, 2012; McLean et al., 2007; Mu et al., 2012; O'Malley et al., 1996; Prugh, Kimmel, & Metcalfe, 1982; Pujala & Koyama 2019; Ritter et al., 2001; Satou, Kimura et al., 2009; Takahashi, Narushima, & Oda, 2002; Temizer, Donovan, Baier, & Semmelhack, 2015; Thorsen & Hale, 2005; Thorsen & Hale, 2007; Thorsen, Cassidy, & Hale, 2004; Trivedi &

Bollmann, 2013; Yao, Li et al., 2016), fin movements (Green & Hale, 2012; Green, Ho, & Hale, 2011; Hale, 2014; Hale, Katz, Peek, & Fremont, 2016), opercular movements (McArthur & Fetcho, 2017) and rheotaxis (Haehnel-Taguchi, Akanyeti, & Liao, 2014; Levi, Akanyeti, Ballo, & Liao, 2015; Liao & Haehnel, 2012; Olszewski, Haehnel, Taguchi, & Liao, 2012; Oteiza, Odstrcil, Lauder, Portugues, & Engert, 2017).

In the realm of more complex behaviors, zebrafish exhibit fear responses and learned fear conditioning (Agetsuma, Aizawa et al., 2010; Amo, Fredes et al., 2014; Duboue, Hong, Eldred, & Halpern, 2017; Okamoto, Agetsuma, & Aizawa, 2012), which can be used to study neural circuitry related to anxiety and associative learning. Zebrafish also show typical circadian activity patterns and have been used to study circuit mechanisms—and consequences—of vertebrate sleep (Gandhi, Mosser, Oikonomou, & Prober, 2015; Kaslin, Nystedt, Ostergard, Peitsaro, & Panula, 2004; Oikonomou & Prober, 2017; Prober, Rihel, Onah, Sung, & Schier, 2006; Yokogawa, Marin et al., 2007; Zhdanova, Wang, Leclair, & Danilova, 2001). Zebrafish have complex social behaviors like shoaling (Buske & Gerlai, 2012; Canzian, Fontana, Quadros, & Rosemberg, 2017; Hinz & de Polavieja, 2017; Saverino & Gerlai, 2008), which have been used as behavioral assays to study the developmental impact of early ethanol and nicotine exposure (Buske & Gerlai, 2011; Fernandes, Rampersad, & Gerlai, 2015; Miller, Greene, Dydinski, & Gerlai, 2013). These are a just sample of behavioral abilities - efforts are underway to categorize the many behaviors in this model (Cachat, Stewart et al., 2011; Kalueff, Gebhardt et al., 2013; Mu et al., 2019).

To illustrate how zebrafish and associated technologies have been used to investigate the neural basis of behavior, we present two case studies in which this animal model's unique advantages have been used to advance our understanding of sensorimotor processing. In each case, the scientific progression parallels (and depends upon) the course of methodological innovation. Furthermore, these two cases follow similar scientific trajectories: initial characterization of stereotyped behavior, followed by efforts to reveal the identity and activity patterns of the neurons involved, with subsequent utilization of that basic understanding to ask even more diverse and sophisticated questions. Regarding each type of behavior, we outline major findings about its neural underpinnings and suggest fundamental principles derived from these discoveries.

## Short-Latency Escapes

*Behavior in goldfish:* Like many animals, fish respond to sudden aversive stimuli with a startle response—a

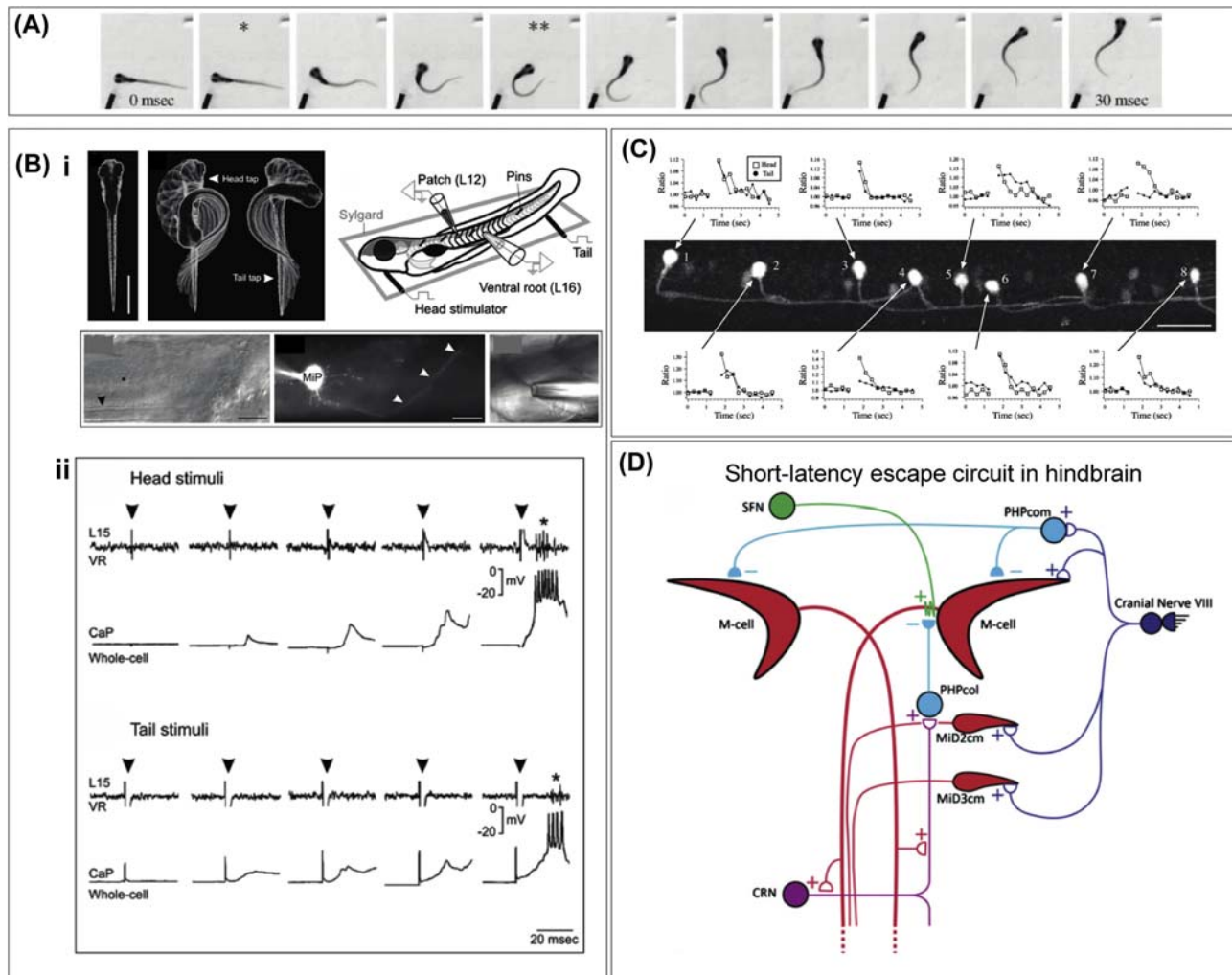
quick, decisive maneuver that orients the animal away from a potentially threatening stimulus (Eaton & Emberley, 1991; Hale et al., 2016). An aversive visual, auditory, or tactile stimulus evokes a short-latency escape maneuver, typically comprised of a sharp C-shaped turn followed by a rapid acceleration away from the threat (Eaton & Emberley, 1991). This behavior was initially well-characterized in adult goldfish, and early work in goldfish identified one of the neurons responsible for driving it: the Mauthner cell (M-cell).

*Identifying premotor neurons in goldfish:* In normal fish, there is one Mauthner cell on each side of the hindbrain. This specialized reticulospinal neuron receives multi-sensory input onto its prominent ventral and lateral dendrites, and projects directly to primary motor neurons in the contralateral spinal cord (Korn & Faber, 2005; Zottoli & Faber, 2000). Electrophysiological recordings in goldfish demonstrated that the M-cell fires a single action potential in response to an aversive stimulus, which drives contraction of the body and tail muscles on the contralateral side (Fetcho & Faber, 1988; Prugh et al., 1982)—evoking the short-latency turn away from the threat. However, following M-cell ablation, goldfish could still generate some short-latency escapes (Eaton et al., 1982), indicating that other neurons must also be involved. Further, anatomical studies suggested additional candidates: two M-cell segmental homologs, MiD2 and MiD3 (Lee & Eaton, 1991; Lee, Eaton, & Zottoli, 1993).

*Identifying premotor neurons in zebrafish:* Like goldfish, larval zebrafish execute short-latency escapes away from threatening stimuli (Figure 46.2A). However, larval zebrafish provided better *in-vivo* accessibility than goldfish (in terms of size and optical transparency) to facilitate further progress in our understanding of escape circuitry. Using backfills with dextran-conjugated calcium indicators to monitor neuronal activity in larval zebrafish, O'Malley et al. (1996) demonstrated that the M-cell and its homologs (MiD2 and MiD3) are active during escapes in response to tactile stimuli to the head, whereas the M-cell alone is active during escapes in response to tail stimulation. This supported a prediction made by Foreman and Eaton (1993) that additional reticulospinal neurons participate along with the M-cell in driving stronger head-evoked escapes (vs. weaker, M-cell-dependent tail-evoked escapes). Further, by refining a non-invasive method for laser ablation of single neurons, Liu and Fetcho (1999) observed that short-latency escapes to head stimuli were robust to M-cell loss alone but were abolished by ablation of the M-cell along with MiD2 and MiD3 reticulospinal neurons—consistent with the original hypothesis.

*Interneurons—action selection:* The M-cell and its homologs provide the shortest synaptic pathway from an aversive sensory input to an escape behavior, but





**FIGURE 46.2** Early advances in the neural basis of short-latency escapes in larval zebrafish. (A) Example of an escape response to a unilateral water pulse delivered to the head. The single-asterisk marks the onset of the response; the double-asterisk marks the frame of the maximal C-bend. Images were collected at 1000 frames/s, and every third frame is shown. Modified from [Liu and Fetcho \(1999\)](#). (B) (i) Top left: Dorsal view of a larval zebrafish (4 dpf) and the kinematics of escape responses to head and tail stimuli, each compiled from frames captured at 1000 frames/s. Note the larger bend in response to a head stimulus. Scale bar = 1 mm. Top right: Schema showing experimental preparation used for whole-cell recordings from the spinal cord, with simultaneous extracellular recordings of tail motor activity. Bottom: DIC (left) and fluorescent (middle) views of a MiP motor neuron in the spinal cord, targeted for whole-cell recording and filled with dye. White arrows mark the MiP axon. Scale bars = 20  $\mu$ m (right) DIC view of extracellular ventral root recording. (ii) Whole-cell recordings from a CaP motor neuron during electrical stimuli of increasing intensity, applied to the head or tail. Simultaneous recordings from the ventral root (VR) are also shown. Black arrows mark truncated stimulation artifacts. Modified from [Bhatt et al. \(2007\)](#). (C) Calcium activity from an array of CiD interneurons in the spinal cord during escapes elicited by head versus tail stimuli. The y axis is the ratio of calcium green to Alexa Fluor 647 divided by the mean ratio from 10 frames collected prestimulus. The escape movement occurs during the gap in the plot, when the cells move briefly out of the focal plane. Note that head and tail stimuli evoke responses in largely the same set of CiDs, consistent with changes in response amplitude being mediated by changes in the magnitude of interneuron activity—rather than the addition of more active neurons. Modified from [Bhatt et al. \(2007\)](#). (D) Summary of short-latency escape circuitry in the zebrafish hindbrain. Modified from [Hale et al. \(2016\)](#).

researchers had reason to suspect that other neuronal populations optimize the functionality of the escape and further shape motor output. For example, the M-cell fires a single action potential, an all-or-none response ([Nissanov, Eaton, & Didomenico, 1990](#))—but the details of the motor output (though relatively stereotyped) vary based on the parameters of the stimulus. Further, early calcium imaging experiments revealed the influence of inhibitory networks in shaping M-cell

activation ([Takahashi et al., 2002](#)). In addition, calcium imaging in zebrafish spinal cord indicated that both primary and secondary motor neurons in the tail respond strongly during escapes ([Fetcho & O'Malley, 1995](#))—although the M-cell only provides direct input to the primary motoneurons. Thus, interneurons must be involved, at a minimum, in modulating motor output and distributing motor commands. Once again, neuronal candidates were identified based on



morphology (Hale, Ritter, & Fetcho, 2001) and backfilled with calcium indicator and imaged during behavior (Ritter et al., 2001). Two spinal interneuron subtypes (the ipsilateral descending CiD and the contralateral descending MCoD) were investigated, and Ritter et al. (2001) discovered that their activity is behavior-specific: CiDs are active during escapes but not spontaneous swims, and MCoDs are active during spontaneous swims but not escapes. This study provided evidence for an additional participant in the escape circuit, but it also demonstrated that different interneuronal populations can be deployed to generate distinct behaviors. Following the development of a fictive swimming preparation in paralyzed zebrafish (Masino & Fetcho, 2005), researchers used whole-cell patch-clamp recordings (shown for motor neurons in Figure 46.2B) to show that individual CiDs (active during escapes) increase their firing rate during stronger escape maneuvers (Bhatt et al., 2007). In the same study, simultaneous calcium imaging from multiple CiDs showed that stronger escapes did not recruit additional CiDs from the population (Figure 46.2C). These experiments, taken together, are consistent with increased motor output strength carried out by modulation of active interneurons' firing rates, rather than changes in the size of the active population.

*Interneurons—visual stimulus selectivity:* Since these early studies, the widespread adoption of classical approaches (such as electrophysiology) and the advent of transgenic methodologies for use in larval zebrafish (Higashijima et al., 2000)—with the subsequent explosion in the number and variety of available transgenic lines—has spurred further progress in our understanding of the neural circuitry underlying short-latency escapes. For example, the use of genetically expressed calcium indicators enabled deeper investigations of the sensory side of the escape behavior—including the responses of tactile sensory neurons (Higashijima et al., 2003) and of the visual circuits involved in generating an escape response to looming stimuli (Dunn, Gebhardt et al., 2016). Looming stimuli elicit C-bend escapes that are abolished in mutants lacking retinal ganglion cells and impaired in larvae with unilateral ablations of tectal neuropil (Temizer et al., 2015). Two-photon calcium imaging in restrained larvae identified looming-specific regions of tectum that might participate in these escapes (Dunn, Gebhardt et al., 2016; Temizer et al., 2015). A study from Yao, Li et al. (2016) used an impressive variety of approaches—including electrophysiology, pharmacology, and optogenetics—to reveal how dopaminergic inputs can act (via modulation of inhibitory glycinergic interneurons) to increase the specificity of visually evoked escapes by facilitating M-cell responses to looming stimuli and suppressing escape responses to other visual stimuli.

*Interneurons—auditory/vestibular and tactile stimulus selectivity:* Other studies have investigated the role of hindbrain interneurons in ensuring optimal function of the escape circuit. For example, spiral fiber neurons receive contralateral sensory inputs and wrap their axons around the axon hillock of the contralateral M-cell (Lacoste, Schoppik et al., 2015), where they form excitatory electrical and chemical synapses (Koyama et al., 2011). Based on studies of mutant fish lacking the hindbrain commissure formed by their axons (Lorent, Liu, Fetcho, & Granato, 2001), spiral fiber neurons have long been thought to play a role in short-latency escape circuitry. Lacoste, Schoppik et al. (2015) proposed that this indirect interneuron pathway ensures that brief, weak stimuli do not evoke strong short-latency escapes—consistent with their optogenetic experiments demonstrating that coincident activation of spiral fiber neurons enhances M-cell responses (and short-latency escape behavior) to weak stimuli or sensory noise. Thus, under ethological conditions, weak dendritic inputs will only elicit an M-cell response if they also excite the spiral fiber neurons (and persist long enough for the direct and indirect excitation to overlap in time).

*Interneurons—laterality:* Koyama et al. (2016) investigated another example of an interneuron motif that optimizes escape circuit function, focusing on feedforward inhibitory neurons located near the M-cell. These neurons receive ipsilateral sensory input and project to both the ipsilateral and contralateral M-cells (Korn & Faber, 1975)—though, importantly, each inhibits the contralateral M-cell more strongly (Koyama et al., 2016). In a study combining electrophysiology, calcium imaging, modeling, and behavior, Koyama et al. (2016) showed how these feedforward inhibitory neurons enhance short-latency escapes by ensuring a quick, lateralized response. Reciprocal inhibition of M-cells (and other feedforward inhibitory neurons) ensures that left and right sensory inputs compete to ultimately produce a strong movement away from a threat, even in the presence of ambiguous stimuli. This work provides a circuit-level account of how a simple, left/right decision is implemented in the brain (see also (Shimazaki, Tanimoto, Oda, & Higashijima, 2018) for a recent careful look at another inhibitory control mechanism in the M-cell network).

*Circuit development:* Because we understand something about the various interneurons participating in escapes, this circuit was used as a test case to determine if hindbrain interneurons are recruited into specific circuits in an orderly way, based on their time of differentiation and positioning during early development. In larval zebrafish, hindbrain interneurons are organized into stripes according to their expression of specific transcription factors (Kinkhabwala, Riley et al., 2011). The neurons in each stripe share the same neurotransmitter

identity and gross axonal morphology. Further, neurons are organized within a stripe according to relative age, with older neurons typically located in the most ventral portion of the stripe. Importantly, the dorsoventral position also correlates with neuronal excitability, suggesting that a neuron's position in a transcription factor stripe might predict its functional role in a motor circuit. Conversely, if you understand the functional role of a specific interneuron, you should be able to predict its location—which stripe it inhabits, as well as its dorsoventral position. Indeed, [Koyama et al. \(2011\)](#) used patch-clamp recordings and cell morphology to confirm this hypothesis for the interneuronal components of the M-cell escape circuit, including the spiral fiber neurons and feedforward inhibitory neurons.

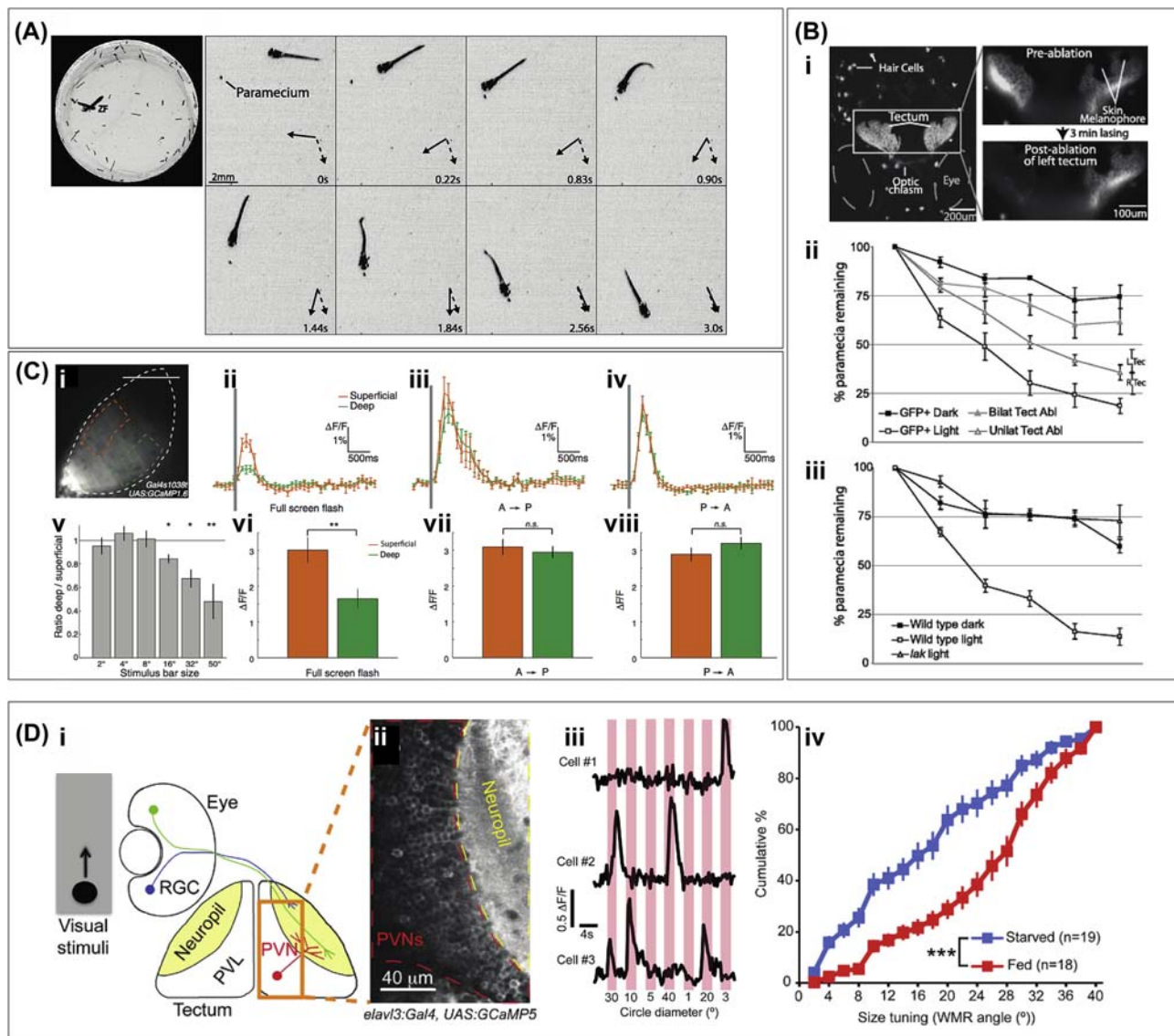
*Conclusion:* Short-latency escape behavior in larval zebrafish has been used to establish basic principles of sensorimotor control, to reveal interneuron circuit motifs that support optimal behavioral output, including one used to implement two-alternative decisions, and to facilitate our understanding of how circuits recruit specific neurons during development (circuit summary in [Figure 46.2D](#)). Further, the pursuit of these objectives propelled many of the methodological innovations that make larval zebrafish such an attractive model for neurobiological study.

## Prey Capture

*Behavior:* Soon after they acquire the ability to swim, larval zebrafish begin foraging for food and pursuing small prey, such as paramecia ([Muto & Kawakami, 2013](#)). Like short-latency escapes, prey capture maneuvers involve both sensory and motor processing—to select and transform appropriate sensory input into patterned muscle activity. An episode of prey capture behavior includes a series of slow swims and small-angle turns by which the larva approaches and re-orientes itself relative to the prey, followed by a strike to engulf the prey ([Budick & O'Malley, 2000](#); [Borla et al., 2002](#); [Patterson et al., 2013](#)). Using a closed-loop virtual reality system to simulate prey capture in restrained larvae, researchers showed that the sequence of orienting turns (J-turns) is always preceded by binocular convergence of the eyes—not to direct the fish's gaze toward the prey, but to create an area of binocular overlap in front of the fish that facilitates prey tracking and capture ([Bianco et al., 2011](#); [Trivedi & Bollmann, 2013](#)). In addition, studies in juvenile and adult zebrafish indicate that prey capture behaviors become more flexible and effective during development, associated with a transition from separate J-turns and approach swims to a single complex maneuver merging orientation and approach ([Westphal & O'Malley, 2013](#)).

*Identifying premotor neurons:* Early studies found that M-cell ablations did not affect prey capture ([Borla et al., 2002](#)), so researchers began investigating other reticulospinal neurons that might be involved in generating prey capture maneuvers. Because the behavior relies heavily on vision—and laser ablation of the retinotectal neuropil causes a prey capture deficit ([Gahtan et al., 2005](#))—candidate reticulospinal neurons would need to receive visual input (directly or indirectly) from the tectum. In zebrafish, the nucleus of the medial longitudinal fasciculus (nMLF) includes two neurons (per side: MeLr and MeLc) that both extend dendrites into the deep output layers of the optic tectum and project to the spinal cord ([Gahtan et al., 2005](#); [Sankrithi & O'Malley, 2010](#)). As assessed by a feeding assay, laser ablation of MeLr and MeLc decreases the number of successful prey captures without disrupting other motor behaviors. Further, a combined unilateral ablation strategy provided evidence that the tectum and the nMLF are part of the same pathway in the prey capture circuit ([Gahtan et al., 2005](#)). Thus, detection of visual prey stimuli by the optic tectum likely activates neurons in the nMLF to initiate or sustain prey capture maneuvers. Indeed, optogenetic activation of the anterior tectum can initiate J-turns ([Fajardo, Zhu, & Friedrich, 2013](#)). Consistent with these results, calcium imaging in freely swimming larvae indicates that anterior tectal activity precedes prey capture maneuvers ([Muto, Ohkura, Abe, Nakai, & Kawakami, 2013](#)), and quantitative analyses of tectal activity in restrained fish indicate that some neurons contain information about both sensory input and motor output ([Bianco & Engert, 2015](#)).

*Sensory processing—size selectivity:* In executing prey capture behaviors, it is important that zebrafish correctly identify potential prey. Larvae must discriminate between a small moving object (which might be suitable prey and should be approached) and a large moving object (which might be a predator and should be avoided). Thus, there should be some mechanism in the sensory circuitry for discriminating between small and large visual stimuli. Using genetically expressed calcium indicators, researchers discovered a region in the deep layers of optic tectum containing projection neurons that are more responsive to small stimuli than to large stimuli ([Del Bene, Wyart et al., 2010](#)). Using pharmacology, laser ablations, and transgenic strategies for blocking synaptic transmission in specific neuronal populations, researchers concluded that tectal inhibitory interneurons are necessary for establishing size filtering in deep layers of tectum—and that interfering with these microcircuits eliminates those projection neurons' size tuning ([Barker & Baier, 2013](#)). However, it is worth noting that other studies using calcium imaging have found evidence for prey stimulus selectivity in the axons of some retinal ganglion cells ([Semmelhack et al., 2014](#)),



**FIGURE 46.3** Advances in the neural basis of prey capture behavior in larval zebrafish. (A) Example of pursuit and capture of a paramecium by a zebrafish larva. Time projection is shown in the upper left panel, illustrating the travel paths of individual paramecia during 3.3s of imaging (200 frames total, imaged at 60Hz). Remaining panels show a single prey capture sequence (3s total), with frames chosen to highlight sequence components: approach swims (0–0.83s), pursuit swims (1.44–2.56s), and the final capture swim (2.56–3.0s). Dashed arrows indicate the heading of the paramecium, and solid arrows indicate the heading of the larva. Note that as the zebrafish pursues its prey, it makes orienting turns that bring its heading into alignment with the path of the paramecium. Adapted from [Gahtan et al. \(2005\)](#). (B) Prey capture relies on visual processing in the optic tectum. (i) Laser ablations were used to eliminate the retinotectal visual pathway. (ii) Prey capture behavior was evaluated by quantifying the fraction of available paramecia remaining over the course of 5h total feeding time. Note that darkness severely disrupts prey capture, and tectal lesions also cause dramatic impairment. (iii) Blind *lakritz* mutants normally cannot hunt, providing further evidence for the importance of visual input. Adapted from [Gahtan et al. \(2005\)](#). (C) Deep layers of the optic tectum neuropil exhibit selectivity for small, prey-like stimuli. (i) Regions of interest highlight superficial (orange) and deep (green) layers of tectal neuropil. (ii-iv) Calcium responses of tectal neuropil to three types of visual stimuli. (v) Ratios of maximum responses in deep and superficial tectal neuropil layers to bars of increasing width. (vi-viii) Average maximum responses of deep and superficial neuropil layers to three types of visual stimuli. Note that deep layers respond less than superficial layers to full-screen flashes and large bars, consistent with selectivity for smaller stimuli. Adapted from [Del Bene et al. \(2010\)](#). (D) Feeding state affects visual processing in the tectum. (i) Schematic diagram of the zebrafish retinotectal pathway. (ii) Calcium indicator expression in the optic tectum was used to observe neuronal activity to prey-like stimuli. (iii) Examples of tectal neuron calcium signals in response to visual stimuli of different sizes. (iv) Comparison of the cumulative percentages of weighted mean response (WMR) angles for tectal neurons in starved and fed larvae. Note that starved larvae have more tectal neurons tuned for small, prey-like stimuli, consistent with a state-dependent modulation of visual processing favoring prey capture behaviors in starved larvae. Modified from [Filosa et al. \(2016\)](#).



raising the possibility that some stimulus filtering may occur before processing in the tectum.

*Stimulus selectivity and behavioral state:* Filosa, Barker, Dal Maschio, and Baier (2016) conducted an important study of how behavioral state might influence sensory processing (Figure 46.3D). They first observed that an ambiguous visual stimulus (intermediate in size) can evoke either prey capture or avoidance maneuvers, but starving the larvae increases the likelihood that they treat the stimulus as prey (and not a threat). Starved fish also have lower cortisol levels, suggesting the involvement of the hypothalamic-pituitary-interrenal (HPI) axis—previously shown to be involved in the regulation of food intake in fish (Bernier & Peter, 2001). Because the HPI axis can modulate serotonin levels (Fox & Lowry, 2013), they pursued a role for serotonin and found that the activity of serotonergic neurons in the raphe nucleus is elevated in starved fish (Filosa et al., 2016). Moreover, the increase in avoidance (in response to ambiguous stimuli) caused by prefeeding is abolished by selective serotonin reuptake inhibitors (SSRIs), and starved fish lacking serotonergic neurons (killed via nitroreductase ablation) behave like prefed fish. These results, taken together, are consistent with a model, wherein satiety activates the HPI axis, thereby decreasing serotonin release from neurons in the raphe nucleus. But what mediates the effect on behavior? The same study also used calcium imaging to observe the effect of starvation on size selectivity in the optic tectum, revealing that starvation induces a serotonin-mediated shift in the population-level tuning of interneurons and periventricular neurons—which may bias motor output in favor of prey capture in starved larvae. Thus, behavioral state modulates how sensory stimuli are represented in the brain and how they are transformed into motor output.

*Conclusion:* Prey capture behavior has provided a window into the neural basis of ethologically relevant stimulus selection, motor sequence generation, and state-dependent sensorimotor processing. These studies capitalized on many of the methodological approaches first pioneered to study the escape circuitry, including electrophysiology, laser ablations, and imaging with genetically encoded calcium indicators. Further, because prey capture (unlike escapes) comprises a sequence of behaviors modified by the stimulus in real time, these studies have innovated in their use of closed-loop virtual reality arenas for calcium imaging in restrained zebrafish. Researchers investigating this behavior can now move to take advantage of increasingly sophisticated tools for the dissection of neuronal connectivity in larval zebrafish that subserve it.

## The Future for Zebrafish in Studies of the Neuronal Basis of Behavior

In spite of the remarkable advances that make zebrafish such a powerful vertebrate model for revealing the neuronal basis of behavior, the glimpses we have provided are just the beginning. The number of labs using fish is growing rapidly, as are the tools that set zebrafish apart from other models. This last section looks to things on the horizon that ensure that the fish model is not going away any time soon as a path to revealing how vertebrate brains work.

### EM Connectivity on Order

Several recent reports make strong use of electron microscopy (EM) to produce serial EM sectioning of the larval zebrafish brain, to reconstruct the connectome of the olfactory bulb, and to reconstruct the connectivity of neurons in the oculomotor system after a functional study by calcium imaging. These point to a fruitful interface between synaptic level EM morphology and functional studies of circuits in zebrafish (Hildebrand, Cicconet et al., 2017; Vishwanathan et al., 2017; Wanner et al., 2016). While a connectome of the entire larval nervous system remains a challenge, mostly because of limitations in automated tracing, it is less wildly out of reach than in other, larger vertebrate brains. The ability to reveal connectivity within regions is critical for constraining circuit models, so we can expect many regional reconstructions in zebrafish to become available soon. Even more fruitful in the short term, however, will be the ability to quickly reconstruct the projections of specific functionally identified neurons, to tie function to exact structural connectivity. As this becomes routine, we can expect that the future will offer the ability to compare the connectivity of identified neurons between animals with different experiences, such as exposure to a specific learning paradigm. This will provide an unprecedented view of how experience reshapes connectivity in neural circuits. If the EM processing and reconstruction becomes more automated and commercialized, it may be possible to simply send tissue and obtain connectivity data from individual animals, much like genomic data.

### Transynaptic Mapping

Viruses that cross synaptic connections offer a potential path to revealing the set of inputs to a class of neurons without the need for an EM reconstruction. Rabies virus-based approaches modified to jump only one synapse already provide information in mammals



about the set of neurons connected directly to a target population (Kim, Jacobs, Ito-Cole, & Callaway, 2016). There remain limitations due to toxicity of the viruses, as well as lingering concerns about whether there is leakage of the labeling to adjacent but unconnected neurons, but the tools are improving substantially in mammals. Similar efforts using vesicular stomatitis virus (rVSV) are underway in zebrafish as part of an effort to extend the range of species available for viral tracing, but these efforts are still in progress (Beier, Mundell, Pan, & Cepko, 2016). As these reach fruition, they will provide a direct path to *in vivo* tracing in zebrafish. An ability to transynaptically express activity indicators or optogenetic constructs, when combined with the fish's transparency, will provide functional and behavioral information for specific components with known connectivity.

### The Physiological Holy Grail—Genetically Encoded Voltage Imaging

The calcium indicators used to monitor neuronal activity *in vivo* are only indirectly coupled to the transmembrane electrical activity that actually contributes to behavior. Efforts to optically monitor membrane potential have a long history, with challenges centering around small signals and phototoxicity. Both issues are a result of the need for the indicators to be localized in the cell membrane, a small and vulnerable region of the neuron, unlike the cytosol where calcium indicators typically reside. Still, there are many efforts underway to improve genetically encoded indicators of voltage, with entire families of them now in existence (Ouzounov, Wang et al., 2017). Improvements are coming quickly, and single-cell resolution *in vivo* has been achieved in some species (Xu, Zou, & Cohen, 2017). While efforts in zebrafish have not yet been resoundingly successful (Kibat, Krishnan, Ramaswamy, Baker, & Jesuthasan, 2016), there is little doubt that the optical access of the model will lead to a burst of voltage imaging if reliable, easy-to-use voltage indicators become available, which seems imminent (Piatkevich et al., 2018; Adam et al., 2019; Abdelfattah et al., 2019). Still, the opposition of membranes of adjacent neurons will make separation of signals from adjacent cells difficult, and deeper imaging with the superior optical sectioning of multiphoton microscopy may not work for voltage indicators—because of the limited number of photons available from restricted regions of the membrane imaged at the kilohertz rates needed to detect action potentials that last a millisecond or so. The voltage indicators will likely prove most useful for sparsely labeled animals.

### Direct to Circuits in Zebrafish

One promise of zebrafish that builds upon prior optical approaches to circuits involves a tool that is still under development but could be revolutionary when combined with the optical access to the larval brain. There are now several new approaches to activate genes using light with cellular specificity in culture, and hints of potential for *in-vivo* application (e.g., (Polstein & Gersbach, 2015)). This ability could prove very powerful when combined with whole-brain calcium imaging, as in a recent combination of whole-brain imaging and laser ablation (Vladimirov, Wang et al., 2018). One could imagine utilizing whole-brain calcium imaging to identify “interesting” candidate neurons, which participate in a particular behavior, and then use light-induced gene expression to cause those candidate neurons to express a genetically encoded label or control element (e.g., optogenetic constructs to turn them on or off, designer ligands for experiments in freely swimming animals, electron-dense markers for EM, fluorescent proteins, or voltage indicators). These candidate neurons may be ones that become active during the behavior or could even be neurons that change their activity after learning. The cells' structure could be revealed by the light-activated expression of membrane-targeted proteins. Inducing expression of optogenetic constructs would allow perturbations in neuronal activity, to test hypotheses about those neurons' specific role in behavior. Light induction of EM markers would even allow later reconstruction of those neurons' connectivity throughout the brain. This would offer the possibility of moving more directly from a specific behavior to formulating and testing predictions about the neural basis of that behavior (Dal Maschio, Donovan, Helmbrecht, & Baier, 2017).

### Circuits and Behavior From Embryo to Adult

Most of the studies of brain and behavior in zebrafish focus on larval fish, typically less than 3 weeks of age. This is a practical choice, as the larvae are small and translucent, so optical tools and targeted recording are easy. The fish must survive on their own after hatching—meaning that they execute a wide variety of behaviors and simple forms of learning - so the larvae have a major place in understanding brains and behavior. A strong understanding of even simple behaviors, such as movement, visual-motor orientation, feeding, and simple forms of learning still elude us on the scale of the whole nervous system.

However, while larval behavior is just as consequential as any other behavior (dead larvae have no

**TABLE 46.1** Advantages and disadvantages of zebrafish by comparison to other model systems for the study of the neural basis of behavior.

	<i>C. elegans</i>	<i>D. melanogaster</i>	<i>D. rerio</i> (larvae)	<i>M. musculus</i>
Number of neurons	302	~135,000	~130,000	~75 million
Body length	1 mm	3 mm	4 mm	7.5–10 cm
Time to sexual maturity	4 days	1 week	12 weeks	10 weeks
Example behaviors studied at the level of neural circuits	Locomotion, Taxis, foraging, mating	Flight, crawling (larvae), foraging, mating, Aggression, visual-motor reflexes	Swimming, escape, visual-motor reflexes, prey capture, phototaxis,	Aggression, mating, fear conditioning, spatial learning, contextual memory, foraging, Maternal care, communication
Optical accessibility	Transparent	Surgical window	Transparent	Surgical window
Electrophysiology	Difficult	Difficult	Feasible	OK
Unique advantages	Full connectome	Defined genetic access to small groups of individually identifiable neurons	Best vertebrate model for optical microscopy.	Most similar to humans. Synaptic tracing viruses. Has a cortex.
Disadvantages	Less behavioral complexity; graded neurons	Opaque cuticle; neuronal computations take place in dendrites	Limited capacity for learning and memory in the larval stage.	Has a cortex. Large scale and high complexity of brain.

offspring!), the fish brain and behavior does change as the fish grows to reach sexual maturity at about 3 months of age. For example, a very small cerebellum in larvae grows to a large and recognizably vertebrate cerebellum in adults. With growth and adulthood, comes more complex social interactions, seemingly more sophisticated motor control and learning, and new behaviors, such as mating. The advantages of the transparency are lost, however, which would seem to undermine the utility of the model later in life.

Recent innovations will likely circumvent that. The development of longer wavelength three-photon microscopy and the attendant ability of long wavelengths to more easily penetrate opaque tissue allows deeper imaging into previously inaccessible regions of adult brains (Ouzounov, Wang et al., 2017). The depth of imaging possible with three-photon microscopy (on the order of 1.5 mm) matches the thickness of an adult zebrafish brain. The combination of three-photon microscopy and some adaptive optical tools with the pigmentless *Casper* fish lines will likely allow optical access anywhere in the intact living adult zebrafish brain. This would make zebrafish the first vertebrate where brain function in behavior can be studied in the same animal from embryo to adult, opening up many questions of how brain circuits and behavior change with maturity and experience, and eventually degenerate with age. [Table 46.1](#).

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