Brain-Wide Neural Dynamics Underlying Looming-Evoked Escapes and Spontaneous Exploration

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Brain-Wide Neural Dynamics Underlying Looming-Evoked Escapes And Spontaneous Exploration

A dissertation presented
by
Timothy William Dunn
to
The Division of Medical Sciences

in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Neurobiology

Harvard University
Cambridge, Massachusetts

April 2015
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Abstract

Behavior is generated via brain-wide coordination of neural circuits. But until recently, it was difficult to analyze neural dynamics at cellular resolution throughout the brain during behavior. With the genetic and optical accessibility of the larval zebrafish, however, we are now beginning to dissect neural circuits on larger scales. Here, I describe the neural origins of two prominent innate behaviors of the larval zebrafish: (1) looming-evoked escape behavior and (2) a self-generated exploratory behavior.

In zebrafish, punctuated mechanosensory stimuli, signaling proximal threats, elicit escape behaviors that rely on a compact neural circuit. Visual identification of threats, however, is more complex: instead of detecting an impulse-like stimulus, danger must be recognized by computations on the spatiotemporal properties of visual scenes. Here, I characterize behavioral responses to visual stimuli simulating predator approach using a high-speed, closed-loop system that enables precise control over the visual environment of free-swimming fish. I report that the visual system alone recruits lateralized, rapid escape maneuvers in response to looming but not static stimuli. Brain-wide calcium imaging isolated the optic tectum as an important visual center processing looming stimuli, with ensemble activity encoding escape latency. Finally, ablations of hindbrain circuitry confirmed that visual and mechanosensory modalities share a premotor output network.
In the absence of specific stimuli, however, animals continue to exhibit rich self-generated behavior. In featureless environments, fish exhibit stereotypical behavioral sequences, which consist of repeated turns in one direction followed by stochastic switches to repeated turns in the other direction. Using whole-brain imaging in behaving animals, we found antisymmetric activity in distinct hindbrain populations that was ipsilaterally correlated with turning and exhibited slow time courses well-matched to behavioral sequences. These populations correspond to the “hindbrain oscillator” (HBO), and cell ablations demonstrated a causal role for the HBO in determining the statistics of spontaneous swimming. We revealed that the HBO comprises separate glutamatergic and GABAergic clusters interacting across the midline, suggesting a mutual-inhibitory circuit motif shaping HBO dynamics and downstream behavior. These findings establish a circuit underlying spatiotemporally structured spontaneous behavior that, in simulations, supports efficient exploration of environments in the absence of explicit sensory cues.
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Acknowledgements

To avoid alienating all of my friends and countrymen: I first thank everyone I have ever known and loved.

But more specifically, I’d like to thank Florian Engert for his undying wit, curiosity, and beauty. I thank my closest collaborator, Eva Naumann, and wish her infinite joy forever. I also thank Misha Ahrens, who took me under his wing from day one and has supported me immensely. I thank James Fitzgerald for his guidance and patience. I thank my parents for giving me this opportunity. And I thank my advisory and defense committees for hanging in there, and for all of their help. Enjoy!
Chapter 1

Larval zebrafish systems neuroscience
1.0 Overview of animal models

The goal of systems neuroscience is to understand how interconnected neural networks in the brain process information and ultimately generate behavior. Studies of brains and behavior, while united under this central tenet, can take many forms, depending on the complexity of the underlying neural networks and the accessibility of these networks to controlled experimentation. Because there is a tight correlation between the complexity of a brain (i.e. encephalization quotient\(^1\)) and the richness of an organism’s behavioral output\(^2-^4\), neuroscientists must weigh the advantages of a system generating interesting output against the disadvantages of that system being harder to measure and interpret.

The *Homo sapiens* brain, on the extreme high end of the complexity continuum, possesses the greatest number of cortical neurons\(^5\) and the largest brain-to-body-mass ratio after normalizing for allometry\(^6,^7\). This brain complexity ostensibly begets a high degree of behavioral complexity: humans are the uncontested kings of intelligence and operate in a high-dimensional behavioral space, exhibiting world-scale social interactions, deep self-expression via language and art, life-long learning of abstract concepts, technology development, and proficient athleticism. But while these features provide ample fodder for systems neuroscientists looking to explain the neural origins of behavior, progress towards understanding precise neurobiological bases is precluded by the same complexity giving rise to this wealth of behaviors in the first place. We currently lack the experimental, analytical, and theoretical tools to interrogate super-complex brains on a network or neuronal basis. Thus, most studies of human brains are relegated to psychology, which produce detailed phenomenological models of behavior and perception but fall short of delivering cellular mechanism and detailed circuit implementation.
The roundworm *Caenorhabditis elegans* (*C. elegans*) brain falls on the lower end of this complexity spectrum. Boasting merely 302 consistently identifiable neurons\(^8\) and 95 muscle cells\(^9\), *C. elegans* has emerged as a flag-bearing model system; its simplicity and amenability to both experimental and genetic manipulation allows for a deep mechanistic understanding of the link between neurons and behavior\(^\text{10}\). Transgenesis and RNA interference can be used to target individual neurons\(^\text{11}\), and the activity of all neurons can be manipulated and recorded in the behaving animal\(^\text{12,13}\). It is also the only organism for which the complete connectome, synapse-level resolution of neuron interconnectivity across the entire brain, has been mapped\(^\text{14}\). These tools have led to leaps in understanding of the general principles underlying locomotor rhythms, chemotaxis, foraging, and primitive spatial learning\(^\text{10}\). The system is so simple, in fact, that researchers are close to simulating the behavior in the complete worm *in silico*\(^\text{15}\). But while these features inspire systems neuroscientists looking to explain the detailed neural mechanisms underlying behavior, progress towards understanding complex actions is all but impossible.

Increasing in complexity, the mouse, *Mus musculus*, has assumed a prominent position as a model system for neuroscientists, owing to its strong mammalian neuroanatomical and genetic homology to the human brain\(^\text{16}\), relatively straightforward genetics\(^\text{17}\), and expanded behavioral repertoire\(^\text{18}\). The mouse brain, however, presents some of the same problems associated with human brain studies: non-superficial neurons and neural circuits are difficult to access non-invasively, typically requiring tissue excavation\(^\text{19}\). Furthermore, the best methods available for monitoring neural activity are restricted to subregions of much larger neural structures, ignoring neural dynamics on large scales\(^\text{20}\); this may limit our understanding of emergent properties\(^\text{21}\). That is not to say we have not learned important principles from studies of the mouse brain and mouse behavior; many brain circuits and computational units have been described in detail and
related to both sensory processing and behavior. Examples include dopaminergic regulation of reward\textsuperscript{22}, decision making\textsuperscript{23}, working memory\textsuperscript{24}, spatial navigation\textsuperscript{25}, olfactory processing\textsuperscript{26}, direction selectivity\textsuperscript{27}, and the vestibulo-ocular reflex\textsuperscript{28}. But, as I will describe in more detail in the following sections, behavior is generated via interactions between many different neural systems; thus, comprehensive algorithmic descriptions of neural circuits and behavior require more coordinated monitoring of neural activity across the brain. At this time, however, the mouse brain is too large and impermeable for this type of collective investigation. There is, therefore, a niche for an animal of sufficient behavioral and neuronal complexity that provides tractable access to its complete neural architecture and activity.

The larval zebrafish, \textit{Danio rerio}, is particularly well-suited for brain-wide investigations of neural circuit function. The larval zebrafish has a small, translucent brain amenable to whole-brain imaging, short spawning time, and large repertoire of lab-reproducible behaviors\textsuperscript{29,30}. Furthermore, it is characterized by rapid neural development and susceptibility to genetic manipulation, and it contains many brain structures and systems homologous to those that exist in mammals\textsuperscript{31}. At five days post-fertilization, larvae have already developed a functional nervous system capable of prey capture\textsuperscript{32}, predatory escape\textsuperscript{33}, and motor learning\textsuperscript{34,35}. First adopted as a model system to study physiology and development due to its early stage translucency\textsuperscript{36}, tools have now been developed to probe brain dynamics\textsuperscript{37}. Transgenic zebrafish now exist that express the fluorescent calcium indicator GCaMP\textsuperscript{38} in all or subsets of neurons in the fish central nervous system\textsuperscript{39,40}. In this dissertation, I will describe how I have used these available tools and careful quantifications of locomotion to advance our understanding of the neural dynamics underlying sensory processing and behavior.
The rest of this introductory chapter will give a more detailed background on why it is important to study brains on large scales, what tools are available to study brain-wide neural dynamics, and how far the field has come towards understanding the brain and behavior of larval zebrafish. In the following two chapters, I will describe the neural origins of two prominent innate behaviors. Finally, I will conclude with a discussion that expands on both studies and provides an optimistic outlook. More specifically,

Chapter 2 will address a sensorimotor transformation from light to rapid escape maneuvers that highlights a neural system tuned to extrinsic stimuli in the environment.

Chapter 3 will report on a self-generated exploratory behavior that may optimize foraging, highlighting a neural system tuned to internal motivational state.

Chapter 4 will discuss and extend these studies to more holistic hypotheses of circuit dynamics and the coordination of behavior.

1.1 The sensorimotor loop, brain states, and a motivation for brain-wide analyses

The relationship between brains and behavior is most often studied using sensory-evoked assays in which the experimenter has precise control over stimulus delivery and variables. This kind of experimental design is convenient because it allows the researcher to develop stimulus-response profiles that help elucidate the underlying neuronal computations that transform sensory input to motor output, computations that enable an animal to interact with the external world. A well-designed psychophysical or behavioral paradigm will typically generate a set of search stimuli\(^1\) that can be used to probe underlying neural processes; if the behavioral experiment has enough power, the results can be combined with electrophysiological or optical measurements of neuronal activity in order to locate relevant brain areas, confirm or deny
equally plausible mechanistic implementations in neural circuits, and test the causal relationship
between specific neuronal activity and behavioral patterns. Furthermore, once a link between a
stimulus and behavior has been established, future studies are justifiably motivated to investigate
only the sensory processing necessarily required to recruit downstream motor outputs. Because
the anatomy of primary sensory systems tends to be simpler, more accessible, and more isolated
than central brain areas, they form a rich substrate through which neuroscientists can begin to
understand neural processing in mechanistic detail\textsuperscript{42,43}.

This sensory-driven approach to understanding circuits and behavior has been used
successfully by neuroethologists to reveal many principles of sensory processing and
sensorimotor transformations. Examples include visual motion computation\textsuperscript{44,45}, wind direction
encoding in crickets\textsuperscript{46}, interaural time differences and sound localization\textsuperscript{47}, sensitization of
\textit{Aplysia} gill-withdrawal reflex\textsuperscript{48}, and the jamming avoidance response\textsuperscript{49}. But studies of sensory
systems and sensory-evoked behavior typically operate under simple models of the external
world that exclude the sensory feedback produced by behavior, or reafference. Interpretation and
processing of these ubiquitous reafferent signals, and linking reafference to prior expectations
and behavior, is a critical function of the brain across species\textsuperscript{50} and thus must be included in any
description of complete system function. The continuous stream of behavior, sensory stimuli
generated from behavior modulo extrinsic environmental stimuli, and subsequent behavioral
responses forms a “sensorimotor loop\textsuperscript{51}” that can be used to deconstruct and formalize
ethologically relevant brain function.

A key insight gleaned from treating the brain as part of a sensorimotor loop, i.e. that the
brain is not a purely reflexive system, is that perception and behavioral responses to sensory
stimuli cannot be separated from prior expectations. Two identical stimuli may be processed
differently depending on behavioral state or context. This is evident colloquially when considering, for instance, that speech is perceived differently when recorded and played back to the speaker, or that a loud sound is unlikely to startle the person that generated it volitionally. The latter example illustrates the utility of neural circuits influenced by action: it is senseless to respond to an otherwise threatening stimulus if one is confident it is not actually threatening. In other words, it is important to separate self- from non-self-generated stimuli in order to maximize responsiveness to relevant phenomena in the external world.

Systems neuroscientists studying these sensorimotor loops look for changes in neural responses to stimuli contingent upon ongoing behavior and learned expectations of reafferent stimulation. From these studies, it appears that a common strategy of the nervous system is to generate an internal representation of expected sensory feedback from intended motor output (a corollary discharge) that can be compared to input measured through sensory receptors. These comparisons are ubiquitous throughout the animal kingdom, come in many different forms, and require the concerted activity of many brain regions. Crickets, for instance, actively suppress the axonal terminals of primary auditory neurons during chirping via an inhibitory interneuron connected to the central pattern generator controlling sound production; this ensures that any environmental stimuli are not drowned out by the cricket’s own stridulation. Electric fish use a similar mechanism to provide an inhibitory negative image of their own electric field while emitting pulses; because these electric fields can change depending on environmental conditions, these fish can also change negative image representation on the fly via cerebellar plasticity. These loops are also present in mammalian systems; in a canonical example requiring corollary discharge from the superior colliculus, primate attention will shift to a future saccadic target before the eyes have moved.
The existence of sensorimotor loops argues strongly for a behavioral component to perception and thus for experiments and analyses that incorporate behavioral factors. The homunculus is not a couch potato; central systems are not passive listeners to the outside world. In a striking illustration of this principle, recent studies have revealed that neurons in primary visual cortex, long probed with visual stimuli in anesthetized animals, more than doubled their sensory-evoked firing rate while animals were running. Furthermore, subsets of neurons were active only during running and were suppressed by visual stimuli. While the function of these motor-related signals remains elusive, it is clear that studying sensory systems in isolation will not suffice to explain circuit function.

In addition to motor-related filtration of sensory signals, brain states also shape perception and behavioral responsiveness. Even at rest, neurons in the brain are not silent. Sensory stimuli are thus delivered to systems with ongoing activity that may influence instantaneous processing. These underlying dynamics are shaped on long timescales by learning, memory, and experience, and on short timescales by attention and motivational states such as arousal and anxiety.

Each of these brain states is useful in that they provide adaptability in a changing and complex world. While learning updates the overarching rules that govern effective behavioral output, attention allows neural systems bombarded by stimuli to choose the most important features to consider at any given moment. These attentional mechanisms have been well-studied and include both top-down regulation of responsivity, where working memory or knowledge of task changes the overall sensitivity to specific stimuli, and “bottom-up salience filtering,” where stimulus signatures are precluded from reaching central processing centers based on competitive context. In both cases, ongoing activity exerts a strong influence on perception and behavior.
Although related, broader motivational brain states can be distinguished from attention on the basis of specificity; whereas attention typically results in the selection of one stimulus over another, motivational states affect processing globally. When one is fatigued, for instance, all thought becomes sluggish and all activity becomes arduous. Moreover, motivational states ordinarily transform overall behavioral responsiveness and are additive to underlying stimulus selection. At a cocktail party, for example, a gentleman will most likely always pick up his name from within the murmur, but his mood, friendly or otherwise, will determine whether or not he engages the speaker. In animal models, these motivational states have been shown to rely on neuromodulators such as acetylcholine, serotonin, hypocretin, and norepinephrine and have profound effects on behavioral responses to sensory stimuli.

From these descriptions, it is clear that the neural response of a central sensory center is dictated not just by the specific properties of the sensory input and neurophysiology of the local neurons and circuits but also by the activity of distal afferents representing behavior and brain state. Likewise, subsequent recruitment of behavioral patterns is also subject to the same global influence. The brain is indeed a highly interconnected and dynamic system. What is the ideal way to study such a synthesis?

The classical approach has been to interrogate subregions or sub-networks independently. The hope is, for instance, that the enhancement in gain of a neuron in V4 can be correlated with an improvement in behavioral performance, which, in a separate experiment, is correlated with activity in the frontal eye fields (FEF) and can be altered with a local perturbation of activity. Suppose though, in a subset of trials while recording from FEF, the animal behaves poorly even when neurons in FEF are activated. The experimenter would then be ignorant of the activity in V4 that might explain this deviation. Perhaps the neuron(s) in V4 were not modulated by FEF on
that trial. Perhaps they were, but some larger population dynamics or network state, influenced by unknown neurons representing motivational state elsewhere, altered the behavioral response. In either case, the experimenter is left with an unsatisfactory explanation of the variability.

Traditional recording techniques also fall short when considering that contemporary models of processing assume that flexible representations of task-related variables and stimuli are distributed across large populations of neurons. Hypotheses of system criticality, phase space dynamics or attractor states, and linear discrimination require large-scale measurements of neuronal activity with high spatial and temporal resolution. Although advances in electrophysiological and optical probes have aided in the testing of these hypotheses, they have not yet spanned the high-dimensional neural space of complex brains.

Together, these examples motivate an experimental preparation that enables a brain-wide examination of neural dynamics. Using pan-neuronal expression of genetically encoded calcium indicators, we can now record the activity of almost every neuron in the larval zebrafish brain during behavior. Because of this coverage, the influence of even the most marginalized neuron or circuit element can be measured. The distributed interactions of tens of thousands of neurons can be visualized as they act to make sense of the external world, all but guaranteeing that the intricacies of sensorimotor loops, brain states, and population dynamics can be captured and incorporated into a comprehensive understanding of the brain. With careful experimental design, investigations in larval zebrafish promise to reveal the functional basis of processing and behavior in unprecedented detail.

1.2 Methods for circuit dissection in larval zebrafish
As a model system, the vertebrate larval zebrafish is well-positioned for mechanistic neural circuit dissection. Its small brain, composed of roughly 100,000 neurons at 5 days of age, and strong homology to mammalian neuroanatomy, promises relative simplicity without sacrificing applicability; computations should be easier to comprehend, and principles are likely to extend to more complex, but evolutionarily related, brains. The zebrafish genome is also easily editable, and generation time is relatively short; stable transgenics and mutants can be created within 3-6 months, accelerating the adoption of new tools and genetically targeted hypothesis testing. Although transgenic fish have classically been made using random plasmid integration via the Tol2 system, limiting specific knock-in and knock-out power, recent advances in genetics, such as CRISPR, have made precise genome editing more tenable. In addition to their genetic accessibility, larval fish also behave robustly in the lab, even when restrained via head-embedding in water-permeable agarose gel; large numbers of fish can be subjected to behavioral assays, and behavior is typically recapitulated under the microscope. Finally, larval fish also have translucent brain tissue; the entire brain can be optically probed in vivo. The experiments outlined in this dissertation capitalize on several different optical tools for circuit dissection.

To start, genetically encoded calcium indicators (GCaMPs) can be expressed under the control of various promoters. GCaMPs were engineered as powerful alternatives to synthetic fluorescent calcium indicators like Fluo-4 and Rhod-3, which rely on relatively direct cellular access and lack cellular specificity. Like synthetic probes, GCaMPs increase in fluorescence, or quantum yield, in the presence of free calcium ions. Because intracellular calcium increases in concentration during strong depolarization events like action potentials, concomitant rises in GCaMP fluorescence can be used as a proxy for neuronal activity and can, under the right
conditions, be deconvolved into spike rates\textsuperscript{73}. GCaMPs were engineered by circularly permuting the green fluorescent protein (GFP) primary sequence and inserting the calcium-binding protein calmodulin and its calcium-dependent binding partner, M13, on the resulting N- and C-terminals, respectively. Because the new N- and C-terminals introduced a break in the barrel structure that provides an energetically favorable environment for the GFP fluorophore\textsuperscript{74}, the calcium-free state of GCaMP exhibited a significant decrease in fluorescence. Only in the presence of calcium, when the affinity of calmodulin for its binding partner, M13, is high, is the barrel structure reconstituted to re-establish green fluorescence. Over several iterative rounds of mutations and screening, GCaMP sensitivity and signal-to-noise ratio have now surpassed those of its synthetic counterparts\textsuperscript{38}.

In larval zebrafish, GCaMP expression can be driven under the control of the pan-neuronal elavl3 promoter, such that almost every neuron in the zebrafish brain contains a sensitive optical reporter of neuronal activity\textsuperscript{39}. The activity of individual neurons throughout the brain can thus be recorded using high-speed scanning two-photon\textsuperscript{35} or light-sheet microscopy\textsuperscript{39}. When combined with simultaneous stimulus delivery and behavioral monitoring, these whole-brain measurements of neural activity can be used to make strong inferences about network processing on large scales. The high dimensionality and large physical size of these data sets, however, presents a problem for analysis; interrogations must be focused and, in the case of light-sheet data, be able to address terabytes of data for individual fish. To solve these challenges, Freeman et al.\textsuperscript{75} have constructed a distributed analysis pipeline that parallelizes data processing over the cloud and provides a suite of tools for deconstructing large data sets. Of these tools, brain-wide dimensionality reduction and regression analysis will be used extensively in Chapter 3.
A second optical tool for circuit dissection in zebrafish is photoactivateable GFP (PA-GFP), which is fluorescent only after exposure to strong 413 nm\textsuperscript{76}, or 780 nm two-photon, light. Successfully used in the mouse, fish, and fly brain\textsuperscript{77}, PA-GFP provides an invaluable tool for supporting functional studies with complementary structural information about connectivity. In zebrafish, pan-neuronal expression of PA-GFP under the $\alpha$-tubulin promoter can be used to activate GFP in individual neurons or small clusters of neurons and trace both dendrites to afferent brain regions and axons to downstream, efferent brain regions\textsuperscript{35}. In some cases, PA-GFP can even be activated in the processes of neurons in hypothetical recipient areas and then traced back to individual cell bodies after it diffuses\textsuperscript{77}. PA-GFP thus provides a powerful tool for following information flow through central circuits. In Chapter 3, I will describe how it has been used to suggest a possible mechanism supporting bistable neural dynamics.

The last tool, used in both Chapter 2 and 3, is focal lesioning of neural tissue with a two-photon laser. The typical fluorescence cycle of a fluorophore begins with photon-energy absorption that kicks an electron into an excited state. After a short interval without continued excitation, this electron falls back to the ground state and emits energy as a red-shifted photon in a process known as a Stokes shift\textsuperscript{78}. At sufficiently high power densities, however, this electron becomes over-excited and, rather than falling back to the ground state and emitting a photon, can induce an ionization cascade of nearby molecules that eventually results in plasma formation and focal tissue destruction\textsuperscript{79}. Because this highly nonlinear process outcompetes linear damage due to heating, it can be used to target individual neurons for ablation. This method is especially effective in zebrafish, where tissue properties ensure a tight point-spread function in the focal plane and thus a high power density\textsuperscript{80}. Indeed, this method has been used to establish causal relationships between specific neurons and behavior in many paradigms\textsuperscript{68,81–83}.\textsuperscript{13}
Together, whole-brain calcium imaging during behavior, PA-GFP tracing, and laser ablations form a triumvirate of experimental methods that can be used to interrogate the neural circuits underlying behavior.

1.3 Larval zebrafish behaviors and brain-wide circuit dissection

The powerful tools available for observing large-scale neural interactions in zebrafish are useful only if we can assign behavioral relevance to the measured neuronal activity. Indeed, a wide-spread criticism of the zebrafish field is that technology has outpaced the underlying questions. While it is now possible to observe the activity of over 100,000 neurons in the behaving animal at high spatial and temporal resolution\textsuperscript{39}, the interpretation of these data needs to be guided by specific hypotheses that relate activity to intended processing and behavior. Thus, the field is experiencing a concerted push to expand and refine the larval zebrafish behavioral repertoire.

The first, and arguably easiest, behaviors to be described in larval zebrafish were innate responses to mechanosensory, acoustic, and visual stimuli. When larvae are challenged with a physical touch or loud sound, they respond with a rapid escape maneuver that can approach angular velocities of up to 10,000 degrees/second and linear velocities of up to 30 cm/s, exceeding even the fastest maneuvers of fruit flies\textsuperscript{84}. Because this behavior shared many similarities with the C-bend escape response in other teleost fish, the basic circuit mechanisms were easily inferred from decades of past literature\textsuperscript{85}. In the case of sound, the behavior requires a two-synapse reflex arc via the eighth nerve from sensory neurons in the ear to the large Mauthner cell in the hindbrain. Depolarization of the Mauthner cell then results in strong contractions of the contralateral musculature via descending projections of the Mauthner cell into
the spinal cord. While studies in zebrafish did little to expand on this basic circuit, the tools unique to larval zebrafish have provided insights into the central regulation of Mauthner cell excitability. Specifically, Yu Mu et al. have demonstrated that the Mauthner cell can be primed by light flashes, presumably to support multimodal sensing of threats. This light-dependent increase in excitability is conferred by dopaminergic input from the hypothalamus that is recruited during stimulus presentation. Although Yu Mu et al. employed methodical patch clamp techniques to map this circuit, they benefitted from the unparalleled optical access to the translucent zebrafish brain and systematically ablated individual cell types in the hypothalamus with a two-photon laser over 150 times in individual fish. Similarly, Lacoste et al. (in press) have used large-scale imaging techniques and focal lesions in transgenic zebrafish to deconstruct the upstream regulation of Mauthner excitability by a set of excitatory neurons called “spiral fiber neurons” that receive multimodal inputs.

Visual-specific behaviors are also well-documented in larval zebrafish. Fish, like most animals, exhibit an optomotor response (OMR), in which whole-field motion elicits swimming behavior that aligns an individual with the direction of optic flow. The neural basis of the OMR in larval zebrafish has been investigated from both the sensory end, with the computation of direction selectivity in the retina and mapping of direction-selective projections, and the motor end, with the identification of specific spinal projection nuclei that mediate individual sensory-evoked turns. But the neural basis of this behavior has also been buoyed by whole-brain imaging studies that document a modular sensory processing architecture that integrates motion binocularly, stabilizes behavioral responses via reciprocal inhibition, and recruits segregated hindbrain nuclei that map onto specific behavioral outputs (Naumann et al., in preparation).
Thus, whole-brain imaging has emerged as an effective tool to dissect ethologically relevant processing on brain-wide scales.

Whole-brain imaging has also been used to map the neurons underlying the larval zebrafish optokinetic response (OKR), which is characterized by smooth eye movements in response to rotating optic flow. Following an examination of the oculomotor integrator, where Miri and colleagues dissected and verified models of network integration by regressing imaged neural activity against individual stimulus- and behavior-derived variables, Portugues et al. imaged pan-neuronal responses to rotating gratings during the OKR in restrained zebrafish. Subsequent regression analysis revealed a brain-wide distribution of phase- and motor-tuned neurons across the brain. This distribution was highly stereotyped across fish, as confirmed with an automated affine registration process that robustly aligned many fish to a standard brain. While this study did not reveal much at the level of circuit mechanism, it did highlight the capacity of the larval zebrafish preparation to study neuronal activity on a brain-wide scale and broke the stereotype that simple behaviors are controlled by simple and compact neural circuits.

One of the more complex innate behaviors exhibited by larval zebrafish is a locomotor sequence used to capture prey during hunting. At five days of age, larval zebrafish cease to rely only on their own yolk stores and begin to actively hunt paramecia, their prey of choice in natural freshwater habitats. When zebrafish detect a paramecium, they respond with a stereotyped behavioral sequence that begins with eye convergence, which maximizes binocular overlap and depth perception, is followed by a series of small turns that orients the fish and stabilizes position, and ends with a strike that, if successful, results in prey capture and ingestion. This behavior is not elicited in the dark and can be evoked with visual stimuli alone, suggesting that it is primarily a visually guided behavior. Imaging of the optic tectum has
dissected individual circuit elements underlying both the specific detection of prey-like (i.e. small, moving) stimuli and the recruitment of similarly tuned “assemblies” that preface, or release, eye converged and subsequent behavioral sequences\textsuperscript{67}. These analyses bear resemblance to seminal studies in the toad visual system\textsuperscript{92}, where behavioral responses to large moving stimuli (i.e. avoidance) and small moving stimuli (i.e. prey-capture) were mapped to specific receptive fields in the optic tectum and accessory visual systems, but expand the understanding of the underlying neural computations and representation to larger and complete sensory systems.

Progress has also been made towards establishing learning assays in larval zebrafish. In classical conditioning paradigms, pairing shocks with light flashes can result in a transfer of unconditioned valence (i.e. escape) to the initially neutral, conditioned visual stimulus, and this learning has been shown to dependent on cerebellar activity\textsuperscript{93}. In another learning assay, Robson and Li (submitted) have shown that larvae can be instrumentally conditioned. When embedded fish are challenged with noxious heat, delivered with a strong infrared laser, they quickly learn which direction they need to flick their tails in order for the laser to turn off. With whole-brain imaging, Robson and Li were able to identify neurons in the forebrain that, as a population, encode relevant learning-related variables. Distributed activity in neurons across the right habenula, for instance, appears to represent success on individual trials, and other neurons in the forebrain provide instructive signals that influence habenular representation. While the mechanisms of operant conditioning are well-studied in other organisms like mice and monkeys\textsuperscript{22,94}, analyses in these high-order systems are currently possible only in small populations, limiting a complete understanding of their processes.
Finally, larval zebrafish also show rapid motor adaption behavior in response to changes in sensory feedback gain. In a pioneering study that exemplifies both the power of whole-brain imaging and the importance of sensorimotor loops, Ahrens et al.\textsuperscript{35} show that, in a closed-loop behavioral feedback system during functional brain imaging, fish will increase swim vigor in response to whole-field motion when during slower-than-expected visual feedback and will decrease swim vigor during faster-than-expected visual feedback. This adaptation represents the critical ability of brains to quickly adapt behavior in response to extrinsic and intrinsic environmental changes that do not meet prior expectations. For instance, if we assume that the goal of the fish is to stabilize its position at a particular location in space when being pushed in a water column (which produces an OMR due to compensatory optic flow), the fish must be able to perform this behavior as environmental conditions, and thus response efficacy, change (e.g. viscosity, distance from moving objects, injury). Ahrens et al. used whole-brain imaging to identify key neuronal players in this computation and measure their distribution and dynamics across the brain.

Advances in both behavioral quantification and imaging technologies in larval zebrafish have begun to reveal principles of neural coordination on brain-wide scales. Using these studies as a foundation, I have expanded our understanding of the neural basis for two novel behaviors in larval zebrafish. First, I will describe how a population of neurons in the optic tectum detects aversive visual stimuli, calculates response timing, and refers processed information to downstream motor circuitry. Second, I will describe a hindbrain nucleus that controls the spontaneous generation of behavior in stimulus-sparse contexts and, additionally, may act as a broad conductor of sensory-evoked behavior as well. Together, I illustrate how close we have come to understanding large-scale neural circuits underlying behavior in a vertebrate brain.
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Chapter 2

Neural circuits underlying visually evoked escapes
2.0 Intellectual contributions

The following chapter is adapted from a manuscript entitled “Neural circuits underlying visually evoked escapes in larval zebrafish,” which, as of April 6, 2015, is under revision for publication at the journal *Neuron*. I am first on the author list, but Dr. Eva A. Naumann, Dr. Misha B. Ahrens, and Dr. Florian Engert also contributed to the manuscript. I performed all experiments and analyses and wrote the paper. Dr. Naumann helped design figure graphics. Drs. Ahrens and Engert edited the manuscript and helped guide the overall methodology.

2.1 Introduction

When confronted with threatening stimuli, organisms respond with stereotyped behavioral patterns, such as freezing \(^1\), aggression \(^2\), or flight \(^3\), that promote survival. The most fundamental of these behaviors is the escape response, which delivers the individual away from assault by bringing the body to a safer location. While these escapes are diverse across phyla, tailored to both anatomy and environmental context – from the omega bend of *Caenorhabditis elegans* \(^4\) to the high-g, in-flight banking of *Drosophila melanogaster* \(^5\) to the nociceptive withdrawal reflex of mammals \(^6\) – they are nevertheless highly conserved and occupy an ancient and essential corner of the ethogram (Eaton, 1984). Indeed, when examined ontogenetically, escape behaviors typically develop before the organism can feed or make coordinated movements \(^7\), highlighting the vital importance of these avoidance programs.

The robustness and stereotypy of escape behaviors are of great utility for studies of sensorimotor computations \(^8–11\). But studies of escape behaviors have often focused on impulse-like mechanosensory stimulation such as a touch or brief auditory buzz, where stimulus control
and behavioral execution is straightforward and where the underlying sensory detection and processing pathways are relatively compact. The visual system, however, is arguably better suited for detecting threatening stimuli, as visual cues can be detected long before the mechanical signatures of an approaching predator reach somatosensory and auditory systems\(^1,12–16\). At the same time, the sensory computations required for the visual detection of threats are potentially richer and more complex, as these must involve the rapid analysis of high-dimensional spatiotemporal sensory streams. Nonetheless, mechanisms of visual escape behavior, typically evoked by signatures of impending collision (looming), have not been well-studied outside of invertebrates\(^17,18\). Here, we use the behavioral, optical, and genetic accessibility of the larval zebrafish, *Danio rerio*, to address the neural basis of visually evoked escapes in a vertebrate animal.

In response to acoustic or tactile stimulation, larval zebrafish perform a fast, high-angle, stereotyped escape maneuver (the “C-bend”) that is conserved across most anamniotes\(^11\). This escape behavior is recruited by a short ipsilateral arc (minimum 2 synapses) from the ear (in the case of sound) to rhombomere 4 of the hindbrain, where a premotor system dominated by the large, morphologically distinct Mauthner cell (M-cell) effects a high-amplitude turn to the contralateral side. While it is not known whether the M-cell and its associated segmental homologues (collectively, the M-system) mediate any visually guided behaviors, studies in goldfish\(^19\) and adult zebrafish\(^14\) show that the M-cell may receive visual input from the optic tectum (OT), the homologue of the mammalian superior colliculus.

In turn, a large body of evidence supports a role for the OT in complex visual processing. The OT, by far the largest contiguous larval visual brain structure, is recurrently connected across its laminar architecture and receives direct input from the majority of retinal projections\(^20\).
in addition to indirect input from accessory visual areas. Neurons in the OT show direction, orientation, speed, and size selectivity and respond to aversive (predator-like) and appetitive (prey-like) visual cues in many animals. Furthermore, OT neurons in birds, tadpoles, and fish respond to looming stimuli. Thus, the OT is well-positioned to mediate visually evoked escape responses by feeding filtered visual input to the hindbrain M-system and associated escape circuitry.

However, so far a causal link between the hindbrain M-system and visually evoked escapes has not been demonstrated. Furthermore, most analyses of tectal processing have remained descriptive and treat single cells in isolation, independent of behavior and the activity of other visual, motion-sensitive midbrain structures such as the pretectum. Given its anatomical and functional position, an understanding of population activity in the OT during a well-defined visuomotor behavior would lead to new insights into how the vertebrate central nervous system isolates behaviorally relevant cues from sensory streams and transforms these into behavior.

In this study, we employ a combination of behavioral and calcium imaging techniques possible only in larval zebrafish to map the sensory and motor systems underlying visually evoked escape behavior and construct a working model of behaviorally relevant stimulus representation in the OT. We establish, for the first time, that larval zebrafish respond to visual stimuli representing object approach with directed C-bend escape maneuvers and describe a convergence in the circuits mediating mechanosensory and visual escapes at the premotor level. In addition, we demonstrate that the OT encodes escape latency across hundreds of neurons, providing a novel basis for ethologically relevant processing in collicular structures. These results outline the circuitry and computations controlling a robust, innate visually guided
behavior and reveal fundamental principles of neural system organization likely prevalent in subcortical visual structures across phyla.

2.2 Looming visual stimuli evoke fast escape maneuvers in larval zebrafish

To test how larval zebrafish respond to looming stimuli, we constructed an arena in which individual freely swimming fish were monitored with a high-speed camera while visual stimuli were presented with closed-loop feedback onto a screen beneath the animal (Figure 2.1A). The arena was large enough to accommodate the full extent of an escape swim without the larvae encountering a wall, allowing for unperturbed estimates of kinematic parameters. We were able to track the position and orientation of the fish at 506 frames per second while updating the visual stimulus in real time at 60 Hz, allowing for precise and stable control of the stimulus in the visual field. This high-speed, closed-loop stabilization was necessary because active swimming and turning would shift a fixed stimulus within the visual field, complicating characterization of the relationship between stimulus input and behavioral output and potentially reducing responsiveness. These locked egocentric stimulus positions generated maximal consistency in visual stimulation across presentations; furthermore, they best matched the conditions used in subsequent imaging experiments. Using this closed-loop behavioral tracking and stimulation system, looming dark spots, which mimic approaching objects, were presented on a neutral gray background to 5-6 days post-fertilization (dpf) larvae as they entered the center of the behavioral arena. These spots started at singular points offset orthogonally from the fish midline and expanded as disks with either constant radial velocity or constant approach velocity. Stimuli were presented either to the left or right side of the animal, remaining exclusively within each respective monocular visual field for at least the first half of the expansion period. These
looming stimuli typically evoked high-velocity, high-angle, long-distance swim maneuvers (Figure 2.1B-C) that we quantified using detailed kinematic analysis (Figure 2.1D).

To better distinguish looming-evoked escape responses from other maneuvers in the larval zebrafish behavioral repertoire (e.g. routine turns or spontaneous swimming), we plotted the maximum instantaneous linear velocities and bend angles of all locomotion events initiated after stimulus onset but before the stimulus had stopped expanding. This analysis revealed a cluster of high-velocity, high-angle events separated from routine turns and swimming, demonstrating that looming stimuli consistently evoke escape-like responses that are distinct from other behaviors (Figure 2.1E). To probe whether these responses were indeed specific to looming stimuli, we also presented spots that appeared instantaneously (flashed stimuli) or spots that dimmed with the same temporal dynamics as the looming stimuli. A response probability metric – the probability of maximum swim velocity exceeding 12.0 cm/s (Figure 2.2A-C) – indicated looming stimuli induced escapes about half of the time (51.0 ± 3.8%), whereas high-velocity escape maneuvers almost never occurred during presentation of dimming and flashed stimuli (3.4 ± 1.1% and 2.0 ± 1.3%, respectively).

To test in more detail which stimulus feature generated the escape behavior during looming stimulus presentation, we evaluated whether object expansion was the key trigger. Expanding disks decrease overall luminance; although larvae did not escape to dimming alone, it is possible that a conjunction of looming and dimming is required for triggering escapes. Therefore, we presented checkered looming stimuli 36, which were subjectively isoluminant over the time course of expansion. These stimuli were equally efficacious in evoking escape responses (57.0 ± 6.1% of the time, Figure 2.1E), providing further evidence that this behavior employs
complex, luminance-independent visual computations to detect expanding borders representing object approach, consistent with looming-evoked escape responses in other species.\textsuperscript{37–39}

Zebrafish escape behavior has so far been described primarily in the context of mechanosensory C-starts.\textsuperscript{40–44} In response to mechanical stimulation, fish quickly bend into a “C” shape followed by a rapid series of tail undulations that propel the fish away from the offending stimulus. To better define looming-evoked behavior and compare it to these C-start escapes, we analyzed 7 different kinematic variables across 4 looming stimulus conditions: black or checkered constant radial expansion and black or checkered hyperbolic expansion. While the former pair simulated a decelerating approach trajectory, the latter pair simulated object approach at constant velocity, the stimulus most commonly used in other organisms.\textsuperscript{45} We found that most kinematic variables tested were indistinguishable across the four stimuli with the exception of maximum bend angle, which varied slightly depending on the temporal dynamics of expansion (Figure 2.1F). Applying the Bonferroni correction, however, eliminates this significance. Thus, all four types of looming stimuli trigger indistinguishable motor programs.

**Figure 2.1 – Kinematic analysis of looming-specific escapes**

(A) Schematic of the closed-loop behavior setup used to present visual stimuli and monitor behavior. Larvae swim in a 9 cm petri dish as visual stimuli are projected via a cold mirror onto a diffusive screen underneath the dish. To monitor behavior, fish are illuminated with IR light while a high-speed camera, equipped with an IR-pass filter, acquires images at 506 fps. Video is analyzed online to detect fish position and orientation in real time. This information is used to update stimulus position with closed-loop feedback so that stimuli remain in a fixed position within the fish’s visual field.
Figure 2.1 (Continued)

(B) A projection of a single looming-evoked behavioral response (106 frames, 210 ms). An expanding, looming disk presented in the left visual field evokes a high-angle, long-distance maneuver to the right.

(C) Each individual trial can be separated into a looming phase (fish images, bottom to top), where the stimulus expands while locked to a fixed position from the fish center of mass (frames separated by 250 ms to illustrate dynamics, black arrowhead denotes stimulus start time), and an escape phase (fish images, top, left to right), during which the fish executes a high-angle, high-velocity escape maneuver (frames separated by the true frame period, 1.98 ms at 506 fps). Heading direction (pink vectors) and center of mass (pink dots) are extracted from each frame and used to characterize behavioral responses. (s), a projection of a spontaneous swim bout that occurs before this fish initiates an escape response, illustrating the need for closed-loop stimulus presentation. (*), The frame corresponding to the extreme maximum change in heading direction (inset, angle $\alpha$), which is typical for visually evoked escape responses. Bottom, plots of heading angle and instantaneous swim velocity isolated from video. High-angle looming evoked responses are also associated with high swim velocities of up to nearly 20 cm/s. A zoom-in of heading direction reveals a high-angle bend followed by a small counterbend (c) and a high-frequency burst swim lasting hundreds of milliseconds.

(D) An example of a looming-evoked escape with a large counterbend. The angle of 20 equally spaced points along the tail relative to the fish body axis can be extracted from high-resolution video, revealing detailed tail kinematics.

(E) Visually evoked high-angle, high-velocity events are elicited specifically by looming stimuli. Top left, a scatterplot of all swim events initiated during looming stimulus presentation ($N = 37$
Fish, n = 315 swim events). Bottom left, a scatterplot of all swim events initiated while presenting dimming (filled circles, N = 26, n = 116) or flashed (open circles, N = 10, n = 1097) stimuli. The cluster (red) of high-angle, high-velocity swim events evoked by looming stimuli is largely absent in dimming and flashed trials. Right, a quantification of escape response probability across multiple stimulus conditions. Escape responses are separated from routine turns and swims using a threshold maximum swim velocity of 12.0 cm/s (dashed red line). Looming black (N = 33 fish and checkered (N = 8) spots elicit escape-like maneuvers much more often than dimming (N = 27) or flashed (N = 6) stimuli. Error bars represent mean ± SEM across fish. **, p < 10^-5, permutation test.

(F) Analysis of 7 kinematic variables across 4 different classes of looming stimuli that vary in either expansion dynamics (linear or hyperbolic) or relative contrast (black or checkered isoluminant). Left, kinematics associated with the initial high-angle bends of classified escapes. Right, kinematics associated with the burst swim phase of behavioral responses. Observed kinematics are similar to those reported for sound- and touch-evoked escapes. While expansion dynamics may affect kinematics slightly, all variables are statistically similar across all 4 conditions after applying the Bonferroni correction (p = 0.008 maximum angle, p = 0.199 bend duration, p = 0.241 bend velocity, p = 0.086 swim distance, p = 0.077 burst frequency, p = 0.125 swim velocity, p = 0.175 counterbend angle, F-statistic (ANOVA) permutation test). Offset points and error bars are mean ± SEM across fish. Each open circle is the mean for a single fish. N = 49 fish, n = 301 responses, black linear expansion; N = 19, n = 192, black hyperbolic expansion; N = 8, n = 65, checkered linear expansion; N = 25, n = 322, checkered hyperbolic expansion.
Figure 2.1 (Continued)
Across all stimulus conditions, looming-evoked behaviors are characterized by at least 3 unique phases. First, larvae initiated a rapid (bend duration 9.4 ± 0.1 ms; maximum bend velocity 19.5 ± 0.2 °/ms), high-angle bend (133.4 ± 2.1°), that quickly reverses heading direction. Second, fish performed a counterbend that re-oriented the body (70.4 ± 1.3°). Third, fish executed a high-velocity burst swim (velocity 16.4 ± 0.2 cm/s; burst (undulation) frequency 62.7 ± 0.9 Hz) away from the starting position (distance 1.5 ± 0.1 cm, mean ± SEM across fish). These kinematics closely resemble the C-start escape behaviors elicited by other, mechanosensory modalities (Budick and O’Malley, 2000; Eaton et al., 1988, Figure 2.2D-F) and are starkly different from the high-angle dark flash o-bend or large spot avoidance behaviors previously described. Thus, this response to looming stimuli is the first description of a rapid escape behavior elicited by a visual stimulus in larval zebrafish.

**Figure 2.2 – Escape velocity threshold calculation and tap-evoked escape kinematics**

(A) A histogram of maximum instantaneous swim velocities for each swim event in Figure 1E, top (looming trials). This histogram is fit well by the sum of 3 underlying Gaussian functions (red). N = 37 fish, n = 315 swim events.

(B) The same histogram as in (A) but with each individual Gaussian plotted in green. The 12.0 cm/s swim velocity threshold used in all subsequent analyses was calculated as the point of intersection between the second and third underlying Gaussian functions.

(C) Residuals for the fit shown in (A) with a normalized root-mean-square deviation (NRMSD) of 2.33%. Because there were multiple fits that varied slightly in peak locations and widths that performed equally well, the reported threshold is the mean of 200 repetitions of the non-linear optimization algorithm used to fit the curves (peakfit function, Matlab).
Figure 2.2 (Continued)

(D) Comparison between tap- and looming-evoked escape kinematics for the 7 kinematic variables analyzed in Figure 1F. For tap-evoked trials, larvae were placed in individual wells of a 6-well petri dish and imaged on the behavioral setup while a brief mechanical pulse was delivered to the behavioral platform. Tap-induced escape responses were comparable to looming-induced escape responses but with some differences. Specifically, tap-induced escape maximum angle 127.02 ± 5.76°, p = 0.3212; burst frequency 46.92 ± 4.78 Hz, p = 0.0154; swim distance 0.80 ± 0.06 cm, p = 0.0077; bend velocity 21.03 ± 0.54, p = 0.1880; swim velocity 16.64 ± 0.78, p = 0.4166; bend duration 8.96 ± 9.42 ms, p = 0.3476; counterbend 67.68 ± 10.03°, p = 0.4071, permutation test. Mean ± SEM across fish. N = 5 fish, n = 11 escape events. All looming-evoked data from Figure 1F were pooled. See main text for the respective values of looming-evoked kinematics. N = 101 fish, n = 880 escape events. Although no kinematic variable reached statistical significance after applying the Bonferroni correction (p < 0.05/7 = 0.0071), swim distance appeared to vary between tap- and looming-evoked escape behaviors, potentially because the looming stimulus followed the fish while the tap stimulus occurred only once. Thus, both stimuli produce escape responses, with some variation in the detailed behavioral kinematics.

(E) Three examples of tap-evoked and (F) looming-evoked behaviors. While the trajectory projections (right side of each black line) are different across each stimulus class (especially in duration and distance traveled), the overall bend kinematics are similar. Frames are separated by the same recording frame period (1.98 ms at 506 fps).
Figure 2.2 (Continued)

A

B

gaussian fit (sum)

gaussian fit (components)

computed threshold

C

NRMSD = 2.33%

D

E

TAP

F

LOOM

max. swim velocity (cm/s)

fit residual

max. swim velocity (cm/s)

max. bend angle (°)

burst frequency (Hz)
swim distance (cm)
bend velocity (°/ms)
swim velocity (cm/s)
bend duration (ms)
counterbend (°)

TAP (T) LOOM (L)

5 mm
2.3 Escape trajectories are dictated by stimulus position within the visual field

The directionality of escape can often be influenced by the location of the eliciting stimulus, reflecting an obvious but important strategy to effectively distance oneself from threats. Touch-evoked escapes in larval zebrafish are coarsely directional \(^{50}\), and looming-evoked behaviors in both flies \(^{51}\) and adult goldfish \(^{52}\) show a dependence on incident angle. To probe whether looming-evoked behavior in larval zebrafish is influenced by stimulus position, we compared the escape trajectories elicited by looming stimuli presented in fixed positions in either the front, back, right, or left visual field (0, 180, 270, and 90° relative to the fish center of mass, respectively). In 33 fish, stimuli in the right visual field consistently evoked escapes to the left, and vice versa. This relationship is readily identifiable when escape trajectories are rotated and aligned onto the body axis for each condition (Figure 2.3A). Despite differences in escape direction (Figure 2.3B, rose plots), the velocity of escape maneuvers is similar across all conditions, as evidenced by plots of fish position 50 ms after escape initiation (Figure 2.3B). Quantification of response preference, (# left turns - # right turns)/(# total turns), across fish formalizes a strong positional dependence for left and right stimuli (0.68 ± 0.09 and -0.69 ± 0.09, respectively) and a lack of directional bias for front and back stimuli (-0.14 ± 0.11 and 0.10 ± 0.10, respectively) (Figure 2.3C). Further analysis reveals that the observed trajectory bias reflects differences in absolute maximum turn angle (100.5 ± 2.3° for left; 100.7 ± 2.2° for right; 114.6 ± 1.5° for front, 94.5 ± 2.0° for back stimuli), with back stimuli eliciting significantly shallower (smaller turn angle) responses (p = 0.0024, permutation test). As indicated, the distance traveled by larvae after 50 ms does not depend on stimulus position (3.61 ± 0.04 mm for left, 3.54 ± 0.04 mm for right, 3.75 ± 0.04 mm for front, 3.58 ± 0.04 for back stimuli; back, front p = 0.1266, permutation test). It is worth noting that even in response to the same stimulus type,
individual trajectories are highly variable; this suggests that larvae might employ a protean evasion strategy \(^5^3\) that makes it harder for predators to predict and foil escapes once they are triggered. Nevertheless, these data demonstrate that larvae utilize a sensorimotor transformation that conserves positional stimulus information and alters escape motor programs accordingly.

**Figure 2.3 – Stimulus position dictates escape direction**

(A) Top, escape trajectories elicited by looming dark spots in the right (blue, \(N = 34, n = 214\)) or left (black, \(N = 33, n = 198\)) visual field. Bottom, escape trajectories elicited by looming dark spots centered in the nasal[back] (orange, \(N = 21, n = 164\)) or temporal[front] (green, \(N = 23, n = 177\)) visual field.

(B) Left, radial plots of fish position 50 ms after escape initiation. Right, rose histograms of the maximum turn angle for all events in (A). While the distance traveled is similar across all 4 conditions, each stimulus evokes a unique distribution of escape angles that underlie the differences in trajectories evident in (A).

(C) Quantification of behavior across all 4 stimulus positions. Top, bar plots of left-right preference \([# \text{left turns} - # \text{right turns}] / ( # \text{left turns} + # \text{right turns})\) for right (blue, \(N = 26\)), left (gray, \(N = 26\)), back (orange, \(N = 18\)), and front (green, \(N = 17\)) stimuli for fish with at least 5 escape responses. Left and right stimuli consistently evoke responses directed away from the starting stimulus position, whereas back and front stimuli evoke responses to the left and right with near-equal probability. Middle, bar plots of mean distance traveled after 50 ms. Bottom, bar plots of mean maximum turn angle across all 4 conditions. Front stimuli consistently elicit higher angle turns when compared to back stimuli. (*) \(p < 0.001\), permutation test. Error bars are mean \(\pm SEM\) across trials.
Figure 2.3 (Continued)
2.4 – Escapes are triggered when stimuli reach a critical visual angle

To probe the effect of stimulus expansion velocity on visually evoked escape behaviors, we next presented a set of 5 stimuli that mimicked disks of constant radius approaching larvae at different velocities. When projected onto a flat surface, these stimuli can be described by functions of spot radius over time arising from fixed ratios of simulated disk radius and approach velocity (R/V, Figure 2.4) 45.

**Figure 2.4 – Simulating object approach at constant velocity**

(A) Left, top-down schematic representation of a disk (black, seen from above) with radius $R$ approaching a zebrafish larva at constant velocity, $V$. Under these conditions, the distance, $d$, between the disk and the larval eye changes as $d(t) = -V \times t$, where $t \leq 0$ and $t = 0$ is the time of collision. As the disk moves closer, the angular image size, $\theta$, and the edge expansion velocity, $\theta_v$, change as functions of time, $\theta(t)$ and $\theta_v(t)$, respectively (see gray bars and dotted lines, representing future positions of the disk if it were to continue on its collision trajectory). If one assumes that the fish has access only to the monocular projection of the disk on the retina, real object approach may be simulated by presenting a disk characterized by $\theta(t)$ and $\theta_v(t)$ on a fixed screen. Right, the functions $\theta(t)$ and $\theta_v(t)$ can be described geometrically and depend on the ratio $R/V$ (red). The absolute size or velocity of an approaching object cannot be determined under this limited schema; only the ratio of size to velocity changes the apparent image expansion profile. This schematic was adapted from 18.

(B) A perspective representation of the behavioral arena used for looming experiments. For this analysis, $\theta$ was calculated using the stimulus diameter parallel to the fish body axis and

(C) $d_{\text{screen}}$ (pink dotted line).
Figure 2.4 (Continued)

(D) When simulating the approach of an object with some fixed $R/V$, and thus a determined $\theta(t)$, the radius of the projected looming stimulus grows as $R_{\text{screen}}(t)$.

\[ \theta(t) = 2 \times \tan^{-1} \frac{R}{d(t)} \]
\[ = 2 \times \tan^{-1} \frac{R}{-V \times t} \]
\[ \dot{\theta}(t) = \frac{d\theta}{dt} \]
\[ = \frac{2R/V}{t^2 + (R/V)^2} \]

\[ \tan \frac{\theta(t)}{2} = \frac{R_{\text{screen}}(t)}{d_{\text{screen}}} \]
\[ R_{\text{screen}}(t) = \frac{R \times d_{\text{screen}}}{-V \times t} \]
Escape latency was strongly modulated by stimulus velocity, with faster stimuli reliably eliciting escapes with shorter latencies (Figure 2.5A). When functions of stimulus image size and edge velocity are evaluated at times of escape onset (minus a fixed processing lag, see Experimental Procedures), an average threshold in angular image size (72.0 ± 1.3°, p = 0.1976, F-statistic (ANOVA) permutation test, Figure 2.5B) but not edge velocity (Figure 2.5C) emerges (p < 10⁻⁵, F-statistic (ANOVA) permutation test). This result is similar to descriptions of looming-evoked escape behaviors in other organisms 36 and suggests that the circuits processing looming stimuli may primarily use stimulus size information when determining when and if an escape should be initiated.

**Figure 2.5 – Stimulus dynamics dictate escape direction**

(A) Records of swim velocity over time for escapes elicited by stimuli simulating approach at 5 different velocities (255 ms, 495 ms, 730 ms, 980 ms, 1450 ms R/V, top to bottom). The ratio R/V is a unique identifier of expansion trajectory [visual angle = θ(t) = 2*arctan(R/(-Vt)), t <= 0, where t is time to simulated collision]. The spots above each trace represent the size of the looming stimulus before a critical size is reached (dark spot), after which an escape is initiated with a fixed delay. Stimuli continue to expand (ellipses) until the end of the allotted trial time.

(B) Plots of visual angle (solid lines, velocity decreasing from left to right) evaluated at the average response latency for each velocity condition (crosses). The visual angle subtending the parallel axis of the stimulus 141 ms before escape initiation is not significantly different across all 5 conditions (p = 0.1976, F-statistic (ANOVA) permutation test). This suggests that escapes are triggered by looming stimuli crossing a critical visual angle. Because it is difficult to estimate the time it takes for visual information to reach the motor system and evoke a response, we
Figure 2.5 (Continued)

determined the fixed delay (141 ms) by minimizing the threshold visual angle standard deviation across all trials (see Experimental Procedures).

(C) Plots of edge velocity, $\hat{\theta}(t)$, evaluated at the average response latency for each velocity condition (crosses). Stimulus edge velocity 141 ms before escape initiation is significantly different across stimulus conditions (*** p < $10^{-5}$, F-statistic (ANOVA) permutation test). This suggests that information about specific edge velocity is ignored when determining escape latency, and only a visual angle threshold is used (E). Dotted lines represent the mean visual angle or edge velocity 141 ms prior to escape initiation across all conditions. For 255 ms R/V, N = 9, n = 11; 490 ms, N = 12, n = 20; 730 ms, N = 18, n = 25; 980 ms, N = 19, n = 31; 1450 ms, N = 21, n = 41. Error bars are mean ± SEM across trials.
2.5 – Looming stimuli are primarily represented in the optic tectum

Our classification of looming-evoked escape behavior allowed us to explore the representation of this novel and ethologically relevant stimulus across various visual brain regions, focusing on identifying likely sites of relevant computation using calcium imaging in pan-neuronal Tg(elval3-GCaMP5G) 5-6 dpf larvae \(^{54}\) (Figure 2.6A). To this end, larvae were fully embedded in agarose and imaged with a two-photon laser scanning microscope during stimulus presentation \(^{55}\) to screen neurons for response selectivity. Stimuli were presented as black disks on a red background to avoid stimulus bleed-through but were otherwise presented as in the freely swimming setup. Looming stimuli and flashed stimuli evoked responses throughout the midbrain, which we segregated into 3 main regions based on anatomical boundaries and functional similarities: the optic tectum (OT), the pretectum/thalamus (PT/TH), and the midbrain tegmentum (MB) (Figure 2.6B). Responses to stimuli were diverse, but, within the scope of this study, were categorized based only on significant differences in activity during looming and flashed stimulus epochs compared to baseline (see Experimental Procedures). This reduction allowed us to analyze stimulus selectivity, an indicator of processing specificity, and form a map of ethologically relevant receptive fields throughout visual processing regions.

To quantify the anatomical distribution of looming-selective neurons across brain regions and fish, we mapped active neurons to a standard fish brain \(^{55}\) after assigning a loom/flash selectivity index (SI, \((z_{loom} - z_{flash})/(z_{loom} + z_{flash})\), see Experimental Procedures) that effectively classified neural responses, with more positive values reflecting greater looming selectivity (Figure 2.6C). This analysis revealed a preponderance of looming-selective neurons in the ventral OT compared to the other midbrain regions we analyzed (Figure 2.6D). Responses in PT/TH were typically smaller and equivalent across both looming and flashed stimulus...
presentations, and activity in MB was generally uncoupled from stimulus presentation. Neurons in the dorsal OT were less responsive to either stimulus. Furthermore, looming-selective activity in the OT typically peaked prior to the end of expansion, consistent with the timing of escape initiations (see examples in Figure 2.6B). On average, the OT was more than twice as selective as PT/TH or MB (0.35 ± 0.01, 0.17 ± 0.01, 0.11 ± 0.01 SI, respectively; OT, PT/TH p < 10^{-5}; OT, MB p < 10^{-5}, permutation test) and contained almost twice as many responsive neurons per unit volume (3.32 ± 0.40, 1.68 ± 0.27, 2.16 ± 0.32 active neurons / 1000 μm³, respectively; OT, PT/TH p = 0.00078; OT, MB p = 0.00992, permutation test). Furthermore, responses in the OT were also lateralized, with neurons in the left OT responding to looming stimuli in the right visual field and vice versa, consistent with the contralateral segregation of retinal streams and providing a putative mechanism for the observed lateralization of escape trajectory. Taken together, these results suggest that the OT serves as the primary looming detector within the larval zebrafish brain.

**Figure 2.6 – Looming-specific neurons in the optic tectum**

(A) Left, schematic of the larval zebrafish brain indicating the positions of the optic tectum (OT) with its neuropil (NP) and cell body layers (stratum periventriculare, SPV), the pretectum/thalamus (PT/TH), and the midbrain tegmental region (MB). Right, a transverse average intensity projection of a 5 dpf Tg(elval3-GCaMP2) larval brain and accompanying sagittal view. (TH), thalamus. (PT), pretectum.

(B) Left, single planes showing anatomy (gray) and activity (blue) in the dorsal and ventral OT, the PT/TH, and MB. Individual ROI numbers correspond to the traces on the right, which illustrate the general pattern of activity observed across midbrain visual areas in response to
Figure 2.6 (Continued)

looming stimuli. Neurons in the dorsal OT (1 and 2) respond weakly to looming stimuli. Neurons in the ventral OT (3 and 4) show more variegated responses but typically respond strongly to and favor looming stimuli. Neurons in PT/TH (5 and 6) respond to both looming and flashed stimuli. Neurons in MB (7 and 8) were typically active spontaneously and non-stimulus-locked. Boxes represent stimulus presentation periods. Traces are re-ordered and concatenated from longer recordings.

(C) Middle, trial-averaged normalized $\Delta F/F$ evoked by looming and flashed stimuli from 1,613 active neurons across 12 fish. Each neuron is sorted according to its selectivity index (see Experimental Procedures) in descending order ($1 =$ looming exclusive, $-1 =$ flash exclusive). Left, the corresponding anatomical location of each neuron, color-coded as in (B). Looming-selective neurons are enriched in the OT. Right, the average normalized $\Delta F/F$ binned across 100 neurons from the sorted list, illustrating the fidelity of our selectivity index. Dotted lines and boxes represent stimulus presentation periods, with start times indicated by arrowheads.

(D) Top, all neurons from (C) mapped to a reference brain and colored according to selectivity index. Dotted lines denote the position of each eye. The arrowhead in the sagittal projection highlights the preponderance of looming-selective neurons in the ventral OT. Differences in the number of OT neurons between the left and right hemispheres reflect a sampling bias; most OT imaging was unilateral. Middle, bar plots quantifying mean selectivity (left) and responsive cell density (right) across the OT ($n = 60$ imaging planes), PT ($n = 34$), and MB ($n = 44$). Bottom, histograms showing the distribution of looming selectivity across neurons in the OT ($n = 973$ cells), PT ($n = 279$), and MB ($n = 361$). The OT shows the highest responsiveness to and
selectivity for looming stimuli. (** p < 10^{-5}, (*) p < 0.01, permutation test. N = 12 larvae. (r) rostral; (c) caudal; (d) dorsal; (v) ventral.
2.6 – Population activity encodes critical image size during looming

If the OT is fundamentally involved in looming processing, activity in the OT should reflect the input-output relationships observed in freely swimming fish. To test this, we presented a set of monocular looming stimuli expanding at 3 different R/V ratios while imaging neural responses in the ventral OT.

Like ventral OT responses to constant radial expansion, activity in ventral OT neurons typically peaked prior to the end of expansion across all 3 velocity conditions (Figure 2.7A, top). The timing of these peaks relative to stimulus onset, however, was strongly influenced by expansion velocity, with responses to the slowest stimulus peaking nearly 8 seconds after responses to the fastest; this trend was reminiscent of the velocity-latency relationship we observed in freely swimming fish. In order to better evaluate this correspondence, we next sought to quantify the dynamics of OT responses to our stimuli. Because single-cell responses are probably not a reliable indicator of holistic OT function, we decided to examine OT activity at a network level. To this end, we performed principal component analysis on 1,816 neurons across 10 fish in order to provide an unbiased estimate of looming representation across the OT neuronal population.

After aligning stimulus size over time with the first temporal principal component, which explained between 44% and 82% of the neural response variance for each velocity, a clear link between the principal component and stimulus size is revealed (Figure 2.7A, bottom). Across all 3 velocity conditions, the OT population consistently signals a common angular image size (81.9 ± 3.2°, mean ± SEM across fish, p = 0.481, ANOVA) during expansion as its activity crosses a fixed threshold (82%, see Experimental Procedures, Figure 2.7B, left). Although threshold image sizes are statistically indistinguishable across simulated approach velocities,
there is an upward trend of the critical angle encoded by the OT with decreasing approach velocity. This may reflect either exacerbated artifacts of our calcium indicator that are not captured by our convolution for slow speeds or a minor contribution from stimulus velocity to PC1 dynamics. Nevertheless, the threshold angular image size represented by the OT population is in close agreement with the critical image size found to trigger the behavior in freely swimming experiments (72.0 ± 1.3°). And not unlike the behavior, the first principal component does not reach threshold at a coherent edge velocity (Figure 2.7B, right). These data argue that the OT is capable of encoding a critical looming visual angle as an ensemble, providing the first example of a putative mechanism for salient expansion encoding across a collicular population.

**Figure 2.7 – neurons in the optic tectum encode critical size**

**(A)** Top, the responses of 110 OT neurons in 1 fish to looming stimuli simulating approach at 3 different velocities (R/V 650 ms, 1300 ms, 3235 ms, top to bottom). The stimulus size over time for each condition, convolved with a calcium impulse response function (CIRF) (τ = 1.6 s) is shown in red. Bottom, the first temporal principal component (PC1, ± SEM across fish) averaged over 10 fish with a total of 1,816 neurons, overlaid on the convolved stimulus size. The spots above each trace represent the size of the looming stimulus before the PC1 threshold (dark spot). Stimuli continue to expand (ellipses) until the end of the stimulus epoch.

**(B)** Quantifications of average stimulus visual angle (left) and edge velocity (right) at PC1 threshold times (82% maximum response, N = 10 fish, n = 1,816 neurons) for all 3 velocity conditions. The colored curves show the convolved stimulus size and edge velocity for each conditions, fanned to show the PC1 dynamics for each individual fish (in experiments, each curve starts at t = 0). Each color value corresponds to the normalized amplitude of the PC1
Figure 2.7 (Continued)

activity during expansion. Note that the transitions to high PC1 activity (red) occur at similar visual angles but not at similar edge velocities. Crosses show the average value of each stimulus variable at the PC1 threshold for each velocity condition. Error bars are SEM for PC1 threshold timing (horizontal) and respective stimulus variables (vertical) across fish. Dotted lines represent the mean visual angle and edge velocity at PC1 threshold across all conditions. Population activity within the OT reaches threshold at a common visual angle but not a common edge velocity, similar to the behavior (Figures 2.5A-C).
2.7 – The Mauthner system dictates looming-evoked escape direction

After looming stimuli are processed by the OT, the OT must recruit a specific motor program that completes the sensorimotor transformation (see proposed model, Figure 2.8A). In flies and locust, this motor program is conveyed by the giant fiber system, but in vertebrates the relevant motor systems have not been fully characterized. In adult teleost fish, the Mauthner cell (M-cell), a large hindbrain spinal projection neuron involved in mechanosensory escapes, responds to looming stimuli \(^{14}\) and receives projections from the OT on its ventral dendrite \(^{19}\). However, the specific role of the M-cell in visually evoked escape behavior has not been tested. Given this history and the kinematic similarities between the escape responses evoked by mechanosensory and looming stimuli, we hypothesized that the M-cell and its segmental homologues, morphologically and functionally similar neurons in rhombomeres 4-6 \(^{42}\), would govern visually evoked escape behavior.

To test this hypothesis, we backfilled the hindbrain reticulospinal system \(^{41,49,57}\) to label the M-cell and its homologues (MiD2 and MiD3) and target them for laser ablation with a two-photon microscope. A short (100 ms to 2 s), high power (~100 mW at sample) laser pulse \(^{58}\) was sufficient to cause a loss of cell morphology and fluorescence specific to the targeted neuron and not its labeled neighbors (Figure 2.8B). Because escape direction is lateralized and easily separated by left-right stimulus position, we performed unilateral ablations of the M-cell and its homologues, using the intact contralateral side as an internal control. Analysis of monocular looming-evoked escapes before and after unilateral ablation revealed a pronounced decrease in maximum turn angle (Figure 2.8C). Only responses contralateral to the ablated M-system (M-cell, MiD2, and MiD3) were perturbed, as evidenced by the significantly smaller average turn angle specific to the affected side (non-ablated side, pre 102.5 ± 3.6°, post 97.7 ± 3.5°, \(p = \))
0.0934; ablated side, pre 99.1 ± 3.3°, post 71.8 ± 3.4°, p = 0.0022). This change is consistent with the laterality conferred by the descending axons of the M-system and is not explained by non-specific perturbations of other spinal projection neurons like the ventromedially located spinal projection neurons, which determine the direction of ipsilateral turns. As a result of this turn deficit, escape trajectories also changed; looming stimuli ipsilateral to the ablated side tended to elicit escapes that followed a more forwardly path (Figure 2.8D). However, the reduction in turn angle and trajectory was not concomitant with an obvious decrease in escape velocity or distance, as evidenced by stick plots of fish position 40 ms after escape initiation (Figure 2.8E). The turn deficit was confirmed on a fish-by-fish basis (Figure 2.8F), and cumulative distribution plots of maximum turn angle before and after ablation also revealed a significant shift in turn angle for the ablated side across all trials (p<10^{-5}).

Other kinematic parameters like escape duration (Figure 2.8G) remained unchanged for responses to both the non-ablated and ablated side (non-ablated side, pre 187.0 ± 9.7 ms, post 170.9 ± 5.1 ms, p = 0.073, permutation test; ablated side, pre 194.7 ± 9.8 ms, post 176.4 ± 5.9 ms, p = 0.101, permutation test), suggesting that an independent population of neurons may control the late phase of the visually evoked escape response. Because it is possible that the ablations affected neurons in the vicinity of the M-system and thus perturbed turn angles non-specifically, we analyzed the distribution of spontaneous swims before and after ablation and found that histograms of spontaneous turn angles before and after ablation are not significantly different (Figure 2.8G, right). As spontaneous turns are thought to be governed by separate premotor circuitry, we conclude that ablations were specific to the intended M-system targets.

In summary, these data demonstrate that the M-cell and its segmental homologues are necessary for the initial, high-angle bend of the visually evoked escape response but not for
subsequent swim kinematics. This provides strong evidence for multi-modal convergence of sensory signals within the M-system of larval zebrafish and is the first study to establish a necessary role of the M-system in visually evoked behavior. The fact that the M-system influences turn angle in our study is surprising, as ablations of the M-cell and its homologues appear only to affect response latency and velocity in mechanosensory escapes\textsuperscript{42,44}. One interpretation of this marked difference is that there is less redundancy in the visually evoked escape pathway than in the tactile and acoustic escape pathways, perhaps reflecting a functional bias towards more reliable modalities. We have established that the M-system assumes an essential role in the sensorimotor transformation from looming stimuli to escape behavior, providing a functional scaffold for the zebrafish to quickly evade threats identified with their eyes alone.

**Figure 2.8** – *Laser ablation of the Mauthner system alters escape trajectory and reduces initial bend angle*

(A) Schematic of the zebrafish brain and hypothesized information flow from the eye, through the contralateral OT, to the contralateral hindbrain Mauthner system (M-system) comprising the Mauthner cell (M-cell) and homologues in rhombomeres 4-6. Unilateral ablations of the M-system alter the bend angle of contralateral escapes (relative to the ablated side and stimulus position, pink) but not ipsilateral escapes (black) compared to pre-ablation responses. While the bend angle is affected (C-bend escape), other escape kinematics are not, suggesting that a significant part of the visually evoked escape motor program is mediated by a separate, non-Mauthner population of neurons (alternative escape).

(B) Two-photon micrographs showing an example of the M-cell (left), MiD2 (center), and MiD3 (right) pre- and post- unilateral ablation. For each fish, the M-cell and MiD2/MiD3 clusters were
ablated together. Ablations were specific to targeted neurons and did not affect nearby cells. Cells were backfilled with dextran-conjugated dye. Scale bar is 20 μm.

(C) The average maximum turn angle during escapes is significantly altered for maneuvers contralateral to the ablated side, with the largest change evident at the first (maximum) bend. The average escape ipsilateral to the ablated side remained unchanged. All traces are aligned to the time point of the first bend. Error is SEM across all trials, N = 8 fish. Pre, non-ablated, n = 60; pre, ablated, n = 73; post, non-ablated, n = 88; post, ablated, n = 66. (*) p = 0.002.

(D) Left, escape trajectories pre- and post-ablation (green and pink, respectively) elicited by stimuli ipsilateral to the ablated side. Right, escape trajectories pre- and post-ablation (green and black, respectively) elicited by stimuli contralateral to the ablated side. Escapes evoked by looming stimuli ipsilateral to the ablated M-system tend to be directed to more forward-bearing directions than their pre-ablation or control (contralateral) counterparts.

(E) Top, stick diagrams representing fish position 40 ms after escape initiation for the ablated and non-ablated sides, pre- and post-ablation. The shift in escape trajectory post-ablation is more apparent on this time scale. Bottom, rose histogram of the maximum turn angle pre- and post-ablation for the ablated and non-ablated sides. Note the shift to smaller turn angles for the ablated side (pink).

(F) Left, quantification of maximum turn angle across fish pre- and post-ablation for the ablated and non-ablated sides. (**) p = 0.002, (n.s.) p = 0.093, permutation test. Right, this change is also apparent in histograms of bend angle across all trials, p <10^-5 ablated side, p = 0.175 non-ablated side, permutation test. The decrease in bend angle contralateral to the ablated M-system is significant. Error is bootstrapped SEM.
Figure 2.8 (Continued)

(G) Left, quantification of escape duration. No significant change is apparent on either side after ablation. Non-ablated, p = 0.073. Ablated, p = 0.101, permutation test. Error bars are SEM across fish. Right, the distribution of spontaneous turns does not change after ablation, providing further evidence of ablation specificity; pre-ablation n = 744, post-ablation n = 911 spontaneous swim events, (n.s.) p = 0.360, permutation test. Error is bootstrapped SEM.
Figure 2.8 (Continued)
2.8 – Discussion

We have shown, to the best our knowledge, the first example of a visually evoked escape behavior in larval zebrafish. This escape behavior is elicited specifically by looming stimuli that simulate the approach of an object or predator and not by flashed or dimming spots, illustrating a highly tuned system for processing image expansion within the zebrafish visual system. High-angle turns elicited by whole-field dark flashes have been described previously, but these maneuvers, labeled o-bends due to the “o” shape the tail assumes during a response, are relatively slow and are not followed by high-velocity burst swims. Furthermore, o-bend directionality appears linked to either turn history or asymmetries in field luminance, not stimulus origin, which is only ambiguously defined given the whole-field nature of the flash. Thus, the dark flash response appears more to re-orient larvae than to propel them away from harm. Given these differences, we believe that looming-evoked escapes represent a separate, novel class of visually evoked behavior in larval zebrafish.

Analysis of the relationship between response latency and approach velocity under our experimental conditions revealed a clear threshold angular image size that is reminiscent of looming size thresholds in other organisms. In locusts and flies, the link between stimulus dynamics and behavior has been traced to a pair of wide-field neurons whose spike rates during looming can be described by a function (typically labeled $eta$) that peaks with a fixed delay before a critical visual angle and the onset of escape. Because our current stimulus set cannot differentiate between a threshold-crossing or peak-finding mechanism for escape initiation, it remains possible that a similar $eta$ mechanism is governing looming computations within the zebrafish brain. And although our OT activity analysis suggests that fish may utilize a threshold, the relationship between peak PC1 timing and peak convolved edge velocity, which
closely resembles *eta*, warrants further investigation. Given the level of anatomical contrast between the wide-field invertebrate looming detection system and the complex, stratified OT in zebrafish, the implementation of an *eta*-based computation, which relies on a non-linear combination of excitation and inhibition, is expected to be unique to collicular organization. However, further behavioral experiments are necessary in order to unambiguously define the specific neural computation underlying looming detection.

Calcium imaging of the main retino-recipient midbrain structures in response to looming and flashed stimuli revealed a preponderance of looming-selective neurons within the ventrocaudal OT. This selectivity may be conferred by the integration of motion-selective inputs from the retina \textsuperscript{61} or from within the OT itself \textsuperscript{22,23}. Although we have not assessed the necessity of the OT for looming-evoked escapes, the high density of looming-selective responses in the ventrocaudal OT provides strong correlative evidence that the OT is involved in processing looming stimuli. The OT is involved in object motion estimation in many animals \textsuperscript{25}, and, in zebrafish, extra-tectal neurons and arborization fields are thought to process other visual cues like whole-field motion and luminance changes \textsuperscript{56}. In addition, a broad survey of tectal receptive fields in zebrafish larvae found that subsets of tectal neurons did respond to looming stimuli, but neither the specificity nor the spatial extent of these responses were described \textsuperscript{24}. Furthermore, electrophysiological studies of tectal and collicular responses to looming stimuli in pigeons \textsuperscript{62}, owls \textsuperscript{29}, and cats \textsuperscript{63} have revealed functionalized classes of looming-responsive neurons. While these studies only tracked single neuron responses, we were able to analyze tectal responses to looming stimuli at a population level, which revealed that ensembles of neurons in the OT are capable of encoding a fixed angular image size that corresponds roughly to the critical visual angle triggering the escape behavior.
In our study, the ventrocaudal OT responds most strongly to looming stimuli. Given the broad responsiveness of neurons within this region and the nature of our stimulus delivery (stimuli were always projected onto the dorsal retina), it is likely that this anatomical specificity arises primarily due to the established retinotopy of the OT\textsuperscript{64} and does not reflect a specialized processing region. However, the broad spatial distribution of activity within the OT does suggest, along with our principal component analysis, that the computation and isolation of looming-related features from the visual scene may operate at a network level before activity is projected out of the OT to recruit downstream motor programs.

While it remains possible that a subset of looming-selective neurons in the OT form a specialized class of looming detectors that drive behavior, a distributed representation of critical visual angle presents several distinct advantages. First, the majority of tectal neuron spatial receptive fields (as assayed by moving spots) are smaller than the critical image size that appears to trigger the escape behavior\textsuperscript{24}. Large stimuli may thus be encoded best by a combination of multiple tectal neurons staggered over retinotopic space. Second, a distributed representation of stimuli may increase the overall flexibility of stimulus representation in the OT. Because animals must extract relevant information from complex visual scenes occupying a large combinatorial space, a functional platform for encoding many different stimuli across the OT population can guide behavior flexibly by feeding any and all processed visual stimuli to downstream decoders as a unique set of activity. The ensemble encoding of critical visual angle during stimulus expansion thus constitutes a small subset of the total stimulus space likely spanned by the OT. Last, high-dimensional representations of stimuli have been shown to improve animal performance on tasks by increasing the degeneracy of available input-output relationships in readout neurons\textsuperscript{65}. Following this logic, the M-system (in our proposed circuit model) would be
free to weigh inputs from the OT over a wide range, ensuring that escapes are triggered reliably across animals and stimulus presentations.

Through laser ablations of the M-cell and its segmental homologues, we demonstrate that the M-system is required for determining bend amplitude in an escape response. While canonical C-start escape responses are preceded by a spike in the M-cell, ablation of the M-system only affects escape latency and bend velocity, not bend angle, when assayed with acoustic or tactile stimuli \(^{42,44}\). These results have cemented an idea of parallel hindbrain escape circuitries that form a redundant pathway for escape behavior. However, ablation of the M-system results in a specific bend deficit in response to looming stimuli, suggesting that visual stimuli recruit only a subset of the available escape circuitry. Because an approaching predator or object on a collision course with the fish will likely stimulate both visual and mechanosensory systems, the observed differences in Mauthner permissiveness may reflect an underlying functional pattern governing multisensory integration at the level of the M-system. How the escape circuitry receives signals from visual areas to trigger an escape is still unclear, however. Anatomical and functional evidence from adult goldfish has suggested a direct pathway from the OT to the ventral dendrite of the M-cell \(^{66}\), but this pathway has yet to be confirmed in larval zebrafish, leaving open the possibility of either a direct or indirect path from the OT. Taken together, our study provides an overview of the circuit mediating a visually evoked escape behavior in a vertebrate model organism and provides an important foundation for future studies of ethologically relevant tectal processing.

2.9 – Experimental Procedures

*Quantifying swim kinematics of freely swimming zebrafish*
Larvae (5-6 dpf) were monitored using a high-speed camera in a 9.2 cm petri dish (VWR). Filtered facility fish water was kept between 3 – 5 mm in height to minimize variability in stimulus visual angle from the point of view of the fish. A high-speed camera (Mikrotron 1362, Mikrotron GmbH) equipped with a lens (CF35HA-1, Fujinon) running at 506 fps captured swim dynamics. Custom-written C# software analyzed fish center of mass and orientation in real time using the higher order central moments of each background-subtracted frame. The extracted fish position and orientation was used to present visual stimuli in a closed-loop manner, with the stimulus locked in a constant egocentric location with the visual field, even as fish were turning or swimming. Visual stimuli were presented with a DLP projector (Dell M109S) and reflected by a 3 x 4 inch cold mirror (Edmund Optics) underneath the petri dish. The petri dish rested on a clear acrylic platform (McMaster-Carr) equipped with a diffusive screen (Cinegel). A 10 x 20 810 nm IR LED array was used to illuminate the arena from below. An IR band pass filter (BP850, Midwest Optics) allowed the IR light to reach the camera, while blocking the visible light from the projector. Trials were initiated whenever the fish was within 200 pixels (1.8 cm) of the center of the dish.

For constant radial expansion trials, the stimulus was centered 0.5 cm to either the left or right of the fish center of mass. Stimuli expanded with a constant radial velocity of 0.5 or 0.6 cm/s until reaching r = 1 cm and disappeared 5 seconds after expansion commenced, always locked at a fixed angle and distance to the fish using the closed-loop control system described above. For flashed stimulus trials, a small, ~13 degree (r = 0.06 cm) spot appeared at time t = 0 and abruptly increased to either ~39 (r = 0.19 cm), 51 (r = 0.26 cm), 74 (close to the measured threshold visual angle during looming, r = 0.41 cm), or 96 degrees (r = 0.59 cm) at t = 2 seconds. For dimming trials, the responses to two different stimuli were pooled: an ~86 degree (r = 0.5
cm) spot and a ~123 degree spot (r = 1 cm) that dimmed with the same temporal dynamics as the
dark looming stimuli, averaged over their respective areas. For checkered trials, a 10 x 10 black
(0, 0, 0) and white (255, 255, 255) checkerboard was mapped to looming spots on a gray (128,
128, 128) background, maintaining subjective isoluminance but resulting in an overall increase
in photometric luminance. For constant approach velocity trials, stimuli were also centered 0.5
cm from the fish center of mass, but the stimuli expanded with R/V = 300 ms (hyperbolic
expansion, see below).

Detailed kinematic analysis of heading and tail angle was performed offline using Matlab
(Mathworks) on background-subtracted frames using the tail tracking algorithm described in 49.
In brief, the background-subtracted image was thresholded and thinned by the function bwmorph
in Matlab. To obtain decimal-precision coordinates for each tail segment from the resulting
image, the integer coordinates for each tail pixel were tuned using a weighted sum of the
intensity of surrounding pixels. A cubic spline was then used to adjust the coordinates, and the
angle of each coordinate was assessed relative to the heading direction of the fish. Maximum
bend angle (maximum deflection from baseline during the first bend), bend duration (time from
rest to the time point of the first bend angle), bend velocity (maximum instantaneous heading
change), counterbend angle (difference in angle between the time of the first bend angle and the
subsequent bend in the opposite direction), and burst frequency (of the first 3 undulations after
the initial bend) were then calculated after performing this tail tracking analysis. Linear velocity
and swim distance were calculated from the recorded fish center of mass after smoothing the x-y
center of mass coordinates with a 5-frame box kernel. Threshold instantaneous linear velocity
(12.0 cm/s) was calculated from the intersection of Gaussian fits (peakfit function, Matlab

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Central) to a histogram of swim velocity, which was trimodal, representing normal swims and fast (escape) maneuvers (see Figure S1).

For trials where stimulus approach velocity varied (Figure 2), we used a set of 5 stimuli: expanding disks with different R/V values (255, 490, 730, 980, and 1450 ms), presented 1 cm to the right of the fish center of mass. Each R/V value describes a fixed temporal profile of expansion that mimics the approach of a disk at constant velocity. If the distance of the disk is described as \( d(t) = -V \times t \), where \( V \) is the approach velocity \( t \leq 0 \) and \( t = 0 \) is the time of collision, then the visual angle subtended by the disk is given by \( \theta(t) = 2 \times \tan^{-1} \frac{R}{-V \times t} \), where \( R \) is the radius of the disk, and the edge velocity is \( \dot{\theta}(t) \). Thus, to present stimuli following a defined \( \theta(t) \), we used \( R_{\text{screen}}(t) = d_{\text{screen}} \times \tan \frac{\theta(t)}{2} = \frac{R \times d_{\text{screen}}}{-V \times t} \), where \( d_{\text{screen}} \) is the distance from the eye to the projection surface. See also Supplemental Figure 2.

For each trial, we first classified responses as escapes if maximum linear velocity surpassed the threshold discussed above (12.0 cm/s). For escapes, we then defined the latency to be the first time point where the heading direction change exceeded 10 degrees from baseline. Only responses that were initiated before the stimulus crossed into the contralateral visual field (in order to restrict our models to monocular information processing in a single tectal hemisphere) and after the stimulus began expanding were considered for latency analysis. Because checkered and black stimuli appear to elicit the same behavior (see Figure 1), we pooled over both black and checkered stimulus trials.

When analyzing the latency data, we sought to evaluate two separate hypotheses that might explain the relationship between instantaneous values of stimulus variables as they changed over time and escape initiation. The first hypothesis was that escapes are triggered by a threshold visual angle, and the second was that escapes are triggered by a threshold edge.
velocity. In order to accept or reject either hypothesis, we tested for a critical angle or velocity by looking for statistical coherence in $\theta(t_{\text{escape}} - t_{\text{offset}})$ or $\dot{\theta}(t_{\text{escape}} - t_{\text{offset}})$ across all R/V trials, where $t_{\text{offset}}$ is a free parameter that estimates the lag introduced by visual and motor processing (which confounds the estimate of a critical value). A critical visual angle, but not a critical edge velocity, was found across R/V conditions ($p > 0.05$, F-statistic permutation test, see statistical analyses below) for $t_{\text{offset}} = 1$ ms to $t_{\text{offset}} = 205$ ms (data not shown). In order to estimate the real critical visual angle used by zebrafish larvae, however, we performed an additional analysis wherein we calculated the standard deviation of the stimulus size evaluated at the time of escape across all trials and stimuli as $t_{\text{offset}}$ was iterated in 1 ms increments. This standard deviation was minimized at $t_{\text{offset}} = 141$ ms, and this value of $t_{\text{offset}}$ was used for presenting the data in Figure 3.

Freely swimming behavior – position

For trials where the stimulus position varied (Figure 2), black looming stimuli on a neutral gray background were presented 9 mm from the fish at angles of 0, 90, 180, or 270° relative to the orthogonal axes passing through the fish center of mass at an expansion velocity of 0.54 cm/s (~61 °/s at 2 mm swim depth). Fish were recorded at 202 frames per second at a spatial resolution of 640 x 480 pixels using an AVT Pike camera. Although the spatial and temporal resolution were lower than in the experiments using the Mikrotron camera (506 frames per second; 1280 x 1024 pixels), the data from these experiments were sufficient to calculate overall heading orientation (the angle of the ellipse major-axis derived from 2nd order central image moments), which revealed significant differences in behavior across the different stimulus conditions. Because the lower temporal and spatial resolution slightly altered our measurements
of instantaneous swim velocity (e.g. a 5-frame box kernel was still necessary to de-noise position measurements even though it represented a ~2.5x longer interval compared to Mikrotron data sets), the threshold swim velocity was recalculated for the Pike data and set to 10.1 cm/s.

Calcium imaging and analysis

To assay neural responses, 5-6 dpf Tg(elval3-GCaMP5G) larvae were paralyzed with alpha-bungarotoxin (1 mg/mL, Invitrogen) and embedded in 2% low melting point agarose before being imaged with a custom-built two-photon laser scanning microscope. Stimuli were presented onto a screen underneath the embedded fish using a DLP projector (Dell M109S), similar to freely swimming experiments. Stimuli were presented in the red channel to avoid contaminating fluorescence signal detection.

For looming selectivity experiments, visual stimuli originated 0.5 cm from the fish in either the left or right visual fields. Black looming stimuli expanded from r = 0 cm to r = 0.625 cm over 1.5, 2, or 4 seconds (~73, 58, 31 °/s, respectively) with the stimulus disappearing after 5 seconds. Flashed spots, r = 0.625 cm, appeared instantaneously at the start of a trial before disappearing after 5 seconds.

For analysis, an activity map for each imaged plane was first extracted using a method adapted from . A square ROI roughly equal to half the size of a cell body was swept across the spatial extent of an imaging movie. For each swept ROI, the spatially averaged fluorescence time series was normalized by the ROI’s mean fluorescence across time and the average fluorescence of all pixels across space and time. This signal was then cubed to amplify peaks, and each square’s average processed signal was assigned to each square’s spatial location. This analysis produced maps of activity that were robust to noise in regions of low baseline fluorescence.
These activity maps were then overlaid on mean fluorescence images (reflecting anatomy) and used to guide manual neuron ROI selection. This method ensured that all selected neurons were active at some point during the recording, regardless of whether or not they exhibited stimulus-coupled fluorescence responses. Neuropil activity was excluded from this analysis.

For each segmented ROI, $\Delta F/F$ (relative to interstimulus intervals) was trial-averaged for both looming and flashed stimulus epochs (from $t = -5$ seconds to $t = 15$ seconds). Trial-averaged signals were then used to calculate a selectivity index for each ROI, where

$$Selectivity\ Index = \frac{Z_{loom} - Z_{flash}}{Z_{loom} + Z_{flash}}.$$  

Here $Z_{loom}$ is the average z-score of the signal during looming epochs relative to interstimulus intervals, and $Z_{flash}$ is the average z-score of signal during flashed epochs. To constrain this index to the range $[-1, 1]$, negative values of $Z_{loom}$ and $Z_{flash}$ were discarded, meaning that only positive deflections from baseline were considered responses. This formulation of the selectivity index reduced contamination from noise and was less influenced by specific response dynamics (e.g. phasic vs. tonic activity) than other methods we explored (e.g. $t$-statistics). Overall, this selectivity index reliably classified looming selective responses (see Figure 3).

To make composite maps of looming selectivity across fish, all imaged planes were registered to a standard Tg(elval3-GCaMP2) brain, which has an expression pattern indistinguishable from Tg(elval3-GCaMP5G), using cross-correlation. In addition, each plane was manually segmented into tectal, pretectal/thalamic, and midbrain tegmental areas using anatomical boundaries as guides. The regional identity of each cell was then assigned from these segmented and annotated maps.

In looming velocity experiments, stimuli were centered 1 cm from larvae in the right visual field, similar to the freely swimming experiments. Stimuli expanded according to 3
different R/V values (650, 1300, and 3235 ms), filling the screen (~160 degrees visual angle) by the end of expansion. Each stimulus disappeared after 15 seconds of presentation. Based on maps of looming selectivity, only select planes in the contralateral ventral OT were imaged in each fish. ROIs in each imaged plane were chosen according to the same protocol outlined above. For PCA analysis, the trial-averaged ΔF/F signals for each stimulus were concatenated, and the resulting signal was used to derive principal components with Matlab’s princomp function (Mathworks). For each fish, the first principal component (PC1) was first normalized to its maximum value across all stimuli. Next, convolved functions of stimulus size and edge velocity (τ = 1.6 seconds, to account for calcium indicator dynamics) were evaluated at the first time point where the normalized PC1 crossed a set threshold value. The output of each function at this time point was used to assess the correspondence between optical variables during population responses. We arrived at an 82% threshold value by evaluating the similarity of encoded image size across stimulus conditions over a range of threshold values (10-100%). An 82% PC1 threshold showed the best coherence in image size as evaluated by 1-way ANOVA. Values of edge velocity remained significantly different over the same range of threshold values.

**Ablations**

Thirty-six hours before M-system ablations (i.e. 3-4 dpf), 2% (w/v) solution of dextran-conjugated Alexa 488 (Invitrogen) was injected into the spinal cord of mitfa-/- (nacre) fish in order to label spinal projection neurons. The behavior of the injected fish was indistinguishable from non-injected larvae, as assayed by looming-evoked escapes and spontaneous swimming. Looming-evoked escapes were elicited by black spots expanding with constant radial velocity in either the left or right visual field, as described previously, before and
after unilateral ablation of the M-system using a two-photon laser \textsuperscript{58}. To more accurately compare behavior before and after ablation, escape velocity thresholds were adjusted before ablation to account for variability across individual fish. During ablations, labeled neurons were targeted with a high-power beam (100 – 150 mW) for intervals of 0.1 – 2 s (repeated until cells were destroyed). This method introduced focal, cell-level lesions that did not appear to affect nearby off-target labeled neurons and processes. Fish were imaged 24 – 48 hours post-ablation to ensure that fluorescence did not recover (i.e. to verify that cells were not merely photobleached). We only analyzed the behavior of larvae in which an M-cell and at least 2 cells in each MiD2 and MiD3 cluster were successfully ablated.

Because half of the fish were recorded with an AVT Pike, and half of the fish were recorded with a Mikrotron 1362, we analyzed fish orientation rather than heading direction and upsampled Pike sequences to Mikrotron frame rates for alignment (see \textit{Freely swimming behavior – position} above). Only the first escape event on any given trial was analyzed in cases where there were multiple escape events. For statistical analyses, escapes were sorted into ablated and non-ablated sides relative to the site of ablation, independent of the position of the eliciting stimulus, which was presented in a random order across the left and right visual fields.

\textit{Statistical analyses}

All means and standard errors were computed as described in the text. For hypothesis testing, we used a resampling method that normalized for differences in sampling density across fish and conditions, preventing the overrepresentation of larvae that contributed more data points than others. For a given distribution (e.g. bend velocity), we sampled \( n \) times with replacement from each fish separately, where \( n \) was the minimum number of data points collected for a
particular quantity across all individuals, and then calculated the mean across fish. As each fish was now represented by an equal number of samples, biases introduced by sampling heterogeneities were reduced. This process was then repeated 100,000 times to converge on a stable mean for the distribution that was used for subsequent hypothesis testing. When testing for significant differences between two distributions, resampling was carried out for both groups separately, and the mean difference was compared to a distribution of shuffled data sampled from both distributions in a (Monte Carlo) permutation test; the percentile of the real mean difference in the shuffled difference distribution provided a 1-tailed p-value accepting or rejecting the null hypothesis of equivalence between the two tested groups. The only deviation from this method was in Figure 2E, where the small sample size per fish for the fastest stimulus produced an artificially low variance that precluded fish-by-fish resampling; instead, all data points across fish were pooled before resampling.

For comparisons made across many groups, resampling proceeded as previously described, but an F-test statistic was calculated rather than a mean difference. The F-test statistic (ANOVA) provides a measure of the statistical similarity of a given quantity across multiple conditions while reducing potential errors resulting from multiple comparisons. For each resampling repetition, as described above, an F-test statistic was calculated across all conditions. After 100,000 repetitions, the mean F-test statistic was compared to the distribution of F-test statistics calculated from 100,000 rounds of shuffling across all groups. The p-values reported correspond to the percentile of the real F-test statistic in the shuffled distribution.

2.10 – References


Chapter 3

Neural control of spontaneous behavioral sequences
3.0 – Intellectual contributions

The following chapter is adapted from a manuscript entitled “Neural control of spontaneous behavioral sequences in larval zebrafish,” which, as of April 6, 2015, is being prepared for submission. I am first on the author list, but Dr. Yu Mu, Dr. Sujatha Narayan, Dr. Eva A. Naumman, Dr. Chao-Tsung Yang, Dr. Owen Randlett, Dr. Alexander F. Schier, Dr. Jeremy Freeman, Dr. Florian Engert, and Dr. Misha B. Ahrens also contributed to the manuscript. I conceived of the project, performed all behavioral experiments, acquired preliminary image data, carried out all modeling, and participated in data analysis for all main figures. Dr. Yu Mu acquired imaging and ablation data. Dr. Narayan identified neurotransmitter phenotypes and traced neurites with PA-GFP. Dr. Naummann conceived of experiments and designed figures. Dr. Yang generated transgenic lines. Dr. Randlett created a standard brain atlas and registered brain images across fish. Dr. Schier and Dr. Engert supervised the work and edited the manuscript. Dr. Freeman developed the behavioral tuning space. Dr. Ahrens supervised the project, conceived of experiments, and wrote and edited the manuscript with me.

3.1 – Introduction

In response to explicit environmental challenges, animals use sensory cues, past experience, and internal states to execute appropriate behaviors. In the absence of salient sensory cues and task demands, however, executing meaningful behaviors remains crucial, as foraging, maintenance of bodily functions, grooming, surveillance, and exploration are important for survival \(^1,2\). These behaviors are internally generated and can be highly structured \(^3-5\), but their neural correlates have not been well-studied \(^2,3\), and the underlying circuits and activity patterns are largely unknown. Spontaneous patterns of neural activity have been mapped in humans \(^6,7\).
and zebrafish on brain-wide scales, revealing structured activity in the absence of task demands and sensory input, but these observations have not been explicitly linked to behavior; thus, the function of this ubiquitous spontaneous activity remains unknown.

To identify neural pathways involved in structured, spontaneous behavior, we first investigated the spatiotemporal properties of internally generated actions by characterizing the spontaneous locomotion patterns of larval zebrafish swimming in a featureless, equiluminant environment. Even without local external cues, fish remain highly active, swimming and turning in discrete bouts with a frequency of $1.0 \pm 0.3$ Hz (mean $\pm$ SD across fish). This behavior appears random, but analysis of turn sequences over time revealed a specific temporal structure where a turn in one direction is likely to be followed by a turn in the same direction, creating alternating “chains” of turns biased to one side. Overall, such a pattern generates conspicuous, slaloming swim trajectories. These are distinct from biased random walks, which evolve randomly at any point in time, and instead show a strong dependency on past behavior. The length of the unidirectional chains was approximately 5-10 turns, lasting about 5-10 seconds, after which the animals initiated a chain in the other direction. Such timescales ($\sim 10$ s) exceed the timescales of individual swim bouts ($\sim 1$ s), indicating that networks upstream of the peripheral motor system likely govern this unique pattern of spontaneous behavior.

Given the large space of putative brain circuits underlying this behavioral program, we employed novel techniques to search for neural populations controlling spontaneous turning. Whole-brain imaging during behavior is a promising method for finding unknown neuronal populations, which, in contrast to conventional recordings from subsets of brain areas, increases the likelihood that neurons underlying a specific behavior will be discovered. To this end, we combined a fictive version of the spontaneous behavior with light-sheet imaging,
enabling fast, volumetric whole-brain imaging at cellular resolution\textsuperscript{12}. By analyzing the relationship between spontaneous brain activity and spontaneous behavior\textsuperscript{13}, we generated whole-brain activity maps of neuronal and neuropil structures that correlated well with the observed locomotor patterns. We revealed anatomically structured neural populations in the hindbrain with activity fluctuating on slow timescales similar to the directional locomotion characterizing spatiotemporal behavioral patterning. Subsequent circuit perturbations established a causal link between these populations and self-generated swim statistics. Finally, we showed that these cells are composed of two glutamatergic clusters and two GABAergic clusters that potentially innervate one another in a mutually inhibitory circuit motif. We suggest that this network spawns innate, volitional behavior and optimizes the statistics of internally generated exploratory behavior.

\section*{3.2 – Fish exhibit a structured spatiotemporal pattern of spontaneous swimming}

To investigate the spontaneous swimming behavior of larval zebrafish, we performed high-speed imaging of animals moving freely in a featureless environment and analyzed their spontaneous swim kinematics. Larval fish were placed in a homogenous, equiluminant environment composed of a water-filled petri dish on top of a diffusive screen onto which uniform dim white light was projected (\textbf{Figure 3.1A, left}). The position and heading direction of individual larvae were then extracted from the acquired video (\textbf{Figure 3.1A, right}). Because larval zebrafish swim in discrete swim bouts, the behavior could be partitioned into a punctuated series of swim bout locations and turn angles (\textbf{Figure 3.1B}). Visual inspection of the data suggests that fish string together repeated turns in one direction before switching to a chain of turns in the other direction (\textbf{Figure 3.1B,C}). This trend can be observed by triggering the data on
a turn and plotting the future swim trajectory, rotated to the initial angle of the fish (Figure 3.1C, right), revealing the influence of previous turn direction on subsequent locomotor events. We quantified this observation by constructing a null hypothesis that the chains of ipsilateral turns arise by chance from a fish choosing randomly to turn left or right. The cumulative turn direction of the real fish, as a function of the number of turns, was significantly different, up to at least five turns, from that of a model fish swimming left and right randomly (Figure 3.1E; turns near the chamber border discarded, see Methods). Furthermore, histograms of chain length showed that long chains were significantly more present in the real data than in the model fish, up to chains of 13 turns (Figure 3.1F). We conclude that freely swimming fish spontaneously chain together turns in the same direction for approximately 5-10 seconds.

Figure 3.1 – Spontaneous orienting behavior is governed by switches in turn state

(A) As fish explore a featureless environment, heading direction (purple vectors) over time is recorded with a high-speed camera. Fish execute discrete spontaneous turns (top right) that comprise long sequences of turns biased in the same direction (middle right). The size and color of the spot (bottom right) denotes the magnitude and direction of the underlying turn, respectively.

(B) Plot of a swim trajectory taken from a much longer recording (from within red box, left). Turns are encoded as described in (A), but symbols are positioned at the points in the trajectory where turns were executed. Note the chains of left and right turns that confer a characteristic slaloming shape to the swim trajectory.

(C) Left, turn states can also be visualized by plots of turn amplitude over time, colored according to turn direction. Notice that the fish tends to turn in streaks. Right, when swim
Figure 3.1 (Continued)

trajectories are triggered, rotated, aligned, and color-coded according to the direction of each preceding turn, streak history is evident as arcs in swim trajectory to each respective side.

(D) Histogram of turn amplitudes from 42,747 swim events across 19 fish. The overall turn distribution is symmetric.

(E) Quantification of average turn history-dependence. Fish tend to turn in the same direction as the previous left->right or right->left turn for 5 swim bouts (p = 0.024, signed rank test compared to a randomly turning fish, red). N = 19 fish. Shaded error is SEM across fish.

(F) Cumulative histogram of streak length (number of turns in the same direction before a switch) for 19 fish, black, compared to randomly turning fish, red. (*) p < 10⁻⁵ rank sum test.
To characterize the neural basis of this behavior, we used light-sheet microscopy to record whole-brain activity in paralyzed zebrafish while recording fictive behavior. We first verified that, in the fictive paradigm, zebrafish exhibit the same pattern of spontaneous behavior as when freely swimming. Fictive behavior was monitored in paralyzed, head-embedded fish using large-barrel extracellular glass electrodes that recorded from motor neuron axons innervating the axial tail musculature (Figure 3.2A). From these electrical signals, forward swims and turns can be decoded, with overall virtual turn distributions closely matching those of freely swimming fish (Figure 3.1D). Thus, “virtual” swim trajectories can be generated from fictive behavior and analyzed like freely swimming behavior (Figure 3.2A,B,C,D). Fictive behavior showed similar signatures of chains as the freely swimming behavior (Figure 3.2B,C), with epochs of virtual left turns alternating with epochs of virtual right turns. The chain length was comparable between freely (Figure 3.1E,F) and virtually (Figure 3.2E,F) swimming fish. Thus, chains of directional locomotion are also present in paralyzed larval zebrafish, confirming that this behavior is recapitulated well in a fictive swimming paradigm (Figure 3.3).

**Figure 3.2 – Spontaneous fictive patterns match freely swimming patterns**

(A) Turns can also be decoded from electrophysiological recordings of ventral root ganglia in paralyzed fish. Turn direction and amplitude is calculated from subtracting the power of recorded bursts in the left channel (green electrode) from bursts in the right channel (pink electrode), weighing the start of a burst more than the end (see exponential filters). (m) muscle; (nc) notochord; M (myotome); VTU (virtual turn unit)

(B) Sequences of decoded virtual turns and virtual swim distances (sum of left and right fictive channels, virtual distance units (VDU)) can be used to plot virtual swim trajectories. The streak pattern observed in freely swimming fish is conserved in fictively swimming fish.
Figure 3.2 (Continued)

(C) Fictive turn amplitude and trajectory history plot for the data in (A) and (B).

(D) Histogram of fictive turn amplitudes from 14,093 swim events in 11 fish.

(E) On average, fictively swimming fish turn in the same direction for 4 swim bouts after a change in turn direction ($p = 0.002$, signed rank test compared to a randomly turning fish, red). N = 11 fish. Shaded error is SEM across fish.

(F) The cumulative probability distribution of fictive streak length is also significantly different from a randomly turning fish. ($* p < 10^{-4}$, rank sum test. The dotted line denotes the longest streak that is significantly different from the random fish distribution.)
**Figure 3.3** – *Analysis of freely and fictively swimming turn states*

(A) Signed cumulative turn angle triggered on left->right or right->left switch events for individual fish (left column) and change in signed turn angle across fish (middle column) for freely swimming (top row) and fictively swimming fish (middle row). A p-value threshold of 0.05, for a signed rank test between the change in cumulative turn angle and a randomly tuning fish (pink line, 0) at each turn since a switch event, is used to determine the average length of turn states in *Figure 3.1* and *3.2*. 
3.3 – Whole-brain maps reveal neural representations of spontaneous behavior

To interrogate the relationship between brain activity and spontaneous turning behavior, we monitored fictive swimming during whole-brain light-sheet imaging of transgenic zebrafish expressing a calcium indicator in almost all neurons. We generated two lines, one with GCaMP6f expressed mostly in the cytoplasm and a second where it is expressed in the nuclei of all neurons, Tg(elavl3:GCaMP6f) and Tg(elavl3:H2B-GCaMP6f), respectively. Using a two-laser setup to create light sheets from the front and from the side of the head, we imaged almost the entire zebrafish brain without invading the visual field with the excitation lasers and were thus able to minimize the effect of brain scanning on behavior 12 (Figure 3.4A). Imaging was performed at a rate of 1-2 brain volumes/second.

To map the relationship between whole-brain activity and behavior, we decomposed the fictive recordings into distinct behavioral events each associated with two variables: one encoding the amplitude or strength and the other reflecting the direction or laterality of each swim bout 14 (Figure 3.4B, left; Methods). These two variables were represented in a two-dimensional space — a behavioral tuning space — describing the strength and angle of the swim bout, analogous to a visual receptive field (Figure 3.4B, middle). A log-polar tiling of basis functions divided this space into regions corresponding to particular ranges of amplitude and angle. A behavioral time-series corresponding to each region was convolved with a calcium impulse-response function 11,15, creating regressors with which to model whole-brain responses (Figure 3.4B, right). Signals from individual voxels (or neurons) were thus described with a tuning field over the behavioral space (Figure 3.4C). The responses of two example neurons are shown in Figure 3.4C, with one tuned to left turns and the other to right turns. The corresponding behavioral tuning fields show preferences for these directions (Figure 3.4C,
right), and these tuning fields can be summarized with two parameters (the central tendency of
tuning in both angle and amplitude).

**Figure 3.4 – Regression analysis and behavioral tuning space**

(A) Schematic of experimental paradigm for fictive swimming combined with light-sheet
imaging (Methods).

(B) Schematic of analysis technique. Left: First, fictive swim signals are converted into measures
of swim amplitude (“Amp”) and turning direction (“Dir” for laterality) (left). Middle: Next,
amplitude and laterality are mapped onto the vertical and horizontal axes of a 2D space. This
space is tiled with 12 basis functions, each representing a region in this 2D behavior space, now
defined in polar coordinates (Methods). Contours are shown for clarity; actual basis functions
overlap by 50%. Right: The signal from each bin is convolved with an impulse response function
to generate a regressor; an example subset of regressors is shown.

(C) Brain activity is regressed against the regressors constructed in (B) to generate a behavioral
tuning function for every voxel. Voxels of two neurons are shown here. Left, relationship
between turn laterality and response for two example example neurons, each dot is a time point.
Middle, time series from the same two example neurons. Black line, ΔF/F; colored line,
prediction of best-fitting model (see panel B). Right, behavioral tuning for the same two neurons,
given by regression coefficients, using the analysis described in panel B; grayscale ranges from
10th to 90th percentile of the coefficient weights.
Figure 3.4 (Continued)
To match neural activity to the pattern of spontaneous turning, we generated whole-brain activity maps for individual fish (Figure 3.5A) by color-coding each voxel for preferred angle. We encoded the predictability of the response, or $R^2$, with brightness so that brighter colors mean more significant correlations to behavior (Figure 3.5A,B,C). These maps revealed the organization of tuning in both neurons and neuropil (for the cytosolic GCaMP6 fish). Neurons linked to spontaneous turning behavior were clustered in (1) a region in the anterior hindbrain just posterior to the cerebellum (rhombomeres 2-3) consisting of four clusters of cells termed the hindbrain oscillator (HBO)\(^8\), (2) diffusely in the caudal-dorsal hindbrain, posterior to the HBO in the area of rhombomeres 6-7, and (3) in an area close to the inferior olive. In addition, in some fish, cells weakly tuned to laterality and strongly tuned to swimming strength were found across the hindbrain, midbrain and sparsely in the forebrain (Figure 3.5A, bottom left).

We next evaluated how consistently these populations were localized across individual fish. We used a nonlinear volume registration algorithm\(^{11}\) to align 7 brains based only on anatomy and found the location of the functionally defined cell clusters to be highly conserved across fish (Figure 3.5B) (see Figure 3.6 for cytosolic GCaMP6 equivalent). Tuning to turn direction was equally consistent (Figure 3.5B, inset). Thus, our analysis reveals tightly anatomically tightly clustered populations of neurons with specific tuning to spontaneous behavior that are strongly stereotyped across animals.

**Figure 3.5** – *Whole-brain analysis identifies the hindbrain oscillator (HBO) as a functionally conserved neural structure that correlates with turning behavior*

(A) Behavioral tuning maps across the brain derived from fitting every voxel with the regressors described in Figure 3.4B. Calcium indicators are either localized in the cytoplasm (left) or in the nucleus (right). The dorsal view is a maximum intensity projection over the whole brain; the side
Figure 3.5 (Continued)

and front views are taken from a maximum intensity projection of 21 slices along the mid-lateral axis and rostral-caudal axis. Arrows in each panel represent the position of slices for the frontal view (top) or lateral view (right). Solid arrowhead, diffusive correlated region in the caudal-dorsal hindbrain. Open arrowhead, inferior olive. Scale bar, 100 μm.

(B) Map of 7 different fish (nucleus localized GCaMP6-fast) registered to a standard brain. Colors represent different fish; brightness represents $R^2$. Bottom, maximum intensity projection. Top, 41-slice projection along the rostral-caudal axis. Arrow represents the position of the slices used for projection. Inset, HBO region with same registration, but the color represents the same laterality as in panel A and the brightness represents the mean $R^2$. Scale bar, 100 μm.

(C) Sample $\Delta F/F$ traces from regions of interest (ROIs) in panel A (left bottom). Left, top to bottom: midbrain, HBO, and caudal hindbrain. Middle, top, signals of swim amplitude (Amp.) and turn laterality (Dir.). Bottom, $\Delta F/F$ from ROIs in the left panels. Right, enlarged view of gray region in middle panel. Responses from ROIs 1-3 and 8-10 show tuning to swim amplitude; ROIs 4,5 to left turns, and ROIs 6,7 to right turns.
Figure 3.5 (Continued)
In transgenic zebrafish expressing the calcium indicator GCaMP6f in the cytosol in all neurons (i.e., not constrained to the nucleus as in the fish of Figure 3.5B), the functionally identified neurons occupy the same locations, co-localize across fish, and are consistent with the locations of the HBO as identified in Ahrens et al. 2013. Analyses identical to those used in Figure 3.5. Scale bar, 100 μm.
Previous analyses of spontaneous whole-brain activity data based only on internal correlations of activity and not on behavior, e.g. ICA, revealed several anatomically localized clusters of functionally coupled neurons and neuropil\(^8,16\). One of these, the HBO, which showed antisymmetric fluctuations on long timescales, matches the networks identified here, as confirmed by ICA analysis on data from Figure 3.5 (Figure 3.7). This shows that the HBO can be identified both through regression to behavior and by analysis of strongly correlated patterns across the brain, implying that directionality in spontaneous locomotion is a dominant mode of whole-brain activity.

We next inspected the activity time series of functionally identified HBO neurons. These neurons showed characteristic tuning to swim amplitude or to left or right turns (Figure 3.5C). The tight relationship between neuronal activity and fictive behavior suggests that the neuronal populations identified here may underlie directionality in spontaneous swimming, such that activity in the right HBO or the left HBO biases turning to the right or the left, respectively. It is worth noting that because neural activity was analyzed at the whole-brain level, it is likely that all populations correlated to the behavior have been identified. Thus, subsequent interrogation of causal relationships between activity of these specific populations and behavior promises comprehensive insights into how the brain directs and coordinates self-generated behavior.

**Figure 3.7 – Recovering the hindbrain oscillator using supervised and unsupervised methods**

(A) Map derived from regression analysis relating behavioral parameters (turn direction and amplitude) to neuronal responses (see Figure 3.5 and Methods), conventions as in Figure 3.5. (B) Maximum projection maps derived from Independent Component Analysis, performed as described in Freeman et al. (2014)\(^16\). Analysis was applied to voxel-wise time series data using
100 principal components and 20 independent components. Spatial maps for 4 out of 20 components shown here, combined into a composite color image by scaling amplitude to a (black, red/green/cyan/magenta) color range, separately for each of the 4 components, and then computing a maximum over the vertical dimension. Location of the HBO is consistent with that from Freeman et al. (2014) and Ahrens et al. (2013).

(C) Temporal components for the same ICA analysis shown in panel B. Fictive swim signal as defined in Figure 3.5. Four colored traced correspond to the four spatial maps in panel B. Inset highlights components (red and green) that recover a region including the HBO; one signal inverted to emphasize correspondence with behavior.
Figure 3.7 (Continued)

(a) [Image of left panel]

(b) [Image of right panel]

(c) [Graph with labeled axes]
3.4 – The HBO is causally linked to spontaneous swim patterning

Having identified neurons correlated to spontaneous turning events, we tested whether these populations play a causal role in behavior. The regression analyses identified three regions: (1) the HBO (horizontal arrow, Figure 3.5A top left), (2) diffusely located cells in the caudal-dorsal hindbrain (closed arrowhead, Figure 3.5A top left), (3) an area near to or overlapping with the inferior olive (open arrowhead, Figure 3.5A top left). We hypothesized that the HBO might be causally related to patterning of spontaneous swimming, motivated by its strong correlation with behavior on long timescales and its highly consistent anatomical location across fish (Figure 3.5B).

Guided by these stereotypical temporal activity patterns as well as the conserved anatomical location of the HBO, we were able to functionally identify the HBO at the single-cell level in each fish. This enabled us to electrically stimulate one side of the HBO in immobilized fish while recording fictive turns (Figure 3.8A). A glass electrode coated with nano-gold particles – making it visible under 930 nm laser illumination – was inserted and guided to the functionally identified area of the HBO. Unilateral electrical stimulation (Methods) elicited increases in calcium fluorescence on one side of the HBO but not the other, concomitant with bursts of fictive turns (Figure 3.8B). The direction of these elicited turns was consistently biased toward the side of stimulation (Figure 3.8C) (N=7, p<0.01, paired student’s t-test).

In a complementary experiment, we used targeted two-photon laser ablation to lesion one side of the HBO while keeping the other side intact. First, fish panneuronally expressing GCaMP6 were filmed as they swam in a featureless petri dish (as in Figure 3.1) to generate a behavioral baseline represented by histograms over turn angles (Methods). Next, fish were embedded in agarose, and the HBO region was imaged with a two-photon microscope. The HBO
was then identified by computing the correlations (or anti-correlations) of the signal in the entire plane to a “seed” region within the HBO (see Methods). The coordinates of about 10 cell body centroids of neurons on one side of the HBO were recorded and ablated with a high-power laser pulse (Methods). The fish were then released from the agarose, and free swimming behavior was tested as before. We found that post-ablation, the fish turned relatively more often toward the direction of the intact half of HBO (Figure 3.9A,B,C) (Left HBO ablation: N = 6, p = 0.011; Right HBO ablation: N = 7, p = 0.025  paired student’s t-test), suggesting that the HBO is necessary for generating directionality in spontaneous turning behavior.

**Figure 3.8 – The HBO drives ipsilateral turning**

(A-C) Electrical stimulation of the HBO enhances ipsilateral turns. (A) schematic of experimental setup for electrical stimulation. (B) Examples and (C) summary of turning behavior without and with electrical stimulation of the medial HBO cluster on one side.

** p < 0.01 (rank sum test)
Figure 3.9 – The HBO is necessary for ipsilateral turning

(A-C) Laser ablation of a subset of cells in the HBO reduces ipsilateral turns. (A) Examples and (B) summary of turning behavior before and after laser ablation of cells in the left medial cluster (green) or right medial cluster (magenta) of the HBO. (C) Histogram of turning angle before and after laser ablation. Inset, frequency difference of turning before and after laser ablation. n.s. not significant.* p < 0.05 (rank sum test)
3.5 – The HBO lies functionally upstream of low-level premotor circuitry

Although ablations shifted the spontaneous turn bias to the intact side, it remained possible that the HBO was functionally similar to output neurons such as the ventromedially located spinal projection neurons (v-cells) in the hindbrain reticulospinal system, a set of premotor neurons directly controlling turning behavior 17-19. To disambiguate the role of the HBO as generating global patterns in spontaneous behavior from neurons that merely generate one-to-one motor output, we examined the magnitude of turns pre- and post-ablation, independent of relative frequency. The average angle in each individual direction was unaffected by the lesion (Figure 3.10A, p = 0.507, ablated side; p = 0.583, intact side, N = 13), reiterating that the HBO is involved in generating turn direction biases but not the overall kinematics of turning.

Furthermore, if the HBO is indeed responsible for inducing temporal correlations in turn direction, one expects an HBO-lesioned fish to switch from a chain of turns in one direction to a chain in the other direction with a probability more consistent with a biased coin-flip than with a temporally correlated process. Indeed, using a $\chi^2$ test to determine the goodness-of-fit between a binomial distribution and observed turn sequences, we found a significant reduction in the $\chi^2$ statistic over fish (Fig. 3.10B, p = 0.035, rank sum test, N = 13). These results indicate that turn sequences in lesioned fish are more similar to string of biased coin-flips than are turn sequences in intact fish, providing additional evidence that the HBO implements a temporally correlated process.
Figure 3.10 – HBO lesions decrease sequence correlations

(A) Mean turn angle to the lesioned or intact side, before and after ablation. Although the relative frequency of turns to the ablated side decreases, fish remain capable of executing normal turns to the ablated side.

(B) Left, empirical cumulative probability functions of streak length before (blue, top) and after (red, bottom) ablation, compared to model fish executing turns at random according to the measured overall turn bias (black). Right, summary of the reduced chi-squared statistic for goodness-of-fit between the observed streak distributions and their respective binomial distributions. n.s., no significance; * p < 0.05 (rank sum test)
HBO activity also appeared to be fundamentally different from v-cell activity, up to the limitations of the calcium-based activity reporter. While both the HBO and v-cells showed similar lateralized correlations with locomotion direction (Figure 3.11A), a direct comparison of HBO and v-cell dynamics shows that the HBO is tonically active on longer timescales, again consistent with the hypothesis that the HBO controls a high-level spontaneous swim pattern (Figure 3.11B, C). In addition, state-related behavioral autocorrelation decays on a similar as, if not faster than, timescale as HBO fluorescence (Figure 3.11D). We also observed unilateral increases in HBO fluorescence following turns (Figure 3.11C), suggesting that motor commands or events may help stabilize ipsilateral HBO activity. In our working model where HBO activity in one hemisphere promotes turns in the congruous direction, this stabilization would support continued ipsilateral turn expression (Figure 3.11F). Consistent with this model, we find that fictive turns executed after long periods without a swim event, i.e. periods concurrent with a slow decay in HBO activity, are relatively more likely to be in the direction opposite the previous turn (Figure 3.11E). Together, these data cement a role for the HBO as a generic generator of behavioral states that bias swim direction.

**Figure 3.11 – Timescales of reticulospinal, HBO and turn state correlations**

(A) Ventromedial cells (v-cells) in the hindbrain reticulospinal system (identified via spinal backfills, white) are weakly correlated with turning behavior. Cyan and magenta show GCaMP6 voxels with laterality indices to the left and right, respectively. Scale bar, 100 μm.

(B) Example traces from 10 v-cells (middle) and 10 HBO neurons (bottom) during fictive swimming. HBO neurons are active on longer timescales than v-cells.
Figure 3.11 (Continued)

(C) Quantification of HBO (red) and v-cell (blue) response dynamics triggered on turns (gray).
Only turns that were not followed by another turn in the same direction for at least 5 seconds
were included to isolate HBO dynamics from correlation in behavior. HBO signals are slower
than v-cell signals, and peak about 1.5-2 seconds after a turn, compared to a near-instantaneous
peak in the v-cell signals, suggesting feedback from (peripheral) motor circuits to the HBO.

(D) Normalized autocorrelation of HBO signals (red), convolved turn laterality (gray), and
convolved turn laterality after shuffling turn identity (dotted). HBO activity and turning is
correlated on similarly slow timescales.

(E) Convolved turning behavior (gray) matches the instantaneous difference between left and
right HBO fluorescence.

(F) Relationship between fictive interbout interval and the probability of making a turn in the
opposite direction from the turn preceding it. The increase in switch probability at longer
interbout intervals is correlated with a decay in HBO activity.

(G) Schematic model of potential HBO circuitry leading to temporally correlated turn sequences.
The HBO provides bias to low-level premotor circuitry on the ipsilateral side and receives
feedback excitation when turns occur. Together with slow internal HBO dynamics reinforced by
contralateral inhibition (see Figures 3.12, 3.13), this feedback sustains the long-timescale
correlations characteristic of spontaneous behavior.
Figure 3.11 (Continued)
3.6 – HBO architecture is consistent with a mutually inhibitory circuit

We next sought to better understand the circuit architecture underlying HBO dynamics. In the hindbrain, cells releasing glutamate, glycine, or GABA neurotransmitter are distributed in laterally alternating rostro-caudal stripes with the most medial stripe, closest to the central canal, being glutamatergic \textsuperscript{20,21}. We combined dense functional imaging with sparse anatomical labeling to identify the neurotransmitter type of the cells composing the HBO in double transgenic lines expressing nuclear-localized GCaMP6f panneuronally and a red fluorescent protein in glutamatergic (Tg(vGlut2a:DsRed; elavl3:H2B-GCaMP6f) \textsuperscript{22,23}) or GABAergic neurons (Tg(gad1b:RFP; elavl3:H2B-GCaMP6f) \textsuperscript{22,24}), allowing us to functionally identify the HBO and overlay this functional map onto the underling neurotransmitter expression pattern (Figure 3.12A-C). In this way, we found that the medial cluster overlaps perfectly with vGlut2a-dsRed expression (Figure 3.12B; inset, orange arrow) indicating that the central (medial) cluster of the HBO is composed largely of glutamatergic cells and are thus excitatory. The lateral clusters lacked vGlut2a-DsRed expression (Figure 3.12B; inset, cyan outline) but overlapped with gad1b-RFP expression (Figure 3.12C, inset, cyan outlines). The inhibitory phenotype of the lateral cluster was further confirmed by immunohistochemistry using an antibody for GABA (data not shown). Thus, the HBO consists of anatomically segregated excitatory and inhibitory clusters of neurons.
(A) Schematic indicating the approximate location of the HBO in the dorsal hindbrain.

(B) ΔF/F correlation map of HBO activity in a Tg(vGlut2a:dsRed; elavl3:H2B:GCaMP6f) fish. Lateral clusters are outlined in cyan; the medial clusters in orange.

(B’) Cells of the medial cluster (orange outline) are positive for vGlut2a (magenta and green in inset, orange arrow) and are therefore glutamatergic. The lateral clusters (cyan outline) are negative (green only in inset, cyan arrow).

(C) ΔF/F correlation map of HBO activity in a Tg(gad1b:RFP;elavl3:H2B-GCaMP6f) fish. Lateral clusters are outlined in cyan; the medial clusters in orange. Dotted lines indicate the location of the midline and the midbrain-hindbrain boundary.

(C’) The cells of the lateral cluster (cyan outline) are positive for gad1b:RFP (magenta and green in inset, cyan arrow) and hence GABAergic. Cells of the medial cluster (orange outline) are negative for gad1b (green only in inset, orange arrow). A single medial cell (yellow outline) appears to be GABA positive indicating that the medial population may not be exclusively glutamatergic.
How might such an arrangement of excitatory and inhibitory neurons lead to the activity patterns observed in the HBO? The strongly antisymmetric activity patterns and the presence of excitatory and inhibitory clusters suggests underlying mutual inhibition, i.e. when one side of the HBO is active, the other side is suppressed. To probe for mutual inhibitory connectivity patterns, we combined functional imaging with anatomical tracing. To that end, we used a double transgenic line expressing a nuclear-localized version of GCaMP6f and photoactivateable GFP (PA-GFP) in all neurons (Tg(elavl3:H2B-GCaMP6f; alpha-tubulin:C3PA-GFP)) \(^{25}\). As before, we first functionally identified the HBO using two-photon imaging (Methods). We then photoactivated PA-GFP in a subset of these cells. The activated PA-GFP accumulates in the cell body and is transported along neurites \(^{26,27}\). After a several-hour-long activation protocol (Methods), we imaged fish with a confocal microscope. Because the calcium indicator was localized to the nuclei and thus did not overlap with the neurite signal, we were able to trace these neurites through relatively large volumes (Methods).

After activating cells in the lateral, GABAergic HBO cluster (Figure 3.13A), we observed labeled neurites on the contralateral side (Figure 3.13B), which crossed the midline ventral to the HBO (Figure 3.13C). These neurites terminated on the contralateral side near the lateral (Figure 3.13D) and medial HBO clusters (Figure 3.13E). Tracing these neurites confirmed the contralateral projections (Figure 3.13F) (N=15 fish, one representative fish shown).

Projections from the medial cluster were also traced. These neurons were found to project ventrally with an initial trajectory that is similar to the lateral cells. However, we did not find neurites crossing the midline, and GFP-positive neurites were never observed in the contralateral hindbrain (N=15 fish; not shown). Hence we conclude that these cells project ipsilaterally. These

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ipsilaterial projections may contact the ipsilateral HBO population to support contralateral inhibition and may synapse onto downstream premotor circuitry in order to affect turn directionality.

Taken together, these anatomical studies suggest a circuit motif in which the two sides of the HBO inhibit one another contralaterally via the lateral GABAergic clusters. This mutual inhibition would support the observed antisymmetric activation necessary for patterning directional motor output.

**Figure 3.13 – HBO neuroanatomy**

(A-F) Processes from the lateral cluster of the HBO cross the midline and terminate in the vicinity of the medial and lateral clusters on the contralateral side. The lateral cluster of the HBO was identified in a Tg(α-tubulin: C3PA-GFP;elavl3:H2B-GCaMP6f) fish and PA-GFP was activated in these cells at 780 nm (Methods).

(A) A representative fish in which PA-GFP is activated in the cells of the right lateral cluster. Activated cells are clearly distinguishable against a background of nuclear GCaMP6 (red arrows).

(B) A coronal slice ventral to the plane of activation showing GFP positive neurites on the contralateral side (red arrows).

(C) A coronal section ventral to that shown in (E) showing a GFP positive process crossing the midline.

(D) Transverse sections at the plane of the activated cells. Processes can be traced traversing ventrally on the activated side and ascending from the midline to terminate medially (E, arrow) as well as laterally (D, arrow).
Figure 3.13 (Continued)

(F) A schematic reconstruction of three cells traced using the Simple Neurite Tracer plugin in ImageJ showing the levels of slices A, B and C. Dotted white lines: midline. Rostral side up. Scale bars: 20 μm
3.7 – The spatiotemporal pattern of spontaneous swimming improves exploration efficiency

We posited that the correlated nature of directional locomotion likely underlies a foraging strategy when external guiding cues are missing. It has been suggested in the engineering literature that a chaotic oscillator\(^{28,29}\) produces winding trajectories that efficiently and evenly cover a space. Although the HBO is probably not a chaotic oscillator, it does exhibit stochastic transitions between long-timescale correlations. Indeed, a two-state Markov model\(^{30}\), which stochastically switches between a “left turn” state and a “right turn” state but exhibits high probabilities of remaining in the same turn state (Figure 3.14A; Figure 3.15, Methods), produced behavior similar to that of real fish (Figure 3.14B). Using this Markov model to simulate trajectories through virtual space (Figure 3.14C) shows that such a scheme covers a restricted area more efficiently than a model fish turning left and right randomly (Figure 3.14D-G). Thus, the HBO circuit may implement a foraging strategy that prevents rapid diffusion into faraway regions where conditions are unknown, and instead favors efficient exploration of the local environment.

**Figure 3.14 – Turn states increase efficiency of local searches**

(A) Spontaneous turn states are well-characterized by a two-state Markov model (Figure 3.15). In an average model fit, fish in the left state, \(S_L\), are much more likely (\(\sim 90\%\)) to turn left than right, and vice-versa. And fish in \(S_L\) or \(S_R\) tend to return to \(S_L\) or \(S_R\), respectively, after a turn.

These transition and emission probabilities can be used to generate turn sequences that show (B) significant correlations up to 5 turns after a left-right switch, similar to freely swimming behavior. \(N = 19\) model fish; \(p = 0.036\), signed rank test.
(C, left, black) Five swim trajectories generated with a Markov model matching the statistics of acquired swim data (P_{transition} = \begin{bmatrix} P_{L\rightarrow L} & P_{L\rightarrow R} \\ P_{R\rightarrow L} & P_{R\rightarrow R} \end{bmatrix} = [0.86 0.14; 0.15 0.85]). Right, blue, five swim trajectories generated with a Markov model randomly emitting left and right turns (all \(P_{transition} = 0.5\)). Notice that the unadjusted “random” fish diffuses farther from the given starting position. The dotted circle represents the mean diffusion distance for the correlated model. All trajectories begin at the center of the circle and facing in the direction of the arrow.

(D) Five example trajectories from the “random” fish after average diffusion has (inset) been matched to the correlated fish by decreasing bout distance.

(E) Plots of exploration efficiency. Left, In this local regime, the “random” fish must turn more and (right) execute more swim bouts to collect randomly distributed virtual resources.

(F) Five example trajectories from the “random” fish after average diffusion has (inset) been matched to the correlated fish by broadening the underlying turn angle distribution.

(G) Plots of exploration efficiency for the “random” fish normalized by turn angle. Left, this “random” fish must turn much more and (right) execute more swim bouts to collect randomly distributed virtual resources.
Figure 3.14 (Continued)

A. Two-state Markov model

B. Cumulative turn direction

C. Correlated fish

D. Diffusion normalized by bout length

E. Total angle turned

F. Diffusion normalized by turn angle

G. Total angle turned
(A) Signed cumulative turn angle triggered on left->right or right->left switch events for individual fish (top) and change in signed turn angle across fish (middle) for the two-state Markov model fish. A p-value threshold of 0.05, for a signed rank test between the change in cumulative turn angle and a randomly tuning fish (pink line, 0) at each turn since a switch event, is used to determine the average length of turn states.

(B) Table of the trained Markov model transmission and emission matrices for each fish in the data set. N = 19 fish.
3.8 – Discussion

We uncovered a circuit that spontaneously generates patterned statistics of directional locomotion, providing, to the best of our knowledge, the first whole-brain mechanistic description of an internally generated behavior. In explicit tasks, responses to sensory cues require intricate neural processing that produce appropriate behaviors, and much of neuroscience is devoted to elucidating the pathways translating sensory input to behavior in well-defined contextual paradigms or sensory environments. However, in the absence of such environmental guidance, animals rely on internal behavioral drive to explore their surroundings in search of momentarily elusive guiding cues. While these implicit behaviors are of paramount importance to animals, the neural underpinnings of these processes have remained largely unknown. The challenge in identifying neural circuits underlying such behaviors lies both in the characterization of the behavior and in locating neural structures implementing any observed behavioral schema. Although spontaneously active single neurons in primates and invertebrates have been studied in concert with behavior, these studies have addressed the generation of individual and specific motor events like saccades, not the regulation of ethologically relevant patterns. Here, we harnessed the power of fast whole-brain imaging to describe, in mechanistic detail, a nucleus in the zebrafish hindbrain generating a simple but vital behavioral algorithm that optimizes foraging when available information about the environment is scarce.

Comprehensive, whole-brain, cell-level imaging was crucial to our discovering the HBO circuit. Lacking a priori hypotheses regarding the location of circuits governing a behavior, the near-complete coverage of this approach helps ensure that neurons with response properties of interest, if present, will likely be identified (depending on the sensitivity of the activity...
reporter\textsuperscript{23} and the design of computational approaches\textsuperscript{13}). Thus, while inputs to the isolated neural populations certainly exist and shape circuit activity, our measurements and analyses suggest that the identified cells are likely to be the complete set of neurons directly involved in generating directional behavior. We decided to causally interrogate the HBO because of its strong stereotypy across fish and tight correlations to the slow switching structure of spontaneous behavior. It is clear that the HBO itself does not project to the spinal cord. However, the more caudal hindbrain neurons revealed in our correlational analysis are in areas rich in reticulospinal neurons\textsuperscript{21}. It is known that some of these caudal cells – which include the v-cells shown to be activated during spontaneous turning\textsuperscript{18} – project to the spinal cord and thus form the output of the circuit governing directionality in spontaneous locomotion. However, it is also possible that no clear division exists between upstream “direction” circuits and subsequent “output” circuits but that they feed back upon one another and act as a recurrently coupled system. Future anatomical tracing and modeling studies will help elucidate this possibility.

The neural populations uncovered by our analysis are involved in setting the direction of spontaneous swimming but may be involved in other functions as well. Activity in the HBO was not strictly oscillatory, in contrast to central pattern generator circuits\textsuperscript{45}, whose dynamics are more stereotyped and immutable. In principle, signals from other motor modalities as well as sensory systems could be integrated into HBO activity fluctuations. For instance, all or part of the cell population may mediate visually evoked turning, as there is evidence that hindbrain populations in the vicinity of the HBO respond to whole-field motion\textsuperscript{14,16}. Furthermore, preliminary observations show that eye movements\textsuperscript{46} and turning in larval zebrafish are correlated (data not shown). Thus, it is possible that both of these motor patterns are represented in and coordinated by the HBO. In the future, it will be exciting to study just how much the HBO
intersects with these auxiliary systems. According to our modeling, the slow fluctuations in turn direction that we observed increase restricted foraging efficiency. Future work can investigate whether this strategy adapts to changes in the environment or internal state. Food restriction or low light levels, for instance, may decrease state length in order to increase diffusion and promote exploration of completely novel environments. Conversely, favorable conditions may increase state length so as to decrease the rate of diffusion while encouraging efficient sampling of the local environment.

In summary, the whole-brain analysis, neural perturbation experiments, and anatomical characterization together reveal a circuit underlying the patterning of spontaneous, self-generated behavior, whose function may be to guide animals through environments where guidance from external cues is lacking, a context where animals must rely only on the internal drive of brain-autonomous activity.

3.9 – Experimental Procedures

Spontaneous Swimming

Larvae (5-7 dpf) were monitored in a 9.2 cm petri dish (VWR). A high-speed camera (Mikrotron 1362, Mikrotron GmbH or AVT Pike) equipped with a lens (CF35HA-1, Fujinon) running at 200 or 100 fps captured swim dynamics. Custom-written C# software recorded fish center of mass and orientation as fish swam spontaneously in the arena. Uniform neutral gray background illumination was delivered with a DLP projector (Dell M109S) and reflected by a 3 x 4 inch cold mirror (Edmund Optics) underneath the petri dish. The petri dish rested on a clear acrylic platform (McMaster-Carr) equipped with a diffusive screen (Cinegel). A 10 x 20 810 nm IR LED array was used to illuminate the arena from below. An IR band pass filter (BP850,
Midwest Optics) allowed the IR light to reach the camera, while blocking the visible light from the projector.

After data collection, swimming was analyzed using Matlab (Mathworks). Swim trajectories (fish center of mass over time) were first smoothed with a 200 ms Gaussian kernel (~20% interbout interval) with \( \sigma = 35 \) ms to reduce noise in recorded center of mass. Swim events were then marked at time points where instantaneous linear velocity crossed a threshold that minimized false positives and negatives. For Mikrotron experiments at 100 fps, this threshold was set to 1.0 mm/s. Turn angle was calculated as the change in heading angle during a swim bout, calculated as the difference between the heading angle 125 ms after and 125 ms before peak swim velocity.

For analyses of cumulative signed turn direction and streak length, we only considered turns that were executed at least 1 cm from the edge of the petri dish in order to eliminate artifacts arising from thigmotaxis (the propensity of fish to hug the walls of an enclosure). The cumulative binary sum of turn sequences triggered on a switch in turn direction were then averaged over all such sequences for a given fish. To determine the last turn from a triggered switch in direction that was reliably in the same direction as the first (i.e. the average length of a turn state), we looked for where the change in turn direction within a sequence across all fish was no longer significantly different from 0 (\( p \geq 0.05 \), signed rank test), which corresponds to the expected value for a fish turning left and right randomly with or without a bias. This point can also be seen as the turn (from a switch) where the average cumulative angle plateaus. Streak length was defined as the number of turns executed in the same direction before a turn in the opposite direction.
Markov model

For each fish, we trained a hidden Markov model with the sequence of all valid turns (i.e., at least 1 cm from dish edge) using a forward-backward Baum-Welch algorithm (hmmtrain, Matlab) to form a Markov model of the experimentally observed turn sequence. This algorithm terminated when the change in the log likelihood that turn sequences were generated from estimated transition and emission probabilities, the change in the norm of the transition matrix, and the change in the norm of the emission matrix were all less than $10^{-6}$. This method produced best-fit estimates for the underlying transmission and emission probabilities explaining the turn sequences for each fish. We then generated sequences of binary turns (hmmgenerate, Matlab) equal in number to the turns used to train the model for each fish. These simulated turn sequences were then used to analyze turn history and streak length, as outlined above.

For simulating exploration (Fig. 3.14), two initial models, a correlated fish and a “random” fish, were used to generate $10^6$ swim trajectories of 40 bouts each. For the correlated fish, emission sequences were generated with $P_{\text{transition}} = [P_{\text{L} \rightarrow \text{L}} \ P_{\text{L} \rightarrow \text{R}} \ P_{\text{R} \rightarrow \text{L}} \ P_{\text{R} \rightarrow \text{R}} ] = [0.86 \ 0.14; 0.15 \ 0.85]$ and $P_{\text{emission}} = = [P_{\text{turn L} \mid \text{L}} \ P_{\text{turn R} \mid \text{L}} \ P_{\text{turn L} \mid \text{R}} \ P_{\text{turn R} \mid \text{R}} ] = [0.85 \ 0.15; 0.10 \ 0.90]$, the best-fit probability matrices for the fish in Figure 1B. For the “random” fish, emission sequences were generated with $P_{\text{transition}} = [0.5 \ 0.5; 0.5 \ 0.5]$ and $P_{\text{emission}} = [1 \ 0; 0 \ 1]$. These Markov sequences were then used to assign direction to individual turn magnitudes, which were drawn according to the turn angle probability distribution derived from all acquired swim bouts in freely swimming fish.

The “random” model fish diffuses on average more rapidly away from the starting point due to the reduced “winding” properties of the trajectories. Assuming pressure to not venture too far afield due to potential dangers in faraway areas, we matched the average diffusion rate of the
correlated and the random model fish after 40 swim bouts. In one random model, diffusion was matched to the correlated fish by decreasing simulated bout length by 24.6%. In the other model, diffusion was matched by broadening the turn angle probability distribution, resulting in a 47.0% increase in mean turn angle. The $10^6$ trajectories for each model, which each started at a common point in space but with a random initial heading direction, were then used to measure how many virtual “resources” were collected by each fish model. Virtual resources were distributed randomly over an area approximately 25 bout lengths x 25 bout lengths in size with density 0.10 resources / bout length$^2$. Resources were counted as collected if simulated trajectories passed within 3.25 bout lengths of a resource position, representing a remote detection radius such as an odor gradient. We then used resource collection as function of bout number or angle turned – each of which is energetically and temporally costly – to assess model exploration efficiency.

**Fictive Behavior**

Larval zebrafish (5-7 dpf) were paralyzed by immersion in a drop of fish water with 1 mg/ml α-bungarotoxin (Sigma-Aldrich) and embedded in a drop of 2% low melting point agarose, after which the tail was freed by cutting away the agarose around it. Two suction pipettes—of diameter 45 micrometers—were placed on the tail of the fish at intersegmental boundaries, and gentle suction was applied until electrical contact with the motor neurons axons was made, usually after about 10 minutes. These electrodes allowed for the recording of multi-unit extracellular signals from clusters of motor neuron axons, and provided a potential readout of intended locomotion $^{9,47}$. Extracellular signals were amplified with a Molecular Devices Axon Multiclamp 700B amplifier and fed into a computer using a National Instruments data acquisition card. Custom software written in C# (Microsoft) recorded the incoming signals.
Fictive swim bouts were processed as described previously, separately for the left and the right channels. To determine fictive turn amplitude and distance, filtered left and right fictive signals at swim bouts were first weighed with an exponential function \( \tau = \frac{\text{bout duration}}{3} \) to emphasize the initial bursts that determine overall turn direction. The power of the right channel was then subtracted from the power of the left channel to arrive at turn amplitude and direction, and the powers were summed to provide a measure of swim vigor or distance. We then analyzed turn history and streak length from these processed turn sequences, as outlined in Spontaneous Swimming above.

**Light-sheet imaging**

The light-sheet imaging experiments were performed according to the paradigm previously described. Zebrafish larva were embedded in a custom made chamber that allowed for electrical recordings of fictive swimming from the tail and access to light-sheet excitation laser beams from the lateral and frontal direction of the fish. The lateral beam was used to scan over the majority of the brain, while the frontal beam scanned over the region between the eyes that was inaccessible to the lateral beam, thus achieving a coverage of about 80% of the entire brain at single-cell resolution. The detection objective was moved with a piezo so that the light sheets were always in the focal plane of the objective. Using this technique the imaging rate was about 2 brain volumes / second, i.e. every cell was imaged every 0.5 seconds. Importantly, the lateral beam rapidly switched off whenever it was located inside a circular exclusion region around the eye, so that whole-brain imaging could be performed without shining the laser beams into the eye. Red background illumination was provided to the fish by projecting homogeneous red light with a mini projector onto a screen underneath the fish (see Vladimirov et al. for
details.) Each experiment lasted between 30 and 60 minutes and thus contained 3000-6000 whole-brain stacks.

*Model-based identification of the HBO in light-sheet data*

We developed a regression analysis to capture the extent to which neuronal responses were related to directionally-specific behavior. First, two one-dimensional parameters were derived from the fictive swim signals: one capturing the instantaneous amplitude of swimming (strictly positive), and another capturing the instantaneous direction (positive for right, negative for left). We noted that, across many experiments, these two parameters tended to fall within the same region of a two-dimensional space (after normalizing amplitude to have a maximum of 1) ([Figure 3.3B](#)). To compute neuronal tuning within this space, we expanded the instantaneous value of the two signals into a nonlinear basis; intuitively, this corresponds to dividing the two-dimensional space into several small wedges each corresponding to a range of directions and amplitudes. We used a polar basis, separably and evenly tiling amplitude (three bins) and angle (four bins). Each basis had a flat top and raised cosine transition region, with 50% overlap; see Simoncelli et al. (1992) and Freeman et al. (2011) for the parameterization of this basis \(^{48,49}\), which is more commonly used to tile the two-dimensional Fourier domain. With this basis, we represented instantaneous behavior with 12 predictor time series, each \(1 \times T\), where \(T\) is the duration of an experiment. These predictors were each convolved with an impulse kernel \(k\) intended to reflect typical calcium dynamics; the kernel had a linear rise of 1 sec and a linear decay of 5 sec; variations of the kernel both in shape (e.g. exponential decay) and timing (0.5 sec rise and 2 sec decay) yielded qualitatively similar maps. Along with a constant offset term, this
yielded a 13 x $T$ predictor matrix $X$. We then used ordinary least squares regression to infer the best fitting coefficients $b$:

$$b = (XX^T)^{-1} X^T r$$

where $r$ is the $T$ x 1 fluorescence time course of either a single voxel or a neuron. The 12 coefficients (ignoring the constant) describe tuning with the two-dimensional behavioral space (e.g. polar wedge plots in Figures 3.3 and 3.4), and $R^2$ from the regression captures prediction accuracy. Computing a weighted angular mean yields a single laterality index, used to determine hue in computational maps (Figure 3.4). Note that a bilinear model could have been used to estimate behavioral tuning and temporal kernel simultaneously, but preliminary analyses showed that tuning was largely invariant to the shape of the temporal kernel.

Identification of the HBO in two-photon data for cell ablation and neuroanatomy

Because the location of the HBO was stereotyped across fish (Fig. 3.4C), it was possible to find the area of the HBO using two-photon microscopy. We imaged single planes in this area, then selected cells with activity profiles similar to the activity time course of the HBO, as identified in the light-sheet data (i.e. fluctuations on the order of 10 s, antisymmetric across the midline). Next, an ROI was drawn manually around a cluster of such identified cells, and the $\Delta F/F$ time course of this ROI was correlated, pixel-by-pixel, to the entire movie. This resulted in an image of correlation coefficients (e.g. Figure 3.12B,C) exhibiting the structure of the HBO. Based on this image, cells were selected for ablation (online) or for overlap with dsRed and RFP (for identifying vGlut2a and gad1b expressing cells).

Hindbrain oscillator lesion experiments
Before HBO lesions, fish were filmed with a high-speed camera and their behavior quantified as before. Next, we embedded the fish and used two-photon imaging to identify the HBO according to anatomical location and function (see Identification of the HBO in two-photon data, above). Next, 10 to 20 hindbrain oscillator cells from either left or right medial cluster were selected, then were laser ablated with the two-photon laser (850 nm, 120 – 135 mW). During the exposure, the laser beam spiraled over a circle of 1 μm. Large brightness increases that indicated a successful lesion were detected automatically using custom software and used to terminate the laser exposure, minimizing the exposure time needed for successful ablation (typically 0.1 - 10 seconds).

**Electrical Stimulation**

Stimulation pipettes were pulled from theta glass capillaries (~10 MΩ, tip diameter ~2 mm), and then coated with nano-gold, which made the pipette visible with 930 nm laser illumination (unpublished data). Zebrafish larvae (5 – 7 dpf) were paralyzed with α-bungarotoxin, embedded in 2% low melting point agarose, and immersed in external solution (in mM: 134 NaCl, 2.9 KCl, 2.1 CaCl2, 1.2 MgCl2, 10 HEPES, and 10 glucose, pH = 7.8). A small piece of skin above the hindbrain was cut open for pipette insertion. Fictive behavior was recorded via two suction pipettes as described in the previous section. A stimulation pipette was inserted through the cut in the skin and targeted to the medial cluster of HBO. To achieve maximal activation of the medial cluster, pipette tip position was adjusted with a maximum range ~ 40 μm (typically 20 μm). A brief electrical shock train (duration: 0.2 - 2 ms, inter-pulse-interval: 20 - 50 ms, number of pulses: 5 - 10) was delivered through the pipette to activate the HBO neurons.
Identification of Neurotransmitter Phenotype

Tg(vGlut2a-dsRed):Tg(elav3:H2B-GCaMP6f) and Tg(gad1b-RFP):Tg(elavl3:H2B-GCaMP6f) fish at 6dpf were embedded in 2% agarose in a 35 mm petri dish. The fish were imaged under a two-photon microscope at 930 nm at the level of the posterior commissure (caudal to the midbrain-hindbrain boundary) in the anterior hindbrain. Several planes about 2 μm apart were imaged in order to find a plane where all four clusters of the HBO were visible. The HBO was functionally identified by using a correlational measure (see Identification of the HBO in two-photon data, above) to construct a ΔF/F map for the various planes. The plane depicting all four clusters was selected and imaged in the red channel (1005 nm). The images were then superimposed to visualize the expression pattern of vGlut2a-DsRed and gad1b-RFP in the cells of the HBO.

Neurite tracing experiments

Tg(alpha-tubulin:C3PA-GFP):Tg(elavl3:H2B-GCaMP6f) fish at 6dpf were embedded in 2% agarose in a 35mm petri dish. The fish were imaged under a two-photon microscope at 930nm at the level of the posterior commissure (caudal to the midbrain-hindbrain boundary) in the anterior hindbrain. Several planes about 2 μm apart were imaged in order to find a plane where all four clusters of the HBO were visible. The HBO was functionally identified by using a correlational measure to construct a ΔF/F map for the various planes. Individual cells of either the medial or lateral cluster were selected on one side of the brain in the plane containing sections of all four clusters. We modified the neurite tracing protocol developed by Datta et al. (2008) to trace projections from a subset of HBO neurons. Cells were selected using a custom
written software and PA-GFP was activated using a protocol for iterative activation: ten 250 ms pulses of 780 nm pulsed infrared laser light were administered over a course of 16 cycles spaced 15 minutes apart for 4 hours. Selective activation was confirmed after each cycle by switching to 930nm and imaging the selected plane for increased fluorescence. At the end of four hours, the fish was transferred to the incubator and kept in the dark for another hour to allow sufficient time for GFP transport along the neurites. Subsequently, the fish was imaged on a Zeiss 710 confocal microscope using a 20x or 40x objective. The confocal stacks were then analyzed using the Simple Neurite Tracer plugin in Fiji (ImageJ).

Transgenic zebrafish

Transgenic zebrafish larvae used in this study were in either casper or nacre background. TgBAC(gad1b:loxP-RFP-lopP-GFP) 22 and TgBAC(slc17a6b:loxP-DsRed-lopP-GFP) 22,24 were used in the absence of Cre-mediated recombination and will be referred to henceforth as Tg(gad1b-RFP) and Tg(vGlut2a-dsRed) respectively. Tg(alpha tubulin:C3PA-GFP) 1-5,9 was used as described previously. (Tg(elavl3:GCaMP6f) jf1 and Tg(elavl3:H2B-GCaMP6f) jf7 lines were generated using Tol2 system and an elavl3 sequence 13. The larvae were reared at 14:10 light-dark cycles according to the standard protocol at 28.5°C.

3.10 – References


Chapter 4

Future directions
Expanding the looming stimulus set

In Chapter 2, I outlined a series of experiments that demonstrated: (1) looming visual stimuli evoked rapid escape maneuvers whose kinematics depend on stimulus position and expansion velocity, (2) escapes are initiated when looming stimuli reach a critical angular image size threshold, (3) neurons in the optic tectum respond to looming stimuli and encode this critical size threshold, and (4) morphologically identifiable neurons in the hindbrain reticulospinal spinal system receive the signal to escape and dictate the initial escape trajectory. This study is unique in the looming field in that it is the first to discuss population-level neural processing in the superior colliculus (optic tectum). My data argue for a novel processing mechanism distinct from the single-neuron computation described in the fly\(^1\) and locust\(^2\). As I mention in the Chapter 2 discussion, however, my current behavioral data match data for escape latency in invertebrates. Thus, while the processing appears to be taking place over many neurons in the optic tectum rather than in a single neuron, it’s possible that the ultimate computation is similar. That is, zebrafish may very well combine excitation as a function of edge velocity \(\theta'(t)\) and inhibition as a function of image size \(\theta(t)\) according to \(\eta(t) = \theta'(t)e^{-\beta\theta(t)}\), the function that has been deconstructed and shown to dictate spike rate as a function of time in locust looming detectors.

In order to test whether zebrafish truly make use of the same functional expression, more behavioral experiments with an expanded stimulus set are required. My previously described stimulus set is underpowered for three reasons. First, all of the hyperbolically expanding stimuli I used for latency analysis are mathematically guaranteed to have a peak in the putative \(\eta(t)\) function; new stimuli can be designed that lack this peak, thus testing for necessity. Second, all of my stimuli expanded continuously and were thus highly correlated in both velocity and size over time; breaking this correlation may better reveal the underlying computation. Third, my
stimuli were presented on an oblique plane at an air-water interface, complicating a truly quantitative description of stimulus parameters. The latter issue can be solved with an approximation of Snell’s law. As for the former two issues, I have arrived, from discussions with Dr. James Fitzgerald, theorist and Swartz fellow, at what I believe will be an illuminating new stimulus set. Rather than simulating true object approach trajectories, we plan to present stimuli with instantaneous velocities chosen randomly with some fixed average correlation time. This random walk in velocity will break stimulus correlations and provide an unbiased and expansive set of stimuli. By triggering on escape events over many trials and fish, an behavior-triggered average of the stimulus can be calculated, generating something analogous to a spike-triggered average for neuronal receptive fields.

Regardless of whether or not the behavior-triggered average filter agrees with the aforementioned $\eta(t)$ function, I can then use it to deconstruct tectal representations in more detail. For instance, preliminary linear modeling of tectal responses has suggested that neurons in the optic tectum are capable of encoding almost any stimulus parameter. Determining the extent and degeneracy of this representation as it relates to ethologically relevant stimulus variables will help test hypotheses of flexible population representations.

\textit{Investigating competitive visual streams}

While work in the larval zebrafish has revealed much about isolated sensory processing of visual stimuli with innate valence (i.e. looming, prey capture, whole-field motion), little is known about how these sensory streams compete for motor output. It is now known that looming stimuli and prey-like stimuli are processed in the optic tectum, and whole-field motion and luminance changes are processed in dedicated visual streams in pretectal accessory visual areas.
By presenting competing stimuli processed through distinct channels (e.g. leftward whole-field motion, which evokes left turns, and looming stimuli in the left visual field, which evoke rightward escapes) and modulating salience parameters (e.g. contrast, speed), we can build predictions for how these stimuli interact to inform behavior. Subsequent whole-brain imaging can be used to search for the site of this interaction.

**The hindbrain oscillator as a central regulator of bout structure**

In Chapter 3, I outlined a series of experiments that demonstrated: (1) larval zebrafish spontaneously swim in a correlated spatiotemporal pattern, (2) these behavioral sequences are conserved under the microscope, (3) a region in the hindbrain, the hindbrain oscillator (HBO), is spontaneously active in antiphase, with each side strongly correlated to strings of ipsilateral turns, (4) the HBO is necessary and sufficient for patterning spontaneous turn sequences, (5) the HBO consists of two medial glutamatergic stripes and two lateral GABAergic stripes, the latter of which mediate contralateral inhibition that may support oscillator dynamics, and (6) correlated turn sequences reduce diffusion and increase the efficiency of local searches. While we describe the relationship only between the HBO and spontaneous swimming, data from others in the Engert, Ahrens, and Aksay labs suggest that at least part of the HBO is correlated with other behaviors and activated by sensory stimuli.

Data from the Aksay lab (personal communication) and Portugues et al.⁶, for instance, suggest that the HBO is correlated with eye movements. Because eye movements are highly correlated with turning (fish look in the direction of a future turn), this is not altogether surprising. But preliminary data from whole-brain light-sheet data sets also suggest that an anterior region of the HBO, along with the GABAergic lateral stripes, is activated by whole-field
motion stimuli that evoke turn bouts. Furthermore, a posterior region of the HBO appears to be correlated with phototaxis stimuli that also drive turns. This coordination across modalities is consistent with what is known about the hindbrain region in which the HBO resides. Rather than existing as a discrete nucleus, the HBO occupies a position in contiguous longitudinal “stripes” of neurons uniquely identified both by neurotransmitter identity and developmental fate. It is possible that neurons along this stripe are organized to communicate efficiently about the instantaneous turn state of the animal.

This hypothesis is supported by preliminary data I have collected in collaboration with Ruben Portugues, where we measured spontaneous turning under different background lighting conditions. We observed that correlations grew stronger with brighter illumination and weaker with dimmer illumination. From this, we may be able to describe global phototaxic navigation using statistical models of turn state alone. That is, what may appear to the naïve observer to be a goal-directed behavior, fish actively sensing and swimming towards a well-lit region and away from darkness, may actually result merely from modulations to the statistics of underlying turn sequences, i.e. changes in HBO activity. Taking this for granted, I, somewhat unconventionally, hypothesize that all basic, bout-related, sensory-evoked behavior may express merely as a change in underlying HBO statistics. This is supported by the aforementioned partial activation of the HBO by whole-field motion; the HBO may act as a central bottleneck, or coordinator, of all zebrafish swim bouts. Future lesions localized to discrete subregions of the HBO will speak to this hypothesis.

**Outlook**
This dissertation is the product of over 5 years of work that began with the very innocent idea that a “detailed analysis of behavior and its neural correlates is critical for furthering our understanding of brain organization and function.” By tackling the zebrafish brain from both the external, sensory end and internal behavioral core, I believe I have contributed much to this original goal. Although with time I have gained a much deeper appreciation of biological complexity, I remain optimistic that the larval zebrafish, with its amazing degree of accessibility and homology, will continue to revolutionize systems neuroscience. Paired with more rigorous theory, advanced quantitative analysis, and improved methods for circuit manipulation, the zebrafish midbrain is certain to reveal far-reaching general principles of visual processing and multimodal integration. And mechanisms of spontaneous activity and behavior may very well lead us to better descriptions of spontaneity in ourselves.

References


