# Neural Mechanisms of Salt Avoidance in a Freshwater Fish

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Neural mechanisms of salt avoidance in a freshwater fish

A dissertation presented

by

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to

The Department of Molecular and Cellular Biology

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Neural mechanisms of salt avoidance in a freshwater fish

Abstract

Salts are crucial for life, and many animals will expend significant energy to ensure their proper internal balance. Two features necessary for this endeavor are the ability to sense salts in the external world, and neural circuits ready to execute appropriate behaviors. Most land animals encounter external salt through food, and, in turn, have taste systems that are sensitive to the salt content of ingested material. Fish, on the other hand, extract ions directly from their surrounding environment. As such, they have evolved physiologies that enable them to live in stable ionic equilibrium with their environment. However, when the environmental salinity changes, this equilibrium is perturbed and the fish’s internal ionic homeostasis is threatened. It would seem advantageous, then, for fish to have also evolved mechanisms to detect and evade undesirable saline environments. Whether this is true and which sensory modalities might be involved is not known.

In this thesis, I attempt to shed light on this puzzle by studying the behavioral and neural responses of the larval zebrafish, a freshwater fish, to salt. First, I develop an assay to determine chemical preferences of larvae, which allows me to identify a robust avoidance response to salt gradients that emerges from the detection of salt increases. I then use calcium imaging techniques to identify the olfactory and lateral line systems as the sensory modalities that most
thoroughly capture external salinity levels. I characterize the response properties of these systems to the environmental parameters that co-vary with NaCl and find that salt detection arises from broadly tuned sensitivity toward monovalent cations.

While the sensory systems can provide representations of absolute salinity, the zebrafish must extract information about relative dynamics in order to guide its behavior. To determine the neural mechanisms responsible for this extraction, I dissect the behavior of the animal in an embedded prep using precisely controlled stimulus delivery. I find that the behavior can arise from a model in which a slowly rising and decaying inhibitory population balances the output of a fast behavior eliciting population. Corroborating this, I use two-photon imaging to identify a salt sensitive population of likely GABAergic neurons that possess persistent activity for nearly two minutes after their initial stimulation. Together, these results allude to a circuit that captures absolute salinity information and transform its dynamics into appropriate behavioral responses.
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Chapter 1

Introduction

“Salt is born of the purest parents: the sun and the sea”

Pythagoras

1.1 Overview

For an animal to survive, its brain must generate behaviors that surmount environmental challenges. Critically, these behaviors must act in accordance with the animal’s physiological limitations to avoid conditions that threaten its homeostasis. In this thesis, I intend to explore how the brain of a fish enables it to avoid experiencing a particular homeostatic challenge - salt imbalance. All animals must maintain a steady internal regime of ion concentrations and threats to this equilibrium must be quickly counteracted. Land animals are often at risk of ionic depletion, and such states will drive them to seek salt-rich food. To facilitate this goal, most land animals have evolved taste systems with salt sensitive channels that can elicit appetitive behaviors. On the other hand, the ionic homeostasis of fish can be directly challenged by their surrounding environment. As such, they likely have unique strategies to avoid undesirable salinities that make use of different chemosensory modalities. The neural underpinnings of these strategies and sensory mechanisms that fish use to optimize salinity are unknown, and represent the focus of my work. This thesis is organized as follows:
The remainder of Chapter 1 is divided into three parts. First, I review the known mechanisms of salt detection in terrestrial model organisms, which are largely found in gustatory systems. I then examine the chemosensitive modalities of fish, focusing on how different chemosensory modalities govern distinct behaviors. Finally, I describe the larval zebrafish and the tools that this model provides that allow me to probe for mechanisms of salt sensing.

Chapter 2 first introduces several tools that I developed to study chemosensation in free-swimming and head-fixed larval zebrafish. In this chapter, I use these tools to identify aversion of larval zebrafish toward increasing salt concentrations and then probe the sensory mechanisms that allow larval zebrafish to detect sodium chloride.

Chapter 3 looks more closely at the behavioral algorithms involved in salt avoidance. In particular, I take advantage of the high temporal control that the head-fixed preparation allows in order to investigate the relationship between the dynamics of salt concentration fluctuations and behavior. From these experiments, I develop a simple model that can explain a number of the observed behavioral phenomenon. I then use two-photon imaging to identify possible sites of the neural implementation of this transformation.

Chapter 4 first discusses the progress that has been made in this thesis toward understanding salt detection and avoidance in fish, before reflecting upon some of the outstanding questions that remain and those that have emerged from this work.

1.2 Salt sensing in terrestrial animals

Terrestrial animals acquire salt by consuming food or water. Ingestion, in fact, is nearly the only way most non-aquatic animals encounter changing salt levels in the environment. Accordingly, the primary sense used to evaluate the nutritional status of consumables, taste, has
evolved high salt sensitivity in most land animals. The molecular logic and circuit mechanisms that enable salinity encoding are diverse and tailored to the behavioral needs of the animal. However, one feature seems to be nearly universal in the models studied so far - the use of separate sensory pathways to detect noxious and appetitive salt concentrations (Figure 1.1). Here, I briefly summarize the mechanisms used for salt detection in the classic terrestrial models of invertebrate and vertebrate neuroscience.

1.2.1 Salt detection in invertebrates

The ability to detect salt has been observed across multiple phyla of invertebrates. With this ubiquity comes a wide range of behavioral responses. For example, the roundworm, *C. elegans*, which naturally lives in soil that can experience fluctuations in moisture and salinity, is very sensitive to salt fluctuations (Bargmann & Horvitz 1991). Sharp increases in NaCl, such as those generated by droplet tests, lead to reversals away from the application (Bargmann et al. 1990). When navigating a sodium chloride gradient, however, the optimal salinity that *C. elegans* seeks to inhabit is dependent upon prior experiences (Saeki et al. 2001; Suzuki et al. 2008). If the worm had previously encountered high NaCl in the presence of food, then it will perform chemotaxis toward sodium or chloride. However, if the worm was in a stressed state, such as starvation, during its encounter with NaCl (Saeki et al. 2001), it will avoid those ions.
Figure 1.1: Salt encoding across model systems: worms, flies, and rodents

*C. elegans* possess several sensory inputs through which it detects salt. Reversal-inducing salinity (> 100 mM) is sensed by the ASH neurons (Hilliard et al. 2002). These chemosensory neurons are polymodal and detect a range of other noxious chemicals, including acids, alkaloids, and heavy metals, among others (Hilliard et al. 2004). Salt sensitivity is endowed to ASH by transmembrane channel 1 (*tmc-1*) (Chatzigeorgiou et al. 2013). In *C. elegans*, *tmc-1* activity is
largely specific to external sodium, as other cation-chloride pairs, such as magnesium chloride, fail to open the channel. On the other hand, when navigating gradients, *C. elegans* uses a pair of gustatory neurons, ASEL (Bargmann & Horvitz 1991) and ASER. While anatomically symmetric, these neurons are tuned to opposing gradient directions, with ASEL responding to increasing salt concentrations and ASER detecting decreasing salt (Suzuki et al. 2008; Ortiz et al. 2009). This pair of neurons works competitively to regulate the activity of an interneurons (AIY, AIA) that promote forward locomotion (Wang et al. 2017).

Many insects also possess the ability to detect sodium chloride. The fruit fly, *D. melanogaster*, for example, is very sensitive to the NaCl concentration in potential tastants. Low concentrations of salt (< 100 mM) promote appetitive responses, including preferentially extending their proboscis toward water droplets with mild salt concentrations over those with sucrose (Zhang et al. 2013). By contrast, high concentrations (> 100 mM) repress proboscis extensions and halt consumption (Zhang et al. 2013; Lee et al. 2017). These behaviors are mediated by the gustatory system. In *Drosophila*, gustatory sensory neurons are harbored within sensilia, hair-like sensory organs found across their body (Montell 2009). The primary site of gustatation is at the labellum, a knob-like structure at distal end of the proboscis. Here, three types of sensilia, each defined by their size (S - short; I - intermediate; L- long), detect appetitive or aversive cues on a potential morsel before its ingestion. All three sensilia types play a role in the identification of both low and high concentrations of salt.

Within the sensilia are representatives of up to four of the five total gustatory receptor neuron types. These sensory cell types are defined by the gustatory receptor they express (*Ppk28*, *Gr64f*, *Ir76b*, *Gr66a*, and, *Ppk23*). While each of these neuron types are sensitive to sodium
chloride, the stimulus-response functions of each neuron-type to different concentrations of salt is unique (Jaeger et al. 2018). It is from the pattern of activity across these cells that the fly is able to decode salinity and distinguish appetitive from noxious salt levels. The water-taste neuron, defined by their expression of Ppk28, exhibits an osmolarity-dependent activity profile, where increasing salt concentrations decrease the firing rate of the neuron (Cameron et al. 2010). The other four neuron types increase their firing rate with NaCl concentration, but with different activation thresholds (Jaeger et al. 2018). The sugar-sensitive neurons, which express Gr64f, are activated by low concentrations of NaCl (50 mM) and optogenetically driving its activity elicits appetitive behaviors (Jaeger et al. 2018). By contrast, neurons carrying Gr66a, which also responds to bitter tastants, are only activated by concentrations of NaCl above 250 mM (Marella et al. 2006). Optogenetic stimulation of these neurons facilitates food rejection (Jaeger et al. 2018). Genetically restriction ablation of Gr66a-positive neurons does not eliminate this aversion (Wang et al. 2004). This is likely due to the continued presence of neurons that express Ppk23, which has a salt-response curve similar to that of Gr66a-positive neurons (Jaeger et al. 2018).

In the mosquito, A. aegypti, salinity plays a role, not only in food consumption, but also in breeding (Matthews et al. 2018). Female mosquitoes lay eggs in still, ion poor water. If her eggs hatch in water that is too salty, the larvae will perish. Therefore, it benefits the mosquito to use a salinity detection mechanism to gate the decision to lay eggs or not. Like Drosophila, mosquitos utilize gustation via their labellum to sample the water quality. In fact, the neurons necessary to gate egg-laying in freshwater express an ortholog of Ppk28, Ppk301, and are also sensitive to water and osmolarity. Unlike Ppk23 expressing neurons in Drosophila, the Ppk301 positive neurons increase their activity with increasing sodium chloride. This means that Ppk301
neurons generate conflicting signals. Water, which promotes egg-laying, activates these cells, as does sodium chloride, which should inhibit egg-laying. Knockout of the Ppk301 channel using Crispr-CAS9 accentuates this conflict, as overall egg-laying is decreased, but the proportion (and total number) of eggs laid in salt water is significantly higher than wild-type sibling controls (Matthews et al. 2018). How these conflicting inputs are disentangled remains a mystery.

1.2.2 Mammalian salt taste

Mammalian interactions with salt are, in many ways, similar to those of Drosophila. In order to counteract the loss of ions and help maintain proper ionic balance, mammals will readily consume food or liquid with low (< 100 mM) concentrations of NaCl. Concentrations that exceed internal physiological levels (> 100 mM) on the other hand, are aversive and inhibit consumption. Like the fruit fly, mammals detect and encode NaCl with their gustatory system. In mammals, the sensory component of the gustatory system begins at the taste buds, which cover the tongue and throat. Two types of cells make up each taste bud, taste cells and the sustentacular cells that envelop them. Within a given taste bud, there may be up to 150 individual taste cells, each a member of one of five classes defined by their receptor expression profiles. Upon stimulation by a tastant, taste cells release either ATP or serotonin from their basal end, which in turn increases the firing rate of the most proximal afferent sensory neurons (Finger et al. 2005; Huang et al. 2005).

Similar to Drosophila, mammals use peripheral mechanisms to discriminate attractive from aversive salt concentrations. This was first noted when researchers observed that application of the sodium channel blocker amiloride to the tongue of rats eliminated their
attraction to low concentrations but not their aversion to high concentrations of NaCl (Schiffman et al. 1983; Heck et al. 1984). Eventually, it was determined that part of this discrepancy was due to low concentrations of sodium chloride driving activity in taste cells that express the epithelial sodium channel $ENaC$ (Chandrashekar et al. 2010). These cells represent a distinct subpopulation of taste cells that is dedicated to detecting attractive salt (Chandrashekar et al. 2010). Epithelial sodium channels are highly specific to sodium, which they allow to diffuse into the cell. This suggests that increasing external sodium chloride directly depolarizes the $ENaC$ positive population of taste cells, which presumably leads to neurotransmitter release. Both conditional knockout mice lacking $ENaC$ positive taste cells, as well as wild-type mice treated with the $ENaC$ inhibitor amiloride, show no attraction toward sodium. This is true even after salt deprivation. In addition, the activity elicited by low concentrations of sodium chloride in the nerve that innervates the tongue is significantly reduced.

Manipulations of $ENaCs$ do not affect the behavioral or neural responses to higher, aversive, concentrations of NaCl. Instead, a separate population of taste cells is responsible for transmitting the valence of these concentrations. This population consists of two distinct types of taste cells. The first are those cells that express the class of T2R3 gustatory receptors, which respond to classically bitter substances. The second are those that express the Polycystic Kidney Disease 2-like 1 (PKD2l1) transient receptor protein, which normally respond to acidic, sour, solutions. Individual deletion of either class of cells reduces but does not abolish a mouse’s aversive response to high sodium concentrations. However, double knockout animals that lack functional bitter or sour responsive cells show now aversion to high salt. Instead, to the double-knockout mice, as sodium concentration of a solution increases, so too does its
appetitiveness, even at concentrations as high as 500 mM (Oka et al. 2013). Together, these results indicate that while attractive salinity is mediated by its own dedicated channel, the aversive flavor of high salt results from hijacking the two classically aversive taste channels, bitter and sour.

Sodium chloride is not the only cation/anion pair that is perceived as salty by mammals. While ENaCs are selective to sodium, humans report that other smalls cations, such as lithium and potassium, also elicit a salt-like percept (Murphy et al. 1981). Of the cations tested, smaller ions were perceived as salty, while the higher the molecular weight (i.e. potassium or rubidium) of the cation, the were more likely it was to be described as bitter. Along these lines, blocking the appetive pathway with amiloride also attenuates the neural activity generated by lithium chloride, but has no effect on the responses to potassium chloride (Heck et al. 1984).

While the cation is considered to be the active component of salt taste, the conjugated anion can also have a strong effect on the perceived saltiness of a solution. Individuals asked to compare the saltiness different sodium/anion pairs reported weaker salty flavor and stronger bitter flavor as the molecular weight of the anion increased (Murphy et al. 1981). Parallel findings have been seen in rodents. One exception to the tendency for anion size to be inversely proportional to reported saltiness is sodium carbonate. In furosemide treated rats, sodium carbonate is over an order of magnitude more appetitive that sodium chloride (St John et al. 2017). While the mechanisms of this so-called “anion” effect are unknown, it is currently hypothesized to result from steric hindrance at the pore of the ENaC channels.
1.2.3 Internal salt monitoring

Behavioral responses to salt concentrations are strongly influenced by the internal state of the animal. Humans that are incapable of ion absorption will desperately seek out salt-rich foods in an attempt to counteract their ion loss (Wilkins & Richter 1940). Similarly, rats that have been sodium depleted, whether by removing sodium from their diet, injecting furosemide, or performing an adrenalectomy to disrupt their sodium regulation, show increased sodium appetite (Richter 1936). This manifests as both increased consumption of salt-rich food, as well as an increased tolerance of normally aversive concentrations of salt. On the other hand, increasing plasma sodium levels by water deprivation leads to increased intake of ion-poor water and rejection of sodium rich fluids. In order to enact these behavioral modulations, it is critical to possess the ability to detect the salt concentration of the internal milieu.

Physiological nutrient detection occurs are at the circumventricular organs of the brain. These are regions of the brain where sensory neural endings are unsheathed by the blood brain barrier and make contact with circulating fluids (McKinley et al. 2003). Three of these regions form afferent connections with the rest of the brain, namely, the area postrema, the organum vasculosum of the lamina terminalis, and the subfornical organ. The latter of these, which is situated within, and well connected to the hypothalamus, is believed to be the primary regulator of salt appetite (Hiyama et al. 2004). Lesion of the subfornical organ disrupts sodium appetite, as operated rats do not increase salt intake following sodium depletion (Weisinger et al. 1990). Furthermore, stimulation of the subfornical organ using chemogenetic (Nation et al. 2016) or optogenetic (Matsuda et al. 2017) approaches spur increased sodium intake by mice.
The sodium sensitivity within the subfornical organ does not directly arise from its neurons, but from the glia. In the subfornical organ, a subset of glia express the sodium-sensitive channel Na\textsubscript{x} (Watanabe et al. 2006; Hiyama et al. 2002). Na\textsubscript{x} responds selectively to sodium levels above physiological levels, reaching fifty percent of peak activity at approximately 170 mM (Hiyama et al. 2004), suggesting it plays a direct role in detecting dehydration. Indeed, mice without Na\textsubscript{x} fail to properly regulate their sodium appetite, and will seek out salt even after water deprivation (Watanabe et al. 2000). The transmission of information from Na\textsubscript{x} activity in the glia to neurons follows an unconventional route (Shimizu et al. 2007). Shimizu et al. (2007) found that the Na\textsubscript{x} channel is coupled to a Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, such that when sodium traverses Na\textsubscript{x}, ATP should be hydrolyzed in order to drive the sodium and potassium exchange. In response to the presumed drop in intracellular ATP, they observed that dissociated subfornical cells increase their uptake of glucose, which, in turn, leads to higher anaerobic respiration and lactic acid production rates. Excess lactic acid is released into the extracellular space, where it is absorbed by GABAergic neurons. This, Shimizu and colleagues find is sufficient to promote increased GABAergic firing, which the author propose is, itself, the result of increased ATP production. These GABAergic neurons are believed to inhibit populations of neurons in the hypothalamus that promote salt-seeking.

1.3 The external chemosensory systems of fish

Land animals use three distinct groups of sensory pathways to detect external chemicals: olfaction, somatosensory chemosensation, and gustation. Each of these pathways operates through distinct neural channels to perform separable functions. Olfactory cues are largely
airborne volatiles that provide the animal information about distant objects and environments. By contrast, somatosensory chemosensors are engaged by compounds directly contacting the skin or other epithelial tissues and primarily alert the animal to potential irritants. Meanwhile, taste is used to evaluate the nutritional quality of food as it enters and engages the digestive system, a task that is often supplemented by the other two chemosensory pathways. For aquatic organisms, however, the lines that separate these classes are often blurred. Because fish live in an aqueous environment, external diffusive chemical cues can simultaneously contact and activate all chemosensitive organs. Despite this, in many fish it appears that the behaviors governed by each modality maintain parallels with their terrestrial counterparts. Here, we review the current state of knowledge concerning the neural circuitry that mediates chemosensation and its dependent behaviors in teleosts. In particular, we focus on zebrafish where appropriate data is available.

1.3.1 Olfaction

Adult zebrafish use olfaction to guide a wide array of innate behaviors that depend on diffuse and distal cues. These range from locating appetitive food sources and avoiding spoiled food (Hussain et al. 2013) to social behaviors including kin recognition (Biechl et al. 2016) and mating initiation (Behrens et al. 2014). Adult fish can also be trained to associate specific odorants with potentially threatening or rewarding spatial environments (Namekawa et al. 2018), suggesting olfactions plays an important role in learning and establishing contextual information. While the olfactory system develops rapidly, with functioning olfactory epithelia emerging by 2 days post fertilization, the range of olfactory driven behaviors in larval zebrafish (< 2 weeks old)
is poorly understood. To date, most research on olfaction in larvae has focused on their responses to a few attractive cues, such as some amino acids and bile salts that mirror those in adults.

1.3.1 The olfactory epithelium

Both developing and mature zebrafish detect olfactory cues with olfactory sensory neurons. These neurons reside in a layer of tissue, the olfactory epithelium, that forms one of two olfactory pits at the rostral end of the fish. While the odorant-detecting projections of all olfactory sensory neurons reach into the mucosa that separates the external world from the outermost layer of tissue, their corresponding cell bodies reside at different layers within the epithelium. The particular location of the soma of an olfactory sensory neuron covaries with its morphological class. Four such classes of olfactory sensory neurons have been described so far (Figure 1), each believed to responsible for detecting separate classes of stimulants (Muller & Marc 1984; Hansen & Zeiske 1998; Ahuja et al. 2014). These are the ciliated and microvillus neurons, which are also found in mammals, as well as crypt and the most recently discovered kappe cells, which are only present in fish and amphibians.

Out of the four known types of olfactory sensory neurons in zebrafish, ciliated neurons express the most diverse set of receptors. In addition to approximately 140 traditional olfactory receptors (OR-type), ciliated sensory neurons also host the family of trace amine associated receptors (TAARs), which, with over 100 different receptors, is expanded in zebrafish compared to other vertebrates (Shi & Zhang 2009). As in mammals, both ORs and TAARs in zebrafish seem to obey the “one neuron - one receptor rule” (Barth et al. 1997; Ferreira et al. 2014) with only a few exceptions (Sato et al. 2007). This is established early in development, with larvae as
young as 3 dpf expressing over half of their possible OR repertoire in non-overlapping sensory neurons (Shao et al. 2017).

At present, though, no ligand/receptor pairs have been identified for OR-type receptors in zebrafish, making it difficult to ascribe precise functions to these receptors. By contrast, an understanding of at least part of the behavioral role that some TAARs play in fish seems closer to grasp. A recent chemical screen managed to identify ligand-receptor pairs for 11 distinct receptors spanning several classes of TAARs (Li et al. 2015). In particular, Li et al. (2016) found that different receptors in this family detected unique biogenic amines. Many of these biogenic amines are released by rotting flesh (Shalaby 1996), an aversive cue to adult zebrafish (Hussain et al. 2013). Furthermore, Hussain et al. (2013) demonstrated that at least one of these amines, cadaverine (which activates TAAR13c and TAAR13d), elicits avoidance behaviors in adult zebrafish. The authors, in turn, propose that at this class of TAARs may act as direct, labeled lines to rapidly promotes aversive responses to specific chemical cues.

Microvillous neurons primarily express V1R and V2R type receptors (Hansen et al. 2004; Saraiva & Korsching 2007). These receptors are homologous to the receptor families of the same names in mammals, where they are primarily expressed by neurons in the vomeronasal organ (Dulac & Axel 1995; Herrada & Dulac 1997). In mammals, these receptors play a critical role in detecting pheromones, and thus play a crucial role in mediating many social behaviors. Of the zebrafish V1R-type receptors, only one (ora1) has been demonstrated to sense a pheromone; one that assists mating (Behrens et al. 2014). Instead, in zebrafish, most microvillous neurons are sensitive to appetitive cues, such as amino acids and bile salts (Lipschitz & Michel 1999) (DeMaria et al. 2013). In fact, without microvillous sensory neurons, adult zebrafish fail to
exhibit otherwise innate attractive responses toward amino acids (Koide et al. 2009). They do, however, retain observably normal social behaviors, such as shoaling, and mating, suggesting these behaviors are largely driven by other modalities, such as vision.

Unique to fish are the crypt and kappe cells (Ahuja et al. 2014). These two OSN types are distinguished from ciliated and microvillous neurons by their superficial placement within the olfactory epithelium as well as their morphology. While ciliated and microvillous neurons both possess a long neurite stalk that carries information from their sensory ends to their more basally situated cell bodies, the apical end of crypt cells features a convex cavity from which cilia and microvilli emerge (Hansen & Zeiske 1998). Less numerous in number than either the ciliated or microvillous neurons, crypt cells express only a single receptor olfactory-type receptor, the v1r-like ora4 (Oka et al. 2012). At present, the ligands for ora4 are unknown. However, a study that compared the olfactory epithelial activity induced by familiar kin-water and unfamiliar kin-water found that crypt cells were only responsive to kin-water the fish had previously experienced (Biechl et al. 2016). These results suggest there may also be some activity-related dynamic expression of receptors. Crypt cells also express a number of other chemosensitive receptors that may provide them with additional functional roles. One such proposed function is the activation of the innate immune system following detection of viral particles through TrkA receptors (Sepahi et al. 2018). In fact, zebrafish with chemically ablated crypt cells displayed increased susceptibility to viral infection.
1.3.1.2 The olfactory bulb

From the epithelia, olfactory sensory neurons project their axons ipsilaterally into the olfactory bulb. In adult zebrafish, the olfactory bulb consists of 140 distinct bundles of neuropil (Braubach et al. 2012), called glomeruli, that are stereotyped and identifiable across animals (Baier & Korsching 1994). Each olfactory sensory neuron sends its axon to one glomerulus (Ngai et al., 1991). While during the development of the olfactory bulb, axon terminals from OSNs carrying related but different sensory neurons may reach into the same proto-glomerulus, a mature glomerulus receives direct, excitatory input only from sensory neurons expressing identical receptors (Dang et al. 2018). Furthermore, in fish, neurons of different morphological class project to anatomically distinct glomerular zones. Ciliated neurons innervate mostly the dorsal and medial glomeruli, while the microvillous neurons predominantly feed into the lateral chain glomeruli (Hamdani et al. 2001; Sato et al. 2005). The more rare crypt and kappe neurons project to single glomeruli each; medial-dorsal glomerulus 2 (Ahuja et al. 2013) and medial-dorsal glomerulus 5 (Ahuja et al. 2014). These innervation patterns reflect the topography of the olfactory bulb’s functional response profiles, with amino acids generating lateral chain activity (Friedrich & Korsching 1997), while broader classes of stimulants generate medial glomerular responses (Friedrich & Korsching 1998).

It is important to note that the olfactory bulb is not just a relay station between the sensory epithelia and higher brain areas. This can be appreciated by noting its internal dynamics. In adult zebrafish, an olfactory sensory neuron’s firing rate stabilizes immediately after chemical exposure (Friedrich & Laurent 2001). By contrast, the activity of individual neurons within the bulb is dynamic and may take hundreds of milliseconds to seconds to reach a new steady state.
(Friedrich & Laurent 2001). Such dynamics implicate the olfactory bulb as the first site of extensive olfactory signal processing. Two of the most important transformations that take place within the olfactory bulb are pattern decorrelation to distinguish odors with similar inputs (Yaksi et al. 2007; Wick et al. 2010), and gain modulation to ensure concentration invariant representations of the same odor (Zhu et al. 2013). These computations are performed through the dense network of interneurons (Wanner et al. 2016) that establish inter- and intra-glomerular connections. GABAergic interneurons between glomeruli, for instance, act to separate correlated patterns (Tabor et al. 2008), such as those generated by similar amino acids, into more easily dissociable signals within a few seconds of processing (Yaksi et al. 2007). Meanwhile, in order to rapidly equalize mitral cell outputs across concentrations, electrically coupled and GABAergic interneurons within a glomerulus dampen strong incoming signals and boost weak signals (Zhu et al. 2013). Longer time-scale gain modulation, however, may be mediated by the population of intrabulbar dopaminergic interneurons, which slowly, over tens of seconds, reduce mitral cell firing rates upon stimulation (Bundschuh et al. 2012).

1.3.1.3 Higher order olfactory regions in the brain

Mitral and tufted cells in the olfactory bulb are responsible for carrying olfactory information to a broad array of target regions in the brain. Using single-cell genetic labelling, a recent study characterized the projections and targets of two genetic lines that label a mutually exclusive but comprehensive set of the mitral cells in the larval zebrafish (Miyasaka et al. 2014). Neurons from all glomeruli, they found, send axonal branches to at least one of five dominant target zones: the dorsal pallium, ventral telencephalon, right habenula, posterior tuberculum or
back into the olfactory bulb. Each of these regions plays distinct roles in the processing of olfactory information.

The olfactory bulb sends more axons to the dorsal pallium than any other target region. In fact, every neuron traced by Miyasaka et al. (2014) sent at least one collateral into this region. Such comprehensive information transfer is consistent with the dorsal pallium’s homology to the piriform cortex in mammals. In mammals, the piriform cortex plays a crucial role in olfactory learning and memory tasks, as well as identity encoding (Roland et al. 2017; Bolding & Franks 2017). The dorsal pallium in adult zebrafish has been similarly implicated in the ability of adult zebrafish to discriminate between odorants.

Most neurons (> 80%) that emanate from the olfactory bulb also send at least one projection to the right dorsal habenula (Miyasaka et al. 2014), a region homologous to the medial habenula in mammals (Amo et al. 2010). The habenula has been hypothesized to play a role in valence extraction from olfactory signals. Excessive exposure of zebrafish to a normally attractive cue seems to recruit the right habenula to drive repulsion. At this point, the lack of studies that identify or utilize strong repellant odorants for larvae, and perform subsequent examination of functional brain activity has hampered the discovery of conclusive results.

1.3.2 Gustation

The primary role of taste in all animals is evaluating the palatability of potential food before its consumption. This system allows animals to separate nutritional from potentially poisonous food and is an active participant in the decision to accept or reject food, accordingly. As in mammals, the functional unit of the peripheral gustatory system in fish is the taste bud
Likewise, the fundamental structure of taste buds is conserved across vertebrates. Depending on the species of fish, a single taste bud may contain between 5 and 70 pear shaped taste receptor cells (Kasumyan & Doving 2003). The apical ends of these cells protrude out of the epidermis, allowing their receptor carrying microvilli to reach into an outer layer of mucous where they can receive chemical information from the external world. Two classes of taste receptors are present in most fish, including zebrafish: T1Rs and T2Rs. As in mammals, the T1Rs and T2Rs have distinct function roles, being responsible for detecting appetitive and aversive tastants, respectively (Oike et al. 2007). In zebrafish, however, amino acids are the only known stimulants of T1Rs, which show little to no sensitivity to sugars (Oike et al. 2007). In addition to lacking the canonical pathway for sugar taste, any ability of fish to salt must at least be partially distinct from those in other organisms. This is because the entire family of epithelial sodium channels, which in land mammals as well as insects is responsible for detecting low concentrations of sodium, is missing from all bony fish (Hanukoglu & Hanukoglu 2016).

Another difference that distinguishes the taste system of fish from that of land animals is the anatomical distribution of taste buds. Whereas mammalian taste buds are localized to the tongue and are therefore almost exclusively involved in evaluating compounds following ingestion, teleosts possess a large population of extraoral taste buds. In addition to the mouth cavity, teleost taste buds are found externally on the lips, fins and barbels, and internally in the esophagus and gills. The precise location and distribution of taste buds varies widely from species to species and often reflects the feeding habits of a given type of fish.

The distributed nature of the taste system suggests a capacity for gustation to collect information about the immediate environment and use it to search for food (Bardach et al. 1967).
Evidence that this information is useful and necessary for environmental optimization has only been observed in one species of fish, the channel catfish *Ictalurus punctatus* (*Kasumyan & Doving 2003*). Instead, long-range chemical detection is believed to fall under the purview of olfaction (*Kasumyan 2018; Koide et al. 2009; Atema et al. 1980*), while extraoral taste buds are used to alert a fish to the presence of nearby food. Presenting a fish with attractive tastants, such as most amino acids, triggers rapid turning toward the source of stimulation (*Kasumyan & Doving 2003*). These reflexive behaviors bring food items into contact with the oral taste buds of the lips which can trigger further ingestive actions, including snapping or sucking actions that bring the object into the fish’s mouth (*Osse et al. 1997*). Even following intake, however, evaluation of the food item is not complete. Within the mouth, food items undergo a retention period, during which mastication causes the further release of chemical cues that the taste buds within the oral cavity and esophagus can detect. These signals are relayed to the brain to decide to either consume or reject in a process known as food-sorting (*Ikenaga et al. 2009*). Food items laced with stimulants of fish bitter taste receptors are rejected by wild-type fish in favor of untreated food (*Aihara et al. 2008*). Genetic ablation of the taste buds expressing this specific G-protein eliminates food preferences (*Aihara et al. 2008*).

In fish, all taste buds are innervated by one of the facial (VIIth), glossopharyngeal (IXth) or vagal (Xth) cranial nerves (*Herrick 1900*). The most prolific of these is the facial nerves which carries information from extraoral taste buds, as well as those on the lips and within the mouth (*Herrick 1905; Atema 1971*). The vagus and glossopharyngeal nerves, by contrast, only innervate internal taste buds within the esophagus and gills, as well as a small fraction of those within the mouth (*Herrick 1905*). These nerves carry information about taste bud activity to their
corresponding sensory lobes in the medulla oblongata, which together form the primary
gustatory nucleus. Here, proper motor actions can be selected based upon the incoming taste
information. For most of these inputs, the synaptic distance that separates gustatory afferents
from their respective motor efferents is short (Kanwal & Finger 1992). For instance, in goldfish
and carp, the primary circuits responsible for food-sorting consist of reflex arcs that are between
one and three synapses long (Ikenaga et al. 2009). Gustatory inputs from the vagal nerve reach
the outermost layer of the radially organized vagal lobe in the nucleus of the tractus solitarius
(nTS). There, they synapse onto glutamatergic interneurons that project inward onto the
branchiomotor neurons at the basal level of the vagal lobe (Ikenaga et al. 2009). These motor
neurons send efferent projections back into the vagal nerve, where they orchestrate the
esophageal muscles that control food intake and rejection (Sibbing et al. 1986).

1.3.3 Somatosensory systems

Irritants on the face of the fish are detected by the sensory neurons of the trigeminal
ganglia. Sensory endings of the trigeminal nerve cover nearly the entire face and respond to a
large range of noxious stimuli. These include mechanical, chemical, and thermal cues. Of these
stimuli, the effects of heat on the trigeminal are the most well characterized (Prober et al. 2008;
Haesemeyer et al. 2018). The multimodal receptor, TRPA1 is partially responsible for endowing
a subset of trigeminal cells with noxious heat sensitivity (Prober et al. 2008). Presumably
through TrpA1 and other receptors, these cells provide the larvae’s brain with a direct readout of
absolute temperature, which can be used by the animal to execute appropriate behaviors
(Haesemeyer et al. 2015; Haesemeyer et al. 2018). More specifically, derivatives of these neural
signals are computed in the secondary projection sites of the trigeminal in rhombomeres five and six. The detection of heat increases then promotes the execution of turning swims, allowing the larvae to avoid high heat.

Unlike taste, chemicals are not transduced into neural activity through an effector cell, and instead affect the nerve endings directly. In these nerve endings, TrpA1 also plays a role in sensing some irritating chemicals, such as mustard oil (Prober et al. 2008). Similar to heat, the trigeminal seems to encode absolute concentrations of chemical stimulants. Noxious levels of carbon dioxide are also sensed and avoided via the trigeminal pathway (Koide et al. 2018), though the receptor is unknown. While it hasn’t been explicitly examined, one can assume that these chemical irritants activate similar circuits and behaviors as heat. Unfortunately, the spectrum of chemical sensitivity in the trigeminal beyond these two chemicals has not been studied in zebrafish. That being said, there has been a study of the sensory stimulants of the trigeminal nerve in the rainbow trout, another freshwater fish (Mettam et al. 2012). In this study, the authors recorded the electrophysiological response of the trigeminal nerve as they applied different putative noxious chemicals. They found that irritants, including acetic acid, carbon dioxide, HCl, and even NaCl elicited spikes. The authors found that the minimal concentration of NaCl to drive activity in the trigeminal was 500 mM - essentially ocean water. Such concentrations would be immediately lethal to zebrafish, and thus, if they avoid salinity it would either be through a more sensitive trigeminal, or other mechanisms.

In addition to the classical chemosensory systems, the activity of the lateral line of at least one species of fish has been reported to be modulated by external chemicals (Kawamura & Yamashita 1983), including amino acids and some salts. The lateral line is generally considered
to be a mechanosensory organ, with its primary function being to detect changes of water flow around the fish. It is essential for detecting and navigating against a moving currents (Oteiza et al. 2017), and, in the absence of visual cues, can be used by fish to locate prey (Yoshizawa et al. 2010). This is performed by hair cells that are similar to those used in the ear for auditory sensing. Clusters of hair cells, called neuromasts, constellate the skin of the larvae and separate into two fields - the posterior lateral line and the anterior lateral line. The posterior lateral line extends from the back of the head all way to the tail of the fish. These neuromasts are necessary for detecting the curl of water flow gradients around the fish (Oteiza et al. 2017). Without them, larvae fail to perform rheotaxis. The anterior lateral line, on the other hand, covers the head and face of the fish, and has a more prominent role in prey and object detections. It is unclear how the chemosensitivity of hair cells may arise. Some of the mechanosensitive receptors expressed in the hair cells also possess chemosensitivity. This includes \textit{tmc-1} (Pan et al. 2018), the channel that endows \textit{C. elegans} ASH neurons with salt-sensitivity. Whether such receptors are also modulated by external ions in zebrafish is unknown.

\textbf{1.4 The larval zebrafish}

The larval zebrafish is a well-suited model organism for improving our understanding of neural circuits and how they process sensory information and generate behavior. Its utility arises from several key features. First, larval zebrafish are transparent. As a result, they are fully amenable to the arsenal of optical tools that have been developed in the last few decades, including single and two-photon microscopy as well as optogenetic neural activation and silencing. In addition, when compared to other vertebrate models, their brain is miniscule. Not only does this allow observation of all neurons within the field of view of standard microscope
components, but with only \(~100,000\) neurons the system’s complexity is several orders of magnitude less than that of other model vertebrate systems. Finally, despite this reduced sized and presumed relative simplicity, there still exists a high degree of homology between these groups. This raises the likelihood that some of the insights gleaned from the brain of a zebrafish will be applicable to the nervous systems of other organisms, including our own.

1.4.1 Genetic techniques that enable circuit dissection in larval zebrafish

The ease of generating transgenic or knockout zebrafish arises thanks to the early development process of the embryo. During mating, a single female releases up to a few hundred eggs over several hours. After fertilization, eggs remain paused as single cell zygotes for nearly an hour. During this time, it is possible to inject genetic material, whether that be RNA or DNA. By injecting into the only developed cell of the animal, one maximizes the probability that the desired genomic modifications will be incorporated into the precursors of the germ-line, and thus be inheritable by future generations.

Most genomic modifications made to zebrafish embryos are either knockouts of genes or transgenic insertions. Historically, loss of function mutants have been made through random mutagenesis (ENU, UV etc…) followed by screens for desired phenotypes. These approaches have been highly successful and led to the discovery of countless genes essential for development and behavior (Patton & Zon 2001). However, recent advances in genomic engineering made by the advent of the CRISPR-CAS systems (Cong et al. 2013) have opened the doors to more targeted approaches (Hwang et al. 2015). Now, specific proteins can be efficiently and immediately knocked out. For instance, specifically targeting dopamine beta hydroxylase to
eliminate adrenergic production enabled the discovery of subtle waking defects that would not have been possible with previous screening techniques (Singh et al. 2015). Further, candidate receptors for external chemicals (such as olfactory receptors) can now be easily rendered inoperative (Horio et al. *unpublished*).

At present, methods for targeted transgenic insertions in fish are less successful than those for generating knock-outs. Efficient introduction of foreign proteins into the zebrafish genome requires a system that not only introduces breaks in the genome, but also facilitates the integration of donor DNA. For zebrafish, the most common such tool is the *tol2* transposon. Discovered in the Japanese rice fish, medaka (Koga et al. 1996), the *tol2* system has proved a powerful tool for teleost transgenics, having been used to introduce novel genes into the genomes of zebrafish (Kawakami et al. 1998), *Danionella translucida* (Schulze et al. 2018), cavefish (Stahl et al. 2019), cichlids (Kratochwil et al. 2017), and killifish (Valenzano et al. 2011). Unfortunately, it is nearly impossible to target genes of interest to specific locations of the genome using the *tol2* system, which uses an essentially random process for integration. As a result, gene-swapping is nearly impossible. As such, most cassettes for *tol2* integration must include a copy of the promoter region to enable expression, and cell-type specificity. Even with its limitations, this approach has proven very successful. For my purposes, one of the greatest achievements of this technique has been the generation of fish that express the calcium indicator GCaMP across nearly all neurons within their brain.

1.4.2 Technologies for circuit observation and manipulation in the larval zebrafish
Brain-wide expression of calcium indicators would be useless without the proper tools to observe them. Fortunately, a number of imaging technologies capable of capturing whole brain dynamics in the larval zebrafish have been developed over the last two decades. The earliest recordings of calcium activity in zebrafish neurons, in fact, precede the opening of their genetic toolbox. These earliest recordings utilized a combination of calcium sensitive dyes, such as Calcium-Green dextran, and either confocal or epifluorescence imaging (Cox & Fetcho 1996; Friedrich & Korsching 1997; Fetcho & O’Malley 1995). In larvae, perhaps the most fruitful preparation, at this time, was the spinal backfill, which labels the roughly 300 motor command neurons in the brain that project into the spinal cord (O’Malley et al. 1996). The reasons for the success of this preparation were two-fold. First, spinal backfills reliably label a stereotyped set of neurons, such that neuron to neuron comparisons can be made across fish. Second, this set of neurons is sparse, such that separating fluorescent signals from individual cells is possible, even with the limited axial resolution of confocal and epifluorescence techniques.

In a fish that expresses a fluorescent indicator pan-neuronally, however, the cone of excitation from a confocal microscope energizes out-of-focus fluorophores. Because of this, at any given pixel, the recorded fluorescence may derive from the emission of multiple cells, making single-cell resolution signal separation difficult. The introduction of two-photon excitation microscopy circumvents this problem. Two photon-microscopy exploits the fact that the resonant energy of a fluorophore can be reached with two synchronously (on the order of femtoseconds) arriving low-energy photons. The requirement that two photons must be present in order to energize a fluorophore means that relationship between excitation efficiency and photon flux scales as a quartic function, rather than the quadratic observed in single-photon
microscopy (Helmchen & Denk 2005). As a result, laser power can be chosen such that the probability of out of focus fluorophores contaminating the emission signal is negligible.

Combining two photon imaging with the aforementioned calcium dyes opened the door for researchers to image large populations of neurons in the spinal cord (Brustein et al. 2003), olfactory bulb (Yaksi & Friedrich 2006), and optic tectum (Niell & Smith 2005). However, it was with the development of calcium sensitive fluorescent proteins, including the GCaMP family (Pologruto et al. 2004), that the appeal of the larval zebrafish as a model for systems neuroscience was made obvious. With genetically encoded indicators, researchers could image specific, reproducible populations of neurons, ranging from those defined by genetic subtypes, such as GABAergic neurons in the olfactory bulb (Li et al. 2005) to nearly all neurons in the brain (Ahrens et al. 2012). Further empowering the preparation is the expanded ability to present stimuli and record behavior from the animal while imaging. Because the excitation light (920-950 nm) is invisible to the fish, visual stimuli can be presented to it without suffering from interference due to the fish also seeing a bright blue laser (Ahrens et al. 2012; Portugues et al. 2014). At the same time one can monitor the behavior of the animal, either by freeing its tail from the embedding material (Portugues & Engert 2011) or by directly recording electrical activity from the ventral root nerve in the spinal cord (Ahrens, Huang, et al. 2013). Together, these aspects of the preparation essentially allow the researcher to observe all components of a sensorimotor transformation.

In addition to its usefulness as a functional imaging tool, the spatial resolution of the two photon microscope makes it a powerful device for manipulating circuits. Single cells can be targeted and ablated by delivering microsecond pulses of high-powered laser (300-500 mW) to
the cytoplasm of the cell (Huang et al. 2013; Vladimirov et al. 2018). In the other direction, with the advent of red-shifted opsins such as ReaChR (Lin et al. 2013), one can optogenetically stimulate single cells by providing pulses of laser power to precisely determined locations (Kawashima et al. 2016; Vladimirov et al. 2018). By combining these single cell manipulation techniques with calcium imaging, the effects of specific on behavior and overall network dynamics can be pieced together (Chettih & Harvey 2019).

One major drawback of two-photon imaging is its slow rate of acquisition. The same absorption inefficiency that provides the technique with its high spatial resolution, also limits the amount of emitted photons per unit time. To generate ample signal to noise requires recording from a single spot for a few microseconds, restricting the amount of space that can be covered with a frame-rate appropriate for calcium signals (0.5-2 Hz). Recently, efforts have been made to accelerate image acquisition such that the entire volume of the larval zebrafish brain can be captured at video-rate. The most popular of these new techniques is light-sheet microscopy (Keller et al. 2008; Ahrens, Orger, et al. 2013). In light sheet microscopy, a cylindrical lens expands the focal point, but only along the axis of the beam. By transforming the focused light from a zero-dimensional point to a one-dimensional line, the number of dimensions that must be scanned is reduced by one. Thus, the time that was previously used to scan a plane can be used to scan a volume. At this point, the most successful implementations of this microscope (Ahrens, Orger, et al. 2013; Panier et al. 2013) have used one or two single-photon excitation beams. As a result, most of these setups fail to achieve the resolution one could obtain with two photon microscopy. Attempts have been made to combine two photon excitation with light-sheet scanning (Wolf et al. 2015). However, the increase in power necessary to produce robust
excitation across the entire axis of focus generates several caveats. For one, the fish is experiencing a constant heat stimulus during the experiment, which may affect embedding stability as well as behavior. More dramatically, the larvae’s eyes are strong heat sinks, and the animal will quickly perish if too much power runs through them. This restricts the volume of the fish that can be scanned, as Wolf et al. (2015) only image the dorsal third of the larvae’s mid and hindbrain.

1.4.3 Salt and the larval zebrafish

Zebrafish are stenohaline freshwater fish, meaning they survive only in a narrow range of low salinities. Swimming into even brackish water can be lethal for them. The reason for this lies in the physiology of their ionoregulatory system. Like all other fish, zebrafish directly exchange ions with their surrounding environment. This task is performed by a set of specialized cells unique to fish and amphibians, called ionocytes. These epidermal cells are situated across the skin of larvae, and gills in more mature fish. For freshwater fish, the primary task of these cells is to extract ions from their hypotonic environment. Because of the large concentration difference between zebrafish and their usual external world, these ionocytes are affixed in a state of ion extraction, which is performed with concurrent removal of hydrogen ions (Yan et al. 2007). As a result, changing the external sodium concentrations leads to a net increased flux of sodium into the fish, potentially at rates too fast for the animal to combat. This combined with the sudden disruption of hydrogen removal disturbs ion equilibrium and can be fatal.

Even for relatively sedentary fish species, such as zebrafish, the ability to avoid saline environments may be crucial for its survival. While most zebrafish spend their lifetime confined
to a small geographic range, their location in the floodplains of the Ganges River in Bangladesh means they are prone to sudden diaspora’s each monsoon season. Further, rising sea levels will bring noxious levels of seawater into many of their current ecosystems. Indeed, reports of increased salinity as well as the abnormal inland presence of seawater fish are already being reported in the Ganges River (Salehin et al. 2018). Such geological changes would have occurred throughout the evolutionary existence of these fish, including as recently as 6000 years ago when parts of the current zebrafish habitat were covered by the sea. Thus, it seems necessary for zebrafish to be capable of optimizing the salinity of their habitat, even if these are through somewhat diffusive processes rather than explicit migration.

In this thesis, I attempt to identify the sensory modalities that fish may utilize to detect salt. In particular, I will exploit the advantages of the larval zebrafish laid out about in order to address this question. This first necessitates identifying whether zebrafish avoid salt, and thus have the capacity the detect salt. Once established, I use the imaging techniques outlined above to identify which sensory regions are involved and what environmental cues might be alerting the zebrafish to increased salinity. From there, I seek to understand how salinity information is processed by higher brain regions to generate appropriate behaviors.
Chapter 2:

Multisensory detection of aversive salt-water by larval zebrafish
Intellectual contributions

The following chapter is adapted from a manuscript in preparation. The author list will be myself, followed by Thomas Panier, Drago Guigianna-Nilo, and Florian Engert. I designed and performed all experiments and analyses, and assembled the text and manuscript. Drago and Thomas built the microscopes used in this study. Florian Engert provided help and guidance throughout.

Summary

An important task for the brain of an animal is optimizing its environmental surroundings for the animal’s physiological limitations. For fish, the salinity of their aquatic environment represents a highly restrictive parameter. Yet, the neural and sensory mechanisms they might use to optimize their surrounding salt content are not known. Here, we use a freshwater fish, the larval zebrafish and observe that it strongly avoids increasing salt concentrations. We use volumetric light-sheet and two photon imaging of calcium activity to identify the lateral line and olfactory systems as sensory modalities capable of capturing changes in external salinity. In particular, we find that in the olfactory system, a subset of neurons respond to increased external monovalent cations by increasing their activity such that it directly reflects the absolute salinity of their environment.
2.1 Introduction

Organisms must keep their internal ionic content within a narrow and stable range. Across vertebrate species, the ion concentrations of plasma is broadly conserved, with sodium and chloride ions present above 100 mM and all other major ions kept below 5-10 mM. However, the mechanisms utilized for osmoregulation are diverse and depend on the animal’s environment. Land animals and marine mammals balance the consumption of food or liquids that contain deficient ions with the intake of ion-poor water and excretion of ions in excess (Matsuda et al. 2017; Buggy & Fisher 1974). By contrast, fish and amphibians supplement fluid consumption and excretion with the function of a specialized system of epidermal cells, ionocytes, that directly exchange ions with their surrounding water (Keys & Willmer 1932; Karnaky et al. 1977). Critically, the direction and mechanism of ion exchange depends on the salinity of the animal’s natural environment. The ionocytes of freshwater fish must acquire salts, while those of marine fish excrete them.

Complicating ionoregulation is the dynamic nature of salinity. As an example, much of southern Bangladesh is occupied by by the flood plains of the Ganges River. During the dry season, the salt content in this region increases from less than 1 ppt to nearly 20 ppt as far as 100 km inland (Salehin et al. 2018). Furthermore, long-term geological changes, including modern global climate change and rising sea levels, can push these fluctuations to intensify and penetrate further inland. Some species of fish, such as three-spined stickleback (Divino et al. 2016) or medaka (Kawamoto et al. 1958; Miyanishi et al. 2016), have the ability to reverse the direction of ion transport and thus can withstand variations in environmental salinity (Mayer et al. 1967). Most, however, cannot. Accordingly, the majority of fish must live within a narrow range of salt
concentrations, as environmental changes from hypotonic to hypertonic (or vice-versa) will have grave consequences for their ionic balance and, in turn, survival.

To prevent ionic disequilibrium, we anticipate that fish have evolved mechanisms for avoiding undesirable salinities. Whether this is true is, at present, unknown. Similarly, the sensory and neural mechanisms that may allow, for example, a freshwater fish to detect, process, and avoid high saline environments are unclear. Most land vertebrates and invertebrates use gustation to detect external salts (Chandrashekar et al. 2010; Oka et al. 2013; Zhang et al. 2013; Lee et al. 2017). This is because terrestrial animals encounter and balance salt via food intake. Aquatic animals, on the other hand, are directly exposed to changes in environmental salinity, which may affect the activity of any number of sensory modalities on their external surface. Therefore, other modalities may be involved in salt sensing. These may include both other chemosensory inputs such as olfaction (Hubbard et al. 2002), or the trigeminal nerve and the dorsal root ganglia, as well as mechanosensory organs such as the lateral line (Katsuki et al. 1971).

In the present study, we use the larval zebrafish as a model to examine the capabilities of fish to sense and avoid the ionic content of their environment. The larval zebrafish was chosen in order to exploit its genetic tractability and optical transparency as a means to characterize how its brain responds to external salt dynamics. Most zebrafish are native to the river basins that surround the Ganges River in India and Bangladesh (Spence et al. 2006; Spence et al. 2008), and occupy soft, ion-poor water, with NaCl concentrations below 1 ppt. As mentioned earlier, however, seasonal variations as well as monsoon-induced diasporas can force zebrafish into water with undesirable salinity. Increasing salinity can lead to elevated stress and cortisol levels,
before eventually becoming lethal (Yeh et al. 2013; Ryu & De Marco 2017). To potentially improve their odds of survival, we predicted that zebrafish possess neural mechanisms that enable the navigation away from increasing salt gradients. Here, we determine that zebrafish can avoid and thus detect salt, and then describe the underlying early neural mechanisms that encode external salinity in the brain.

2.2 Larval zebrafish avoid increasing NaCl concentrations

In order to test our hypothesis that larval zebrafish avoid saline environments, we developed an assay to determine their place preference in a chemical gradient (Figure 2.1a and experimental methods). In this assay, four larvae are placed in separate lanes bookended by two agar pads, one made from filtered fish water, and a “source” made from fish water plus the chemical being tested. Diffusion of NaCl from the source rapidly generates a monotonically increasing salt gradient that remains present throughout the duration of a 1 hour experiment (Figure 2.1B). At the end of the experiment, the NaCl concentration peaks at approximately 15 percent of the initial agarose concentration.

In control experiments, we use unsupplemented fish-water in the source, and observe that larvae spend approximately even amounts of time across all segments along the length of the arena (Figure 2.1C,D). When we add 400mM NaCl to fish water in the source pad, larvae are significantly more likely to be found away from the high salt (Figure 2.1C). We quantify this aversion with a traditionally defined preference index (Figure 2.2A,B). Early in the experiment, before much salt has diffused from the agarose, fish in both the control and the 400 mM experiments have an average preference index near 0 (Figure 2C). However, after 15 minutes,
Figure 2.1. Larval zebrafish avoid increasing environmental salinity

A. Schematic depiction of the rig used to perform chemical place preference assays. Four larvae are placed into individual lanes with agar gels. B. Development of the salt gradient over time relative to the concentration of salt added to the test agarose gel. C. Sample experiments when 0 mM (top) or 400 mM (bottom) are added to the source agarose. Individual dots demarcate position of larvae at every 50th frame. D. Histogram of positional occupancy by larvae when the source gel contains either 0 mM (n = 30) or 400 mM NaCl (n = 24) added (Mann-Whitney U test p<0.001). E. Histograms of turning angles when the larvae are swimming toward or away from NaCl in the half of the arena nearest the test gel. Turn angles between 20 and 50 are significantly increased when the larvae is swimming toward 400 mM NaCl than away from it (p < 0.01). F. Average turn angle as the larvae swims toward or away from the test gel as a function of their position in the arena (p <0.01). G. Results of monte-carlo simulations of effects of salt changes on turn angles recaptures the spatial distribution of larvae in a simulated linear salt gradient.
Figure 2.1. Larval zebrafish avoid increasing environmental salinity
Figure 2.2: Supporting data for Figure 2.1

A) Schematic depiction of the preference index calculation. Briefly, the preference index is calculated from the difference in time spent on the side with salt from that with fish water divided by the total time. B) Sample preference indices for source containing 0 additional NaCl and when 400 mM NaCl is added to the source agar. C) Evolution of preference indices over the course of the experiment. D) Sample positional trace of a fish experiencing no gradient along the axis that bisects the two agar pads. E) Sample positional trace of fish along the same axis as D, but for a fish experience a gradient generated from 400 mM NaCl. F) Polar histogram of orientations of fish in no gradient. Fish facing 270 degrees are facing the source. G) Same as in F, but for all fish in a gradient generated from 400 mM NaCl H) Difference in average turning angle of bouts that following an increased salt concentration compared to those that followed a decreased salt concentration for different concentrations of source salt. I) Preference indices toward different concentrations of NaCl that results from simulating fish according to our algorithm.
Figure 2.2: Supporting data for Figure 2.1

A

\[ \text{Preference index} = \frac{\text{time}_{\text{NaCl}} - \text{time}_{\text{control}}}{\text{time}_{\text{NaCl}} + \text{time}_{\text{control}}} \]

B

C

D

E

F

G

H

I

\[ \text{Distance from NaCl (cm)} \]

\[ \text{Time (min)} \]

\[ \text{Distance from NaCl (cm)} \]

\[ \text{Time (min)} \]

\[ \text{Angular difference} \]

\[ \text{Preference index} \]

\[ \text{Source} \]

\[ \text{NaCl added (mM)} \]

\[ \text{Preferences for 0 mM and 400 mM NaCl} \]

\[ \text{Preference index as a function of time for 0 mM and 400 mM NaCl} \]

\[ \text{Data and Simulation for angular difference and preference index} \]

\[ \text{Source} \]

\[ \text{NaCl added (mM)} \]
while the control remains neutral, NaCl promotes a strong aversive behavior in zebrafish larvae (PI = -0.5) that remains stable for the remainder of the experiment. Similar time courses are seen in all examined NaCl concentrations (ranging from 50 to 400mM), with preference indices decreasing with higher salt content.

In order to avoid high salt concentrations, the larval zebrafish must employ some behavioral algorithm that is a spatial and/or temporal function of experienced salinity. To determine this transformation, we dissected the kinematic parameters of the larvae’s movements and how they are influenced by the animal’s position in the arena. We first note that in both the control and high salt conditions, larvae spend the majority of the experiment swimming steadily toward one end of the arena (Supplemental Figure 2.2D,E). This is reflected in the orientation of the fish, which is biased along the axis of the arena (Supplemental Figure 2.2 F,G). As fish swim up the salt gradient, however, we observed that they are more likely to reverse directions than those swimming away or in control condition. A possible strategy the larvae may use to promote such reversals is a biased random walk where the larvae responds to increases in salt concentration by turning more frequently (Block et al. 1982; Haesemeyer et al. 2015). In order to test this hypothesis, we compared the distribution of reorientation angles during bouts of two types: those that followed a swim toward the salt, and those that followed a swim away from the salt. When the control and test pads are identical, larvae do not alter their turn statistics between these two cases (Figure 2.1E). By contrast, if the source is supplemented with salt, then larvae are significantly more likely to execute a 20-40 degree turn if the previous bout brought them closer to the salt rather than away from it (Figure 2.1E). Furthermore, we find that the average turn angle is only affected by the salt concentration as the larvae moves up the gradient (Figure
2.1F, Figure 2.2H), suggesting that increases in salinity, rather than absolute salinity, drive turning.

We also examined a number of other behavioral parameters as a function of either the previous turn direction or the alignment of the larvae relative to the gradient, including swim speed and time between bouts. However, none of these kinematic parameters exhibit trends that correlate with the stimulus. The absence of other relationships between kinematic parameters and the stimulus leads us to suppose that the larvae are indeed performing a simple biased random walk to avoid high salinity environments. To test whether such a simple algorithm is sufficient to explain the larvae’s avoidance behavior, we performed simulations utilizing the turn and swim statistics of the larvae and our proposed rule (A1: Experimental Methods). We find that, indeed, this simple rule recaptures the avoidance (Figure 2.1G) and observed linear relationship between aversion and NaCl concentration (Figure 2.2I).

2.3 Activity in the olfactory and lateral line systems reflects external salinity

We next wished to understand how the brain of the larval zebrafish represents environmental salinity. In order to screen the brain for regions that are sensitive to sodium chloride, we wished to use calcium imaging techniques. Such techniques require the head of the animal to be immobilized. Therefore, we designed a stimulation setup (Figure 2.3A) that enables us to rapidly and reversibly present different chemicals to the rostral end of the larvae (Figure 2.4), while the head and body are embedded in an agarose gel. To acquire a behavioral readout from the animal during our experiments, we remove the agarose around the tail of the animal. We find that, consistent with the free-swimming experiments, larvae respond to increases of
Figure 2.3. A preparation to probe responses to salinity pulses in head-fixed larvae

A) Schematic of preparation used to stimulate head-fixed larvae. Solenoid valves digitally control gravity flow through a manifold that leads to a 300 uM diameter tube facing toward the larvae. NaCl ions in red (not to scale). B) Sample data from an experiment where a larva is stimulated with 10 second pulses of 100 mM NaCl at random intervals. C) Probability that the larvae will exhibit a behavioral response to an NaCl pulse of a given concentration (red) and inter-pulse spontaneous behavior rate (gray) to flowing fish water. D) Probability of a bout event occurring within two second bins for different concentrations of salt relative to the onset of a pulse.

Consistent with our proposed algorithm, the larvae seem to be most sensitive to the onset of a randomly applied salt (Figure 2.3B) in a concentration dependent manner (Figure 2.3C).
sodium chloride pulse, as the response probability in the first two seconds of a pulse is significantly higher than in the last two seconds of a pulse (Figure 2.3D).

Our primary goal was to determine which sensory processing regions most accurately reflect external salinity information. To this end, we used a custom-built lightsheet microscope (Figure 2.5A and experimental methods) to perform volumetric imaging of the larval zebrafish (Figure 2.5B) while stimulating the fish with pulses of different concentrations of NaCl (Figure 2.5C) and simultaneously tracking its tail (Figure 2.5E). After imaging, we segmented the fluorescence from each plane into activity units (Figure 2.5D) using a previously described correlation-based algorithm (Portugues et al. 2014). It should be noted that these activity units may consist of individual cells, neuropil or combinations of cells and neuropil, as our segmentation algorithm relied exclusively on correlation across time and did not incorporate anatomical features. Parallel to segmentation, we registered all imaged volumes to the reference coordinate system of the online Z-brain atlas (Randlett et al. 2015) and used the generated transformations to map all activity units onto the Z-brain. Doing so allows us to localize activity units to community standardized anatomical regions and to use those regions to analyze the activity units within select subregions of the brain.

From our behavioral experiments, we know that the larval zebrafish brain represents changes in salinity. There are two ways this representation may emerge - either directly from the
Figure 2.4. Supplemental material for Figure 2.3

A) Example frames of 10 nM fluorescein pulses. Red circle indicates region analyzed in B and C. Dashed white outline surrounds the fish. Time is relative to the beginning of the fluorescein pulse, and red text indicates presentation of fluorescein. B) Fluorescence over the course of thirty minutes with 30 second interstimulus intervals. C) Average fluorescence change during a pulse.

sensory inputs via cellular processes such as sensitization, or within the brain through neural transformations of an absolute salinity encoding. To determine whether the zebrafish brain carries information about absolute salinity, we searched for the activity units whose calcium traces most closely mirror the dynamics of the delivered NaCl concentrations. In selecting a metric to quantify this relationship, we did not wish to invoke any priors or biases, such as a linear or monotonic relationship between stimulus and activity. For this reason, we chose to calculate the mutual information shared between each activity unit and the stimulus to identify putative NaCl encoding populations (A1: Experimental Methods).
A map of the average mutual information across fish reveals a prominent hotspot of efficient NaCl encoders within the olfactory bulb (Figure 2.5F). To determine which regions of the brain contain the most efficient NaCl encoding units, we segregate each activity unit into each of the 220 brain regions defined in the Z-brain atlas. We then both calculate the average mutual information of all activity units within a given region and identify the maximum single unit mutual information, and average these values across fish (Figure 2.5G, Figure 2.6). Of the sensory ganglia, we find that the most precise representation of salt concentration occurs in the afferents of the rostral-most neuromasts, followed closely by the olfactory epithelium. In both the lateral line nerve and the olfactory epithelia, we observe concentration dependent activity that are steady across trials (Figure 2.5H,I). The activity within these regions was essentially identical in trials where the animal behaved compared to those where it did not (Figure 2.5K,L).

We should also make note of a few other regions that contain high mutual information with the salt concentration (Figure 2.6). The preoptic area, for example is likely responsible for coordinating a number of stress responses. In addition, rhombomeres 5&6 and the cerebellum seem to be active with increased salt. These regions, as was found in Haesemeyer et al. (2018) may be involved in generating the appropriate behavioral responses. However, for the remainder of this chapter, our goal is to understand the sensory systems that introduce salt information into the brain, and what features of salinity are represented. Therefore, we will focus the rest of this work on the early olfactory and lateral line systems.

With brain-wide, volumetric imaging, we identified the olfactory system as an effective site of salinity encoding. We next sought to understand how olfactory activity is shaped by salinity, both in terms of the dynamics within the olfactory system and the specific
Figure 2.5. Volumetric imaging reveals NaCl levels can be extracted from activity in the olfactory system and lateral line

A) Schematic of the lightsheet microscope. B) Sample z-slices taken within a stack. Stacks were collected at 1 Hz. Imaging experiments captured either the rostral 2/3rds or caudal 2/3rds of the fish. C) Arrangement of salt pulses during experiment. Zebrafish experienced escalating concentrations of NaCl pulses in five minute blocks. D) Example calcium signal from an activity unit in the olfactory bulb. E) Example tail trace during imaging experiment. F) Average mutual information at each voxel of the z-brain across 15 fish. G) Average (bar height) and peak (bar color) mutual information within different regions of the brain as defined by masks available with the z-brain atlas. A full list of regions and their names is provided in Figure 6. H) Stimulus triggered responses of top NaCl encoding units from the olfactory epithelia, averaged across fish, and their locations within the Z-brain. Responses are separated into trial where the fish swam (blue) or did not swim (purple). Mean +/- SEM across fish. I) Stimulus triggered responses of top NaCl encoding units in the nasal neuromast averaged across fish as in H. J) Average peak intensity across fish at different concentrations within the olfactory epithelia separated by whether the fish swam in a pulse. K) Average peak intensity across fish at different concentrations within the nasal neuromast separated by whether the fish swam in a pulse.
Figure 2.5. Volumetric imaging reveals NaCl levels can be extracted from activity in the olfactory system and lateral line.
Figure 2.6. Supporting data for Figure 2.5.
Average and maximum mutual information in each Z-brain region as in Figure 5G. Regions are sorted by the peak mutual information across fish of the activity unit within that region with the highest mutual activity with the stimulus. Only the top 50 regions are shown.
environmental variables that drive it. To address these questions, it is first necessary to determine whether inputs into the system are specific olfactory sensory neurons or a global increase in the activity of all externally contacting olfactory sensory neurons. The optical sectioning of the light-sheet microscope was insufficient for us to answer this question with confidence. Therefore, we turned to two-photon microscopy (Figure 2.7A) and delivered one of two concentrations of NaCl, 25 and 50 mM, that were randomly selected at the onset of each pulse. We compared the amount of activity in the olfactory bulb and epithelia (Figure 2.7B), finding that very few overall units in the epithelia are responsive (Figure 2.7C), though this does broaden in the bulb (Figure 2.7D). Furthermore, more units are recruited with increasing concentration of NaCl.

We also wished to examine whether either of the two major classes of olfactory sensory neurons - ciliated and microvillus - possess different capacities for NaCl-sensitivity. Thus, in addition to the experiments in fish with brainwide GCaMP6s expression, we performed calcium imaging experiments in two lines: OMP:Gal4/Uas:GCaMP6S (Figure 2.7F) and TRPC2:Gal4/Uas:GCaMP6S (Figure 2.7H), which label ciliated and microvillous neurons, respectively (Sato et al. 2005). In both genetic classes we found that fewer than 95% of activity units in the epithelium are sensitive to NaCl (Figure 2.7G,I). These results inform us that while specific olfactory sensory neurons are sensitive to environmental salinity, such sensitivity is not restricted to one type of sensory neuron.
Figure 2.7. Neural responses to NaCl within the olfactory epithelium are sparse

A) Schematic depicting the two-photon microscope setup used to image the olfactory bulb and epithelia. B) Region of the brain imaged in this figure and all subsequent figures (green). C) Sample slice averaged over time. Shadings indicate segmented regions - olfactory bulb (yellow) and epithelia (red). D) Average fraction of activity units in the olfactory epithelia of HuC:GCaMP6s positive fish that were deemed to be active during 25 mM or 50 mM pulses and after applying same criteria to shuffled traces. E) Average fraction of activity units in the olfactory bulb of HuC:GCaMP6s positive fish that were deemed to be active during 25 mM or 50 mM pulses and after applying same criteria to shuffled traces. F) Maximum intensity projection of two-photon stack of OMP:Gal4/UAS:GCaMP6S positive fish with location of NaCl responsive units overlaid (red). G) Average fraction of activity units in the olfactory epithelia of OMP:Gal4/UAS:GCaMP6S positive fish that were deemed to be active during 25 mM or 50 mM pulses and after applying same criteria to shuffled traces. H) Maximum intensity projection of two-photon stack of TrpC2:Gal4/UAS:GCaMP6S positive fish with location of NaCl responsive units overlaid (red). I) Average fraction of activity units in the olfactory epithelia of TRPC2:Gal4/UAS:GCaMP6S positive fish that were deemed to be active during 25 mM or 50 mM pulses and after applying same criteria to shuffled traces.
Figure 2.7. Neural responses to NaCl within the olfactory epithelium are sparse.
2.4 Salinity shifts baseline olfactory activity into new steady state, while neuromasts desensitize

We next wished to determine whether all activity units within the olfactory bulb and neuromast represent absolute salinity or may also encode features of the stimulus dynamics (i.e. increases in salt concentration). In all imaging experiments performed so far, however, we only present the fish with ten second pulses of increased salt. While useful for screening, this duration is too brief to capture the full dynamics of the circuit, as nearly all units seem to be still ramping their activity at the end of the pulse. Accordingly, we imaged in the olfactory system (Figure 2.8A) and neuromasts and extended the stimulus to 60 second long pulses of 50 mM NaCl (Figure 2.8B). In the olfactory epithelia and bulb, we observe that the net activity continues to increase with time (Figure 2.8D,E). The neuromasts behave differently. Instead of continuing to ramp, the net activity slowly desensitizes after about 20 seconds (Figure 2.8F).

It is unclear how monolithically these units are behaving within these regions. In particular, the olfactory bulb may perform additional filtering of the incoming epithelial dynamics (i.e. low-pass filtering or adaptation as in the neuromast). In order to describe any distinct types of responses, we attempt to classify the kinetics of each unit’s response to prolonged sodium chloride exposure. We do this by fitting three exponentials to each stimulus triggered average trace. Namely, these are an on-rise, an on-decay, and an off-decay. Units that respond to the onset of the stimulus will have strong on-decay components (i.e short half-lifes), whereas those driven by absolute salinity will have strong on-rise and off-decay components.

Following removal of salt, the off-decay times with a given region are restricted to a fairly narrow range - close to that of the GCaMP6s indicator (Figure 8F,G). The rate of this
Figure 2.8. Divergent dynamics across the olfactory and lateral line systems
A) Sample slice averaged across time from the region of the brain being imaged. Regions that are analyzed - olfactory bulb, epithelia, and rostral neuromasts are shaded B) Sample activity units from each of the regions from one experiment. Fish receive random pulse lengths separated by one minute each. C) Average activity across NaCl responsive units in the olfactory epithelia for each of the stimulus duration (mean +/- sem across 4 fish). D) Average activity across NaCl responsive units in the olfactory bulb for each of the stimulus durations (mean +/- sem across 4 fish). E) Average activity across NaCl responsive units in the bulb for each of the stimulus durations. F) Distribution across the olfactory bulb of activity decay half-lifes after stimulus removal for each of the stimulus durations (mean +/- sem across 4 fish). Inset illustrates scatterplot of off-rates after a 10 second and a 20 second pulse. G) Distribution of decay times after all pulses across each of the three regions. H) Distribution of rise half-lifes after the start of a 60 second pulse across each of the three regions (mean +/- sem across 4 fish). I) Average stimulus triggered calcium activity for activity units with different on-rise rates (mean +/- sem across 4 fish). Each trace is normalized to its peak activity around the end of the salt pulse (mean +/- sem across 4 fish). J) Distribution of decay half-lifes after the start of a 60 second pulse across each of the three regions. K) Average stimulus triggered calcium activity for all units with on-decay half-lifes less than 25 seconds (mean +/- sem across 4 fish). Units are grouped according to the region in which they are found.
Figure 2.8. Divergent dynamics across the olfactory and lateral line systems
decay is unaffected by the preceding duration of salt, indicating there are no accumulation effects. While the range of possible decay times is small, it does not appear, that all units within a region draw from the same distribution. The decay times from a single activity unit are highly correlated with each other, even following different stimulus durations, indicating that the variance of the off-decay rate distribution mostly arises from differences between units. We observe more variance between different regions, as activity units from the neuromasts, olfactory epithelia and olfactory bulb are described by distinct distributions. Surprisingly, the olfactory bulb represents an intermediate between the fast decaying neuromasts, and slower olfactory sensory neurons.

Unlike the off-decay times, the on-rise times that followed an increase in salt were highly variable across units, particularly in the olfactory bulb and olfactory epithelium (Figure 2.8H). As we anticipated from the average activity within each region, the olfactory units are notable for the long tail, as units across fish can be found with rise half-lifes extending over a minute. The duration of the on-rise time weakly predicts the off-decay time, supporting the idea that the resistance that restricts their firing rates from changing too quickly varies across units (Figure 8I). Such resistance appears to be highly symmetric with respect to increases and decreases in salinity. Whether this is cell intrinsic or a feature of the circuit wiring is unclear. Between the olfactory bulb and olfactory epithelia, the distributions of on-rates a very similar, consistent with most olfactory bulb activity being driven from the epithelia. The neuromasts, by contrast, rise more quickly, with > 90% of rise half-lives less than 30 seconds.

Only in the neuromasts do a large fraction of the activity units possess an appreciable desensitization component (Figure 2.8J). However, if we examine the small fraction units within

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the olfactory bulb that have desensitization time constants less than 30 seconds, we find that their activity strongly reflects that seen in the neuromats (Figure 2.8K). The major deviation being a strong overshoot during the decay back to baseline. By contrast, the epithelia lack any appreciable desensitization dynamics, suggesting they are not arising directly from the olfactory sensory information, but from elsewhere in the brain.

The above results suggest that the bulk activity in olfactory bulb directly reflects a low-pass filtered value of the absolute external salinity. If this is true, then we would expect decreases in salt concentration to drive decreases in olfactory bulb activity. To explore this possibility, we compared the activity of the network elicited by 50 mM NaCl to its activity when the external salinity decreases (Figure 2.9A). We dropped the salinity by presenting deionized water. Briefly removing ions reduces the overall network activity (Figure 2.9B), and the response of individual units is strongly anticorrelated with their response to 50 mM NaCl (Figure 2.9C). This result is consistent with the idea that rather than alerting the larvae to an increase in salinity, the bulk activity in this olfactory network reflects the absolute external salinity.

If the behavior of the animal reflects olfactory activity, then we predict that free-swimming larvae travel toward the environment with the lowest salt concentration, no matter the absolute level. This may even include swimming toward deionized water. To test this, we used our free swimming assay to determine the preference of larvae toward agar pads made of either fish water or from deionized water (Figure 2.9D). In order to generate a gradient, we filled the arena with different dilutions of fish water (1:1, 1:10, and deionized). Consistent with
Figure 2.9. Lowering external salinity reduces olfactory activity
A) Example unit from the olfactory bulb during experiment with 10 second pulses of either 50 mM NaCl (red) or deionized water (blue). B) Average activity of NaCl-sensitive neurons in response to 50 mM NaCl and deionized water. C) Violin plots of the correlation of each unit’s response to sodium with their response either a separate pulse of sodium, or to deionized water (Median +/- 25% and 95%). D) Schematic of the free swimming experiments performed here. Each experiment bookends the lanes with agar made from deionized water and agar made from fish water (F.W.). Different dilutions of fish water were added to the lanes in order to generate different gradients. E. Preference indices of larvae toward deionized water in different dilutions of fish water (Mean +/- SEM, n = 8 for all conditions).
Figure 2.9. Lowering external salinity reduces olfactory activity
the neural activity pattern, we observe that larvae travel toward deionized water, with preferences increasing as the gradient sharpens (Figure 2.9G).

2.5 Olfactory activity is most effectively driven by small monovalent cations

So far, we have determined that activity within the olfactory bulb appears as a slowly integrating reporter of external NaCl. However, it remains unclear whether this network is driven specifically by sodium and/or chloride or is tuned to one of the other environmental shifts that co-occurs with NaCl fluctuation. To clarify the circuit’s activation mechanism, we wished to disentangle the response properties of the olfactory system to three categories of parameters that covary with NaCl - 1) osmolarity, 2) conductivity, and 3) specific ion levels (i.e. sodium or chloride). In each experiment, we presented the fish with random pulses of either a “test” chemical or 50 mM NaCl, which was used to identify the NaCl-sensitive glomeruli. We observed that the NaCl circuit does respond to equimolar concentrations of mannitol, telling us that these cells are not osmolarity sensors (Figure 2.10A,B,E). By contrast, a comparable monovalent ion, such as potassium also strongly drove this network (Figure 2.10C,D,E). To test whether it may be chloride that is driving this activity, we imaged the response of the olfactory system to sodium conjugated with another anion - ascorbate. This pairing drove the network as well. By contrast, 25 mM magnesium chloride, a divalent cation of similar molecular weight to sodium, only partially drove the network equally sensitive to other small monovalent cation/anion pairs (Figure 2.10E,F,G). Further, doubling the concentration of magnesium chloride did not drive the network any higher (Figure 2.10E,H,I). Testing the behavioral preference to these chemicals reveals a pattern of avoidance and neutrality that reflects the olfactory bulb activity (Figure
Figure 2.10. NaCl-sensitive olfactory bulb units are most sensitive to small monovalent cations

A) Sample activity trace from an olfactory bulb unit while the fish is stimulated with pulses of 100 mM mannitol and 50 mM NaCl. B) Stimulus triggered average of the activity unit in A in response to mannitol and NaCl. C) Sample activity trace from an olfactory bulb unit while the fish is stimulated with pulses of 100 mM mannitol and 50 mM NaCl. D) Stimulus triggered average of the activity unit in C in response to mannitol and NaCl. E) Distribution of the the correlation of each activity unit’s NaCl response to its response to different chemicals. Bars in violin plots indicate median +/- 25%. F) Scatter plot of unit’s response to 50 mM NaCl versus 25 mM MgCl$_2$. G) Average activity induced across the olfactory bulb by 50 mM NaCl and 25 mM MgCl$_2$. H) Scatter plot of unit’s response to 50 mM NaCl versus 50 mM MgCl$_2$. I) Average activity induced across the olfactory bulb by 50 mM NaCl and 50 mM MgCl$_2$. 
Figure 2.10. NaCl-sensitive olfactory bulb units are most sensitive to small monovalent cations
2.11A). Together, they suggest that the olfactory network is mostly driven by small monovalent cations.

We next examined the effect of cation size on olfactory activity. Overall, we found that the closer in molecular weight to sodium the ion was, the more strongly the network was activated (Figure 2.11B). Large cations, such as N-methyl-D-glucamine chloride (NMDG), produced activity patterns that were only weakly correlated with that of sodium chloride. This suggest, perhaps, that porous leak channels might be depolarizing neurons via direct ion flow during cation exposure. To test this, we examined whether NMDG could still depolarize olfactory neurons in deionized water. If leaky channels are the mechanism of action, then NMDG is likely too large to pass through them and directly activity the cells. Therefore, the activity we do see during NMDG exposure would arise from the molecule indirectly increasing the flux of other ions, such as sodium. When those ions are removed, NMDG should have no effect on the activity of the network. Instead, we see that in deionized water, NMDG still causes depolarization, albeit reduced (Figure 2.11C), suggesting it is capable of driving activity directly. Furthermore, we also examined whether using gadolinium to specifically block cation channels (Elinder & Arhem 1994) would eliminate the olfactory response. However, we found no change in olfactory sensitivity at even very high concentrations of gadolinium (Figure 2.11D). We find these results to be most consistent with a receptor, possibly a TRP channel, whose activity is modulated in the presence of cations. However, without directly recording ion flux, neither scenario can be ruled out.
Figure 2.11. Supporting data for Figure 10.

A) Preference indices toward different cation/anion pairs for larvae in the free swimming assay from Figures 1 and 2. B) Correlation of activity unit’s NaCl response with different cations sorted by molecular weight (25-75). C) Average calcium activity in the olfactory bulb during pulses supplemented with 50 mM NaCl, 50mM NMDG, deionized water, or deionized water plus NMDG. D) Average calcium activity in the olfactory bulb during pulses supplemented with 50 mM NaCl or 50 mM NaCl with 1mM gadolinium chloride.
Figure 2.11. Supporting data for Figure 10.
2.6 Discussion

Traditionally, the sensation of saltiness is considered to be transmitted to the brain through taste. This reflects the fact that land animals, such as ourselves, primarily encounter salt through ingestion of food or drinking water. For teleosts and other aquatic species, however, saltiness is a critical environmental variable; one that can assign restrictive boundaries upon their livable environments. Accordingly, the concentrations of salt that need to be avoided in the environment may be very different from those that are generally considered non-consumable. In land animals, salt does not become aversive until it approaches physiological concentrations, around 100 mM. For zebrafish, these are highly deleterious environmental concentrations, and indeed we see that they will avoid environments with much lower salinities. Even increases as low as 10 mM NaCl are enough to drive avoidance responses, necessitating an alternative mechanism. It is possible, that when evaluating food items, zebrafish still find physiological salt concentrations appetitive, as has been observed in other fishes (Kasumyan & Doving 2003). If this is the case, then it makes sense that other modalities may be involved in mediating aversion to environmental salt.

Here, we found that olfactory sensory neurons carry the most information about absolute external salt, while activity in the lateral line is more correlated with changes in salinity. We should note here that the time constants of the lateral line desensitization are slower than the behavioral adaptation, and thus may not be directly relevant for gradient navigation. Instead, it may play an important role in gain modification. The lateral line is necessary for detecting minute changes in water flow that can be triggered by small currents (Oteiza et al. 2017) or even prey items. However, following Weber’s law, increasing the baseline firing rate - as occurs in
increased salt may render the animal insensitive to these critical cues. Therefore, by adjusting
the hair cell activity back to baseline, may be reenabling the detection of more subtle mechanical
stimuli. Dopaminergic and cholinergic neurons project to the neuromasts of the lateral and help
to prevent overstimulation during swim events. It is possible that one or both of the efferent
populations are also capable of reducing the basal firing rate if the hair cells are too active.

It is unclear why the lateral line desensitizes but not the olfactory system. One would
assume that such gain modulation is also important for olfactory detection. But, perhaps it is
necessary for the fish to have some ongoing information about absolute salinity to coordinate
hormone and neuropeptide release, and evolution has deemed the olfactory system expendable.
Or it may just be that increased activity is unused, and evolution has not pressured the animal to
 circumvent it.

We also do not know how the lateral line desensitizes but not the olfactory sensory
neurons. It may be the result of bottom-up efferent projections, as mentioned earlier.
Alternatively, the molecular mechanisms that endow olfactory sensory neurons with salt
sensitivity may be distinct from those of the hair cell. A present, we can only speculate about the
nature of these mechanisms. It is possible that the additional ions are simply raising the resting
potential of the neurons. However, this would necessitate essentially equal membrane
permeability to lithium, sodium, and potassium to explain the similar activity profiles. Instead it
seems more likely that the presence of cations has allosteric modulatory effects on native
receptors.
Chapter 3

A circuit mechanism for salt adaptation and gradient detection
Summary

In the previous chapter we determined that zebrafish larvae identify increases in salinity to drive avoidance behaviors. Yet, the sensory activity we observed does not evoke an obvious mechanism for this process. Here, we characterize the relationship between the dynamics of the salt stimulus and the animal’s behavior to propose a model for the necessary derivative calculation and resulting behavioral response. Our model features two integrating populations that are driven by salt and compete to determine a turn rate. These two populations are characterized by different dynamics, one is fast and excitatory, while the other is inhibitory and features activity that persists for up to a minute after the stimulus is removed. Notably, this model is an entirely feedforward function of the stimulus dynamics and does not depend on feedback from the animal’s actions. We then perform two photon imaging and identify populations of cells in the hindbrain with properties that suggest they act as the neural substrate for our model.
3.1 Introduction

Organisms use changes in their environment to make decisions and select appropriate behaviors. This is seen across all realms of life, from bacteria navigating chemical gradients to humans having increased perceptual awareness of sudden movements. Each of these actions depends upon the detection of a temporal derivative in their environment. Among animals, *C. elegans* has perhaps one of the best understood circuits for utilizing gradient detection. Briefly, as the worm navigates a salt gradient, it makes use of two gustatory neurons, ASEL and ASER that can individually detect increases and decreases of salt concentration, respectively (Suzuki et al., 2013). Activity from these opposed channels then compete to either activate or inhibit an interneuron, AWA, that controls reversals, which in turn enables navigation away from undesirable areas. Even in this system, though, the precise cellular mechanisms by which ASEL and ASER perform their derivative calculations is unknown.

In the previous chapter, I showed that zebrafish larvae respond to increases in salt concentration. In particular, following an increase in the surrounding salt concentration, the larvae will attempt to redirect their swimming by elevating their turning frequency. At this point, it remains unclear how the brain might be detecting changes in salinity. From imaging experiments, I find stable representations of salinity in the olfactory bulb, and desensitization in the lateral line. The rate of hair cell desensitization, however, is too slow to act as a behaviorally relevant derivative function. Thus, unlike *C. elegans*, the zebrafish must be using circuit rather than molecular computations to identify changes in salinity. Here, I will attempt to identify these neural implementations that underlie the detection of salinity changes.
This first necessitates a more thorough investigation of the nature of the computation involved in detecting derivatives. In the previous analyses, I treated each swim event as a sharp boundary, where the stimuli before and after that event are compared to directly to each other. It is possible that the brain of the larval zebrafish is performing a similar action, and is somehow caching the pre-bout information and directly comparing this to the value that emerges afterward. Such implementations have been proposed in other behaviors (Oteiza et al. 2017). Alternatively, the use of multiple long pass filters can be used to extract derivatives directly from the stimulus dynamics, without any concern for the behavior the animal. Here, I propose a model and identify potential circuit implementations of those latter concept.

3.2 Larvae adapt to quickly repeated pulses of salt

In order to carefully characterize the transformation of salinity concentration fluctuations into behavior, it is necessary to have precise spatiotemporal control of the stimulus being delivered to the fish. Unfortunately, in my free swimming preparation, I am limited by the timescales and noise generated during the setup of the system. Therefore, I will use the head-embedded preparation to dissect the relationship between stimulus dynamics and behavior. To begin, I further analyzed the head-embedded behavioral data collected in the previous chapter. Briefly, in each of these experiments, one larvae was exposed to pulses of a single concentration of sodium chloride that arrive at random interstimulus intervals (Figure 1A,B). Larvae respond to these pulses with vigorous tail bends indicating avoidance attempts (Figure 1C). As seen in Chapter 2, as the concentration of salt increases, so too does the probability of a
Figure 1. Larvae adapt to closely timed pulses of salt

A) Cartoon depicting head-embedded preparation and salt pulses  
B) Sample experiment with 10 mM NaCl pulses delivered at random intervals (10 s, 20 s, 30 s, 45 s, 60 s, 90 s, 120 s, 180 s).  
C) Sample experiment with 100 mM NaCl pulses delivered at the same randomly selected intervals as A.  
D) Example of a 100 mM pulse with two swim events occurring within it (corresponds to asterisk in B)  
E) Probability (mean ± sem) that at least one swim event occurred within a pulse of a given concentration across all interstimulus intervals (red) plotted against the probability that swim will occur in a 10 second window between pulses (this plot is reproduced from chapter 2).  
E) (95% CI).  
F) Probability a larvae will respond to a given concentration of NaCl separated by the interstimulus interval preceding that pulse.  
G) Probability within in a pulse (2 second bins) that the larvae will respond to 100 mM NaCl given the preceding interstimulus interval.  
H) Cumulative probability of the fish swimming at least as a function of time within a pulse across different concentrations given a 180 s interstimulus interval. All curves are fit with an assumed poisson swim rate (dashed lines).  
I) Cumulative probability of the fish swimming at least as a function of time within a pulse across different concentrations given a 10 s interstimulus interval.  
J) Estimates of swim rates as fit in H) and I) for each concentration separated by different interstimulus intervals.
Figure 1. Larvae adapt to closely timed pulses of salt.
swim event (Figure 1D). Further, the probability of a response is highest just consistent with the larvae being more responsive to the change in salt rather than the absolute salinity.

If the animal uses a perfect derivative transformation to detect salinity changes, then I expect to observe the larvae respond with an equal rate to the onset of each salt pulse. However, when I analyze the probability of a swim event occurring within a pulse as a function of the time that has passed since the last encounter, I see that this is not the case. Instead, as the interstimulus interval decreases, the larvae becomes significantly less likely to respond (Figure 1E). Further, when the fish does respond, the average latency is longer following shorter interstimulus interval times (Figure 1E). This is consistent with a transformation that utilizes a slowly decaying history of salt concentration to detect changes, similar to a low-pass filter. In order to quantify this process, I assume that swim events follow a poisson process, and that this rate is modulated as a function of salt concentration (Figure 1F). The extracted poisson rates are lower across concentrations with shorter interstimulus interval times (Figure 1G,H), such that they are nearly indistinguishable from water unsupplemented by salt if only 10 seconds has passed. This suggests that fish may be adapting its behavior such that the output of the sensorimotor transformation elicited by a given concentration becomes equivalent to the baseline rate.

3.3 The controllability of the stimulus does not directly influence the animal’s response rate

One possible explanation for the reduced behavioral responsiveness is the emergence of a helpless-like state. In mammals, the presentation of inescapable stressors leads to extended periods of quiescence and unresponsiveness to future stress. This phenomenon has been proposed to occur in invertebrates and other lower vertebrates, including drosophila and
Figure 3.2. Inability to control the stimulus does not affect the larvae’s NaCl induced swim rate

A) Behavior and stimulus from the six minutes of an experiment with escapable 100 mM salt pulses. B) Behavior and stimulus from the six minutes of an experiment with inescapable 100 mM salt pulses, where the stimulus extends for 10 seconds after each swim attempt. C) Effect of the amount of time the stimulus persists after each turn on future response rates. Dashed line indicates escapable (0 seconds) swim probability. D) Evolution of swim probability over time for three experimental conditions, escapable, inescapable (10 second pulse extension), and playback of escapable stimuli. E) Behavior and stimulus of a fish receiving the same stimulus as the fish in A). F) Probability the fish has swam at least once given that it has experienced t seconds of salt within a pulse for the three conditions. G) Swim probability of fish in the escapable and corresponding playback controls compared to the expected value generated from the distribution of pulse durations and escapable swim rate in F). Grey lines connect individual fish response probabilities in the playback controls to their yoked fish from the escapable experiments (p > 0.7, one-sided t-test).
Figure 3.2. Inability to control the stimulus does not affect the larvae’s NaCl induced swim rate
zebrafish (Lee et al. 2010; Yang et al. 2013). I sought to determine whether such feedback about the utility of the its own behavior could explain the larvae’s adaptation by testing whether endowing larvae the ability to control salt removal increases their future salt induced swim rate. To this end, I performed online behavioral analysis during the experiment to detect when the larvae was performing a swim maneuver. I used the extracted timing of these maneuvers to control the the ending of a salt pulse. For experiments with virtually escapable stimuli, flow was switched from high salt to fish water immediately after the larvae attempted a swim. Larvae in these conditions persistently responded to salt pulses (~65%) throughout the experiment (Figure 2B,C). For larvae tested with the inescapable condition, I do not end the salt pulse immediately after the swim. Instead, in order to avoid coincidences between behavior and salt decreases, I extend the salt pulse by set number of seconds every time the fish swam. As predicted, larvae in these conditions were significantly less responsive to individual salt pulses than those in the escapable experiments (Figure 2B). Two seconds of continued sodium chloride after each swim is enough to drive the response rate to a floor of 30%. Larvae reach steady-state response rates early in the experiments, including within the first 5 minutes for fish exposed to 10 seconds of extended salt (Figure 2C).

From these results, though, it is unclear whether the difference I see between the two paradigms is related to the connection (or lack thereof) between the animal’s behavior and the stimulus dynamics. Such a relationship would be derived either from learned helplessness reducing behavior in the inescapable condition, or operant learning increasing responsiveness in the escapable condition. Alternatively, the difference between the two conditions may purely be a function of the amount of salt presented. In order to discriminate between these two scenarios, I
performed a third cohort of experiments, namely, playback controls. During an escapable experiment, the larvae being tested generates a unique pattern of salt pulses. In the playback control, I copy this pattern of NaCl pulses and present it to a different larvae. This paradigm presents the playback control animal with an identical amount of salt as the corresponding animal from the escapable condition. At the same time, the randomness of the animal’s response times ensures that the behavior and stimulus will be dissociated. I observe that in playback experiments, the overall probability of the fish responding to a pulse is closer to that of the inescapable cohort than their counterpart escapable stimuli. However, in the playback experiments, the average time the larvae has to respond to a pulse is much shorter. While the escapable experiments a larvae has 10 seconds to respond, their yoked counterparts are experiencing, on average, less than six second pulses. To account for this discrepancy, I examine the response probability as a function of how much time has elapsed within a pulse. Doing so reveals almost no difference between the two equivalent stimuli. In fact, the playback group’s low response rate is nearly identical to the rate that is expected from both the distribution of response latencies and the distribution of pulse durations generated during the escapable experiments (Figure 2G). Therefore, the behavioral discrepancy distinguishing the escapable pulses and their corresponding playback controls should be interpreted as a regression to a mean generated by equivalent sensorimotor functions.

Inescapable salt does not drive zebrafish larvae into a classically defined helpless state; one that arises from ineffective behavior. However, the reduced salt triggered swim rate may still be a general behavioral reduction, brought about, for instance, by excessive salt exposure reducing the health or energy of the fish. To test this, I examined whether the difference in
**Figure 3.3. Adaptation to NaCl does not generalize to other stimuli**

A) Schematic depiction of the experiments testing optomotor response interleaved with different types of 100 mM salt pulses (escapable in this example). B) Probability of a swim in response to motion pulses interleaved between inescapable or escapable salt pulses. Grey box indicates first five minutes of experiment where no salt pulses were presented. C) Scatter of the optomotor response probability versus salt response probability of individual fish (dots) for the two types of pulses (red versus blue). D) Scatter of the individual fish’s optomotor response probability between salt pulses compared the optomotor response probability before any salt has been presented. E) Cartoon depiction of experiment where escapable 5 mM putrescine pulses are presented randomly between a given type of salt pulse. F) Response probability to 100 mM NaCl and 5 mM putrescine during the two experimental groups. G) Cartoon depiction of experiment where escapable 100 mM NaCl pulses are presented randomly between a given type of 5 mM putrescine pulse. H) Response probability to salt and putrescine during the two experimental groups.
Figure 3.3. Adaptation to NaCl does not generalize to other stimuli
behavior seen between the two paradigms transferred to other modalities. In particular, I examined the effects of increased salt levels on the optomotor response, and on their escape attempts to another olfactory stimulus, putrescine (Figure 3A and Figure 3E).

In testing the optomotor response, I interleaved pulses of leftward or rightward motion between pulses of salt (Figure 3A). As before, a given fish received either only escapable or only inescapable pulses. To obtain a baseline optomotor response rate, I began each experiment with 5 minutes of motion pulses presented every 15 seconds. I find that increased salt duration does not affect the optomotor response at any stage of the experiment (Figure 3B,C), and that the optomotor response rate does not change from baseline (Figure 3D). The only disruption I observe is that fish responding frequently to salt, as is common early in the escapable experiments, have fewer optomotor responses. In fact, the response probability to the two stimuli is anti-correlated (Figure 3C). This may be a result of fatigue.

To determine whether salt adaptation cross-adapts with other olfactory stimuli, I tested the effect of increased salt exposure on the larvae’s response rate to an aversive odorant, putrescine (Horio and Herrera, unpublished). In these experiments, I presented a given fish with either 100 mM NaCl or 5 mM putrescine. To minimize the probability that excessive putrescine exposure itself leads to adaptation, only one in five pulses were of putrescine, and these pulses were always escapable (Figure 3E). The remaining pulses contained 100 mM NaCl, and a given fish received either only inescapable (10 s extensions) pulses or only escapable pulses. I observe, that while the increased salt exposure decreases the fish’s response rate in future salt pluses, it has no effect on the larvae’s swim rate in putrescine (Figure 3F). This indifference is reciprocal, as presenting either inescapable or escapable pulses of putrescine does not alter the response rate
of the larvae to NaCl (Figure 3G,H). Together with the results from the optomotor response experiments, this allows us to conclude that when the larvae adapts to sodium chloride, it is adapting specifically to salt.

3.4 Adaptation provides the fish a dynamic baseline

I next wished to determine how adaptation affects the response rate to different concentrations of sodium chloride. If adaptation is, indeed, one part of the mechanism that underlies gradient detection, then I expect that a larva adapted to one concentration of sodium chloride to still be sensitive to higher concentrations. I predict that in such a case, the larva’s response rate would be proportional to the difference between the new concentration and the adapted one. Because the increased salt exposure in the preceding experiments does not seem to drive the animal into a helpless or injured state, I can use the escapable/inescapable paradigm to test this idea. In particular, I adapt the animal to one concentration of salt using the inescapable rule. I then test its response to a second, “test” concentration that is escapable, thereby minimizing the adaptation to the test concentration (Figure 4A). Consistent with my hypothesis, I observe that adaptation to increasing concentrations of salt is correlated with an animal’s response rate to a given “test” concentration decreasing (Figure 4B). I find that the size of this depression is such that the response rate is fully defined by the difference between the test and
Figure 3.4. Larvae respond equally to equal magnitude salt changes

A) Cartoon depiction of the experimental design. Inescapable pulses of one concentration ("adapted") represent eighty percent of stimuli. The other 20% are escapable pulses of the "test" concentration. B) Plot of the response probability of larvae to test concentrations (x-axis) following adaptation to either 25, 50 or 75 mM NaCl (shades of red). C) Same response probabilities as above but plotted as a function of the difference between the "test concentration" and the "adapted" concentration.

adapted concentrations of salt (Figure 4C). These results are consistent with the notion that the animal’s adaptation is simply adjusting its estimate of a baseline salinity.
3.5. A simple competing integrator model can explain the behavior

In earlier experiments, I found that the fish’s brain contains a representation of the, essentially, real-time salinity that does not desensitize at the sensory level (Chapter 2). Therefore, it seems probable that adaptation takes place through network rather than cellular dynamics. Accordingly, I wished to formulate a simple hypothetical circuit model that could qualitatively explain the observed relationships between salinity dynamics and behavior. Importantly, such a model must generate an impulse response function that lasts for nearly three minutes. This suggested at least one component must act as a low pass filter with a long time constant. In order to reduce the swim rate over these time courses I ascribed this slow component as inhibitory (I).

In order to generate a derivative, I propose a second, excitatory ramping population (E) with much faster kinetics. The difference between the I and E populations then sets the poisson bout rate (Figure 3.5A). As activity in E increases, the bout increases, but so too does the inhibitory population, which eventually catches up to E to shut down behavior.

I modeled each population as simple differential equations (Figure 3.5B) and generated a poisson rate from a logistic transformation of the difference between E and I. To test whether this model can capture the behavioral dynamics, I ran simulations of a number of the behavioral experiments (Figure 3.5C). Before I could run these simulations, however, I needed to fit the free parameters. This model requires five free parameters, the time constants for the integrators and the three logistic terms (Figure 3.5B). I chose to optimize these terms by fitting the the expected bout rates generated by the model in response to different concentrations and interstimulus intervals, to the actual bout rates I observed in the experiments (Figure 3.1J). This optimization settled on half lifes for the E and I populations of approximately 5 and 40 seconds, respectively.
Once these parameters were set, I ran this model through virtual versions of the preceding experiments. First, I wished to determine whether this model could generate not only the increase swim probability with higher concentration pulses, but also the corresponding salt concentration dependent decrease in swim rate during the interstimulus periods. Such results were faithfully recaptured - likely a result of the slower inhibitory population still being active after the excitatory population has turned off. Similar, within the pulses, I observe that the frequency of virtual bouts is much higher during the first few seconds of the pulse than towards the end. This means that the model is generating a derivative on the relevant timescales.

Next, I wished to see whether the learned helpless phenotypes could be explained with this system. Thus, I designed three simulations that mirror the experiments - one where a virtual extends the salt by 10 seconds, a second where the bouts immediately cause the salt to be turned off, and a third where the pattern of salt pulses is played back. Not only did the simulation recapture the respective response probabilities, with escapable simulations retaining a high bout rate, while the other two are notably reduced, but also the dynamics. These steady-state dynamics are reached well within the first five virtual minutes. Finally, I sought to determine whether this model was creating a dynamic baseline that retained similar swim rates. Here, I ran simulations that mirror the experiments that test the effects of adaptation to one concentration on the response rate to different concentrations. As in the experiments, I find that the simulated swim rate is determined by the difference between the test concentration and the adapted concentration.
Figure 3.5. A simple competing integrator model can explain the behavioral dynamics
A) Cartoon depiction of our model. B) Mathematical description of our model. Note the free
parameters indicated by greek lettering. C) Sample simulation data of a virtual inescapable
experiment. Depicted our the salt concentrations (red) the ensuing excitatory (green) and
inhibitory (magenta) activity levels which generate virtual bouts (black dots). D) Virtual bout
rates that result from fitting to the experimentally derived bout rates. This is the only data that is
used to fit the free parameters. E and F) Simulation (n = 20) of the experiments in Figure 3.1. E)
depicts bout probability within and between pulses of different concentrations. F) Bout
frequency within different time bins of each pulse. G) Simulations (n =20) of the experiments in
Figure 3.2. Probability of swimming within the three types of 100 mM pulses across time. H)
Simulations (n=20) of the experiments in Figure 3.4.
Figure 3.5. A simple competing integrator model can explain the behavioral dynamics
3.6 The hindbrain contains neurons with persistent salt-induced activity

I next wished to discover whether any neural correlates of the two components of the model exist in the brain. This initially requires identifying at least two separable populations of neurons with very distinct dynamics. Most crucially, I need to identify a set of neurons with activity that persist after a salt pulse for up to two or three minutes. Previous work from the Engert lab has identified the anterior hindbrain as an important site for transforming sensory inputs into more behaviorally relevant signals (Naumann et al. 2016; Haesemeyer et al. 2018). Such transformations have been proposed to include calculating derivatives (Haesemeyer et al. 2018), albeit on shorter time-scales (10s of seconds) than observed here. Therefore, I hypothesized that the neural implementation of my proposed adapting circuit motif might be located in this region. Accordingly, I performed two photon imaging of the hindbrain while stimulating the fish with pulses of 50 mM NaCl (Figure 3.6A). Since the goal of these initial imaging experiments is to identify whether there are distinct populations of neurons that can be defined by their relative time constants, I presented each 10 second pulse three minutes apart from the previous one, so as to fully capture the decay dynamics (Figure 3.6B).

After imaging, I generated activity units by segmentation with a previously described algorithm as in Chapter 2 (Portugues et al. 2014). To characterize the kinetics of these unit’s responses, I first determine the stimulus triggered average response of each unit (Figure 3.6C) and subsequently fit an exponential to the decay back to baseline following the removal of salt. Broadly, units could be separated into two classes, ON-type where activity increases during salt exposure, and OFF-type, where activity decreases upon salt exposure.
**Figure 3.6. The hind brain contains neurons with lingering post-salt activity**

A. Region of the zebrafish brain we imaged (green). B) Example units and stimulation pattern. 50 mM NaCl pulses are separated by 180 seconds. Neurons one and two are examples of what I define as an ON-type while unit 3 is an OFF-type. C) Stimulus triggered average of the three neurons from B. All ON-type activity units (10254 from five fish). Units are sorted by the time constant of an exponential fit to the activity decay following salt removal. D) All OFF-type units (257 from five fish). Units are sorted as in C. E) Distribution of off-decay constants for all activity units. Fifteen seconds will be used as the cut-off to distinguish units with fast and slow kinetics. F) Fraction of ON-type units with fast and slow kinetics. G) Fraction of OFF-type units with fast and slow kinetics. H) Maximum intensity projection of the subregion of the Z-brain that contains prominent fast (green) and slow (magenta) ON-type units. I) Maximum intensity projection of the subregion of the Z-brain that contains prominent fast (green) and slow (magenta) ON-type units. J) Anatomical locations of the GABAergic Gad1b stripe 1 (magenta) and glutamatergic Vglut2 stripe 2 (green). K) Distribution of decay half-times for units within these regions. L) Average half-life across units in these regions for all fish (individual fish are indicated with gray lines, n = 5).
Figure 3.6. The hindbrain contains neurons with lingering post-salt activity.
Within these classes, I observe a wide range of kinetics (Figure 3.6D,E). Most units fall within a tight distribution centered around a half life of eight seconds, comparable to the sensory inputs in Chapter 2. However, distinct from the sensory regions, there also exists a long tail of decay-rates (Figure 3.6F). In both the ON and OFF type classes, I observe units that have activity that persists for greater than a minute following a salt pulse, possessing kinetics that closely match those of the model (Figure 3.6C). To simplify classification, we separate slow and fast units based on a half-life thresholded at 15 seconds. Among ON type units, we observe that around 75% of the units have fast kinetics (Figure 3.6G), while the relationship is flipped for OFF type units (Figure 3.6H).

To identify these neurons, I turned to the Z-brain atlas (Randlett et al. 2015) and its annotated list of anatomical regions. In order to use this tool, I first registered all imaged brains onto the atlas using previously described techniques. Using the resulting transformations, I mapped the half-life of each unit onto the atlas, and averaged across fish. For ON-type cells, we saw a striking separation of fast and slow units, with the slow units forming a horseshoe-like pattern within the prominent neuropil region of the rhombencephalon (Figure 3.6I). Fast decaying units, meanwhile, are situated amongst the cell bodies between these horseshoe, as well as in the neuropil that surrounds it. For OFF-type units, I only observed a few small anatomical clusters within the cerebellum were observed across fish (Figure 3.6J). I do not currently have a hypothesis for what role they are playing in this sensorimotor transformation.

The pattern of slow acting units, and the medial stripe of fast acting units reflects the anatomical locations of two stripes of GABAergic and glutamatergic cells, respectively (Figure 3.6K). To examine whether slow units were enriched within the inhibitory stripes, we analyzed
the units that fell within the masks corresponding to those aforementioned stripes, Gad1b stripe 1 and Vglut2 stripe 2. We observe that only the GABAergic stripe contains a noteworthy fraction of slow acting units (Figure 3.6L). Similarly, the average half-life of units within the GABAergic region is significantly higher than those in the glutamatergic region. While this is an imprecise method for determining the genetic identification of the different functional classes of units, these results lend support to the idea that inhibitory slow acting neurons are working to hamper fast excitatory neurons. If confirmed using genetic labels, such results would provide a clear substrate for the neural implementation of our proposed model of adaptation.

3.7 Discussion

Here, I have proposed a simple model for the generation of behavior in response to increases in salinity. Crucially, this model does not depend on feedback from the animal’s behavior. In my original description of the free swimming behavioral algorithm in Chapter 2, turning decisions were framed as if they were a consequence of the animal’s behavior. Yet, this does not have to be the actual implementation, as clearly in the gradient navigation the behavior and salt dynamics are entirely correlated. With the tail-free set up I can break this correlation and even investigate the effects of feedback from the behavioral consequences on future swim rates. To do so, we used the classical paradigms of learned helplessness experiments, inescapable stress, escapable stress, and playback of escapable stress regimes. While we observe changes in the animals salt-induced swim rate, these can entirely be explained by the duration of salt experienced.
An important caveat must be mentioned before any conclusions made from these experiments can be applied to the free swimming algorithm. In the embedded preparation, the overall behavioral activity of the animal is much lower, and thus more salient saline stimuli must be used to drive any behavior. Thus we are increasing swim rates by presenting the animal with concentration leaps that it would not experience during gradient navigation. The behaviors generated in response are also much more extreme than those that they use during navigation. Thus, it seems reasonable to assume that we are working with a perhaps slightly more intense version of the same transformation, where the smaller turns in response to smaller changes in salt simply do not match the threshold for swims imposed by the agarose immobilization.

The neural implementation of competing integration

While I have identified potential neural counterparts to the components of my antagonistic integrator model, the precise neural implementation of its dynamics is still unknown. This can be highlighted by a few key differences between the model and what we have observed so far in the functional data. For one, in our model, we have a single set of dynamics that acts to regulate behavior. By contrast, even within fish, slow acting neurons can possess a wide range of decay constants. Determining how these are combined to limit behavior on the minute scale is crucial for generating a more accurate mechanistic understanding. One possible way forward with the analysis of this circuit is to classify more carefully the timing of the activity loss. While the trial averaged responses mirror an exponential decay, at the individual trial level activity may drop as a step function that occurs at random times (Zoltowski et al. n.d.). Pooling of these activity steps may ultimately underlie the adaptation.
Rhombomere 5/6 as an integrator of noxious stimuli

In our experiments we identified the neurons surrounding Rhombomere 5/6 as the key site for adaptation. This site has previously been implicated in the detection of changes in temperature (Naumann et al. 2016; Haesemeyer et al. 2018), and is also activated by noxious odorants (Horio and Herrera et al., unpublished). This suggests the region is an important site for the integration of multiple modalities of noxious stimulants. One important difference though between the experiments here and in Haesemeyer et al. (2018) are the timescales that being used. While adaptation to salt persists for up to a minute, the dynamics in response to heat are much faster. It is possible that these differences in timescales may reflect the expected temporal fluctuations of these stimuli in nature. Salt and other chemical gradients are most likely to be experienced through long range detection of diffuse particles. Changes in their concentration will happen slowly as the fish meanders up the gradient. Thus, integrating over these long timescales might be advantageous. By contrast, fluctuations in heat that can be avoided are more likely to happen instantaneously as the fish either gets too close to a hot material, or moves from a shaded location to one exposed to the sun. Accordingly, it may be necessary to calculate changes on shorter timescales. Further, based on our examination of cross-adaptation with putrescine, it seems that separate circuits must indeed be utilized by different noxious stimuli. Exactly how the brain separates these stimuli and generates unique timescales is unclear, and requires additional circuit features to be modeled and discovered.
Chapter 4
Conclusions and outlook

“Salt is good, but if it loses its saltiness, how can it be made salty again?”

Jesus Christ, Luke 14:34-35

4.1 Summary

Salinity is an important environmental variable for aquatic animals, especially fish. For most fish, their physiology is only well-suited to operate in a narrow range of ionic environments. Spending too much time outside their ideal realm can be deleterious or even lethal. In this thesis I have shown that, in the larval stages, zebrafish possess the ability to avoid such noxious environments. They utilize a seemingly simple behavioral rule that allows them to minimize the salinity of their environment. Namely, increases in ionic content lead to increased variance in their behavior - a classic motif of gradient navigation. However, the sensory systems that feed into the brain and ultimately generate these behaviors are complex. At least two sensory modalities, the olfactory system and the lateral line, are capable of providing their brain with complete information about the salinity content. Among these channels, we see divergences in the dynamics of this information, with some parts of the olfactory system accumulating activity over the course of at least a minute, while the neuromasts quickly ramp and begin to desensitize after 20 seconds. How these modalities are weighed and combined to generate behavior remains unknown. It is likely that no single modality is responsible for generating the behavior, and that
there are instead multiple, distributed sites of integration that the fish can use to detect changes in salt concentration.

While it is unclear what modalities are ultimately responsible for generating the behavioral responses to salt, I used activity in the olfactory system as a lens to probe the transduction of salinity changes into neural activity. I found that potassium, and to a lesser extent lithium and rubidium, have a similar impacts upon sodium-sensitive cells as sodium itself. I also see that osmolarity alone does not. Thus, it appears that salinity information is being transmitted to the larvae’s brain via the presence of small monovalent cations. Finally, I should note that not all externally exposed neurons are inherently salt-sensitive. In fact, most olfactory sensory neurons do not respond to changes in salt concentration. This argues against the idea that simple changes in resting membrane potential alone are responsible for the resulting calcium signals. It could be that distinct populations of neurons express varying levels of channels that make their resting potential more or less sensitive to external cations.

From these representations of absolute salinity, increases in salt must be extracted. By dissecting the relationship of the stimulus dynamics with the animal’s behavior in a head-fixed preparation, I derived a model that was 1) fully dependent on the stimulus and did not include feedback from the behavior and 2) built from two integrating components, an excitatory fast population and an inhibitory slow population. Critically, this slow population should have dynamics that keep it active for at least a minute after the encounter with high salt. I have found that such slowly-evolving components exist within the brain. These units are found within a predominantly GABAergic region of the mid-rhombencephalon, and neighbor a population of fast-acting units in a glutamatergic neuron heavy region of the brain. From our simulations of
this feasible model, we find that this simple circuit motif can lead to the emergence of several phenomenon, that on first glance appear suggestive of higher cognitive processing, such as learned helplessness or behavioral evaluation. Instead, it seems to be a careful tuning of the behavior rate with the expected dynamics that allows these more complicated appearing to emerge.

Rather than tying up all possible loose ends, I believe this work has helped to open a veritable pandora’s box of puzzles to be solved. Here, I would like to discuss a few of what I find to be the most interesting questions that should be addressed in future work.

4.2 What are the molecular mechanisms for salt sensation

As determined in chapter 2, salinity activates a number of the external sensory systems of the larval zebrafish. However, I have uncovered little about the cellular and molecular mechanisms that generate these responses. In addition to describing a novel method of salt transduction, understanding these mechanisms would potentially open up genetic control over the inputs and allow for more carefully tuned interrogations of circuit activity through optogenetic or chemogentic means. If there are distinct genetic mechanisms that distinguish the two modalities one could even use such approaches to determine how these multiple senses are combined and interact at a circuit level. Of course, there may only be one molecular mechanism for salt sensitivity that is universal to the lateral line and olfaction. Or, within modalities, there may be a jumbled set of sufficient pathways.

In my work, I found that essentially all olfactory sensory neurons respond in similar ways to a diverse set of environmental variables that reflect different components of the environment
that change with increasing sodium chloride. Namely, monovalent cations similar in molecular weight to sodium seem to be the driving factor. The lack of diversity of response types argues for a somewhat streamlined set of transduction mechanisms, at least within the olfactory system. Further, the high correlation between potassium and sodium activities suggests that passive resting membrane potential changes are not the underlying cause. Typically, neural cell membranes are orders of magnitude more permeable to potassium than sodium, and thus potassium chloride would be expected to have a greater impact on resting potential than sodium. That being said, the molecular makeup or ionic resistances of these sensory neurons are not known. Thus, it remains, possible that they are equally conductive to these two cations, while also being impermeable to chloride (to avoid a balancing hyperpolarization).

One alternative is that salt sensitivity comes through the actions of externally exposed receptors. These could be receptors specific to salts, or receptors that bind other molecules but are allosterically modulated by monovalent ions. In mammals, three proteins are believed to be responsible for transmitting salinity information within the taste system, ENaCs, TRPM5, and PKD2l1. Fish have lost all members of the ENaC family, so any attraction to salt must be mediated by a separate mechanism. Of the two classically aversive receptors, TRPM5 is expressed only in the taste cells of the gills and pharyngeal arches (Kastenhuber et al. 2013). It may play a role in promoting the behavioral responses we observe, but is unlikely to endow the neuromasts or olfactory sensory neurons with salt sensitivity. Similarly, PKD2l1 is also expressed in taste buds, as well as sensory neurons within the spinal cord (Sternberg et al. 2018), but not in either of the lateral line or olfactory epithelia. It should be noted that while these taste receptors may play a role in generating an aversive response to salt, the concentrations normally
required to activate these receptors in mammals is far higher than the concentrations that the larvae are initially sensitive too. If the fish homologs of these proteins mirror the mammalian salt sensitivity, than the may instead play a more important role in consumption related behaviors.

In chapter two, I found that both olfactory sensory neurons as well as the hair cells of the lateral line respond to sodium chloride. Both sensory inputs seem to have similar patterns of activity. Thus, it is reasonable to expect that the protein or proteins that confer sodium excitability are present in both cell types. For example, in zebrafish and other fish, Calcium Sensing Receptor (CaSR) is expressed in both the neuromasts and olfactory systems (Hubbard et al. 2002; Kwong et al. 2014; Lin et al. 2018). Found in all vertebrates, CaSR is a classical seven transmembrane domain G protein coupled receptor that is necessary for proper homeostasis. In particular, CaSR is necessary to inhibit ion reabsorption when internal salts are too high. Its sensitivity to different ions is complex (Quinn et al. 1998). It is directly activated by divalent cations, such as magnesium and calcium. Monovalent cations, on the other hand, modulate the activity of the receptor such that they become less sensitive to calcium. Thus, while providing a mechanism for endowing sensitivity to cations, CaSR achieves this in a way that is not completely congruent with the neural activity data.

4.3 How do other sensory processing actions remain robust?

In the presence of sodium chloride, the activity of at least two sensory channels increases - the mechanosensitive lateral line and olfaction. Both of these channels play an important role in sensing features of the environment other than salt. However, it is unclear whether salt perturbs the the normal functions of these channels. For example, let us consider the lateral line. In the
presence of increased external sodium chloride, the activity of hair cells in the neuromasts also goes up. According to Weber’s law, by increasing the absolute magnitude of the baseline rate, the larvae will be less sensitive to small changes in water flow. In order to maintain comparable sensitivity to mechanical stimuli in different salinities, then, it seems advantageous to have a feedback system in place that adapts the excitability of hair cells if they get too high. Such feedback may come in the form of efferent projections from dopaminergic nuclei in the midbrain (Bricaud et al. 2001). Increased dopamine concentrations near hair cells drives their baseline firing rate higher (Toro et al. 2015). Thus, desensitization could arise from an inhibitory feedback mechanism that projected from afferents of the lateral line to the efferent dopaminergic populations.

Similar principles should apply to olfaction. Yet, we do not see widespread desensitization to salt. This suggests that olfactory fidelity may be impaired in high salt environments. Thus, it would be interesting to first determine whether behavioral response to odorants such as putrescine or cadaverine are affected by salinity. If so, then it must be determined whether the initial representations at the olfactory epithelial level are preserved, or whether there are higher level circuits responsible for this robustness.

4.4 How does sodium deprivation affect behavior?

In all of the experiments performed, larval zebrafish prefer low salt concentrations. This includes experiments where the larvae shuttled between standard fresh water and deionized water. Such behavior can actually be harmful to the fish, as water fully lacking ions will also eventually be lethal. Perhaps such situations are rare enough that no evolutionary pressure exists
to develop two-sided optimization behaviors. That being said, there is still room to determine whether or not there is any flexibility in the low salt preference. None of these experiments took into account the internal state of the animal, or attempted to perturb it. In mammals, sodium deprivation leads to an increased appetite for sodium, although the concentration that drives the strongest attraction remains unchanged. To determine whether zebrafish alter their sodium preference, it would make sense to attempt to mirror the experiments performed in rats. While it is difficult to truly sodium deprive the animal via appetite, one could use a diuretic such as furosemide, to deplete sodium from the

Another possible strategy is to disrupt the homeostatic mechanisms fish use to extract sodium chloride from the environment. Sodium is acquired by the ionocytes, specialized cells across the skin that regulate ionic flux into and out of the animal. At these cells, sodium is brought into the animal through sodium/hydrogen antiporters (Chang et al. 2013). Lower pH environments make this process more difficult and energetically costly, leading to sodium loss (Kwong & Perry 2016). The fish then must switch to a more active process, expending more energy, to make up for loss sodium ions. In acidic conditions then, it would be advantageous for the animal to seek out salt when encountering acidic water.

I have collected preliminary data along these lines. Using 96 well plates to screen the behavioral responses across salinities and pH values, we note that under neutral conditions, animals increase their locomotor activity with increasing salinity (Figure 4.1A). As the pH drops, though, the relationship between behavior and salinity changes. With zero additional sodium chloride, decreased pH causes the behavior to increase. However, if both the pH drops and salinity increases, then behavior will reach baseline again. This suggests that at low pH,
Figure 4.1. Low pH alters sodium avoidance behavior

A) Average activity of larval zebrafish in a 96 well plate in different salt concentrations (x-axis) for different pH levels (colored bars). B) Histogram indicating spatial frequency of larvae in the free swimming assay when agar pads made from 200 mM NaCl in pH 7. C) Histogram indicating spatial frequency of larvae in the free swimming assay when agar pads made from 200 mM NaCl in pH 4.

zebrafish may prefer higher salinities. Testing the behavioral preference toward salt at neutral and acidic pH supports this idea (Figure 4.1B,C).

The origins of this shift are unclear. It may simply be a factor of changing the sensitivity of the sensory inputs. Hydrogen ions can be a potent allosteric modulator of receptors and channels, and thus may be acting to interfere with the sodium induced activity. Along these lines, the larvae may have separate sensory channels for pH and salt, but when they are coactive, the salt signals decrease the activity of generally aversion encoding neurons, for instance those in rhombres 5/6. Alternatively, the depletion of sodium may itself alter the state of the animal. Internal sodium is sensed by periventricular organs in the hypothalamus, and their activity may be capable of shifting the desired salinity.
4.5 How does evolution match sodium sensing with physiological requirements?

A somewhat similar question, but taking place over a much longer timescale concerns how different species of fish adjust their salinity preference. Across the diverse spectrum of fishes, salinity tolerance and intolerance have evolved numerous times. These evolutionary transitions can take place on surprisingly rapidly. This is most readily observed and studied in the three-spined stickleback, where subpopulations have transitioned from marine to lake-dwelling over the course of only 10,000 years (Divino et al. 2016). Coinciding with shifting salt tolerances must be evolution of their behaviors to ensure that their chemotaxis and physiological needs are matched. However, it is unclear whether this necessitates two independent genetic pathways to evolve. The stickleback, with its cross-population fertility offers a unique opportunity to study this question by looking for correlations between salinity preference behaviors and salinity tolerance. Identifying such connections or independence would open the door for QTL mapping and GWAS studies to identify the underlying genetic pathways and requirements - including potentially identify receptors that endow salt sensitivity. Such experiments may lead to answers concerning how physiological evolution drivers behavioral evolution or vice versa.

In addition, no matter the physiological tolerance, changing salinities affects the function of a number of chemosensory modalities, that presumably must remain robust. For instance, most marine fish are highly sensitive to olfactory cues, so their olfactory systems must have physiological mechanisms to remain sensitive in these environments. More interestingly,
perhaps, are euryhalines, such as salmon and medaka, which travel between fresh and marine waters. During these transitions, their sensory systems will experience changes in excitability that will dampen their ability to detect, for instance, minute olfactory cues. Thus, it would seem advantageous for such species to have in place mechanisms to modulate their baseline excitability. Similar to the modulation we observe on the neuromasts of zebrafish, these could be molecular, or circuit mechanisms.

4.6 How do different neurons develop the time constants necessary for computation?

In order to detect changes in environmental salinity, we propose a model that requires neurons with multiple time constants for ramping and shunting their activity. From our imaging experiments, we find that such populations of neurons do exist. In fact half-lifes can range from a few seconds to well over a minute. What are the origins of these disparate dynamics? This question has two parts to it. First are the circuit mechanisms that activate these regions and are presumably upstream of these GABAergic populations. Second, are the molecular mechanisms concerning the retained excitability.

For the first question, some clues have been uncovered. Across experiments, I have observed another region that also operates on long time scales are the adrenergic regions of the medulla and area postrema which envelope the posterior rhombencephalon. I performed two-photon guided laser ablations of these regions (Figure 4.2A). After ablations, I compared the response rate in escapable and inescapable conditions. Compared to the control, I observed a marked increase in the behavioral activity of the animals, even during escapable experiments (Figure 4.3B,C). These results suggest an attractive mechanism by which norepinephrine increases the excitability of GABAergic neurons. Since norepinephrine is typically released
Figure 4.2 Ablation of the area postrema increases response rate.

A) Sample anatomy stack of area postrema before (top) and after ablation (bottom). B) Sample area postrema ablated fish being exposed inescapable 100 mM pulses (red). C) Average response probability to escapable and inescapable conditions for fish with sham or area postrema ablations (n = 6 for all groups).

diffusely rather than via synapses, the lingering presence of this neuromodulator may help to keep these neurons persistently active. Of course, this still begs the question, why do only the GABAergic neurons have persistent activity. The mostly likely reason is specific expression of subtypes of adrenergic receptor. Alternatively, the rates of norepinephrine washout may be different in the microenvironments that surround these populations of neurons.
Appendix 1

Experimental methods

Animal Husbandry

Unless otherwise noted, all fish used were embryos from crosses of HuC:GCaMP6s positive parents. Embryos were raised at 27 degrees celsius. For the first 24 hours embryos developed in embryo water plus methylene blue. Afterward, larvae were exclusively raised in filtered (200 um pore size) facility water. Water was exchanged everyday, and larvae were fed live paramecia starting at 4 days post fertilization. Experiments were performed on fish between 6 and 7 days old. All experiments followed institution IACUC protocols.

Free-swimming place-preference assay

In order to test whether larval zebrafish avoid salts, we sought to develop a rig that would allow us to determine a larvae’s place preference within a chemical gradient. Previous studies have examined chemical preference in adult zebrafish by using flow chambers. These strategies, however, introduce two main confounds that we wished avoid. First, any chemical avoidance behaviors will be convolved with rheotaxis and the optomotor response, and switching chambers forces the larvae to behave in opposition to its rheotactic drive. Second, these arenas only create a very steep local gradient at the chamber boundaries, which may not provide the information necessary for the larvae to find its preferred location.
Agar pads are made from three percent low melting point agarose. Agar is poured into premade casts designed to fit the arena. After the agar settles, it is cut out and added to the arena, which is then filled with water, lane by lane. We confirmed the presence of a gradient by using a refractometer to measure the osmolarity of the water in 15 minute intervals at the quartiles of the arena’s length. After the fish are added the initial background image is calculated for 20 seconds and the experiment begins. We define a preference index as the difference in time spent on the half of the arena close to the “test” pad from the time spent away divided by the total length of the experiment. As such, the preference index ranges from one for larvae that spend all of their time on the side proximal to the NaCl and negative one for those that spend all of their time distal to the salt.

Behavior analyses

All analyses were performed with custom written MATLAB code. Preference indices were calculated based upon the position of the fish along the axis perpendicular to the two agar pads according to the following formula:

\[
P.I. = \frac{t(salt) - t(control)}{t(salt) + t(control)}
\]

where \(t(salt)\) and \(t(control)\) are the time spent in the half of the arena closest to the salt and control, respectively. To analyze kinematic parameters, bouts were segmented automatically from the absolute speed of the fish.
Free swimming simulations

For simulations, we assumed larvae chose bouts from one of two types of swim events - straight swims and turns. We described the heading angle changed during these swims by a gaussian, $\mathcal{N}(0,4.5)$, and lognormal, $\mathcal{N}(3.5,0.6)$, distribution for straight swims and turns, respectively. For every swim, larvae choose a heading angle turn from a probability distribution that is a linear combination of the above distributions. As the virtual fish navigates it experiences changes in salt concentrations. We simplify the gradient by estimating it as linear increase from the control agar pad to the source that rises linearly with time. We fit slopes to reach a peak concentration of 15% of the source at the end of the experiment. Following each bout, we redraw the heading distribution, based upon the change in salt concentration. As the change in salt increases, we apply a higher weight to the turn distribution. with a higher weight for the distribution. This weight is assumed to be a linear function of the the change in salt concentration caused by the previous bout. Bouts are assumed to take place at a frequency of 0.8 Hz and move an average distance of 1 centimeter.

Head-embedded chemical stimulation and behavior tracking

At present, the designs available for performing calcium imaging of the brains of freely-swimming zebrafish larvae (Kim et al. 2017) do not offer the same resolution as generated by traditional methods that require the brain to be immobilized, such as two-photon point scanning and light sheet microscopy,. Therefore, we designed a preparation that would enable simultaneous stimulation of a head-immobilized larvae with salt and recording of its behavior. In this preparation we use gravity to control fluid flow that is directed to the rostral end of the fish
by a narrow, 360 μm diameter perfusion pencil tip (AutoMate Scientific 04-360). Multiple solutions were directed to the perfusion tip through an 8-channel manifold that ensured rapid liquid volume exchange (AutoMate Scientific 04-08-zdv). Solution outputs were regulated by solenoids (Cole Palmer EW-01540-01) that were digitally controlled by an Arduino® during the experiment such that at all times one and only one solution was being presented to the fish. Presenting a continuous stream of flow both attenuates behavioral responses to sudden changes of flow velocity and hastens the removal of salt at the end of a trial compared to diffusion alone. The dynamics of the pulse were assessed by imaging pulses of 10 nM fluorescein under an epifluorescent microscope (Olympus® MVX10).

In order to track the tail, midline position points were estimated from the contour of the fish and angles between the points extracted. During live bout extraction, a running variance of the cumulative angle was kept over 200 ms. Whenever this passed a specific threshold, a swim event was called, and used to determine the solenoid status.

Light-sheet microscopy

Volumetric imaging experiments were performed with a custom-built single-photon lightsheet microscope similar to that described previously (Panier et al. 2013). One difference however, is that we used used a transparent specimen chamber and holder to enable tail tracking via a camera below the fish. For imaging, Nacre -/- larvae positive for GCaMP6s expression under the HuC promoter (Kim et al. 2017) were embedded in agarose. In order to stimulate the fish, we removed that agarose surrounding the nose. To allow behavioral monitoring, we also removed the agarose that surrounds the tail. Larvae were illuminated with a 488 nm digitally
scanned sheet that swept through 200 um of depth with 4 um steps at 1 Hz. During the experiment, fish were stimulated with 10 s of a given concentration of NaCl (25, 50, 75, 100 mM) separated by 40s of water flow. Each concentration block lasted for 5 minutes and was separated by 5 minutes.

**Two-photon microscopy**

Two photon microscopy experiments were performed with a custom-built two photon microscope described previously (Huang et al. 2013). *Nacre* -/- larvae positive for GCaMP6s expression in the brain were embedded in 1.8% agarose, and the tail and nose were freed as done during light-sheet experiments and embedded behavior experiments. Larvae were imaged with a spectra-physics *Mai-tai* at 950 nm with 10 mW of power at sample. Volumes spanning the olfactory bulb were imaged plane-by-plane at 8 micron steps.

**Image Analysis**

After imaging experiments, all data was segmented into activity units that approximate cells. Segmentation was performed using an algorithm based on one described previously (Portugues et al. 2014). Registration to the reference brain was performed using CMTK as described previously (Randlett et al. 2015). The following parameter set was used:

```
a -w -r 01 -v -T 8 -X 32 -C 6 -G 24 -A '--accuracy 1.6' -W '--accuracy 6.4'
```

The relationship between the stimulus and each activity unit was determined by calculating their mutual information. To do so each signal was normalized to range from 0 to 1
and then binned into 10 equal sized units. The same was done to the stimulus signal after convolving with the GCaMP6S kernel. Mutual information was then defined as follows:

$$\sum_{x=1}^{10} \sum_{y=1}^{10} P(X \cap Y) * ln\left(\frac{P(X|Y)}{P(X)P(Y)}\right)$$

Here, x and y represent stimulus and activity bins.

For two photon imaging experiments, we defined units as active based upon their coherence across trials. Namely, we asked for each trial, what is the correlation of the units activity with the average across trial. All units with an average correlation across trials greater than 0.7 was deemed active. This threshold was determined by the value above which the probability of being seen in shuffled data is less than 0.01.

In the sixty second long exposure experiments, we determined on-rise and decay-times by using least squares regression to fit w,x,y, and z in the following:

$$Signal = w * exp\left(\frac{-1}{x} * time\right) + y * exp\left(\frac{-1}{z} * time\right)$$

Here, w is bound by [-Inf,0) and y is bound by (0,Inf] so that x and z correspond to the on-rise and on-decay times, respectively. In order to determine off-decay time constants across all experiments, a single exponential was fit to each signal for the 40 seconds immediately after the stimulus was removed.
Appendix 2

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