A Screen of Brain Regions Involved In Prey Capture and The Spitting Behavioral Response In Danio rerio

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Abstract

Previous studies have shown that zebrafish exhibit prey capture behavior, and that the inferior lobe of the hypothalamus (ILH) shows activity during that behavior (Muto et al. 2017). Additionally, the habenula has been recognized to be crucial to the decision-making process in vertebrates (Hikosaka 2010, Cheng et al. 2014). The fish have also been observed to spit out inedible materials after mistakenly attacking it (Muto et al. 2017, Kim et al. 2019). Another study has shown that experience changes the circuitry of the larval zebrafish brain in regards to prey capture behavior (Oldfield et al. 2020). Based on these observations, we set up a screen to observe neural activities in different areas of the larval brain. Specifically, we chose to examine the forebrain, vagus nerve, habenula, and ILH. We predicted that the habenula would show a difference in neural response activity based on how much prior experience the fish had with prey capture. This project explored two different concepts:

1) Whether having prior experience doing prey capture behavior altered neural activity levels in these different brain regions.

2) Which of these brain regions were activated during spitting behavior.

The behavioral tests were conducted on larval fish 5-8 days post fertilization (dpf) that contained GAL4 gene trap lines and crossed with UAS:GcAMP lines. Due to the short duration of the experimental period, we were unable to obtain data pertaining to spitting behavioral responses. However, we were able to observe baseline brain activity in the larval zebrafish brain with no prior experience in prey capture behavior. Future experiments and more time would lend itself to furthering these results and being able to generate a more complete observation of both the prey capture behavior in the brain as well as spitting behavior.

Background

Prey Capture Behavior in Zebrafish

Prey capture behavior has been observed in zebrafish from as early as 4 days post fertilization (dpf) (Muto et al. 2013). However, sometimes the fish mistakenly attack inedible materials, and then spit them out (Muto et al. 2017, Kim et al. 2019). Prior research has shown that zebrafish have a spitting behavioral response to ingesting microplastics (Kim et al. 2019).

Our study aims to elucidate which brain regions are involved in the spitting behavioral response, first examining the regions activated during the prey capture behavior. Based on some observations from a previous prey hunting behavioral study, we suspect that the habenula is a primary candidate for this behavioral response (Oldfield et al. 2020, Muto, unpublished observation).

The inferior lobe of the hypothalamus (ILH) brain region has been associated with prey capture behavior (Muto et al. 2017; Figure 1). To further understand prey capture behavior and the spitting response after ingesting inedible matter, we chose to look at neuronal firing in the forebrain, vagus nerve, habenula, and ILH. Since this study expanded upon the previous work from Muto, et al. from 2013, 2016, and 2017, many of the methods are directly used or adapted for our purposes. These five regions were readily available to study because there were pre existing, robust transgenic fish lines already characterized by the Kawakami lab. We chose to examine the ILH as a positive comparison for neural activity because data from Muto et al. 2017 had already shown activity in the ILH during prey capture behavior.

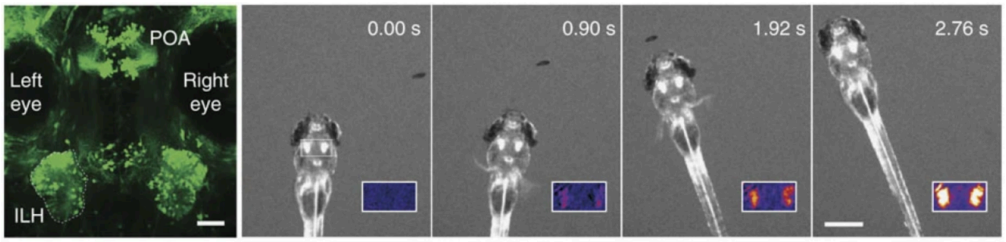

Figure 1: The inferior lobe of the hypothalamus (ILH) is activated during prey capture behavior. “UAS:EGFP reporter gene expression in the ILH in hspGFFDMC76A Gal4 fish at 5 d.p.f. The left ILH is encircled by dotted lines. ILH: the inferior lobe of the hypothalamus, POA: preoptic area. Scale bar, 50 μm.” Figure and quoted caption from Muto, et al. 2017.

The forebrain is known for its involvement in higher order information processing, and a prior study has shown that the forebrain is “recruited” during prey capture, specifically the telencephalon and habenula; as such, we were interested to see to what extent the forebrain as a whole was involved for spitting behavior (Oldfield et al. 2020). The same study has shown that experience changes the circuitry of the larval zebrafish brain in regards to prey capture behavior (Oldfield et al. 2020). Therefore, all prior evidence suggests that we will probably see a change in response levels during prey capture behavior between animals that have done it before, and those of which the trial is their first time exhibiting prey capture behavior.

The vagus nerve has a conserved topography between zebrafish and mammals, and is responsible for a variety of functions; our primary interest is its involvement with swallowing and digestion (Barsh et al. 2017, Isabella et al. 2020).

Perhaps most intriguing, the habenula is involved in decision making processes in vertebrates (Hikosaka 2010, Cheng et al. 2014). The habenula in zebrafish is a homolog of the mammalian habenula (Amo et al. 2010, Cheng et al. 2014), so we suspect that it has a similar role in integrating information central to the process of decision making. As referenced earlier, we know that the habenula is already involved in prey capture behavior (Oldfield et al. 2020), so we are interested in seeing if it is even more active when the zebrafish makes a decision to first “capture” an inedible microplastic, and then spit it out.

Ingesting Microplastics and Paramecium

With the current impact of humans on the environment, many studies have examined the physiological impacts of microplastics on zebrafish (Kim et al. 2019, Bhagat et al. 2020, Xu et al. 2021). Based on these studies, we know that zebrafish can show a preference for ingesting food rather than microplastics, yet still ingest microplastics when they are present (Kim 2019). These fish have also been observed to spit out non-food items after showing prey capture behavior (Muto & Kawakami 2017, Chen et al. 2022). The process of selecting which foods to consume requires input and integration of information from multiple sensory processing systems, and often reflect preferences towards valuable nutrients (Boyer et al. 2013). As such, it would be interesting to observe first a zebrafish larvae's decision to ingest non-food items, and then see how the neural firing responses and behavior changes after that experience. However, like any behavioral study, the animals may not always do what we intend for them to do. With that in mind, we set up our experiment to also examine how the neural responses for prey capture behavior and prey selection changes with prior prey capture experience.

Since we rear our larval fish with paramecium, we decided to test behavioral prey capture with both microplastics and paramecium. Larval zebrafish have a highly stereotyped behavior for prey capture, and commonly hunt paramecium at this early stage (Oldfield et al. 2020, Muto et al. 2017, Bianco & Engert 2015, Bianco et al. 2011). When the fish visualizes the prey, it first gets into position by orienting itself towards the prey, then swims forwards to get into striking range (Oldfield et al. 2020, Muto et al. 2017, Bianco & Engert 2015, Bianco et al. 2011). Once in range, the eyes noticeably converge on the prey, and the fish will quickly "dart" forwards to make the final capture (Oldfield et al. 2020, Muto et al. 2017, Bianco & Engert 2015, Bianco et al. 2011).

Methods

Transgenic Zebrafish

Transgenic zebrafish lines were created and maintained in the Kawakami lab by first fertilizing zebrafish eggs, and then injecting a DNA construct into them during the single cell stage (Muto 2017). This study uses the adult progeny of these lines to create UAS:GcAMP lines of different regions of the brain. We mated GAL4 gene trap lines with the genotypes gSAIzGFFD2269A (forebrain), gSAIzGFFM2146B (vagus nerve), hspGFFDMC76A (ILH), gSAIzGFFM3856A (habenula), and gSAIzGFFM707A (habenula) with transgenic UAShspzGCaMP6s fish. The UAShspzGCaMP6s fish had a GFP marker in the lenses of their eyes. Unfortunately, we were unable to use fish with a homozygous *nacre* background, due to the short term time constraints of the study, and the fact that our preexisting UAShspzGCaMP6s fish were heterozygous for *nacre*. The GFP images were generated on a confocal fluorescence microscope, and fish lines of interest were identified via the ztrap database, as well as through literature review.

To identify the offspring with the GcAMP6s phenotypes of interest, we monitored expression patterns from 1dpf to 5dpf, and manually sorted larvae by fluorescent phenotypes at 5dpf. We selected larvae with both expression of GFP in the lens, and also weak green fluorescence in their corresponding brain region of interest. We then selected for larvae with the least pigment cell development.

Spitting Response/Prey Capture Assay

Figure 2 shows most of the materials we used to set up the behavioral assays. First, we picked a larva and transferred it into system water in a 60cm petri dish. We added one drop of 10x tricaine (overall, 9 drops of system water, 1 drop of 10x tricaine) to anesthetize the fish. In the 60cm petri dish lid, we added a thin layer of 1.5% low melting temperature agarose (created using system water). While the agarose was still warm, but not hot to the touch, we carefully used a clean plastic Pasteur pipette to add a zebrafish larva to the dish. We introduced as little liquid as possible by first tapping the pipette so that the larva would swim downwards within the pipette. Using an illustrated needle (etsukihari), we kept the larva dorsal side up and in the middle of the petri dish while the agarose cooled. We kept adjusting the fish to maintain this orientation until the agarose hardens. Once the agarose hardened, we poured a thin layer of system water over the agar so that the fish would not dry out during imaging.

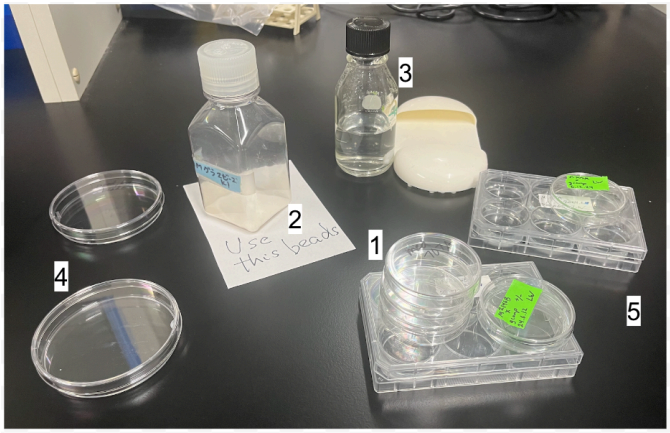

Figure 2: Overview of most of the materials needed for the spitting assay.
1) Medium petri dishes
2) Microplastic beads
3) 1.5% low melting temperature agarose (created in 100mL aliquots)
4) Extra petri dishes for paramecium washes, and anesthetizing fish in tricaine.
5) 6 well plates for sorting fish by phenotype
6) tricaine 10x (not pictured)

Once the fish is set up, the larvae were imaged under an epi-fluorescence microscope (Imager.Z1, Carl Zeiss, Germany) at 10x magnification. Images were taken at 10fps for 2 minutes. Larvae were imaged first with no prior prey capture experience, then while being given paramecium to eat for the first time, then after being exposed to paramecium. Figure 5 shows a breakdown of the experimental treatment pairings. The two fish lines with gSAIzGFFM707A and GcAMP, and gSAIzGFFM2146B and GcAMP were also given microplastic to see if they would try to ingest it. Around 1mg of microplastic was administered with no prey capture experience, and also post-paramecium, but not at the same time as paramecium. Imaging and behavioral assays were performed anywhere from 5-8dpf, with the majority being recorded on day 5 for better imaging quality through the developing pigment cells.

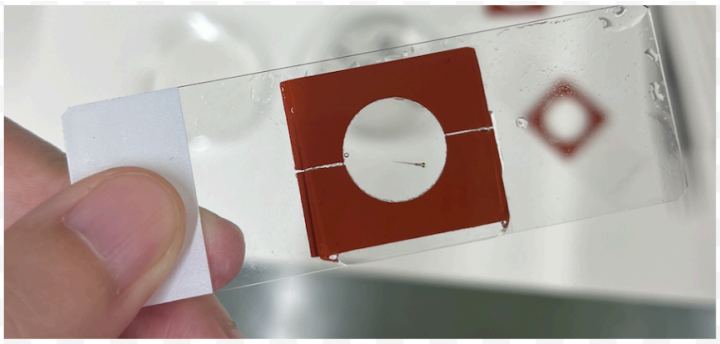

Figure 3: Setup for the free-swimming calcium imaging.

Neuronal GcAMP Imaging in Free-Swimming Larvae

This experiment used the protocol developed by Muto & Kawakami (2016). We used the same zebrafish larvae lines as the spitting response assays, at 5-8dpf. First, gently pipette a zebrafish larvae into a 2mm deep dish that is 2cm diameter. Wash

some paramecia with system water, and then try to isolate a singular paramecium. Pipette the paramecium into the dish. Carefully place a coverslip over the dish, so that the fish is free swimming, and the paramecium remains within the dish. Figure 3 shows the setup of the 2cm dish with a zebrafish larva inside. Using the same epifluorescence microscope as the spitting assay, use 5x zoom to find the fish. Record the GcAMP response by moving the dish to follow the fish. If the quality looks good, zoom into 10x magnification and conduct more recordings. Record until the fish eats a paramecium, which will take lots of patience.

Results

Obtaining Transgenic Lines

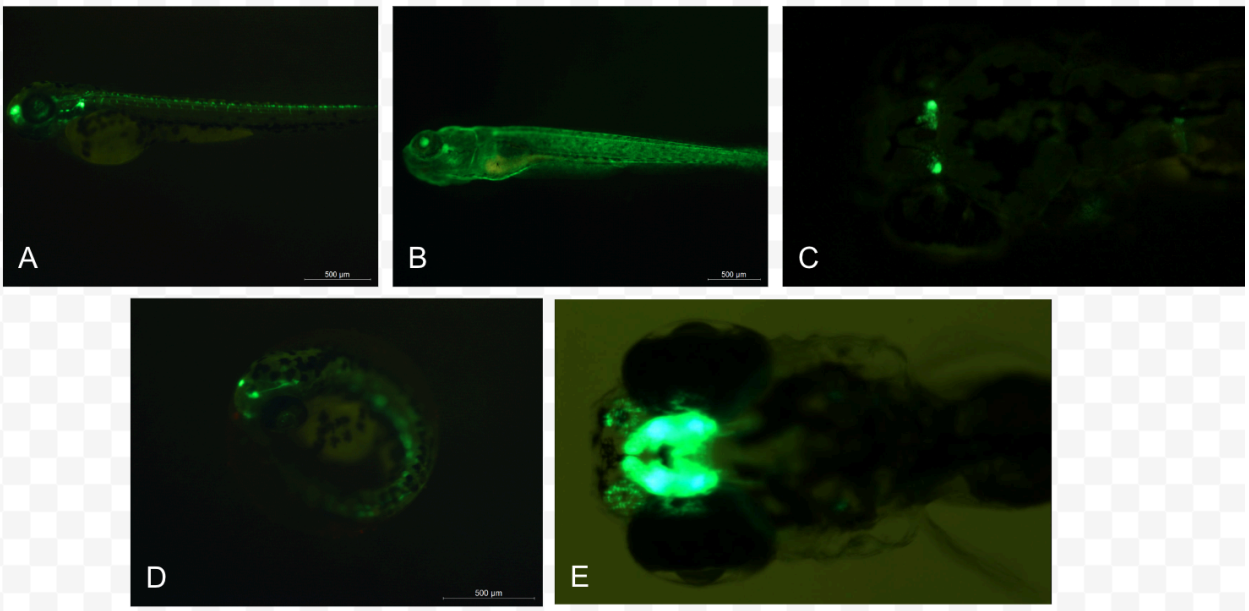

Figure 4: Transgenic fish imaged during larval days 3-5. The best images were used in this figure. All lines for this study were crossed with UAShspzGCaMP6s. A) Transgenic line gSAIzGFFM2146B (vagus nerve) at 5x zoom, 3 dpf. B) hspGFFDMC76A (ILH) at 4x zoom, 5 dpf. C) gSAIzGFFM707A (habenula) imaged at 5dpf*. D) gSAIzGFFM3856A (habenula) imaged at 6.3x zoom, 3 dpf. E) gSAIzGFFD2269A (forebrain) imaged at 5 dpf*. Dorsal views are shown for images C and E.
*images taken from ztrap database, due to a lack of good quality images generated during the study.

As seen in Figure 4, we were able to obtain all 5 transgenic lines of interest. Due to the available transgenic lines' ages, we were able to obtain more data for certain lines than others. The two habenula lines (gSAIzGFFM3856A and gSAIzGFFM707A) and the vagus nerve (gSAIzGFFM2146B) were able to breed the best with our GcAMP6s line, so most of our data comes from these three lines.

Prey Capture Imaging

The larvae displayed neural activity, and we were able to gather baseline data during the duration of the IRES program. Due to the extensive amount of time and patience required for behavioral studies, we were unable to visualize fish attempting to eat the microplastics. There

were no instances where the zebrafish chose to ingest the microplastics, out of 6 trials between the lines gSAIzGFFM707AxUAShspzGCaMP6s, and gSAIzGFFM2146BxUAShspzGCaMP6s (N=2 for gSAIzGFFM2146BxUAShspzGCaMP6s, and N=4 for gSAIzGFFM707AxUAShspzGCaMP6s). Figure 5 shows the experimental groups we created for imaging. These groups were used for both the imaging sessions in agarose, and the

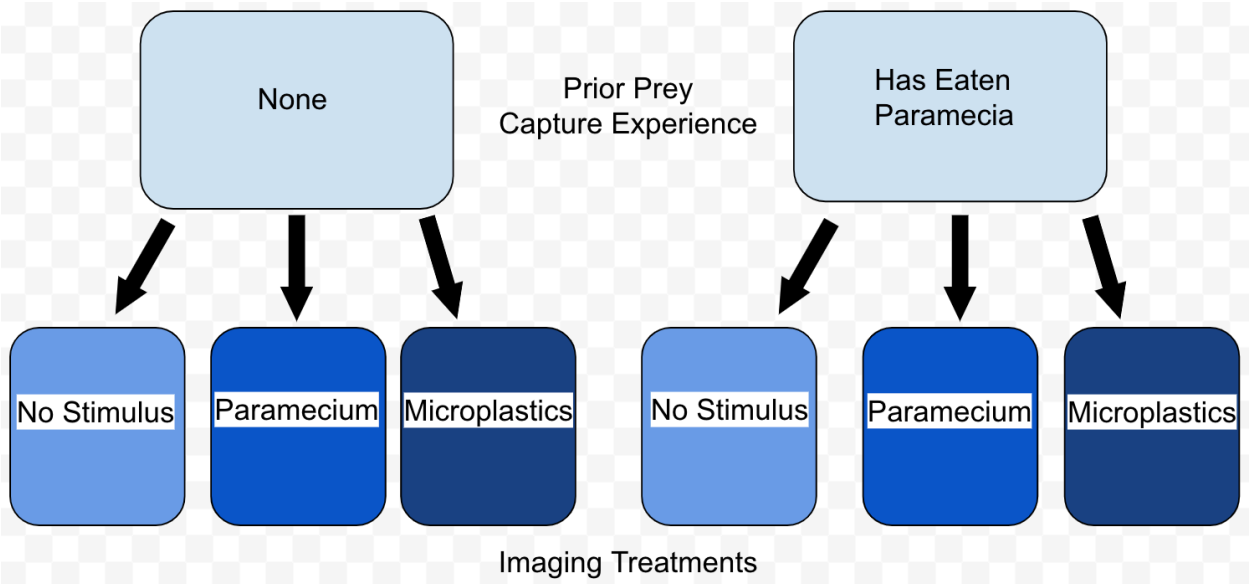

Figure 5: Flowchart of spitting response/prey capture behavioral test design.

free-swimming prey capture videos. Unfortunately, we were unable to conduct very many free-swimming videos (N=3, where each N is one day's worth of imaging sessions). We were also unable to obtain data pertaining to spitting behavioral responses. However, we were able to observe baseline brain activity in the larval zebrafish brain with no prior experience in prey capture behavior.

The imaging sessions where we were able to observe good neuronal activity happened to be the imaging sessions where we ran the no prior experience paired with paramecia, the no prior experience paired with no stimulus, and the prior experience with the paramecia. Figure 6A shows a zebrafish larvae with no experience with a single

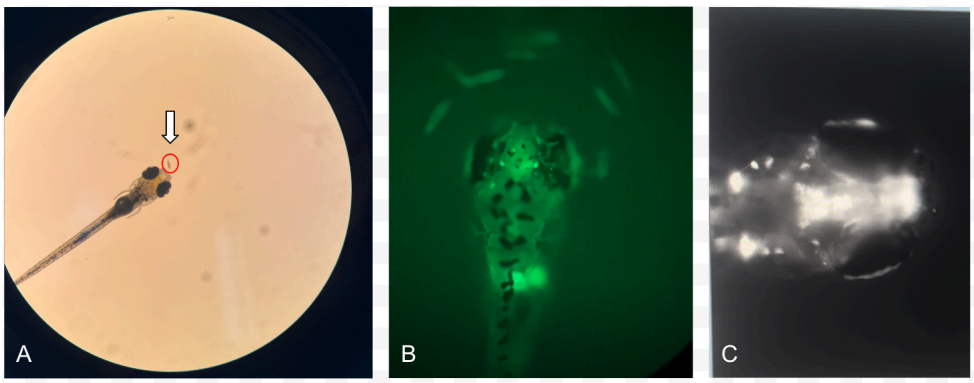

Figure 6: A) Zebrafish larvae (5dpf) with a single paramecium embedded in agarose under the confocal fluorescence microscope without the fluorescent setting. Imaged at 2.5x zoom. The white arrow with the red circle point to the paramecium. B) GcAMP imaging for zebrafish larvae from the gSAIzGFFM707AxUAShspzGCaMP6s line embedded in agarose with several paramecia swimming around the head. Imaged at 5x zoom on the epi-fluorescence microscope. C) Vagus nerve GcAMP expression (gSAIzGFFM2146BxUAShspzGCaMP6s) with no stimulus at 10x zoom. Imaged at 5dpf.

paramecia embedded in low melting temperature agarose. Figure 6B shows a different larvae embedded in agar with habenula GcAMP expression and several paramecia around it. The best image quality we were able to obtain during this study shows the vagus nerve transgenic line (gSAIzGFFM2146BxUAShspzGCaMP6s) during calcium imaging with no prior experience and no stimulus. The baseline expression here was very strong, yet it is unclear what the neurons are firing in response to (Figure 6C). This, however, would serve as an excellent background expression level for more trials that test this line's response to eating paramecia for the first time. Unfortunately, we were unable to obtain those results because of poor imaging quality when conducting those trials.

Discussion

This research is just the beginning of understanding how the larval brain changes from zebrafish gaining prey capture experience. We predicted that we would see activity in the ILH, forebrain, and habenula. However, since this is an exploratory screen, we were also interested in whether fish that had prior experience eating paramecium would show more activity in the vagus nerve, given its involvement with eating in humans. Future experiments and more time would lend itself to furthering these results and being able to generate a more complete observation of both the prey capture behavior in the brain as well as spitting behavior. Since we were unable to observe any trials where the larva attempted to ingest the microplastic, we still do not know which brain regions are involved during spitting behavior.

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