UNIVERSITY OF CALIFORNIA

Los Angeles

Chemically-induced Rapid Sensitization in Restrained Larval Zebrafish (*Danio rerio*)

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Joseph Bassam Alzagatiti

2018

ABSTRACT OF THE THESIS

Chemically-induced Rapid Sensitization in Restrained Larval Zebrafish (*Danio rerio*)

by

Joseph Bassam Alzagatiti

Master of Science in Physiological Science

University of California, Los Angeles, 2018

Professor David L. Glanzman

The larval form of the zebrafish (Danio rerio) holds significant promise as a model vertebrate system for understanding the neural mechanisms of behavior. This promise arises from two particularly attractive properties of zebrafish larva: their translucence, which permits robust optogenetic manipulations, and their possession of a relatively simple neural circuitry, which facilitates cellular analysis of behavior. In particular, past studies successfully demonstrated behavioral changes in fish as young as five days post fertilization (DPF) via transient receptor potential A1 (TRPA1) ion channel activation mediated by the chemical irritant allyl isothiocyanate (mustard oil - MO). In the present study, we found that 10 µM total bath concentration of MO significantly increased the duration of tail activity responses in 5 and 6 DPF, agarose-restrained larval zebrafish compared to control treated animals. Further, we recapitulated this sensitizing effect in agarose-restrained older fish, 12 DPF, and to our knowledge, uniquely, in fish as young as 3 DPF. Together, these results indicate the immense promise that zebrafish hold as a neurobiologically tractable animal model for the study of learning and memory, especially non-associative memory.

The thesis of Joseph Bassam Alzagatiti is approved.

Stephanie White

Alan Grinnell

David L. Glanzman, Committee Chair

University of California, Los Angeles

2018

Dedicated to my dear family, both metaphorical and literal,

for orienting me to pursue Truth

Nihil Supernum

TABLE OF CONTENTS

LIST OF FIGURES

Introduction

The complexity of most mammalian systems yields challenges to reductionist understandings in learning and memory systems. However, zebrafish (*Danio rerio*) as a relatively new animal model in the field of learning and memory possess an unique position as easily-studied, neurally-reduced vertebrate models (Roberts et al. 2013). In particular, this position arises from three points: the animal's large clutch sizes; a mechanistically reduced nervous system with homology to vertebrates that is amiable to genetic and pharmacological manipulations (Best et al. 2008) (Goldsmith 2004) (Guo 2004); and physiological translucency in the larvae stage of development that allows for *in vivo* optogenetic imaging and neuromodulation (Knafo and Wyart 2015).

Furthermore, studies have demonstrated short-term and long-term memory paradigms like habituation and classical conditioning in zebrafish larvae (Aizenberg and Schuman 2011)(Roberts et al. 2011) (Roberts et al. 2016) (Valente et al. 2012). Similar to habituation, sensitization is another form of non-associative learning available to an organism that is characterized by a transient increased physiological response upon the application and removal of some form of noxious stimulus or agent. Specifically, a chemical irritant that can reliably and robustly induce sensitization in larval zebrafish will profoundly aid in further understanding of mechanisms underlying learning and memory, especially in an organism that can readily absorb neurotropic chemicals in bath application (Wolman et al. 2011). For larval zebrafish, TRPA1 (transient receptor potential) receptors are implicated in mediating chemosensation, and of particular interest, is the activation of said receptors in zebrafish upon bath application of the natural chemical irritant, mustard oil or MO (allyl isothiocyanate) (Prober et al. 2008).

Therefore, combining the promise of the larval zebrafish animal model with a known chemical irritant, MO, we sought to determine if MO could reliably induce sensitization in semi-restrained larval zebrafish, aged 5 to 6 days post-fertilization (dpf), in order to also capitalize on the ability to do *in vivo* optogenetic imaging in restrained specimens. As such, we first determined an effective bath concentration of MO (10 uM) to induce a transient increase in tail locomotion of 5 to 6 dpf animals, then we investigated whether this effect could be recapitulated in 3 and 12 dpf animals. Subsequently, we investigated the duration of the MO locomotor response to ascertain a time point to choose for our sensitization experiments, that is the expectation of enhanced physiological response in absence of MO. After we confirmed sensitization in the semi-restrained 5 to 6 dpf animals in both tail locomotion and heart rate, we desired to verify that TRP receptors contribute to the expression of this sensitization utilizing a non-selective TRPA1 receptor inhibitor, ruthenium red (RR). Finally, utilizing an optogenetic strain for full-brain imaging of larval zebrafish expressing GCaMP6 pan-neuronally, we tentatively determined a potential area for a neuronal correlate of this sensitization memory.

Materials and Methods

Animals

Animals for experiments obtained from the University of California, Los Angeles zebrafish core facility and vivarium. Zebrafish eggs were collected after standard breeding protocols. Briefly, adult males and females were paired for breeding, and fertilized eggs were collected the subsequent day. Any dead animals and debris were removed, and the fertilized eggs placed into E3 (embryo solution) solution (5 mM NaCl, 0.33 mM MgCl2, 0.33 mM CaCl2, 0.17 mM KCl, 10-5% methylene blue, pH 7.2) and maintained in an incubator (28.5º C) to allow the embryos to develop. Animals were cleaned every day, continuously removing dead animals and

debris, while refreshing with clean E3 solution. Wild-type TL zebrafish ranging from 3 to 12 dpf were utilized for experimentation. All animal procedures and experimental protocols were approved by the Chancellor's Animal Research Committee (ARC) of the University of California, Los Angeles.

Behavioral and Optogenetic Paradigms

Tail Locomotion with MO Application in Semi-restrained Zebrafish Preparations

For dosing response to MO, a semi-restrained preparation of 5 to 6 dpf larval zebrafish was used. Specifically, animals restrained in 3% low melting point UltraPure™ LMP Agarose (Invitrogen) on a 50 mm glass-bottom dish with 8 mL of E3 solution, and had the agarose around both the head and tail removed to allow for exposure to MO and tail locomotion, respectively. Animals were acclimated on a light box (Gagne Inc., Johnson City, NY) for 20 minutes. After acclimation, all animals were treated with 100 uL of E3 (as a control, vehicle solution) delivered by a pipette to the head of the animal as a pretest application. After 1 minute, each fish was treated with either 100 uL of E3 or 100 uL of MO diluted to 1, 10, 100 uM in E3 (effective bath concentration) as a post-test application. Tail locomotion during the first 30 seconds after all treatments was recorded using a high speed camera (Casio HS Exilim EX-ZR800 at 120 fps; Casio™ America, Dover, NJ), oriented above the dish containing the semi-restrained fish preparation. Duration of tail locomotion for all experiments was determined by a trained observer, blinded to the conditions of each behavioral recording.

For developmental ontogeny with MO, fish aged 3, 5, and 12 dpf were semi-restrained as mentioned previously. Animals were acclimated for 20 minutes on a light box, then all were given 100 uL of E3 as a pretest. Subsequently, after 1 minute, those in the experimental group received an ejection of 100 uL of MO that yielded an effective bath concentration of 10 uM,

while those in the control received 100 uL of E3 as a post-test. Animal locomotion after all treatments was recorded for 30 seconds using a high speed camera positioned above the animal, and the behavioral recording analyzed by a trained observer.

For duration of response to MO, a semi-restrained preparation with 5 to 6 dpf was also used. Animals were set and prepared in 3% low melting point as previously noted. Animals were acclimated for 20 minutes on top of a light box. Upon the end of acclimation, all animals were exposed to an ejection of 100 uL E3. Then, all animals received an ejection of 100 uL of MO calibrated to an effective 10 uM MO bath concentration. MO was washed out after 1 minute using a Rabbit-Plus™ peristaltic pump (10 mL per minute cycle; Rainin Instrument Co. INC). Tail locomotion was recorded using a high speed camera for 30 second intervals at 0, 5, 10, 15, and 30 minutes after MO washout as post-tests. Determination of tail locomotion duration performed as mentioned above.

For MO sensitization tail locomotion experiments, 5 to 6 dpf were semi-restrained in low melting point as noted above. Animals were then acclimated on a light box for approximately 30 minutes. Then, a 30 second recording with a high speed camera was taken as a pretest. 5 minutes after this recording, either an ejection of E3 or MO (10 uM bath concentration) was given to the animal for 30 seconds. After a 1 minute washout of MO manually with transfer pipettes, then 2 minute wait without disturbance, a 30 second recording was captured. Tail locomotion for the behavioral recordings was analyzed as above paradigms.

All values for tail locomotion reported as normalized duration in seconds, post-test minus pretest durations.

Heart Rate with MO Application in Semi-restrained Zebrafish Preparations

5 to 6 dpf larval zebrafish were semi-restrained in 3% low melting point agarose. In this preparation, only the agarose near the head of the animal was removed to allow for exposure to MO. The fish were placed under a dissecting microscope to acclimate for 30 minutes. After acclimation, a baseline heart rate was determined via visual observation for 30 seconds, and this value was multiplied by 2 to yield beats per minute (bpm). Upon subsequent baseline heart rate observation, either E3 or MO (10 uM bath concentration) was added for a period of 1 minute. Then, after 1 minute of MO washout and 2 minutes of wait without disturbance, another visual observation was taken for 30 seconds as a post-test.

For ruthenium red (RR) experiments, similar procedures to the mentioned heart rate protocols used. However, RR or E3 solutions were washed into the dish 3 minutes before the first baseline heart rate measurement.

All values reported as normalized heart rate fold changes with post-test divided by pre-test heart rates.

Optogenetic Imaging in Semi-restrained Zebrafish Preparations

Tg(elavl3:GCaMP6s) strain of zebrafish, a generous gift from Dr. Misha Ahrens Lab, were used (Ahrens et al. 2013). Briefly, GCaMP6s, a slow-kinetic binding fluorophore-calmodulin complexed protein, expressed pan-neuronally in almost the entirety of the central nervous system of the animal via the ubiquitous promoter *elavl3* (Chen et al. 2013). Transgenic fish were semi-restrained in 3% low-melting point agarose with only the agarose near the head removed to allow for application of MO. Semi-restrained animals were placed into a compound, Zeiss confocal microscope, acclimating for 30 minutes. Utilizing high-speed, line-scanning microscopy, we captured images of a volume (200 X 140 X 100 microns) of the zebrafish

hindbrain at 5 volumes per second at 200 frames per second at similar time points to those taken in the MO sensitization tail locomotion experiments with an ejection of E3 at the start of each 30 second interval recorded. However, instead of a manual washout, the peristaltic pump was used.

Fluorescence values were normalized by selecting a region of basal fluorescence and dividing an optical density measurement by that value to yield fold increases in fluorescence.

Pharmacology

Mustard Oil (allyl isothiocyanate) purchased from Sigma-Aldrich (St. Louis, MO). Ruthenium Red (RR) also purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis

Where appropriate, statistical analyses of repeated measures, one-way, or two-way analysis of variances (ANOVA) were carried out followed by Tukey post-hoc tests when statistical significance was present. Further, Student's *t*-test was utilized, where appropriate, between comparison of two group experimental paradigms.

Results

MO Dosing Response

In a semi-restrained larval zebrafish preparation of 5 to 6 dpf animals, we discovered that animals experienced significantly increased tail flick durations with bath concentrations of 10 or 100 uM MO ($F [3, 40] = 27.11$; $p < 0.05$). Furthermore, Tukey's post-hoc tests indicate that 10 uM and 100 uM groups hold true statistical significance in terms of increased tail locomotion durations as compared to 0 uM (E3 control group) and 1 uM MO (mean normalized duration of

tail locomotion: 0 uM; -0.09 ± 0.22 s; 1 uM, 0.05 ± 0.31 s; 10 uM, 3.14 ± 0.30 s; 100 µM, 1.57 ± 0.32 s) (**Figure 1**).

MO Tail Locomotor Enhancement Recapitulates in Development of Larval Zebrafish

At this juncture, we chose 10 uM of MO bath concentration as our effective dose with the consideration that the peak locomotor effect is present at this concentration as well as a lower dose of a noxious stimulus to use in the semi-restrained fish preparation.We then sought to demonstrate at what age larval zebrafish begin to exhibit behavioral responsiveness to application of MO. After applying E3 or MO to larval zebrafish aged 3 to 12 dpf, we then performed a two-way ANOVA to examine if any significant interactions existed between the age of the fish and exposure to MO. The two-way ANOVA revealed significant interaction between fish age and MO exposure in normalized tail locomotion duration ($F [2,44] = 3.99$; $p < 0.05$). Further, one-way ANOVA with Tukey post-hoc tests showed that there were no statistical differences for groups exposed only to E3 at any age ($F [2,23] = 0.06$; $p = 0.95$: 3dpf, -0.03 ± 0.09 s; 5dpf, 0.01 ± 0.08 s; 12 dpf, -0.05 ± 0.12 s), but for the groups exposed to MO, a significant effect was observed at all ages, becoming more pronounced with age ($F [2,21] = 3.87$; $p < 0.05$: 3dpf, 1.24 ± 0.19 s; 5dpf, 2.39 ± 0.54 s; 12dpf, 3.09 ± 0.59 s), verified by a Tukey post-hoc test yielding a statistical difference between 3 and 12 dpf ($p < 0.05$) (**Figure 2**)

MO Duration of Response in 5 to 6 dpf Larval Zebrafish

Given that translucency and cellular resolution for optogenetic imaging is most prominent at 5 to 6 dpf (Ahrens et al. 2013) as well as our findings of an effective bath concentration of 10 uM MO holds a rather pronounced effect as early as 5 dpf, we decided to investigate the duration of the MO induced increase in tail locomotion upon its removal. We

observed that an increase in tail locomotion duration post MO washout persisted for approximately no greater than 15 minutes and receded by 30 minutes with a less variable increase within the first few minutes (< 5 minutes) after MO removal (normalized means of tail locomotion duration in seconds: 0 min, 2.713 ± 0.3995 s; 5 min, 2.595 ± 0.9197 s; 10 min, 2.682 ± 0.7742 s; 15 min, 1.455 ± 1.446 s; 30 min, 0.633 ± 0.421 s) (**Figure 3)**.

MO mediates Short-term Sensitization of Tail Locomotion in Larval Zebrafish

Building on our aforementioned results, we chose a 5 to 6 dpf semi-restrained preparation to demonstrate MO short-term sensitization of the duration of tail locomotion. After a 30 minute acclimation and a pretest recording of 30 seconds with a high speed camera, MO (10 uM) was ejected into the dish towards the head of the zebrafish, then manually washed out with transfer pipettes, and finally a post-test recording of 30 seconds was taken 3 minutes after MO exposure and wash. We witnessed that the change in duration of locomotion of the MO exposed group was significantly greater than those exposed to E3 alone upon evaluation with an unpaired Student's *t*-test (E3/Control, 0.38 ± 0.80 s; MO, 5.13 ± 1.69 s; $p < 0.05$) (**Figure 4**).

MO mediates Short-term Sensitization of Heart Rate in Larval Zebrafish

To further validate our demonstration of MO-induced short-term sensitization in a semi-restrained preparation of larval zebrafish, we proceeded to replicate the short-term sensitization effect by modulating heart rate as a correlate to activation of sympathetic nervous system that often accompanies behavioral sensitization (Bouwknecht et al. 2000). After acclimating animals on a dissecting microscope for 30 minutes and taking a baseline heart rate measurement, MO or E3 was applied to the animal for 30 seconds, then washed out manually with transfer pipettes, and a final heart rate measurement was taken. After normalizing post-test to pretest values of

bpm, we noticed that animals exposed to MO had a significantly higher fold increase in normalized heart rate as compared to those exposed to E3 after evaluation with an unpaired Student's *t*-test (E3/Control, 1.0158 ± 0.0076 bpm; MO, 1.2193 ± 0.1785 bpm; $p < 0.0001$). (**Figure 5**).

RR Pharmacological Blockade of MO-mediated Short-term Sensitization

After elucidating robust short-term behavioral sensitization in semi-restrained larval zebrafish in two behavioral paradigms, duration of tail locomotion and heart rate, we then advanced to determining if TRPA1 receptors play a role in MO-induced sensitization. For our behavioral paradigm, we decided to utilize heart rate due in part to consistently yielding the sensitization effect without high variability in experimental collections. For TRPA1 receptor blockade, we chose 10 uM RR, bath concentration, which has been shown previously to antagonize TRP receptors in larval zebrafish without serious health effects (Prober et al. 2008). To ascertain a TRPA1 blockade effect, baseline heart rate was measured after a 30 minute acclimation, E3 solution or RR was washed in 3 minutes before the application of MO (10 uM). We found that upon application of RR, MO-induced sensitization is significantly blocked, implying that TRPA1 pathways play a role in facilitating the observed behavioral changes after use of an unpaired Student's *t*-test (E3-MO, 1.1602 ± 0.0113 bpm; RR-MO, 1.0137 ± 0.0393 bpm; $p < 0.0001$) (**Figure 6**).

Potential Neuronal Correlate for MO-induced Short-term Sensitization

To exploit the inherent translucency advantages of the larval zebrafish and investigate potential neuronal correlates to behavior, we employed a transgenic strain, Tg(elavl3:GCaMP6s) that possess a calcium-sensitive fluorophore complexed protein (GCaMP6) expressed under the

pan-neuronal *elavl3* promoter (Dunn et al. 2016). We examined mostly restrained larval zebrafish, aged 5 to 6 dpf, with only the head segment freed from agarose in a confocal microscopy setup to image hindbrain segments of the fish before and after exposure with washout of MO (10 uM) or exposure to E3 and washout. Upon visual inspection and normalization of fluorescence of these limited recordings, we tentatively determined that a region of the hindbrain located caudally to the *commissura infima Halleri* seems to show a fold increase in fluorescence sometime after MO removal (normalized fluorescence values of fish receiving MO 2.14 compared to 1.46 for animals receiving E3 alone; N=1 per group) (**Figure 7**). Further, previous data has shown neurons in this area to be associated with neurons responsible for swim commands and induction of swim locomotion repertoire (Arrenberg et al. 2009).

Discussion

The present study set out to investigate the capabilities of MO as a reliable and robust sensitizing agent that could chemically induce behavioral short-term sensitization in semi-restrained larval zebrafish as well as discern a potential cellular-molecular pathway for the expression of this memory.

As such, our results, taken together, indicate that a bath application of MO (10 uM) is effective as a sensitizing stimulus as well as capable of inducing behavioral short-term sensitization mediated by TRPA1 receptors upon ejection and removal in semi-restrained preparations of larval zebrafish with optogenetic evidence of potential expression via swim command neurons. In particular, we found that the sensitizing effect is most prominent with 10 and 100 uM concentrations, choosing 10 uM as a safer option for further experimentation in larval zebrafish, and recapitulating such an effect in 3 to 12 dpf aged zebrafish with the effect becoming more profound with age. We then determined the duration of MO-induced

sensitization to be no greater than 15 minutes after MO washout, and validated the short-term sensitization with a more stringent tail flick paradigm, yielding a resultant of increased tail locomotion duration even after MO washout. After, we ascertained an increase in autonomic nervous system activation as another measure of MO mediated sensitization via measuring and normalizing heart rate to MO ejection and washout. Finally, utilizing the heart rate paradigm, we observed a potential role of TRPA1 receptor in facilitating MO-induced sensitization with RR, a known TRPA1 receptor inhibitor, to block this sensitization; and gathered some preliminary optogenetic evidence of increased fluorescence in a swim command neuron region that may mediate the expression of this sensitization.

However, some points of consideration do linger for future investigations: the most pertinent relating to the efficacy of the TRPA1 blockade and interpretation of the optogenetic evidence, especially in regards to physiological necessity and sufficiency of these cellular-molecular mechanisms in mediating the MO-induced behavioral sensitization. In particular, for the TRPA1 blockade with RR, the drug is known to be non-selective (Vincent and Duncton 2011), and it is possible that MO was resistant to washout. To address this matter, use of optogenetic TRPA1 strains of zebrafish would enable more finely tuned control over the TRP channel as well as improving the link between TRPA1 as a necessary and sufficient sensory component in the induction of the MO mediated sensitization (Kokel et al. 2013). On another point, the interpretation of the optogenetic data should be made with caution, both due to the sample size as well as the contribution of the region to the MO-induced sensitization. Specifically, one must determine if this region is a necessary and sufficient for the expression of the behavioral sensitization and/or whether other brain regions are implicated and may compensate. In order to overcome these obstacles, a two-pronged approach may be employed:

full-brain, 3D volumes taken with adequate fidelity will allow for a more robust exploration of neuronal populations that contribute to this non-associative memory (Randlett et al. 2015); secondly, photoablation of the identified region (Kohashi and Oda 2008) and/or optogenetic manipulations of the area with pan-neuronal expression of channelrhodopsin (Douglass et al. 2008) or halorhodopsin (Arrenberg et al. 2009) with accompanying examination of behavioral and memory deficits will provide more evidence to the exact role that this particular set of swim command neurons plays in the expression of the MO-induced sensitization.

And yet, the ability to chemically induce a memory in a vertebrate analog with the potential for *in vivo* optogenetic neuromodulation and imaging sets the stage for robust and rigorous cellular-molecular investigations of any and all forms of learning and memory.

Figures

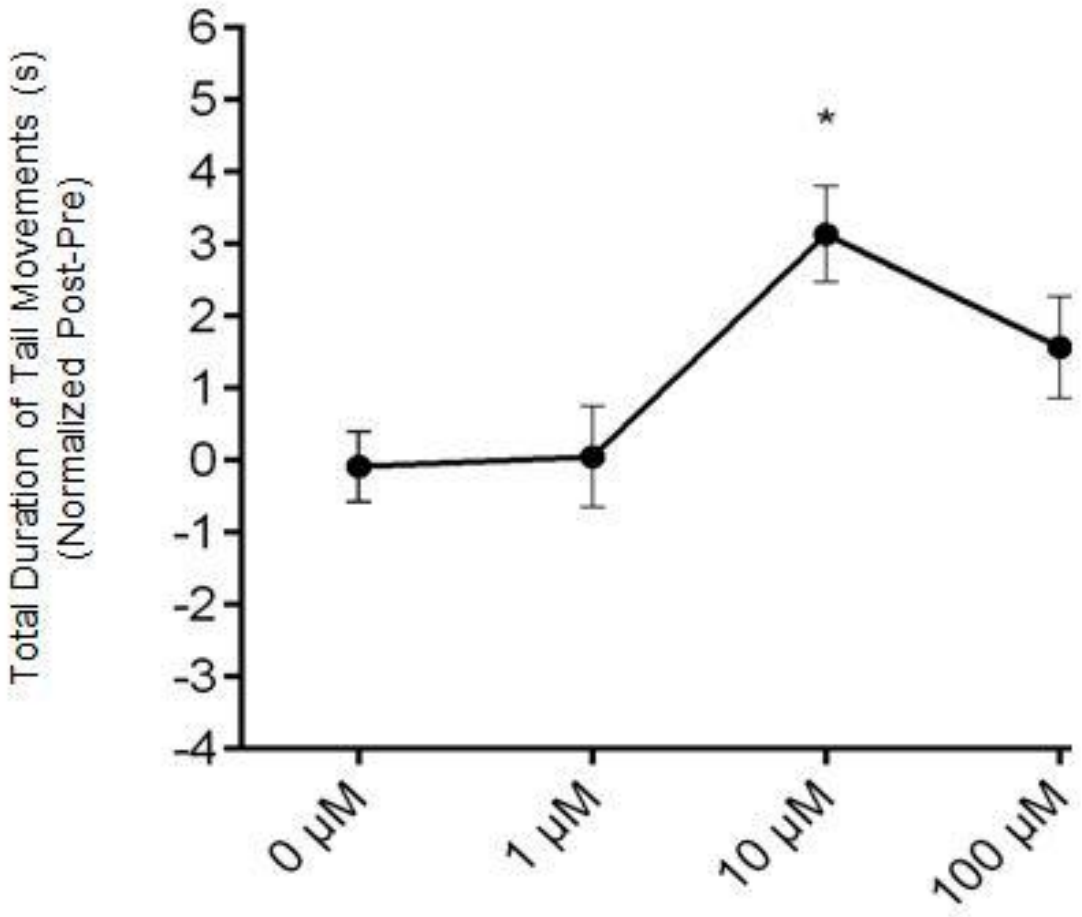


Figure 1. MO Dosing Curve. Mustard oil significantly enhances tail locomotion duration at specific concentrations (n = 11 per group). As indicated by one-way ANOVA, concentrations of MO 10 uM and 100 uM significantly enhanced swimming duration ($F [3, 40] = 27.11$; $p < 0.05$). Further, Tukey post-hoc tests indicated that 10 uM and 100 uM total swimming time were significantly greater than both 1 uM or 0 uM (E3-control group) ($p < 0.05$). Data represented as means ± SEM. (Asterisk indicates chosen effective bath concentration for all other experimental paradigms).

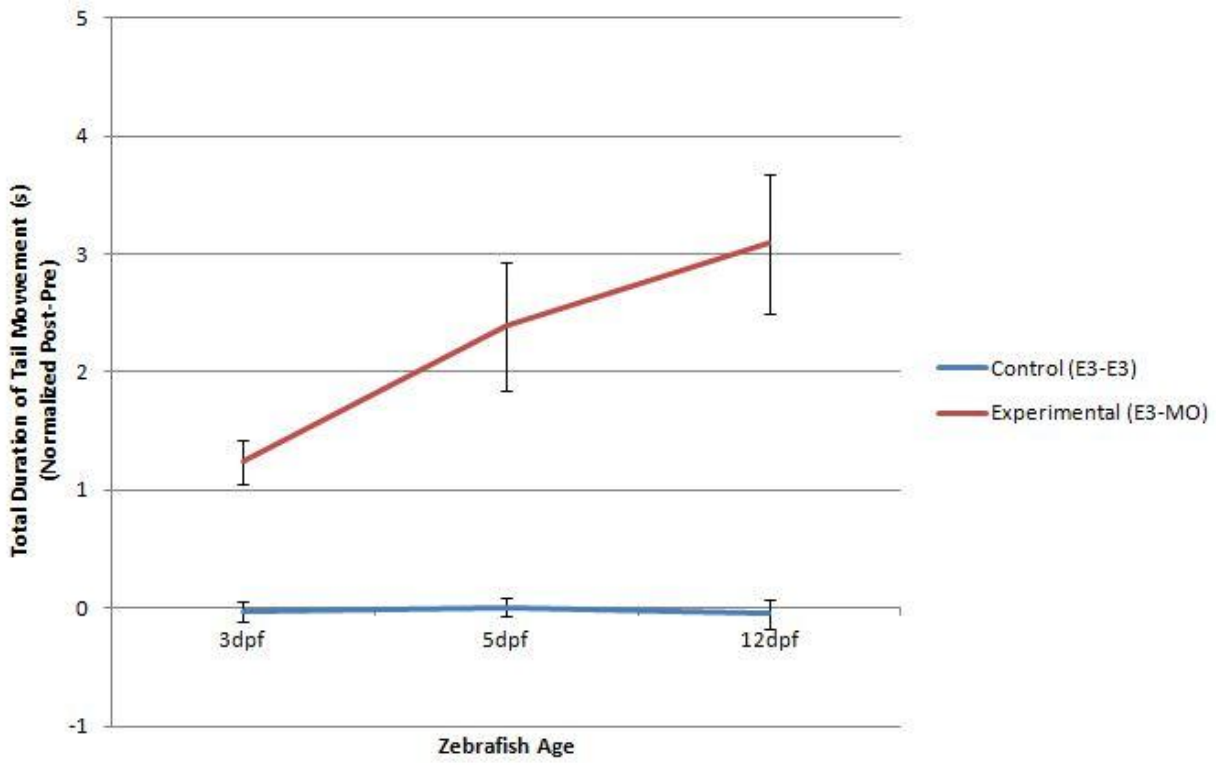


Figure 2. MO enhancement of tail locomotion recapitulated through larval fish development. Larval zebrafish were tested at three different ages: 3, 5, and 12 dpf (n = 8 for 3 dpf group; n = 7 for 5 dpf group; n = 9 for 12 dpf group). Relevant two-way and one-way ANOVAs showed that 10 uM MO enhances locomotion at all ages with a Tukey post-hoc test indicating statistical differences between 3 and 12 dpf ($p < 0.05$), while no statistical differences were observed in any age group only exposed to E3 ($p = 0.95$). Data represented as means ± SEM.

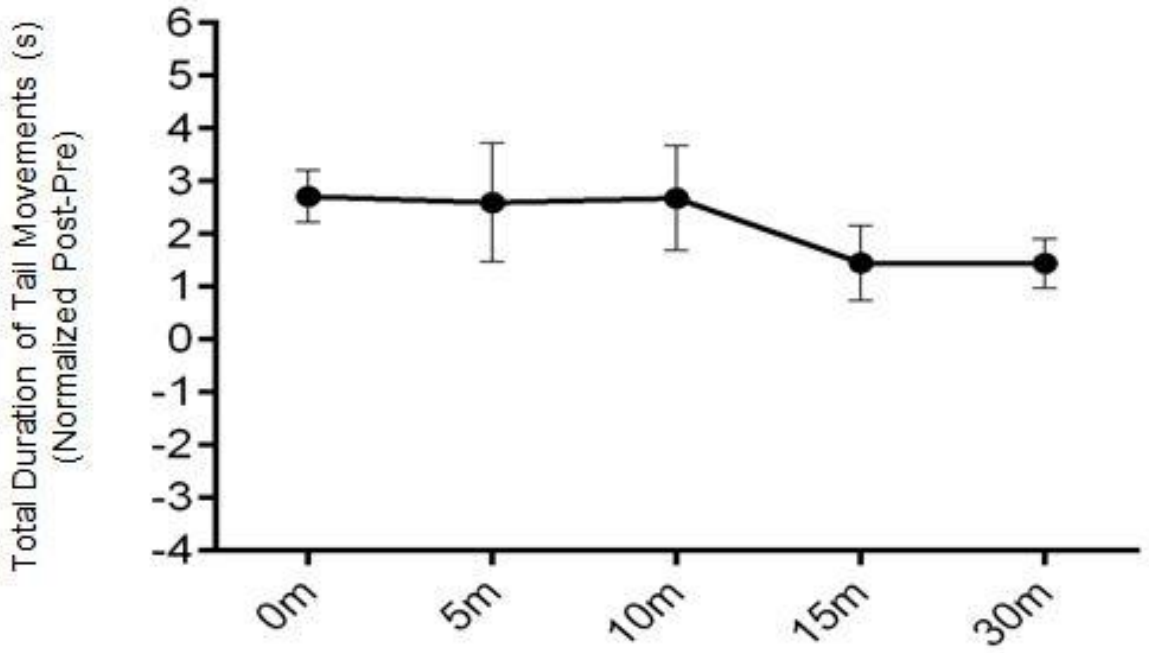


Figure 3. Duration of enhanced MO response without stimulus. Duration of increased tail locomotion in bath persists for no greater than 15 minutes in 5 to 6 dpf semi-restrained larval zebrafish (n = 10 per time point). Normalized means of tail locomotion duration in seconds ± SEM: 0 min, 2.713 ± 0.3995 s; 5 min, 2.595 ± 0.9197 s; 10 min, 2.682 ± 0.7742 s; 15 min, 1.455 ± 1.446 s; 30 min, 0.633 ± 0.421 s.

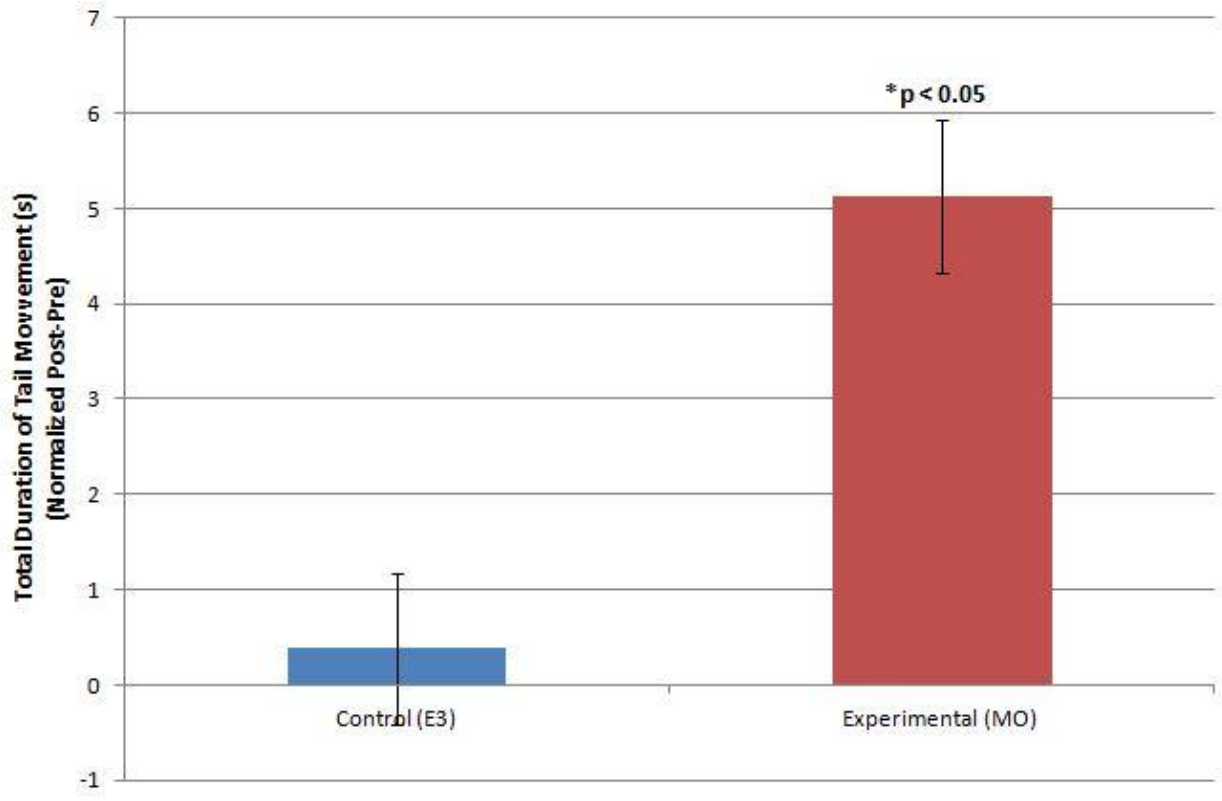


Figure 4. MO-induced short-term sensitization of tail locomotion. Enhanced duration of tail locomotion 3 minutes after MO exposure and removal in 5 to 6 dpf semi-restrained zebrafish (n = 8 per group). Animals exposed to MO demonstrated statistically significant increases in tail locomotion duration as compared to those only exposed to E3 (control solution) alone (MO, 5.13 ± 1.69 s; E3/Control, 0.38 ± 0.80 s; $p < 0.05$). Data represented as means ± SEM.

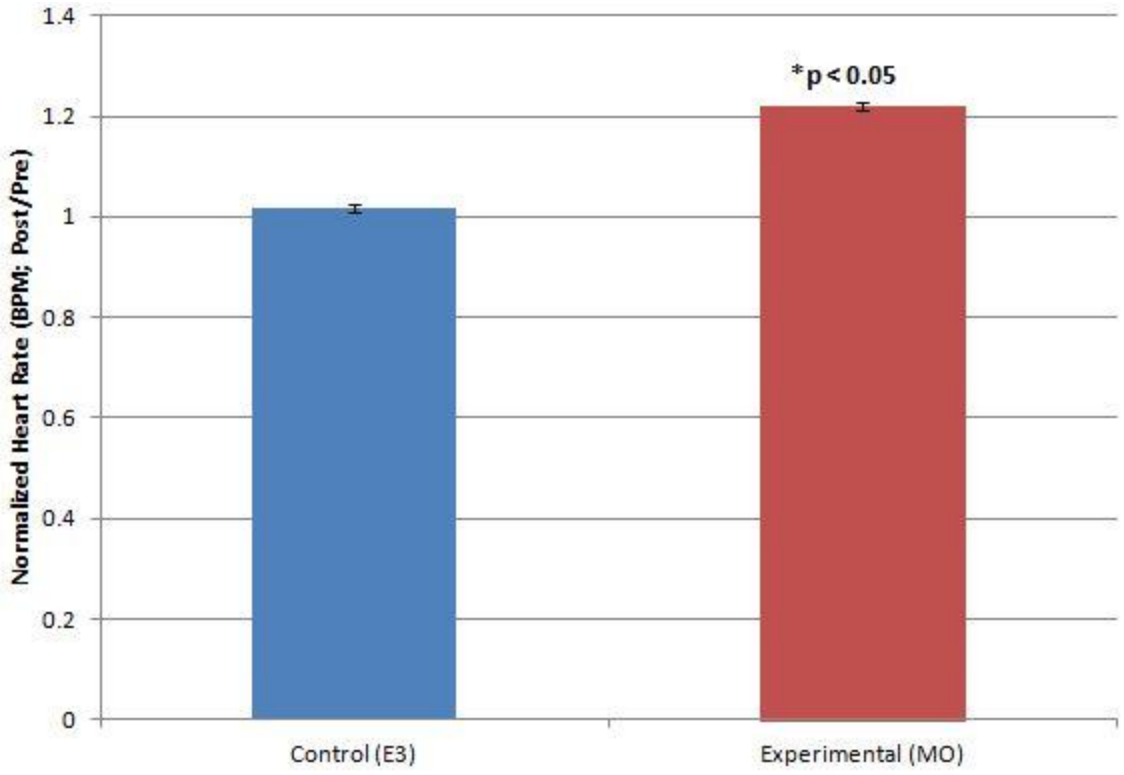


Figure 5. MO induces sensitization of heart rate. Sensitization of heart rate used as a correlate to activation of the autonomic nervous system (n = 8 per group). Semi-restrained animals aged 5 to 6 dpf experienced a significant fold increase in normalized heart rate after MO exposure and washout as compared to animals exposed to E3 (control) solution alone (MO, 1.2193 ± 0.1785 bpm vs. E3/Control, 1.0158 ± 0.0076 bpm; $p < 0.0001$). Data represented as means ± SEM.

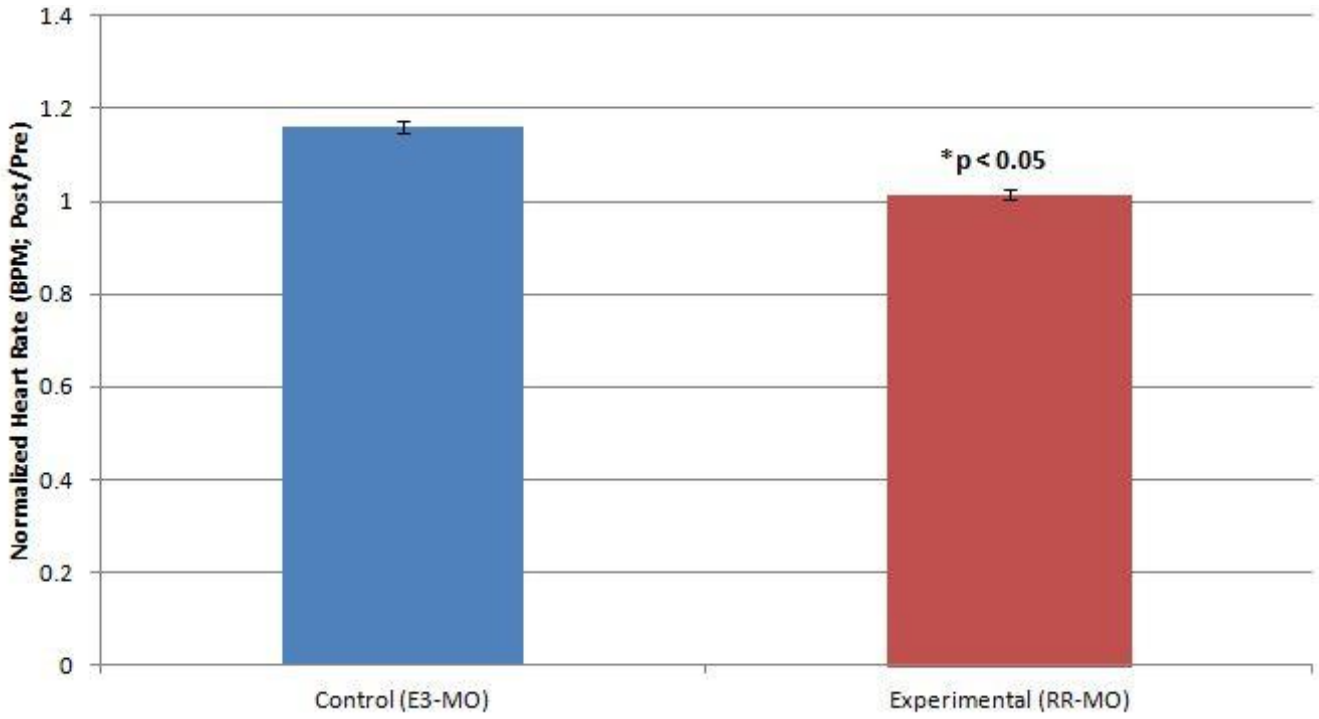
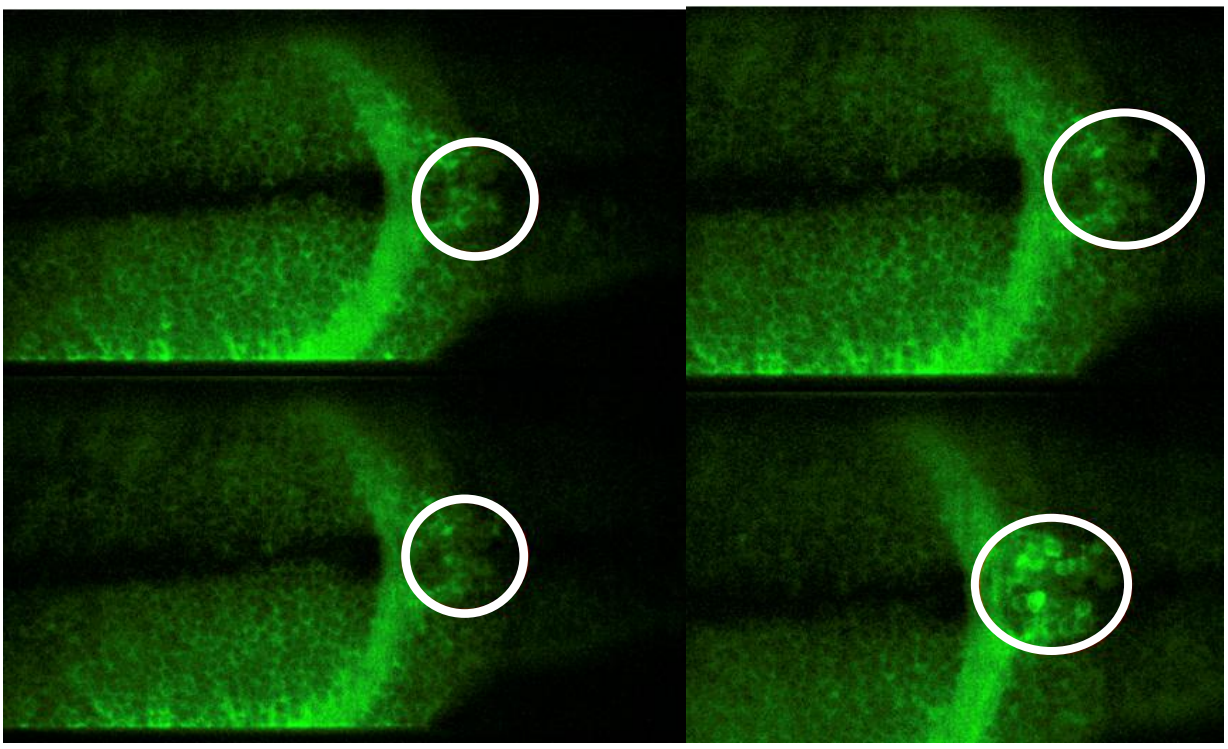


Figure 6. MO-induced sensitization requires TRP channel activation. Utilizing a semi-restrained preparation of 5 to 6 dpf larval zebrafish, we measured fold changes in heart rate in either the presence of MO alone or with ruthenium red, TRPA1 inhibitor (n = 8 per group). We found that MO exposure and washout alone induces sensitization, while ruthenium red with MO significantly reduces the fold increases in heart rate, indicating a role for TRPA1 activation (E3-MO, 1.1602 ± 0.0113 bpm; RR-MO, 1.0137 ± 0.0393 bpm; $p < 0.0001$). Data represented as means ± SEM.



E3 Alone **MO with Washout**

Figure 7. Potential neuronal correlate for the expression of MO-mediated sensitization. Utilizing Tg(elavl3:GCaMP6s) in aged 5 to 6 dpf, restrained larval zebrafish (n = 1 per group), pretest (top panels) and posttest (bottom panels) recordings were taken with all images oriented to rostral on the left-side of each image and caudal on the right-side of each image, and a single time point processed for all images (5 millimeters = 25 microns). Optical density of the circled region was normalized to a nominal fluorescing region with more complexed green presence indicating higher neuronal activity. In doing so, we observed that the post-normalized value after MO exposure and washout shows approximately a 2 fold change in fluorescence; a normalized optical density value (2.14) greater than all other values measured (Pretest E3/control = 1.44 ; Post-test E3/Control = 1.46; Pretest MO with Washout = 1.53).

References

Ahrens, M. B., Orger, M. B., Robson, D. N., Li, J. M., Keller, P. J. Whole-brain functional imaging at cellular resolution using light-sheet microscopy. Nat Meth. 2013;10(5):413-20. doi: 10.1038/nmeth.2434.

Aizenberg M, Schuman EM (2011) Cerebellar-dependent learning in larval zebrafish. J Neurosci 31:8708-8712.

Arrenberg, A. B., Del Bene, F., Baier, H. Optical control of zebrafish behavior with halorhodopsin. PNAS. 2009;106(42):17968-73. doi: 10.1073/pnas.0906252106.

Best, J. D., & Alderton, W. K. (2008). Zebrafish: An in vivo model for the study of neurological diseases. Neuropsychiatric Disease and Treatment, 4(3), 567.

Bouwknecht JA, Hijzen TH, van der Gugten J, Dirks A, Maes RA, Hen R, Geyer MA, Olivier B (2000) Startle responses, heart rate, and temperature in 5-HT1B receptor knockout mice. Neuroreport 11:4097-4102.

Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS (2013) Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499:295-300.

Douglass, A. D., Kraves, S., Deisseroth, K., Schier, A. F., Engert, F. Escape behavior elicited by single, channelrhodopsin-2-evoked spikes in zebrafish somatosensory neurons. Current Biology. 2008;18(15):1133-7. doi: 10.1016/j.cub.2008.06.077.

Dunn TW, Mu Y, Narayan S, Randlett O, Naumann EA, Yang CT, Schier AF, Freeman J, Engert F, Ahrens MB (2016) Brain-wide mapping of neural activity controlling zebrafish exploratory locomotion. Elife 5:e12741.

Goldsmith, P. (2004). Zebrafish as a pharmacological tool: the how, why and when. Curr Opin Pharmacol, 4(5), 504-512. doi: 10.1016/j.coph.2004.04.005

Guo, S. (2004). Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish?. Genes, Brain and Behavior, 3(2), 63-74.

Knafo, S., & Wyart, C. (2015). Optogenetic neuromodulation: New tools for monitoring and breaking neural circuits. Ann Phys Rehabil Med. doi: 10.1016/j.rehab.2015.05.001

Kohashi, T., & Oda, Y. (2008). Initiation of Mauthner-or non-Mauthner-mediated fast escape evoked by different modes of sensory input. The Journal of Neuroscience, 28(42), 10641-10653.

Kokel, D., Cheung, C. Y., Mills, R., Coutinho-Budd, J., Huang, L., Setola, V., . . . Peterson, R. T. (2013). Photochemical activation of TRPA1 channels in neurons and animals. Nat Chem Biol, 9(4), 257-263. doi: 10.1038/nchembio.1183

Prober, D. A., Zimmerman, S., Myers, B. R., McDermott, B. M., Jr., Kim, S. H., Caron, S., Schier, A. F. Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function. J Neurosci. 2008;28(40): 10102-10110. doi:10.1523/JNEUROSCI.2740-08.2008.

Randlett O, Wee CL, Naumann EA, Nnaemeka O, Schoppik D, Fitzgerald JE, Portugues R, Lacoste AM, Riegler C, Engert F, Schier AF (2015) Whole-brain activity mapping onto a zebrafish brain atlas. Nat Methods 12:1039-1046.

Roberts, A. C., Bill, B. R., Glanzman, D. L. Learning and memory in zebrafish larvae. Front. Neural Circuits. 2013;7:126. doi:10.3389/fncir.2013.00126.

Roberts, A. C., Pearce, K. C., Choe, R. C., Alzagatiti, J. B., Yeung, A. K., Bill, B. R., Glanzman, D. L. Long-term habituation of the C-start escape response in zebrafish larvae. Neurobiology of Learning and Memory. 2016;134:360-8. doi: 10.1016/j.nlm.2016.08.014.

Roberts, SC, Reichl, J, Song, MY, Dearinger, AD, Moridzadeh, N, Lu, E, Pearce, K, Esdin, J and Glanzman, D (2011). Habituation of the C-Start Response in Larval Zebrafish Exhibits Several Distinct Phases and Sensitivity to NMDA Receptor Blockade. PLoS ONE 6: e29132.

Valente, A., Huang, K. H., Portugues, R., & Engert, F. (2012). Ontogeny of classical and operant learning behaviors in zebrafish. Learn Mem, 19(4), 170-177. doi: 10.1101/lm.025668.112

Vincent, F; Duncton, MA (2011). "TRPV4 agonists and antagonists". Curr Top Med Chem. 11 (17): 2216–26. doi:10.2174/156802611796904861. PMID 21671873.

Wolman MA, Jain RA, Liss L, Granato M (2011) Chemical modulation of memory formation in larval zebrafish. Proceedings of the National Academy of Sciences of the United States of America 108: 15468–15473.