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Los Angeles

Noxious Stimuli-Induced Sensitization of Thigmotaxis in Larval Zebrafish

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Duy Truong Ly

2018

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ABSTRACT OF THE THESIS

Noxious Stimuli-Induced Sensitization of Thigmotaxis in Larval Zebrafish

by

Duy Truong Ly

Master of Science in Physiological Science

University of California, Los Angeles, 2018

Professor David L. Glanzman, Chair

Chronic pain and anxiety disorders are known to be intimately connected in humans. Occasionally, the sensation of pain and the associated emotional affects like anxiety linger past the end of the triggering stimulus or even in the absence of a trigger. Central sensitization has been posited as a simple form of memory that may underlie the persistence of idiopathic disorders like fibromyalgia. In the present study, we examined the effect of a known chemical irritant, allyl isothiocyanate (mustard oil), and electric shock on freely swimming zebrafish larvae (5 and 12 DPF). We found that mustard oil (MO, concentration = 10μM) and electric shock induced thigmotaxis, an anxiety-related behavior. Future work should determine the specific changes induced by MO or shock in the behavioral circuits that mediate swimming in the zebrafish. The results of this future work may contribute to our understanding of the neural basis of pain and anxiety disorders.

The thesis of Duy Truong Ly is approved.

Peter M. Narins

Mark Arthur Frye

David L. Glanzman, Committee Chair

University of California, Los Angeles

2018

Dedicated to my family in the United States and Vietnam,

For allowing me to stand on your shoulders

And see into the beyond

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I would like to first acknowledge my advisor, Dr. David Glanzman, for impressing the wonders of neuroscience on me. After taking his class on the neurobiology of learning and memory, I developed a deep fascination for understanding the complexity of the nervous system in biological terms. Importantly, he provided me with the independence to explore my research interests and tempered my naïve enthusiasm with valuable critique. With the laboratory resources and intellectual freedom at my disposal, I made great strides in my ability to think critically and overcome obstacles with my own ingenuity.

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Also, I would like to extend gratitude to Dr. Peter Narins and Dr. Mark Frye for their wonderful critique as members of my master's thesis committee. Their insightful remarks provided a much-needed outside perspective on the possible interpretations of my research.

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friendships with Kim and Gaby, as they positively shaped my formative experiences as a new researcher and teacher.

Most importantly, I want to thank my parents and relatives for their continuous support through the years. As the first person to enter a four-year university in the United States in my extended family, I received the utmost care and attention from family members who prioritized my success above their own. Although I can never repay their love and kindness, I will pay it forward to the siblings and cousins who follow in my footsteps.

I came into the Physiological Science MS program unsure of a career path to pursue, but I am leaving it with a burning passion for neuroscience and academia. I truly believe I have found a career that can inspire my lasting devotion and ensure my long-term happiness.

Introduction

Investigations of learning and memory may provide significant insight into the mechanisms of persistent pain and anxiety disorders. Central sensitization induced by a noxious painful stimulus shares many commonalities with the neural mechanism of LTP, which is required for various forms of learning in mammals (Ji et al. 2003). Sensitization of amygdala neurons due to neuropathic pain has been shown to induce anxious behavior in rats (Jiang et al. 2014). In addition, pain and emotional affects may possess a reciprocal relationship with the amygdala acting as the key interaction site (Neugebauer et al. 2004). The neural circuits mediating the interaction between pain and emotional states are not fully understood but using zebrafish as a model organism for such studies may prove fruitful.

In recent times, zebrafish have demonstrated significant value for studying the effects of drugs (Goldsmith 2004), neurological diseases (Best et al. 2008), and learning and memory (Roberts et al. 2013). Additionally, zebrafish have unique experimental advantages for neurobiological investigations of behavior. They possess relatively simple neuronal circuits despite being a vertebrate organism; and the larvae are translucent, which facilitates optogenetic manipulation (Knfao and Wyart 2015). Previously, larval zebrafish have demonstrated the capacity for different forms of learning, including habituation (Roberts et al. 2011) and classical conditioning (Hinz et al. 2013).

In the present study, we examined whether allyl isothiocyanate (mustard oil, MO), a natural chemical irritant that has been confirmed to activate TRP1A (transient receptor potential) receptors in larval zebrafish (Prober et al. 2008), or electric shock can induce behavioral sensitization of thigmotaxis, or wall-hugging behavior in the open field test. Thigmotaxis behavior was shown to be a behavioral indicator of anxiety and attenuated by the anxiolytic drug

diazepam (Schnorr et al. 2012). We found that MO, at a concentration of 10 µM, induced sensitization of thigmotaxis. Interestingly, 100 µM MO did not induce sensitization. To explore the role of neuromodulatory systems in learning, we tested the necessity of dopamine in this learning paradigm. Dopamine had previously been shown to modulate anxiety in zebrafish (Kacprzak et al. 2017). We found that a bath application of Haloperidol (HAL), a high-affinity D2 receptor antagonist, at a concentration of 20 µM, did not affect the sensitization of thigmotaxis.

We also investigated whether an electric shock stimulus could induce behavioral sensitization of thigmotaxis. We distinguished induction from sensitization since we examine the behavior a certain amount of time after the end of the stimulus. Electric shock had been used previously in studies of sensitization for invertebrates (Walters 1987) and vertebrates (Pilz et al. 1999). We demonstrated that a single electric shock to the water bath induced sensitization of thigmotaxis in larval zebrafish and that a competitive NMDAR antagonist, D-APV, blocked the induction of thigmotaxis.

Methods

Animals

The zebrafish (*Danio rerio*, wild type TL strain), at 5 or 12 days post fertilization (DPF), was used in all experiments. Using standard methods, we cared for and maintained the adult fish at the UCLA Zebrafish Core Facility. The adults were bulk mated and embryos were collected the next morning. Embryos were placed into a Petri dish (density of approximately 80 embryos per dish) containing E3 medium for zebrafish embryos (5mM NaCl, 0.33 mM $MgCl_2$, 0.33 mM $CaCl_2$, 0.17 mM KCl, 10-5% methylene blue, pH. 7.2) and kept in a darkened incubator at

28.5°C for 5 days. The embryo medium (E3) was changed daily, and sick or dead embryos were removed from the Petri dish. All experimental procedures were approved by the Chancellor's Animal Research Committee (ARC) of the University of California, Los Angeles.

Behavioral Recording and Training

MO-Induced Sensitization of Thigmotaxis Paradigm

Larvae fish were randomly selected and plated in small dishes (20 per dish) filled with 12 mL of embryo medium (E3). Plates of freely swimming fish were placed side by side and left to acclimate for one hour (Figure 1).

After acclimation, fish received either a vehicle solution of E3 or drug (MO). The final concentration of MO in the 12 mL dish was 1 μM, 10 μM, or 100 μM MO. After washout of MO, the fish were transferred to a larger plate and allowed to acclimate for 30 minutes before analysis of thigmotaxis. For analysis, the distance of all 20 fish to the plate edge in millimeters (mm) was averaged for one n, or data point (Figure 2).

To assess the effect of dopaminergic neuromodulation on sensitization of thigmotaxis, fish were initially left to acclimate in E3, DMSO, or Haloperidol (HAL) before receiving the dose of vehicle solution or MO.

Shock-Induced Sensitization of Thigmotaxis Paradigm

Larvae fish were randomly selected and plated in medium-sized petri dishes (20 per dish) filled with 100 mL of E3. Electrodes A and B were placed on opposite ends of the petri dish (Figure 8). Plates of freely swimming fish were placed side by side and left to acclimate for one hour.

After acclimation, a pretest recording of thigmotaxis was taken at t = -10 seconds relative to the shock stimulus. At t = 0 seconds, a one-second duration 60V, 1 Hz electric shock was applied across electrode A and B for the experimental group. The control group did not receive a shock. The post-test recording examined thigmotaxis at t = 15, 30, 45, and 60 seconds after the shock (Figure 9).

To assess the effect of an NMDAR antagonist on sensitization of thigmotaxis, fish were initially left to acclimate in E3 or D-APV before receiving the shock stimulus.

Drug Treatment

Drugs were obtained from Sigma (St. Louis, MO). Allyl isothiocyanate (MO), haloperidol (HAL), and D-APV were dissolved in control medium (E3). In experiments using MO, larvae were placed into petri dishes containing the control medium (E3) or HAL before the drug was added in the appropriate concentration to the dish following acclimation. In experiments using shock, larvae were placed into petri dishes containing the control medium (E3) or D-APV before the shock was applied.

Statistical Analysis

Data were analyzed for significant differences using unpaired Student's T-tests, One-Way ANOVAs, and Two-Way Repeated Measures ANOVAs. Tukey's HSD tests were used for post-hoc analysis of significant ANOVAs.

Results

To determine if mustard oil could induce behavioral sensitization of thigmotaxis, we first assessed the time course of thigmotaxis exhibited by 5 DPF larvae, which are exposed to 10 µM MO for 30 seconds before washout of the drug (Figure 3). The MO group exhibited high thigmotaxis compared to that of the control group at 30 minutes after washout, prompting us to replicate the result by only assessing thigmotaxis at the 30-minute time point. Indeed, MO induced significantly higher thigmotaxis in 5 DPF larvae than control (E3) solution (Figure 4). To compare behavioral sensitization of thigmotaxis across different ages, we assessed MO-induced sensitization of thigmotaxis in older 12 DPF larvae. Similarly, MO also induced significant sensitization of thigmotaxis in the older fish (Figure 5).

Next, we began characterizing other parameters of MO-induced sensitization including the effectiveness of different MO concentrations and whether the learning required dopaminergic neuromodulation. Using the same previous experimental paradigm, we observed that MO concentrations of 1 and 5 µM were insufficient to induce sensitization of thigmotaxis. Interestingly, the MO concentration of 100 µM also failed to induce sensitization (Figure 6). After acclimating the fish in the high-affinity D2 receptor antagonist, haloperidol (HAL), we performed the same experimental protocol and observed that a bath application of 20 µM HAL did not significantly affect MO-induced sensitization of thigmotaxis (Figure 7). A 20 µM concentration of HAL was determined to be non-lethal and modulated larval locomotion through bath application (Irons et al. 2013).

Finally, we examined whether electric shock applied to the water bath could induce sensitization of thigmotaxis. A single electric shock of 60-V to the water bath induced sensitization of thigmotaxis in 5 DPF larvae (Figure 10). When we performed the same

experimental paradigm with older 12 DPF larvae, the shock was insufficient at inducing sensitization of thigmotaxis (Figure 11). To investigate the molecular mechanisms of shock-induced sensitization of thigmotaxis, we acclimated fish in a bath application of 200 μM D-APV before applying the shock stimulus. D-APV significantly blocked the induction of thigmotaxis (Figure 12).

Discussion

Our results demonstrated that a single trial of noxious stimulus could induce behavioral sensitization of thigmotaxis in larval zebrafish. A chemical irritant like mustard oil, which activates the TRP1A receptors in zebrafish, and electric shock were both capable of inducing the behavior. We observed that the MO concentration of 100 μM failed to induce sensitization of thigmotaxis. There may be two possible explanations: the high concentration of mustard oil was impairing fish health and the ability to display thigmotaxis behavior, or the high concentration may be inducing receptor desensitization of the TRP1A receptors (Akopian et al. 2007). Although haloperidol, a high-affinity D2 receptor antagonist, did not block the MO-induced sensitization of thigmotaxis, it appears that dopamine signaling is still important for the expression of anxiety-related behaviors in zebrafish (Kacprzak et al. 2017). Future work should examine the specific neural circuits which incorporate dopamine or other neuromodulators to understand the basis of anxious behavior.

One alternative interpretation of our shock-induced thigmotaxis data is that the shock facilitated active place avoidance learning in the larvae. However, we believe this is unlikely due to the lack of significant shock-induced thigmotaxis in 12 DPF larvae, which have greater capacity for learning than 5 DPF larvae. After demonstrating that electric shock could induce

sensitization of thigmotaxis, we attempted a pharmacological dissection of the molecular mechanisms mediating the sensitization. D-APV, a potent NMDAR antagonist, was able to block the induction of thigmotaxis. However, we are uncertain whether the D-APV blocked the sensitization of thigmotaxis or just the sensory input of the electric shock stimulus. Future experiments with specific administration of D-APV in certain brain regions can determine the true explanation.

The study presented here may prove significant for future investigations in the neural basis of pain and anxiety disorders. We have demonstrated the induction of an anxiety-related behavior using a noxious stimulus and a simple experimental paradigm. The behavioral sensitization observed in the study here will be relevant for the study of persistent pain, which shares mechanistic commonalities with memory (Woolf 2011). Indeed, zebrafish has begun to attract attention as a model for nociception studies (Malafoglia et al. 2013). Furthermore, future experiments can examine the specific changes in the neural circuitry mediating this behavioral expression of anxiety. Larval zebrafish, which are translucent and possess a simple neural circuitry, provide the significant advantage of visualizing whole-brain neural circuit activity using in vivo optical imaging. For future studies, researchers should examine the amygdala (Perathoner et al. 2016) or habenula (Mathuru and Jesuthasan 2013) for sites of neural plasticity that mediate the motor program of thigmotaxis. The mechanistic insights gathered from such studies will be significant for our understanding of anxiety in humans.

Figure Legends

Figure 1: Experimental Timeline for MO-Induced Sensitization of Thigmotaxis.

Protocol for Sensitization of Thigmotaxis. Acclimation period of 1 hour followed by an application of either vehicle (E3) or drug (MO). Washout of drug is performed for 1 minute. Analysis of thigmotaxis is conducted 30 min after washout.

Figure 2: Quantification of Thigmotaxis.

Quantification of Thigmotaxis. The formula is used to identify distance of each individual fish from the plate edge in millimeters (mm). The distance is averaged across 20 fish to obtain one n, or data point, per plate.

Figure 3: Time Course of MO-Induced Sensitization of Thigmotaxis in 5 DPF Zebrafish.

Time Course of Sensitization for Control (E3) vs. MO (10µM) groups (n=10 per group). There was a significant interaction between the variables of group vs time (*p = 0.015) with the Greenhouse-Geisser Correction.

Figure 4: MO-Induced Sensitization of Thigmotaxis in 5 DPF Zebrafish.

Control (E3) vs. MO (10 µM) Thigmotaxis. Mean ± SEM distance to edge in each group (n = 8 per group). The mean distance from edge was significantly less in the MO group compared to the Control (**p = 0.0044) based on an unpaired Student's T-test.

Figure 5: MO-Induced Sensitization of Thigmotaxis in 12 DPF Zebrafish.

Control (E3) vs. MO (10 µM) Thigmotaxis. Mean ± SEM distance to edge in each group (n = 8 per group). The mean distance from edge was significantly less in the MO group compared to the Control (*p = 0.0135) based on an unpaired Student's T-test.

Figure 6: Dose-Response Comparison of MO-Induced Sensitization of Thigmotaxis in 5 DPF Zebrafish.

Thigmotaxis in Control (E3), MO (1 µM), MO (10 µM), and MO (100 µM). Mean ± SEM distance from edge in each group (n = 10 per group). There was a significant difference across groups (p = 0.0021) according to a One-Way ANOVA. A Post-Hoc test with Tukey's HSD showed a significant difference between Control (E3) and MO (10 µM) (*p < 0.05).

Figure 7: Effect of HAL on MO-Induced Sensitization of Thigmotaxis in 5 DPF Zebrafish.

Effect of Haloperidol (HAL) on Sensitization of Thigmotaxis. The concentrations of MO and HAL were 10 µM and 20 µM, respectively. The mean distance to edge across groups was significantly different (p = 0.003). A Post-Hoc test with Tukey's HSD showed a significant difference between HAL-E3 and HAL-MO groups (*p < 0.05).

Figure 8: Experimental Setup for Shock-Induced Sensitization of Thigmotaxis.

Two electrodes were placed on opposite ends of a 150-mm diameter petri dish. A 60-V electric shock is applied across Electrode A and B.

Figure 9: Experimental Timeline for Shock-Induced Sensitization of Thigmotaxis.

Protocol for Sensitization of Thigmotaxis. Acclimation period of 1 h followed by no shock for the Control group and a 60-V shock for the Shock group. The pretest recording is t = -10 seconds relative to the shock. The post-test recording is at t = 10, 30, 45, and 60 seconds.

Figure 10: Shock-Induced Sensitization of Thigmotaxis in 5 DPF Zebrafish.

Control vs Experimental (Shock) Thigmotaxis. Mean ± SEM distance to edge in each group (n = 8 per group). The mean distance from edge was significantly less in the Shock group compared to the Control (*p = 0.014) based on a Two-Way Repeated Measures ANOVA.

Figure 11: Shock-Induced Sensitization of Thigmotaxis in 12 DPF Zebrafish.

Control vs Experimental (Shock) Thigmotaxis. Mean ± SEM distance to edge in each group (n = 8 per group). There was no significant difference in the mean distance from edge in the Shock group compared to the Control (p = 0.451) based on a Two-Way Repeated Measures ANOVA.

Figure 12: Effect of D-APV on Shock-Induced Sensitization of Thigmotaxis in 5 DPF Zebrafish

Control vs Experimental (Shock) Thigmotaxis. Mean ± SEM distance to edge in each group (n = 5 per group). The concentration of D-APV was 200 µM. The mean distance from edge was significantly less in the Shock Alone group (**p = 0.002) based on a Two-Way Repeated Measures ANOVA.

Figure 1

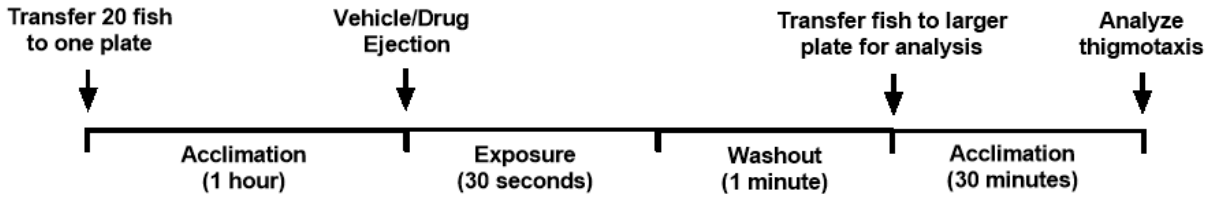
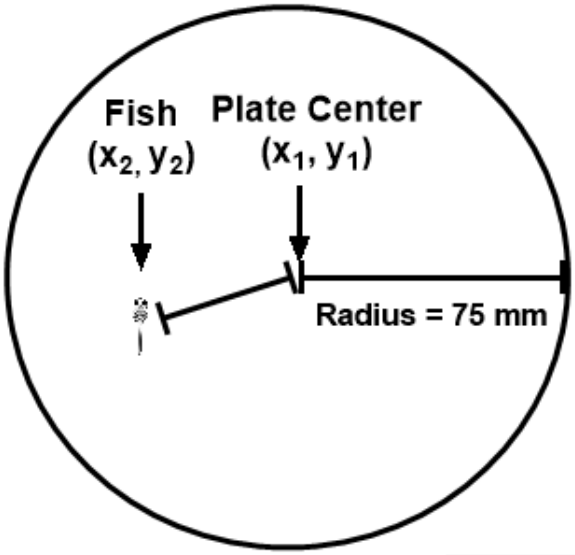

Transfer 20 fish to one plate
Vehicle/Drug Ejection
Transfer fish to larger plate for analysis
Analyze thigmotaxis
Acclimation (1 hour)
Exposure (30 seconds)
Washout (1 minute)
Acclimation (30 minutes)

Figure 2



Distance from Edge: $75 - \sqrt{(x2 - x1)^2 + (y2 - y1)^2}$

Figure 3

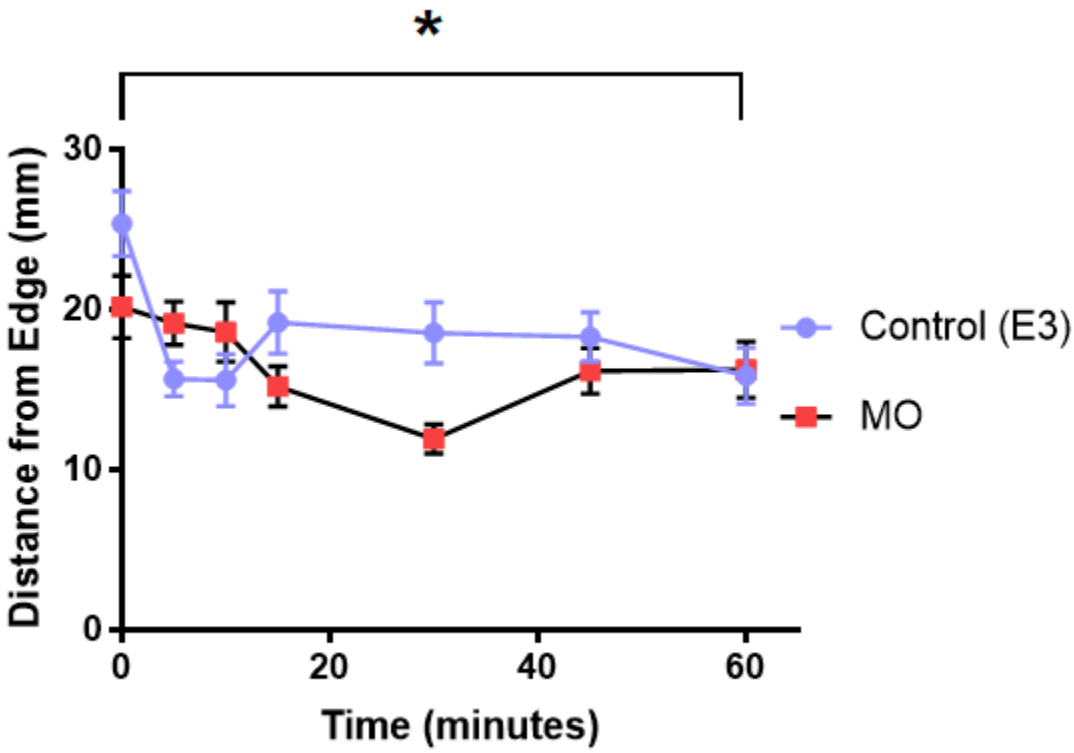

*
Distance from Edge (mm)
30
20
10
0
0
20
40
60
Time (minutes)
Control (E3)
MO

Figure 4

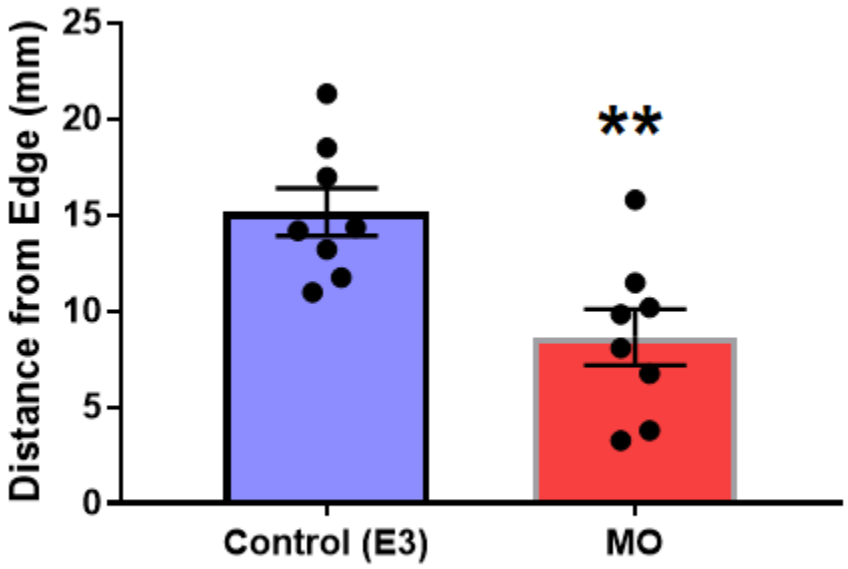


Figure 5

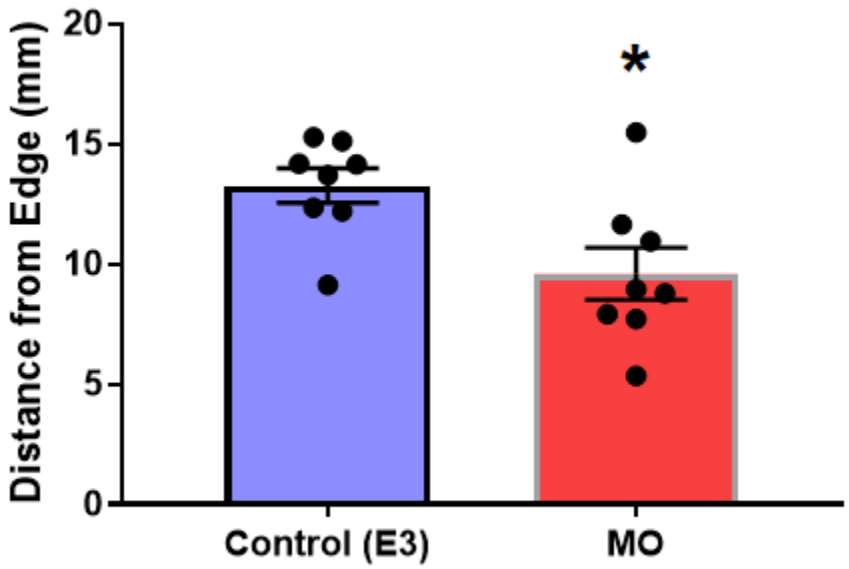


Figure 6

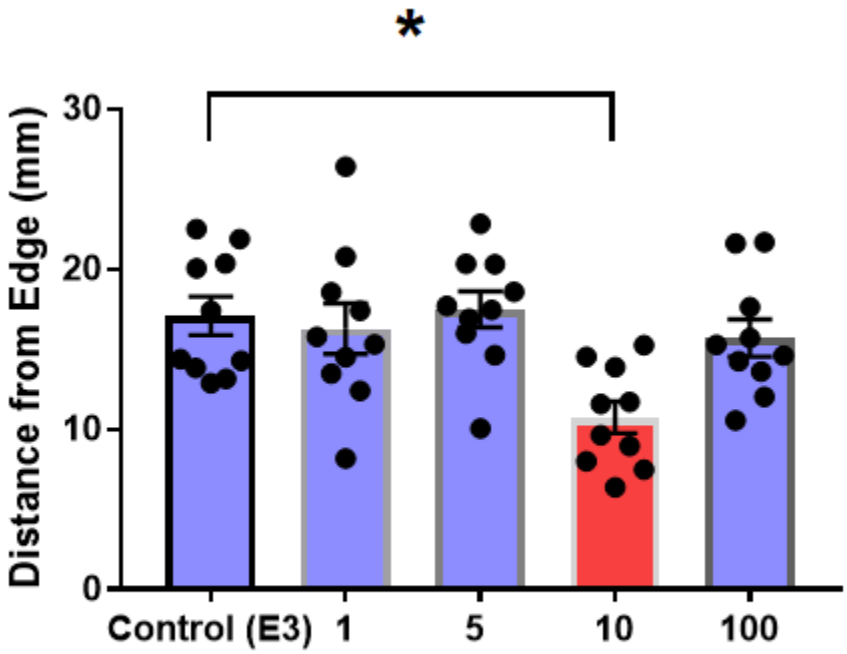


Figure 7

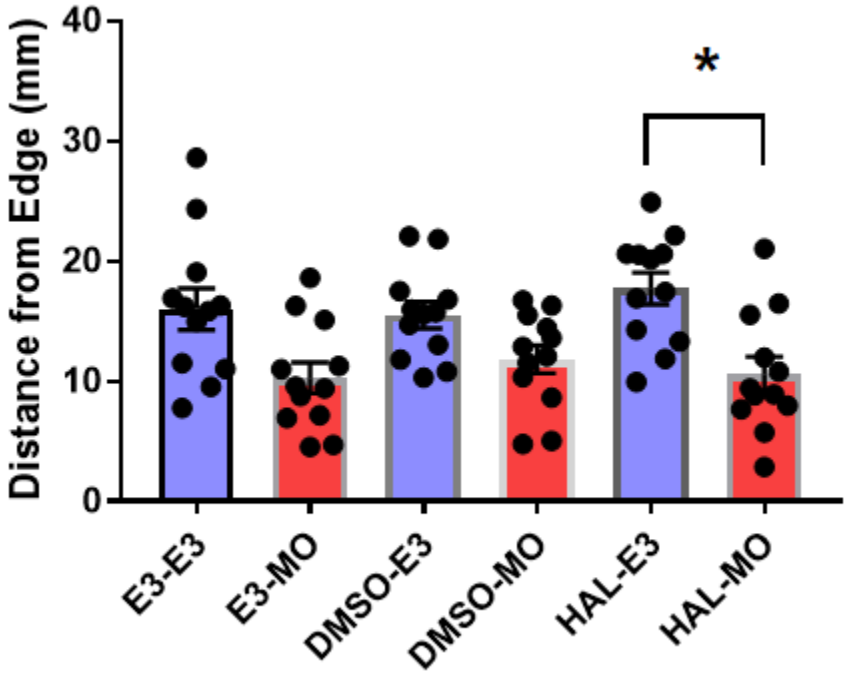

Distance from Edge (mm)
40
30
20
10
0
*
E3-E3
E3-MO
DMSO-E3
DMSO-MO
HAL-E3
HAL-MO

Figure 8

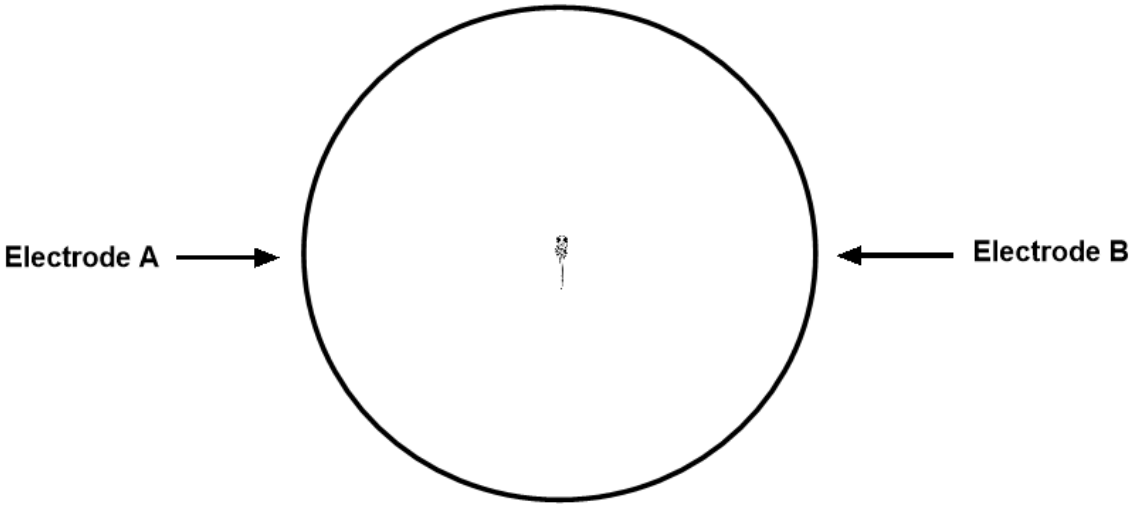


Figure 9

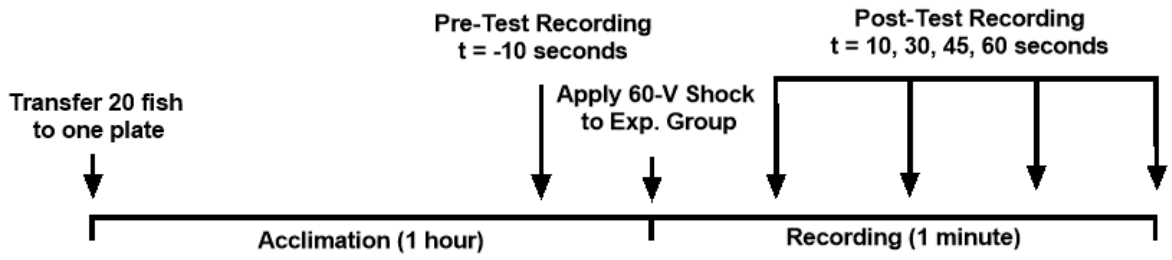

Pre-Test Recording
t = -10 seconds
Post-Test Recording
t = 10, 30, 45, 60 seconds
Transfer 20 fish
to one plate
Apply 60-V Shock
to Exp. Group
Acclimation (1 hour)
Recording (1 minute)

Figure 10

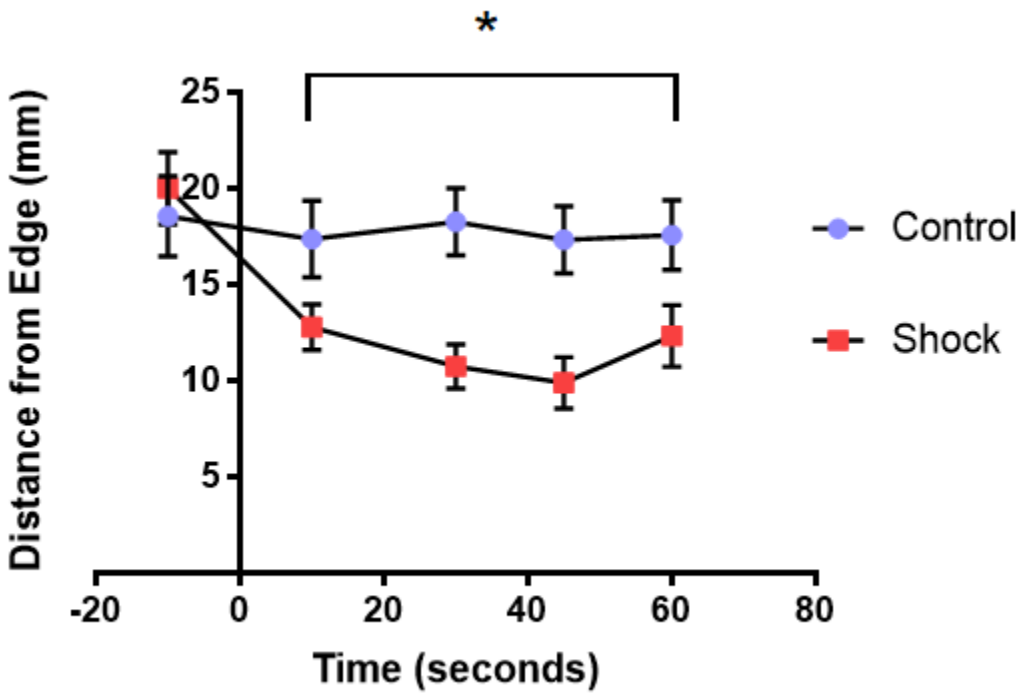

*
Distance from Edge (mm)
25
20
15
10
5
-20
0
20
40
60
80
Time (seconds)
Control
Shock

Figure 11

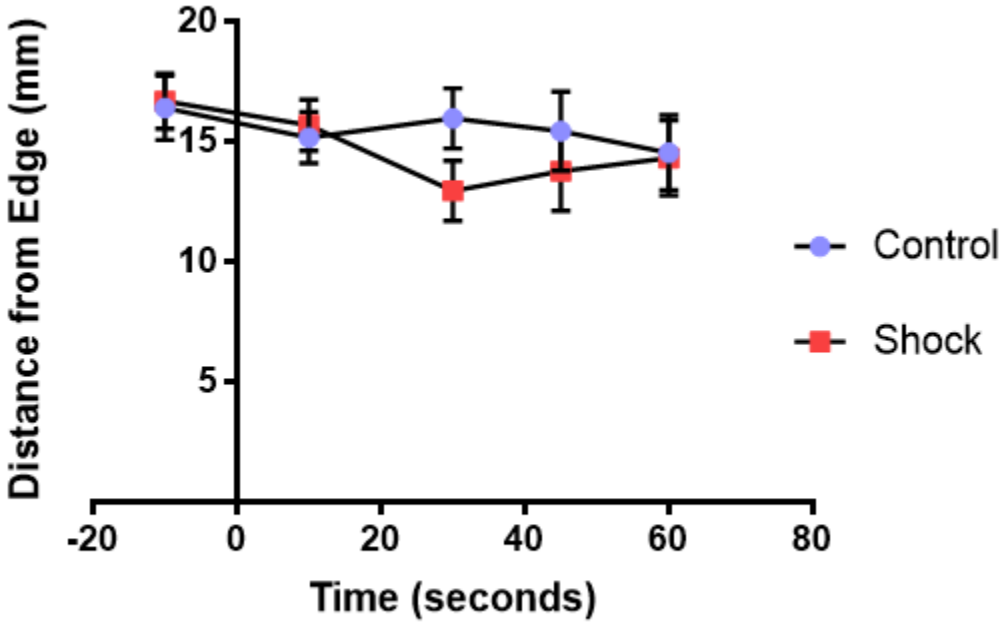
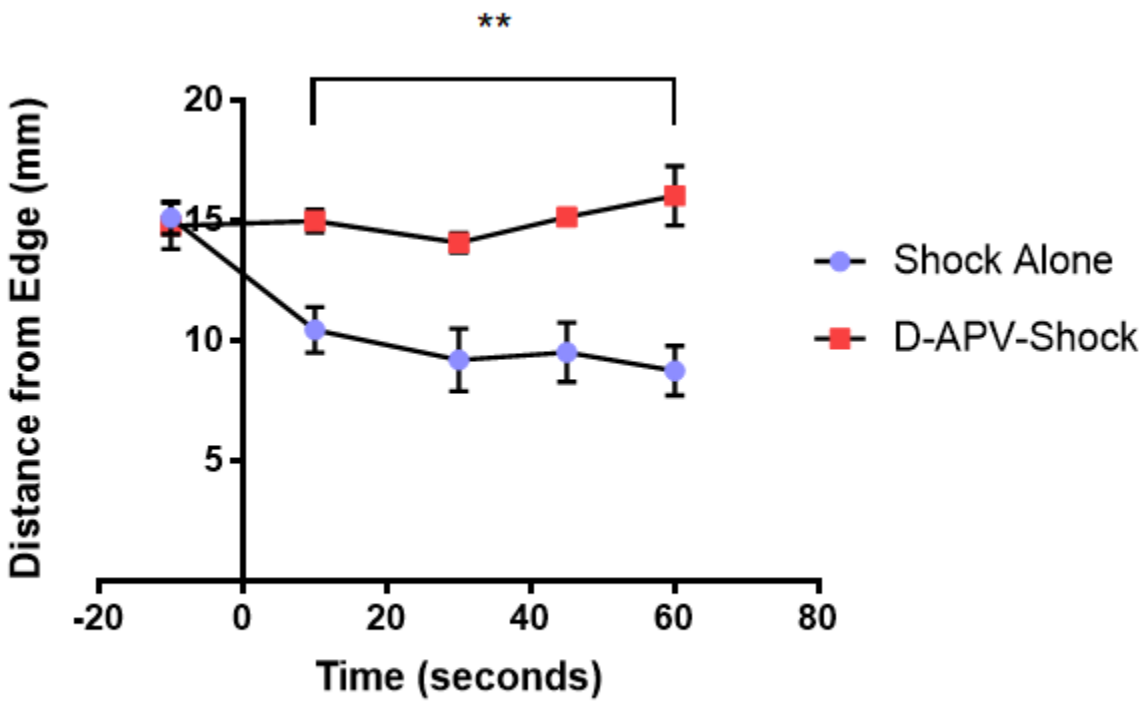

Distance from Edge (mm)
20
15
10
5
-20
0
20
40
60
80
Time (seconds)
Control
Shock

Figure 12


**
Distance from Edge (mm)
20
15
10
5
-20
0
20
40
60
80
Time (seconds)
Shock Alone
D-APV-Shock

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