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Allyl Isothiocyanate-Induced Rapid Sensitization of Heart Rate in Larval Zebrafish (*Danio rerio*)

A thesis submitted in satisfaction of the requirements for the degree Master of Science in

Physiological Science

by

Asif Al Razee

2019

ABSTRACT OF THE THESIS

Allyl Isothiocyanate-Induced Rapid Sensitization of Heart Rate in Larval Zebrafish (*Danio rerio*)

by

Asif Al Razee

Master of Science in Physiological Science

University of California, Los Angeles, 2019

Professor David L Glanzman, Chair

Among the most protuberant behaviors during early developmental stages in the zebrafish is changes in heart rate. In particular, past studies demonstrated behavioral sensitization by producing a range of behavioral modifications and often activating the autonomic nervous system. Previous work has shown that allyl isothiocyanate (AITC or mustard oil, MO) most likely elicits its sensitization effects through activation of TRP channels expressed on the trigeminal and Rohon Beard sensory neurons in larval zebrafish. To determine if AITC exposure activates the sympathetic nervous system in larval zebrafish, we used heart rate as a proxy for the activation of the autonomic nervous system. To confirm that our behavioral sensitization was induced by activation of TRP channels, we used Ruthenium Red (RR), which was previously shown to antagonize these receptors in zebrafish. In the present study, we found that 10 µM total bath concentration of AITC significantly increased the heart rate activity in 5-day post fertilization (dpf), agarose-restrained larval zebrafish compared to control treated animals. On the other hand, 10 µM total bath concentration of RR exposure prior to 10 µM AITC exposure significantly decreased the heart rate activity, whereas, the equal concentration RR exposure after the AITC exposure had no suppressive effect on AITC induced increased heart rate in 5

DPF, agarose-restrained larval zebrafish. Further, we used behavioral pharmacology to dissect the molecular underpinnings of the sensitization memory. To determine whether the different neural circuits contributing to enhanced heart rate depend upon one or many different neuromodulators and ascertain how these neuromodulators influence distinct neural circuits we have conducted various extracellular receptor blockade experiments. We used D-APV, a competitive NMDAR antagonist, MK801, a non-competitive NMDAR antagonist, Methiothepin, a selective serotonin receptor antagonist, Haloperidol, a dopamine D2 receptor antagonist, Mecamylamine, a non-competitive nicotinic acetylcholine receptor antagonist, Propranolol, a non-selective beta-adrenergic receptor blocker and Atropine, a non-selective muscarinic acetylcholinergic antagonist. We discovered that AITC induced sensitization is accompanied by increase in heart rate, which implicates serotonin, dopamine receptors as well as β-adrenergic, nicotinic and muscarinic acetylcholine receptors from cardiac autonomic nervous system, but NMDA receptors don't seem to play any role in this short term memory. Together, these results indicate the enormous potential that zebrafish hold as an animal model for the study of learning and memory, especially non-associative memory.

The thesis of Asif Al Razee is approved.

Mark Arthur Frye

Mansoureh Eghbali

David L Glanzman, Committee Chair

University of California, Los Angeles

2019

DEDICATION

This thesis is dedicated to my family, specially my parents, spouse and children for being

supportive of my academic endeavors.

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INTRODUCTION

Both associative and non associative learning leading to procedural/ nondeclarative memory formation in invertebrates and vertebrates have been documented in situ. Researchers have demonstrated a form of non-associative memory, sensitization, an enhanced behavioral response due to aversive or arousing stimuli in diverse organisms (Cai et al., 2012; Carew et al., 1971; Duerr and Quinn, 1982; Fendt et al., 1994; Koch, 1999; Krasne and Glanzman, 1986; Groves et al., 1969; Rankin et al., 1990; Thompson and Spencer, 1966; Watkins et al., 2010). Particularly the invertebrate *Aplysia californica* served as a great study model for various non-associative learning and memory, such as long-term sensitization and long-term habituation of gill and siphon withdrawal reflex (Bailey and Chen 1983). The advantage of using *Aplysia californica* as a model organism is the rapid behavioral screening through easily visible motor movements as well as it's massive neurons for in-vitro study. By manipulating these advantages researches have made remarkable progress toward understanding biochemical correlates of behavioral sensitization (Byrne and Hawkins, 2015; Cleary and Byrne, 1993; Glanzman et al., 1990; Glanzman et al., 1989; Hegde et al., 1997; Hu et al., 2015; Kaang et al., 1993; Martin et al., 1997; Rajasethupathy et al., 2012; Sugita et al., 1992; White et al., 1993; Xu et al., 1994). Many molecules, synaptic plasticity, and structural plasticity are essentially the same in non-associative and associative form of memory in both invertebrate and vertebrate animal models (Dash et al., 1990; Frank and Greenberg, 1994; Kandel, 2012; Byrne and Hawkins, 2015; Hawkins and Byrne, 2015). However, it is very difficult to study the molecular, cellular, and systems level basis of memory formation and consolidation in vertebrate organisms, since they have complex neuronal circuitries, large number of neurons and require vigorous behavioral screens. Zebrafish (*Danio rerio*), a vertebrate class organism, has become a powerful

model to study genetic and neural circuits that regulate behavior. This is due to a number of key advantages that zebrafish possesses, such as, large clutch sizes (100+ eggs per breeding pair), relatively simple neural circuitry with only ~100,000 neurons in 5-day post fertilization (dpf) larvae that facilitate cellular analysis of behavior; and translucence, which allows for in-vivo experimentation and optogenetic imaging (Roberts et al., 2013; Ahrens et al., 2013; Meyer and Smith, 2006; Sagasti et al., 2005). Zebrafish larvae can be also exposed to various pharmacological substances in a bath application; therefore, behavioral screenings can be performed quickly and efficiently (Goldsmith et al., 2004). As a vertebrate class organism, zebrafish are considered analogous models for human neurobiological disease (Guo, 2004) and human learning and memory (Best, 2008).

Although our understanding of the biochemical correlates associated with behavioral sensitization is drawn largely from observations of various defensive withdrawal behavior in *Aplysia californica* and other invertebrate organisms, some studies have investigated sensitization of physiological systems, such as cardiovascular system and their associated behaviors. These experiments have shown that sensitizing stimulation can alter physiological function as well as behaviour (Levy and Susswein 1993; Levy et al. 1994, Krontiris- Litowitz et al. 1989, 1994, 1999). Among the most protuberant behaviors during early developmental stages in the zebrafish is heart rate fluctuations. In order to utilize the advantages of zebrafish larvae as a model organism to elucidate the cellular and molecular mechanisms involved in non-associative learned behavior, it is critical to discover novel forms of memory that they can express at ~5-dpf.

To develop a simple and robust protocol for eliciting varying heart rates, we utilized mustard oil (allyl isothiocyanate, AITC) as a sensitizing stimulus for 5-DPF zebrafish larvae.

AITC has been shown to activate Transient receptor potential (TRP) ion channels, which are responsible for detecting a range of thermal, chemical and mechanical stimuli (Christensen and Corey, 2007). Ruthenium red (RR), on the other hand, has been identified as a noncompetitive antagonist that act as an inhibitor against all TRP channels (Cahusac, 2009). A particularly interesting TRP channel is TRPA1, which has been linked in detecting noxious chemicals in mammals and *Drosophila* (Bautista et al., 2006), in mediating mechanotransduction in mammals and *C. elegans* (Kindt et al., 2007). Zebrafish larvae perceive a variety of thermal, chemical and mechanical stimuli through trigeminal and Rohon-Beard sensory neurons, whose cutaneous peripheral axons innervate the head and trunk, respectively (Sagasti et al., 2005). Therefore, we hypothesized that the increased activation of trigeminal mechanoreceptors from short-term exposure to AITC would sensitize larvae zebrafish and increase their heart rate compared to the normal heart rate. Conversely, exposure to ruthenium red would block the AITC induced sensitization of heart rate in larval zebrafish (5 DPF).

Previous studies in *Aplysia californica* investigating the role of neural mechanisms on induction and maintenance of sensitization-induced increase in heart rate employed nerve conduction blockade during sensitization. These studies have suggested that like sensitization of defensive behaviours, sensitization-induced increase in heart rate depends on functional conductive neural pathway (Krontiris- Litowitz et al., 1989, 1994, 1999). Experiments with 5-HT1B receptor knockout have shown to impede sensitization induced increase of heart rate in mice (Robert Meas et at, 2000). Using laser ablation technique, researchers demonstrated the role of the Mauthner neuron in zebrafish escape response (Satou, C., 2009). In the current study, we further used behavioral pharmacology to dissect the molecular underpinnings of the AITC induced sensitization memory. To determine whether the different neural circuits contributing to

enhanced heart rate depend upon one or many different neuromodulators and ascertain how these neuromodulators influence distinct neural circuits we have conducted various extracellular receptor blockade experiments. We used D-APV, a competitive NMDAR antagonist; MK801, a non-competitive NMDAR antagonist; Methiothepin, a selective serotonin receptor antagonist; Haloperidol, a dopamine D2 receptor antagonist; Mecamylamine, a non-competitive nicotinic acetylcholine receptor antagonist; Propranolol, a non-selective beta-adrenergic receptor blocker and Atropine, a non-selective muscarinic acetylcholinergic antagonist.

MATERIALS AND METHODS

Animals

After collection, zebrafish eggs were put into E3 water (5 mM NaCl, 0.33 mM MgSO₄, 0.33 mM CaCl₂, 0.17 mM KCl, 10^{-5}% methylene blue, pH 7.2) and placed in an incubator (28.5 °C). Behavioral experiments were performed on the TL strain of zebrafish obtained from the UCLA core facility.

Behavioral protocols

Experiments measuring heart rate in restrained fish

Larval zebrafish (mixed sex), 5-dpf, were fully restrained in 3% low melting point agarose and placed inside of a cell culture dish (50 mm diameter) to which E3 (9 mL) was added after the agar had hardened. Each fish was positioned laterally to facilitate observation of heart rate. Once the agarose solidified, a small window of agarose above the head was cut away to allow complete access of solutions to the skin on the head. The fish were then placed under a dissecting microscope and allowed to acclimate for 30 min. A baseline heart rate was determined

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with visual observation for a period of 30 s. 30 s after this baseline observation, 75 µL (10 µM) of either AITC or E3 was added to the bath for 1 min. While the fish was being exposed to AITC or control solutions, another measurement of heart rate was taken 30 s after the initial exposure. After the 1-minute exposure to AITC or control solutions, a manual wash of AITC or control solutions from the bath was performed for 1 min using E3 and plastic Pasteur pipette. Two minutes after the washout (Four min after the initial exposure to AITC or E3), a final measurement of heart rate was taken. Similar methods were employed for experiments involving Ruthenium Red (RR) except the RR (10 µM) or control solutions (E3) were washed into the bath 4 min prior to AITC application or were washed into the bath as AITC or control solutions were being washed out of the bath.

Extracellular receptor blockade experiments measuring heart rate in restrained fish Similar to above methods, larval zebrafish (mixed sex), 5-dpf, were fully restrained in 3% low melting point agarose, to which E3 (9 mL) was added. Once the agarose solidified, a small window of agarose above the head was cut away to allow complete access of solutions to the skin on the head. Then the fish was exposed to either 75 µL of E3 or DMSO or drug (an extracellular receptor blocker) with appropriate concentration. The drugs used as extracellular receptor blocker included: 100 µM DAPV (a competitive NMDAR Antagonist), 100 µM MK801(a Non-competitive NMDAR Antagonist), 10µM Methiothepin (a selective antagonist of serotonin receptors), 20 µM Haloperidol (a dopamine D2 receptor antagonist), 30µM Mecamylamine (a non-competitive nicotinic acetylcholine receptor antagonist), 100µM Propranolol (a non-selective beta-adrenergic receptor blocker), 100 µM Atropine (a non-selective muscarinic acetylcholinergic antagonist) and 100µM Propranolol plus 100 µM Atropine. The fish were then placed under a dissecting microscope and allowed to acclimate for

60 min. A baseline heart rate was determined with visual observation for a period of 30 s. 5 minutes after this baseline observation, 75 µL (10 µM) of either AITC or E3 was added directly to the fish head for 1 min. While the fish was being exposed to AITC or control solutions, another measurement of heart rate was taken 30 s after the initial AITC exposure. After the 1-minute exposure to AITC or control solutions, a manual wash was performed for 1 min using E3 and plastic Pasteur pipette. Two minutes after the washout (Four min after the initial exposure to AITC or E3), a final measurement of heart rate was taken.

Pharmacology

Sensitization was elicited with the chemical irritant AITC (mustard oil) for 30s to 1 min. To block transient receptor potential (TRP) channels, we used Ruthenium red (RR:10 µM). AITC and RR were obtained from Sigma (St. Louis, MO). The drugs for extracellular receptor blockade experiments were obtained from Sigma (DAPV, MK801, Mecamylamine, Propanolol), Tocric, Minneapolis, MN (Methiothepin), SC Biotechnology, Greenville, SC (Haloperidol) and Cayman Chemical, Ann Arbor, MI (Atropine).

Statistical analyses

Statistical comparisons were conducted using Analyses of variance (ANOVAs). For experiments measuring the same fish over time, repeated measures between groups ANOVAs were used. A Tukey HSD test was used for all post-hoc analyses.

RESULTS

Allyl isothiocyanate-induced behavioral sensitization activates the autonomic nervous system and is dependent upon TRP channels

Induction of behavioral sensitization often results in the activation of the autonomic nervous system besides modifying a range of behavioral patterns (Bouwknecht et al., 2000; Krontiris-Litowitz, 1999). To determine whether AITC exposure activates the sympathetic nervous system in larval zebrafish, we used heart rate as an indicator of autonomic nervous system activation. Accordingly, we measured heart rate in larvae fully restrained in agarose before, during, and after application of AITC. Each larva was fully restrained in agarose in a small petri dish (50 mm diameter) to which 9 mL E3 was added after the agar had hardened. A small window of agarose above the head was cut away to permit ready access of solutions to the skin on the head. After acclimating for 30-min, 75 µL of either AITC or E3 was added to the bath; the final concentration of AITC in the dish was 10 µM (Fig. 2A1). After a 1-min exposure period, the AITC/E3 was washed out of the holding dish. The normalized heart rate of larvae was significantly enhanced in the presence of AITC (AITC$_{HR}$ group = 1.164 ± 0.031 beats per min [BPM]), as well as at the 4-min test (~2 min after the aversive agent was washed out of the bathing solution) (AITC$_{HR}$ group = 1.219 ± 0.021 BPM) compared to a group of larvae exposed only to E3 (E3$_{HR}$ group, initial measurement = 1.009 ± 0.003 BPM; measurement at the 4-min test = 1.016 ± 0.009 BPM) (Fig. 2A2). Thus, the 1min exposure to AITC induced short-term sensitization of heart rate in zebrafish larvae.

Previous research indicated that AITC activates TRP channels expressed in the trigeminal and Rohon Beard sensory neurons in larval zebrafish (Prober et al., 2008). To confirm this, we used 10 µM ruthenium red (RR), which has been shown to antagonize TRP channels in zebrafish

(Prober et al., 2008) (Fig. 2B1). Bath application of RR (10 µM) for 4 min prior to AITC exposure blocked the increase in the normalized heart rate in the presence of the irritant (RR-AITC group =1.011 ± 0.010 BPM; E3-AITC = 1.143 ± 0.009 BPM), as well as the sensitization of the heart rate observed after washout of AITC (4-min test: RR-AITC group = 1.014 ± 0.018 BPM; E3-AITC group = 1.160 ± 0.011 BPM) (Fig. 2B2). This confirms that the AITC induced sensitization of zebrafish heart rate is due to the activation of TRP channels located within the trigeminal and Rohon Beard sensory neurons.

To test the possibility that AITC was not being completely washed out from the bath or AITC activating TRP channels lead to a prolonged sensory neuron activation in the absence of the chemical irritant we performed another experiment in which RR was added to the petri dish after exposure to AITC (Fig. 2C1). Previous research has shown that RR effectively antagonizes AITC-induced activation of TRP channels even when the irritant AITC is applied prior to the onset of RR treatment (Prober et al., 2008). Application of RR following the washout of AITC did not affect the enhanced heart rate produced by the irritant (AITC-E3 group: measurement in irritant = 1.183 ± 0.015 BPM; 4-min test = 1.192 ± 0.019 BPM vs. AITC-RR group: measurement in irritant = 1.175 ± 0.016 BPM; 4-min test = 1.176 ± 0.021 BPM) (Fig. 2C2). Thus, the AITC induced enhancement of heart rate was not due to incomplete washout of this aversive agent or sustained activity of sensory neurons resulting from prolonged TRP channel activation.

Extracellular receptor blockade experiments measuring heart rate in restrained fish

To determine the role of various extracellular receptors in AITC induced short term sensitization of heart rate, different neurotransmitter receptor blockade experiments were

employed. The drugs that were used to block receptors associated with AITC induced heart rate sensitization included 100 µM DAPV (a competitive NMDAR Antagonist), 100 µM MK801(a Non-competitive NMDAR Antagonist), 10µM Methiothepin (a selective antagonist of serotonin receptors), 20 µM Haloperidol (a dopamine D2 receptor antagonist), 30µM Mecamylamine (a non-competitive nicotinic acetylcholine receptor antagonist), 100µM Propranolol (a non-selective beta-adrenergic receptor blocker) and 100 µM Atropine (a non-selective muscarinic acetylcholinergic antagonist). Zebrafish larvae was restrained in agarose in a small petri dish and a small window of agarose above the head was cut away to permit ready access of solutions to the skin on the head. Then the fish was exposed to either 75 µL of E3 or DMSO or an extracellular receptor blocker (drug) with appropriate concentration added to the cell culture dish. The fish were then placed under a dissecting microscope and allowed to acclimate for 60 min. A baseline heart rate was determined and after a 5 min wait period 75 µL (10 µM) of either AITC or E3 was added directly to the fish head for 1 min. While the fish was being exposed to AITC or control solutions, another measurement of heart rate was taken 30 s after the initial exposure. After the 1-minute exposure to AITC or control solutions, a manual wash was performed for 1 min using E3. Two minutes after the washout (Four min after the initial exposure to AITC or E3), a final measurement of heart rate was taken. (Fig. 3B)

Lack of an effect of NMDAR blockade on AITC induced heart rate sensitization

Previous research indicated that bath application of DAPV completely blocked NMDA receptors in the brain of zebrafish larvae. (Nam RH., 2004, Edwards WG., 2002). Bath application of DAPV (100 µM) for 60 min prior to E3 exposure had no effect in the normalized heart rate (Con-Apv group =1.016 ± 0.004 BPM; Con-E3 = 1.016 ± 0.005 BPM), as well as the

heart rate observed after washout (4-min test: Con-Apv group = 1.016 ± 0.005 BPM; Con-E3 group = 1.017 ± 0.005 BPM) (Fig 4A). Thus bath application of DAPV did not modify heart rate in a nonspecific manner.

Bath application of DAPV (100 μM) for 60 min prior to AITC exposure also had no effect in the normalized heart rate in the presence of the irritant (Sens-APV group =1.186 ± 0.021 BPM; Sens-E3 = 1.182 ± 0.023 BPM), as well as the sensitization of the heart rate observed after washout of AITC (4-min test: Sens-APV group = 1.196 ± 0.021 BPM; Sens-E3 group = 1.192 ± 0.023 BPM) (Fig 4B). Thus, blocking NMDAR by the 60 min exposure to DAPV did not impede sensitization of heart rate in zebrafish larvae.

Previous research indicated that bath application of MK801 blocked NMDA receptors in the brain of zebrafish larvae (Gaspray et al., 2018). Bath application of MK-801 (100 μM) for 60 min prior to E3 exposure significantly increased the normalized heart rate (Con-MK801 group =1.042 ± 0.008 BPM; Con-E3 = 1.014 ± 0.005 BPM), as well as the heart rate observed after washout (4-min test: Con-MK801 group = 1.048 ± 0.010 BPM; Con-E3 group = 1.020 ± 0.006 BPM) (Fig 4C).

Bath application of MK801 (100 μM) for 60 min prior to AITC exposure had no effect in the normalized heart rate in the presence of the irritant (Sens-MK801 group =1.166 ± 0.020 BPM; Sens-E3 = 1.162 ± 0.024 BPM), as well as the sensitization of the heart rate observed after washout of AITC (4-min test: Sens-MK801 group = 1.167 ± 0.013 BPM; Sens-E3 group = 1.174 ± 0.018 BPM) (Fig 4D). Thus, blocking NMDAR by the 60 min exposure to mk801 did not impede sensitization of heart rate in zebrafish larvae.

Serotonin receptor blockade attenuates AITC induced heart rate sensitization

Previous research indicated that administration of methiothepin reduced heart rate by blocking NMDA receptors in the preoptic area of conscious rats (Szabo et al., 1996). Bath application of Methiothepin (10μM) for 60 min prior to E3 exposure had no effect in the normalized heart rate (Con- Methio group =1.007 ± 0.004 BPM; Con-E3 = 1.011 ± 0.003 BPM), as well as the heart rate observed after washout (4-min test: Con-Methio group = 1.009 ± 0.004 BPM; Con-E3 group = 1.010 ± 0.003 BPM) (Fig 5A). Thus bath application of methiothepin did not modify heart rate in zebrafish in a nonspecific manner.

Bath application of Methiothepin (10 μM) for 60 min prior to AITC exposure significantly decreased the normalized heart rate (Sens-Methio group =1.003 ± 0.008 BPM; Sens-E3 = 1.170 ± 0.008 BPM), as well as the heart rate observed after washout (4-min test: Sens-Methio group = 1.010 ± 0.004 BPM; Sens-E3 group = 1.175 ± 0.007 BPM) (Fig 5B). This confirms that blocking serotonin receptor attenuated AITC induced heart rate sensitization.

Dopamine D2 receptor blockade impedes AITC induced heart rate sensitization

Previous research indicated that bath application of haloperidol reduced heart rate by blocking Dopamine D2 receptors in the zebrafish larvae (Parker et al., 2014). Bath application of Haloperidol (20μM) for 60 min prior to E3 exposure had no effect in the normalized heart rate (Con- Halip group =1.012 ± 0.004 BPM; Con-E3 = 1.012 ± 0.002 BPM), as well as the heart rate observed after washout (4-min test: Con-Halip group = 1.012 ± 0.004 BPM; Con-E3 group = 1.012 ± 0.003 BPM) (Fig 6A). Thus bath application of haloperidol did not modify heart rate in zebrafish in a nonspecific manner.

Bath application of Haloperidol (20μM) for 60 min prior to AITC exposure significantly decreased the normalized heart rate (Sens-Halip group =1.103 ± 0.049 BPM; Sens-E3 = 1.205 ± 0.013 BPM), as well as the heart rate observed after washout (4-min test: Sens-Halip group = 1.128 ± 0.045 BPM; Sens-E3 group = 1.220 ± 0.011 BPM). (Fig 6B). This confirms that blocking dopamine D2 receptor attenuated AITC induced heart rate sensitization.

Autonomic nervous system blockade impedes AITC induced heart rate sensitization

Previous research indicated that administration of haloperidol elicited a biphasic effect on heart rate by blocking nicotinic acetylcholine receptor in rat model (Jutkiewicz et al., 2013). Bath application of Mecamylamine (30μM) for 60 min prior to E3 exposure had no effect in the normalized heart rate (Con- Mec group =1.013 ± 0.002 BPM; Con-E3 = 1.012 ± 0.004 BPM), as well as the heart rate observed after washout (4-min test: Con-Mec group = 1.011 ± 0.007 BPM; Con-E3 group = 1.014 ± 0.006 BPM) (Fig 7A). Thus bath application of mecamylamine did not modify heart rate in zebrafish in a nonspecific manner.

Bath application of Mecamylamine (30μM) for 60 min prior to AITC exposure significantly decreased the normalized heart rate (Sens-Mec group =1.013 ± 0.004 BPM; Sens-E3 = 1.176 ± 0.017 BPM), however had no effect on the heart rate observed after washout (4-min test: Sens-Mec group = 1.194 ± 0.015 BPM; Sens-E3 group = 1.172 ± 0.032 BPM) (Fig 7B). This confirms that blocking nicotinic acetylcholine receptor impedes AITC induced heart rate sensitization.

Previous research indicated that bath application of propanolol significantly reduced heart rate by blocking beta-adrenergic receptor in zebrafish larvae (Finn et al., 2012). Bath application of Propanolol (100μM) for 60 min prior to E3 exposure had no effect in the normalized heart rate

(Con- Prop group =0.980 ± 0.019 BPM; Con-E3 = 1.010 ± 0.005 BPM), as well as the heart rate observed after washout (4-min test: Con-Prop group = 0.969 ± 0.022 BPM; Con-E3 group = 0.997 ± 0.008 BPM) (Fig 7C). Thus bath application of propanolol did not modify heart rate in zebrafish in a nonspecific manner.

Bath application of Propanolol (100μM) for 60 min prior to AITC exposure significantly decreased the normalized heart rate (Sens-Prop group =0.820 ± 0.034 BPM; Sens-E3 = 1.211 ± 0.007 BPM), as well as the heart rate observed after washout (4-min test: Sens-Prop group = 0.788 ± 0.053 BPM; Sens-E3 group = 1.234 ± 0.008 BPM) (Fig 7D). This confirms that blocking beta-adrenergic receptor attenuated AITC induced heart rate sensitization.

Previous research indicated that bath application of atropine significantly increased heart rate by blocking muscarinic acetylcholinergic receptor in zebrafish larvae (Mann et al., 2010).. Bath application of Atropine (100μM) for 60 min prior to AITC exposure decreased the normalized heart rate (Sens-Prop group =1.090 ± 0.059 BPM; Sens-E3 = 1.162 ± 0.008 BPM), as well as the heart rate observed after washout (4-min test: Sens-Prop group = 1.108 ± 0.063 BPM; Sens-E3 group = 1.185 ± 0.007 BPM), however the data were not significant. (Fig 7E). The persistent bradycardia that we observed by the bath application of propranolol is blocked here by the muscarinic antagonist atropine.

We further investigated the role of sympathetic and parasympathetic branches of autonomic nervous system in AITC induced heart rate sensitization by exposing the zebrafish larvae to both propranolol and atropine together. Bath application of both 100μM Propanolol and 100μM Atropine for 60 min prior to AITC exposure decreased the normalized heart rate (Sens-Prop group =1.080 ± 0.001 BPM; Sens-E3 = 1.822 ± 0.004 BPM), as well the heart rate observed after washout (4-min test: Sens-Prop group = 1.131 ± 0.009 BPM; Sens-E3 group =

1.195 ± 0.007 BPM), however the data were not significant. (Fig 7F). This data validates the rescue effect of atropine on AITC induced heart rate sensitization of larval zebrafish that have been shown in the previous experiment to significantly reduce due to propranolol exposure.

DISCUSSION

In the current study we have described a novel form of nonassociative learning in zebrafish larvae, sensitization (Cai et al., 2012; Carew et al., 1971; Duerr and Quinn, 1982; Fendt et al., 1994; Koch, 1999; Krasne and Glanzman, 1986; Groves et al., 1969; Rankin et al., 1990; Thompson and Spencer, 1966; Watkins et al., 2010) of heart rate. Here, we demonstrated that a brief exposure to 10μM of allyl isothiocyanate (AITC or mustard oil, MO) significantly activated the TRP1A receptors in 5-dpf larval zebrafish inducing rapid sensitization of heart rate and exposure to 10μM of ruthenium red (RR) inhibited the TRP1A receptors in these larval zebrafish blocking heart rate sensitization or desensitized the fish decreasing its heart rate. Previous studies have reported that AITC activates the TRP1A receptors in the Trigeminal and Rohan Beard sensory neurons in zebrafish head (Prober et al., 2008). However, researchers have not explored heart rate behavior due to increased sensitization through TRPA1 receptor agonist AITC. Moreover, in the current study we demonstrated the AITC induced enhancement of heart rate was not due to incomplete washout of this aversive agent or sustained activity of sensory neurons resulting from prolonged TRP channel activation, but it was rater due t the induction of short term sensitization (Fig. 2C2).

The heart rate sensitization is different from the phenomena observed in other studies of behavioral sensitization, since enhancement of heart rate it is not an evoked response. Heart is myogenic and the contraction is not instigated by sensory stimulation, but rather modified by it.

The current study together with previous studies on zebrafish and *Aplysia californica* suggest that physiological systems respond to sensitizing stimulus and that in physiological systems sensitizing stimuli can facilitate spontaneous phenomena as well as elicited phenomena (Mann et al., 2010; Krontiris- Litowitz et al. 1989, 1994). The enhancement in heart rate produced by AITC (Fig. 2A2) confirms the activation of the autonomic nervous system. It has been reported previously that zebrafish uses both parasympathetic and sympathetic branches of the autonomic nervous system early during developmental stages to regulate cardiac output (Mann et al., 2010). Although the persistence of rapid increased heart rate in the larvae following washout of AITC is attributable to autonomic nervous system (ANS) activity, other factors may also contribute. Previous studies have shown that noxious stimuli similar to those used in current study elicit the release of paracrine or endocrine factors which may act as mediators to increase heart rate following sensitization. (Koester and Koch 1987; Dieringer et al. 1978; Koch et al. 1984; Krontiris- Litowitz et al. 1989, 1994).

In *Aplysia californica*, the heterosynaptic modulatory input from monoaminergic interneurons facilitate various behavioral sensitization. The sensitization-related neuronal changes are mediated by a modulatory neurotransmitter, serotonin (Brunelli et al., 1976; Glanzman et al., 1989; Hochner et al., 1986a; Hochner et al., 1986b; Marinesco and Carew, 2002). Accordingly, we explored whether AITC-induced release of one or more monoamines within the CNS mediates sensitization of heart rate in zebrafish larvae. Moreover, we investigated the role of sympathetic and parasympathetic neuromodulators on AITC-induced sensitization of heart rate. To ascertain how these neuromodulators influence distinct neural circuits we have conducted various extracellular receptor blockade experiments (Fig. 3A). In line with several previous researches, NMDAR blockade with both DAPV and MK801 have shown

no effect on AITC induced rapid heart rate sensitization (Fig. 4B, 4D) (Wood et al, 2015). Blocking serotonin receptor with methiothepin attenuated AITC induced rapid heart rate sensitization, whereas methiothepin did not modify heart rate in zebrafish in a nonspecific manner (Fig 5A, 5B) (Szabo et al., 1996). Dopamine D2 receptor blockade by bath application of haloperidol impedes AITC induced rapid heart rate sensitization, although dopamine did not modify heart rate in zebrafish in a nonspecific manner (Fig 6A, 6B) (Parker et al., 2014). The current study showed that autonomic nervous system appears to play a major role in initiation and maintenance of AITC induced rapid heart rate sensitization. Previous research indicated that administration of haloperidol elicited a biphasic effect on heart rate by blocking nicotinic acetylcholine receptor in rat model (Jutkiewicz et al., 2013). In line with that, bath application of mecamylamine reduced short term sensitization of heart rate in the presence of stimulating agent, but the heart rate was elevated after the washout procedures. The rapidly enhanced heart rate following AITC induced sensitization was severely blocked by the beta-adrenergic antagonist propranolol (also a very weak 5HT 1A/1B/2B receptor antagonist), suggesting that this response is mediated by the sympathetic nervous system (Finn et al., 2012; Mann et al, 2010). On the other hand, the AITC induced increase in heart rate was insignificantly reduced by the muscarinic antagonist atropine. Bath application of both propranolol and atropine blocked the bradycardia that was evident from treatment with propranolol alone, suggesting that this response is mediated by the parasympathetic system (Mann et al, 2010).

In the current study some possible sources of error include the agarosing of zibrafish larvae that could have had different thermal stimulation, loud noises and any vibration to the acclimatizing apparatus. The freeing procedures for head might have also lead to increased sensitization to some fishes. The freeing of head might not have been done properly leading to

not exposing the fish head to the various drugs might also lead to erroneous results. Future experiments using optogenetics together with laser ablation of serotonergic neurons and specific subsets of serotonin, dopamine, nicotinic, muscarinic, adrenergic receptor blockade as well as intracellular receptor blockade should help to clarify the role of sensitization in the prolonged elevation of larval heart rate after washout of AITC.

In conclusion, our current study elucidated a simple, robust protocol for allyl isothiocyanate-induced rapid sensitization of heart rate in larval zebrafish. We also demonstrated ruthenium red induced desensitization/ inactivation of TRP receptor leading to reduced heart rate. Further we report that this AITC induced heart rate sensitization implicates serotonin, dopamine receptors as well as β-adrenergic, nicotinic and muscarinic acetylcholine receptors from cardiac autonomic nervous system, but NMDA receptors don't seem to play any role in this short term memory. Together, these results indicate the enormous potential that zebrafish hold as an animal model for the study of learning and memory, especially non-associative memory.
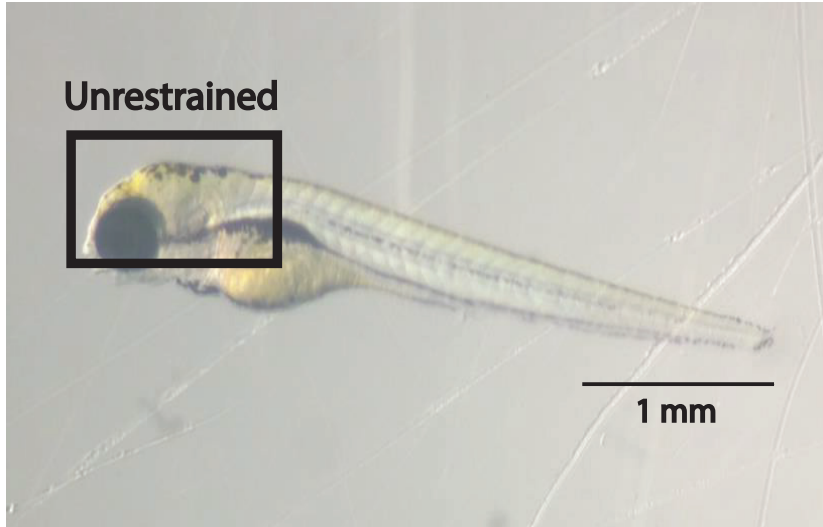
Figure 1. Semi restrained preparation of AITC induced heart rate paradigm. Representative image of 5 dpf fish larvae positioned laterally in cell culture dish to facilitate observation of heart rate. Dorsal portion of head was freed for experimental manipulation.
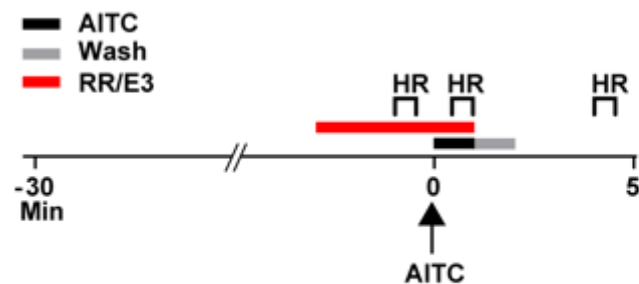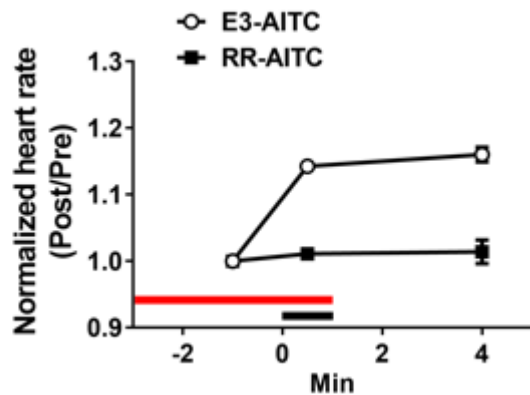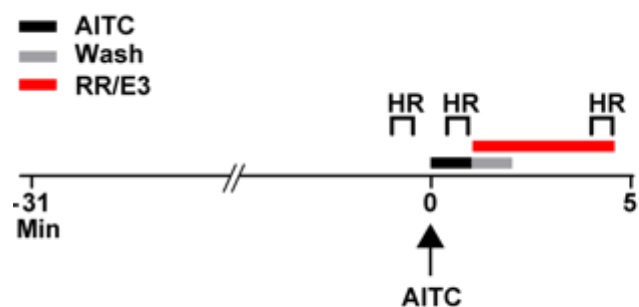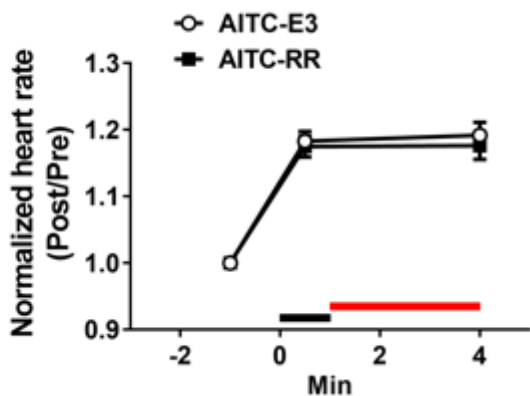
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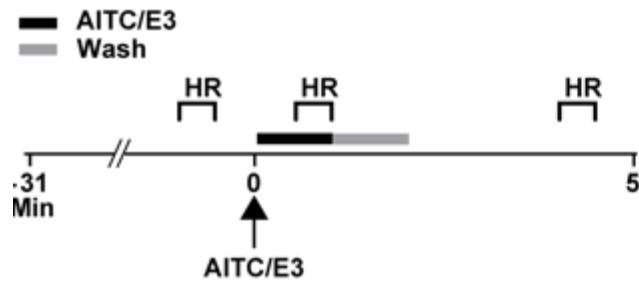
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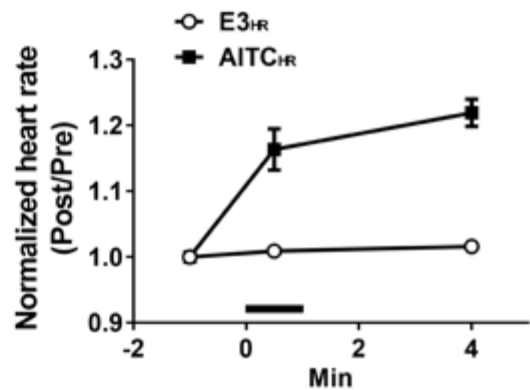
2B1.

2B2.

2C1.

2C2.

Figure 2. AITC causes a post-exposure increase in heart rate in restrained zebrafish larvae that depends on TRP channels. (A1) Experimental protocol investigating the effect of AITC/E3 on zebrafish heart rate. The experiments were performed on larvae fully restrained in agar. The skin on each larva's head was exposed to AITC/E3 by removal of a small section of agar. Heart rate was measured 1 minute before, during and 3 minutes after exposure to mustard oil or E3. There

was a 1 minute washout after 1 minute exposure to AITC/E3. The pretest HR was used to normalize the response to the chemical irritant. (A2) Effect of AITC/E3 on larval heart rate. A repeated-measure Two-way ANOVA revealed a significant overall effect ($F_{[1,14]}=78.68$, $p < 0.05$) that indicated that the group (AITC-HR; $n= 8$) exposed to mustard oil demonstrated a normalized increase in heart rate in the presence of the chemical irritant and after it was washed out compared to a group (Con-HR; $n = 8$) that was only exposed to E3. (B1) Experimental protocol investigating the effect of ruthenium red (RR) on AITC induced heart rate sensitization. The fish was exposed to RR (10 µM) for 4 min prior to the onset of a 1 min exposure to AITC/E3. After the exposure to the AITC/E3, the RR and AITC/E3 were washed out of the petri dish. Heart rate was measured 1 minute before, during and 3 minutes after exposure to AITC or E3. (B2) The normalized mustard oil-induced enhancement of heart rate was reduced/blocked by bath application of RR (10 µM) 4 min prior to the AITC/E3 exposure. A repeated-measures, two-way ANOVA indicated that the fish exposed to RR 4 min prior to AITC application (RR-AITC, $n = 8$) exhibited a significantly lower heart rate than did fish (E3-AITC, $n = 8$) not exposed to RR, both when AITC was present in the bath and after washout of the irritant ($F_{[1,14]}=67.06$, $p < 0.05$). (C1) Experimental protocol investigating the effect of RR on AITC-induced sensitization of heart rate. The RR was applied to the bath for a 3.5-min period beginning at the onset of the washout of AITC/E3. The baseline heart rate was measured 1min before the onset of AITC/E3; the second heart rate measurement was taken 30 s after the onset of AITC/E3; and the third 2 min after the end of washout. (C2) Lack of an effect of RR on AITC-induced sensitization of larval heart rate if RR is not present before or during AITC exposure. The AITC induced sensitization is not due to residual activation of AITC or prolonged TRP channel activation in the absence of the chemical irritant. A repeated-measures, two-way ANVOA indicated that the heart rate of larvae exposed to RR after AITC exposure (AITC-RR, $n = 8$) did not differ significantly from that of AITC-treated larvae not exposed to RR in the presence of AITC or 3 min after the initiation of wash out procedures (AITC-E3, $n = 8$; $F_{[1,14]}=0.35$, $p > 0.05$).
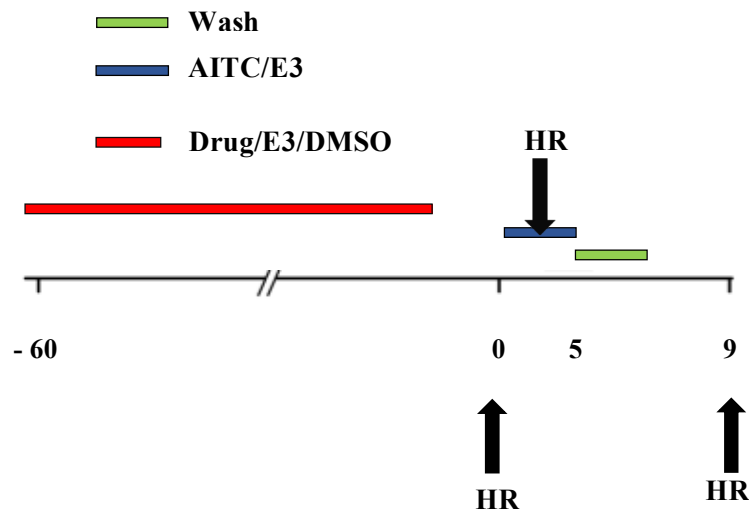
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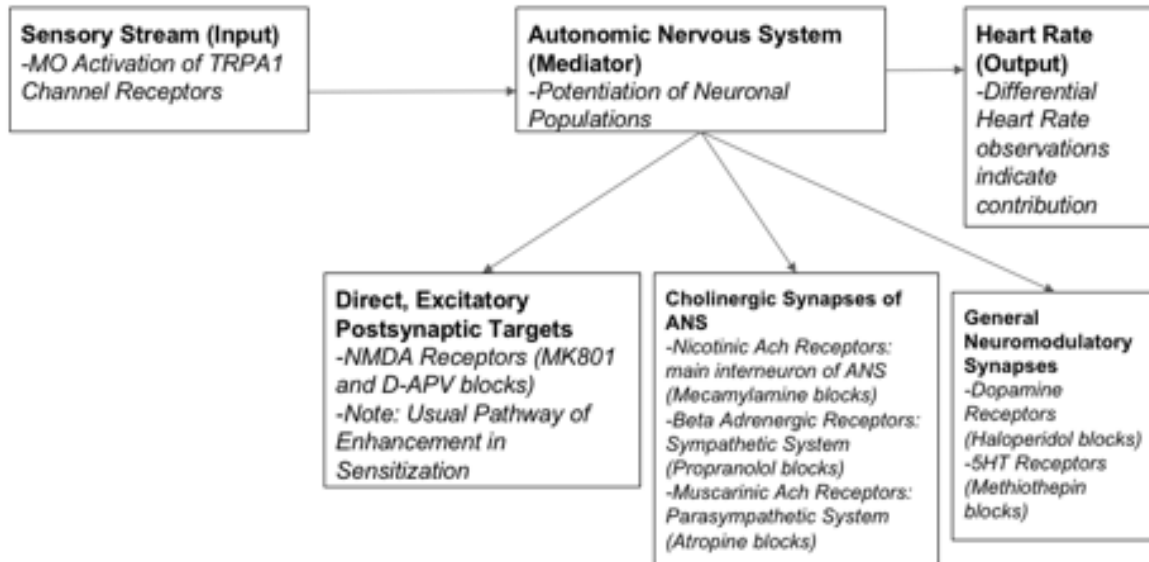
3B.

Figure 3A. General overview of the neuromodulation workflow. 3B. Experimental protocol investigating the effect of various neuromodulation (receptor blockade) on AITC induced heart rate sensitization on zebrafish larvae. The experiments were performed on larvae fully restrained in agar. The skin on each larva's head was exposed to the different receptor antagonists and AITC/E3 by removal of a small section of agar. The fish was exposed to different antagonists/E3/DMSO for 60 min prior to the onset of a 1 min exposure to AITC/E3. After the exposure to the AITC/E3 for 1 minute, the antagonist/DMSO and AITC/E3 were washed out of the petri dish for another minute. Heart rate was measured 1 minute before, during and 3 minutes after exposure to AITC or E3. The pretest HR was used to normalize the response to the chemical irritant.
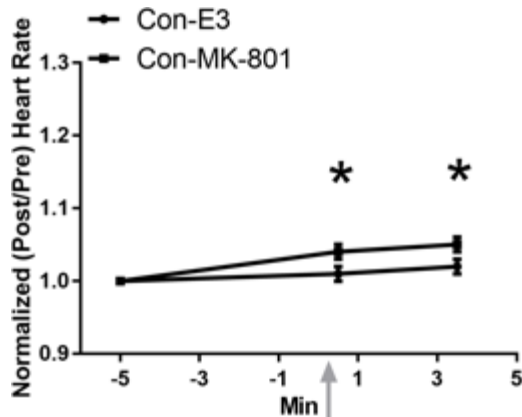
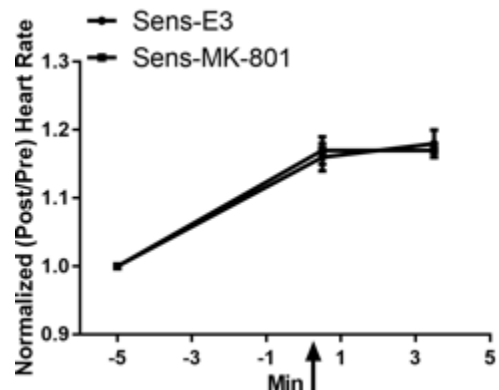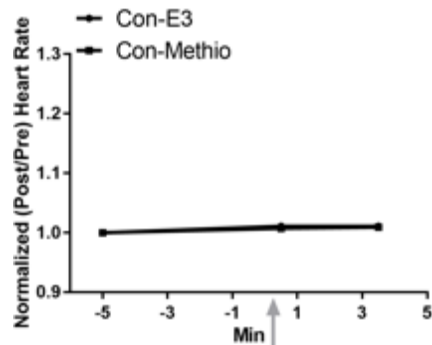**4A.**
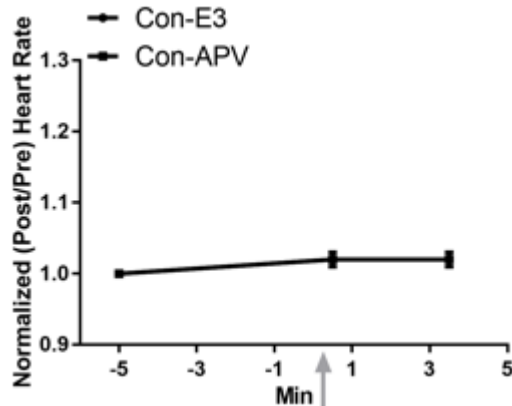
4B.
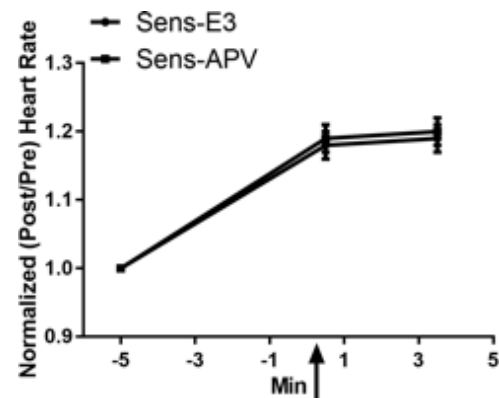
4C.

4D.

Figure 4. The effect of NMDAR blockade on AITC induced heart rate sensitization. (A) Effect of D-APV (Competitive NMDAR Antagonist)/E3 on larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to D-APV (Con-APV, $n = 8$) did not differ significantly from larvae not exposed to D-APV while the fish was being exposed to control solutions or 3 min after the initiation of wash out procedures (Con-E3, $n = 8$; $F_{[1,14]}=0.082$, $p > 0.05$). (B) Lack of an effect of D-APV on AITC-induced sensitization of larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to D-APV (Sens-APV, $n = 8$) did not differ significantly from larvae not exposed to D-APV while the fish was being exposed to AITC or 3 min after the initiation of wash out procedures (Sens-E3, $n = 8$; $F_{[1,14]}=0.062$, $p > 0.05$). (C) Effect of MK-801 (Non-competitive NMDAR Antagonist)/E3 on larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to MK-801 (Con-MK-801, $n = 8$) increased significantly from larvae not exposed to MK-801 while the fish was being exposed to control solutions and 3 min after the initiation of wash out procedures (Con-E3, $n = 8$; $F_{[1,14]}=65.08$, $p < 0.05$). (D) Lack of an effect of MK-801 on AITC-induced sensitization of larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to MK-801 (Sens- MK-801, $n = 8$) did not differ significantly from larvae not

exposed to MK-801 while the fish was being exposed to AITC or 3 min after the initiation of wash out procedures (Sens-E3, $n = 8$; $F_{[1,14]}=0.49$, $p >0.05$).
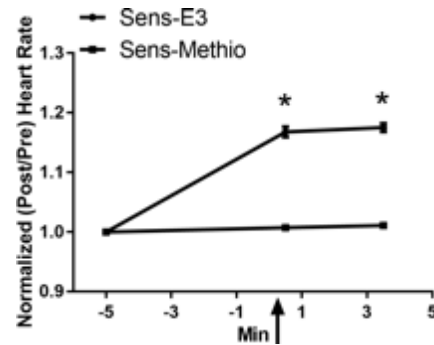
5A.

5B.

Figure 5. The effect of Serotonin receptor blockade on AITC induced heart rate sensitization. (A) Effect of Methiothepin (Selective antagonist of serotonin receptors)/E3 on larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Methiothepin (Con-Methio, $n = 8$) did not differ significantly from larvae not exposed to Methiothepin while the fish was being exposed to control solutions or 3 min after the initiation of wash out procedures (Con-E3, $n = 8$; $F_{[1,14]}=0.028$, $p >0.05$). (B) Effect of Methiothepin /E3 on AITC induced larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Methiothepin (Sens-Methio-801, $n = 8$) decreased significantly from larvae not exposed to Methiothepin while the fish was being exposed to AITC and 3 min after the initiation of wash out procedures (Sens-E3, $n = 8$; $F_{[1,14]}=37.26$, $p <0.05$).
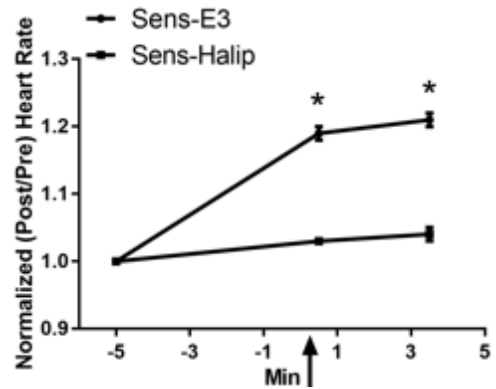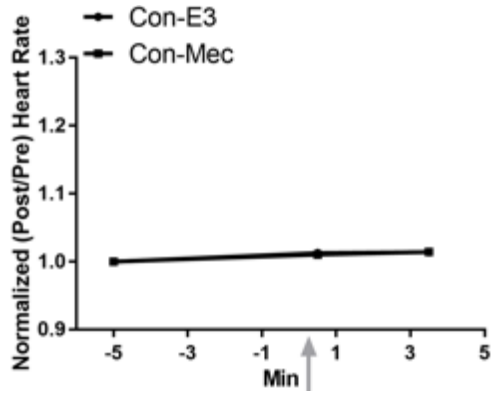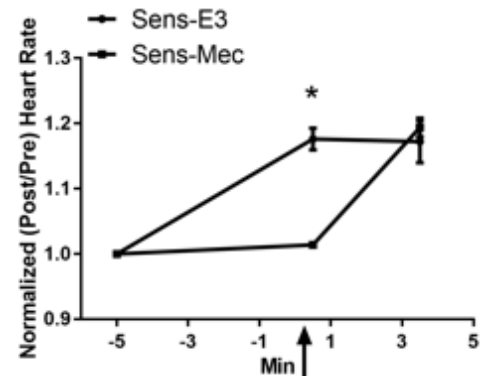
6A.
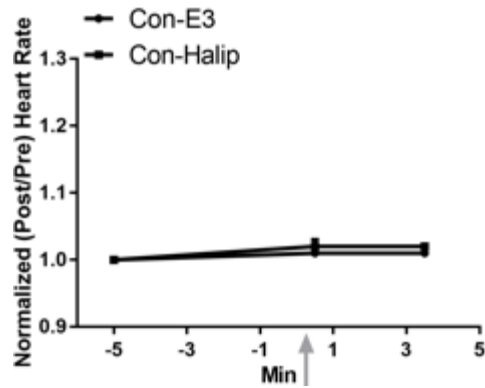
6B.

Figure 6. The effect of Dopamine receptor blockade on MO induced heart rate sensitization. (A) Effect of Haloperidol (Dopamine D2 receptor antagonist)/E3 on larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Haloperidol (Con-Halip, $n = 8$) did not differ significantly from larvae not exposed to Haloperidol while the fish was being exposed to control solutions or 3 min after the initiation of wash out procedures (Con-E3, $n = 8$; $F_{[1,14]}=0.78$, $p > 0.05$). (B) Effect of Haloperidol /E3 on AITC induced larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Haloperidol (Sens-Halip-801, $n = 8$) decreased significantly from larvae not exposed to Haloperidol while the fish was being exposed to AITC and 3 min after the initiation of wash out procedures (Sens-E3, $n = 8$; $F_{[1,14]}=0.53.07$, $p < 0.05$).
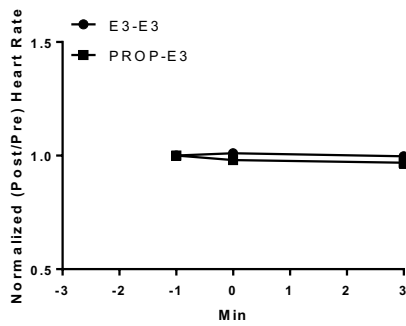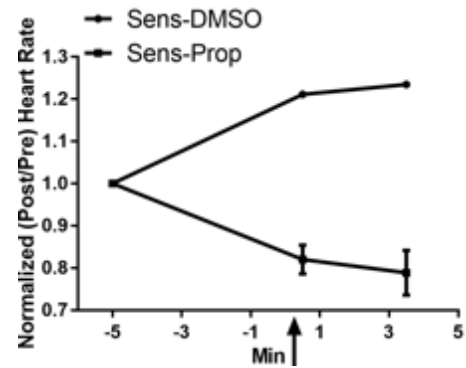
7A.

7B.

7C.

7D.

7E.

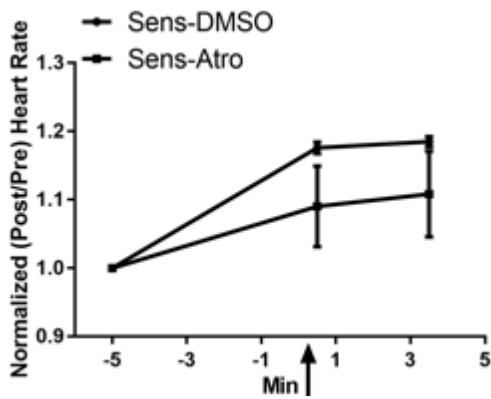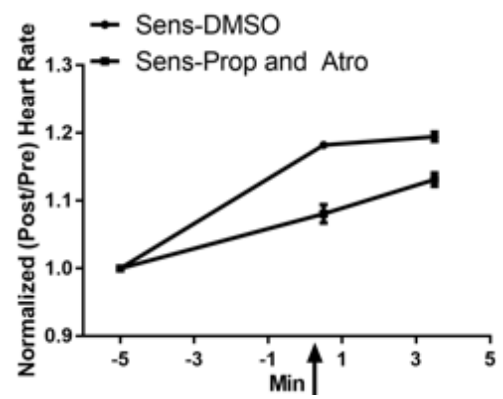**7F.**

Fig 7. The effect of blocking autonomic input on MO induced heart rate sensitization.

25

(A) Effect of Mecamylamine (Non-competitive nicotinic acetylcholine receptor antagonist) /E3 on larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Mecamylamine (Con-Mec, $n = 8$) did not differ significantly from larvae not exposed to Mecamylamine while the fish was being exposed to control solutions or 3 min after the initiation of wash out procedures (Con-E3, $n = 8$; $F_{[1,14]}=0.39$, $p >0.05$). (B) Effect of Mecamylamine /E3 on AITC induced larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Mecamylamine (Sens-Mec-801, $n = 8$) decreased significantly from larvae not exposed to Mecamylamine while the fish was being exposed to AITC, but did not differ significantly 3 min after the initiation of wash out procedures (Sens-E3, $n = 8$; $F_{[1,14]}=59.70$, $p <0.05$). (C) Effect of Propranolol (Non-selective beta-adrenergic receptor blocker) /E3 on larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Propranolol (Prop-E3, $n = 8$) did not differ significantly from larvae not exposed to Propranolol while the fish was being exposed to control solutions or 3 min after the initiation of wash out procedures (E3-E3, $n = 8$; $F_{[1,14]}=2.34$, $p >0.05$). (D) Effect of Propranolol /E3 on AITC induced larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Propranolol (Sens-Prop, $n = 8$) decreased significantly from larvae not exposed to Propranolol while the fish was being exposed to AITC and 3 min after the initiation of wash out procedures (Sens-E3, $n = 8$; $F_{[1,14]}=84.08$, $p <0.05$). (E) Lack of an effect of Atropine (Non-selective muscarinic acetylcholinergic antagonist) on AITC-induced sensitization of larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Atropine (Sens-Atro, $n = 8$) did not differ significantly from larvae not exposed to Atropine while the fish was being exposed to AITC or 3 min after the initiation of wash out procedures (Sens-E3, $n = 8$; $F_{[1,14]}=8.53$, $p >0.05$). (F) Lack of an effect of Propanolol and Atropine together on AITC-induced sensitization of larval heart rate. A repeated-measures, two-way ANVOA indicated that the normalized heart rate of larvae exposed to Propanolol and Atropine (Sens-Prop and Atro, $n = 8$) did not differ significantly from larvae not exposed to Propanolol and Atropine while the fish was being exposed to AITC or 3 min after the initiation of wash out procedures (Sens-E3, $n = 8$; $F_{[1,14]}=12.06$, $p >0.05$).

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