Rapid effects of sex steroids on PGF2α approach response in zebrafish (*Danio rerio)*

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Abstract

Steroids are molecular messengers that regulate a variety of bodily functions, including behaviors and sexual motivation. Steroids have long been known to induce behavioral effects in days or weeks, but more recently have been discovered to effect these changes within seconds. Research surrounding these so-called "rapid" effects of steroids on sensory systems is lacking. I took advantage of the sophisticated sex pheromone system in zebrafish to study this system, and the male zebrafish approach response to the pre-ovulatory pheromone prostaglandin F2α (PGF2α). Sexually mature male zebrafish were treated with vehicle, T, or 11-ketotestosterone (11-KT) for 40 minutes, and then immediately evaluated for time spent near vehicle or PGF2α administered to their test tank. Initial data suggests that T-treated fish spent more time near the pheromone, indicating that T may rapidly modulate this response in zebrafish. There were no significant differences between treatments. Further investigation, with higher steroid and pheromone concentrations and a slightly different experimental design, is underway to confirm how T and/or 11-KT modulate(s) the male zebrafish approach response to PGF2α.

Project Objectives

Classically, steroids have been thought to act in the body by binding to intracellular receptors and inducing gene transcription, protein translation and ultimately behavioral responses. However, because this process – termed the "genomic" mechanism due to its interactions with the genome – takes time, these genomic behavioral effects usually take days or weeks to appear. More recent research has discovered a novel non-genomic or "rapid" mechanism of steroids in which steroids bind to membrane receptors, activate second messengers and initiate phosphorylation cascades. This process can induce or modulate behavioral responses within minutes or even seconds. Rapid effects of steroids on motor systems and even sexual motivation have been widely studied, but less research has focused on rapid effects of steroids on sensory systems, and whether and how these effects occur. To investigate these questions, I take advantage of the intricate pheromonal communication system crucial for synchronous gamete release and in turn, fertilization, reproduction and proliferation.

Prostaglandin F2α (PGF2α) is a pre-ovulatory pheromone released by several male cyprinid species, including both goldfish (*Carrasius auratus*) and zebrafish (*Danio rerio*). Male goldfish, and more recently male zebrafish, have been shown to approach this pheromone (Sorensen, et al. 1988; Yabuki, et al. 2016). Previous work in the Thompson Lab has shown that in goldfish, testosterone (T; formally methyltestosterone) rapidly modulates the male approach response to PGF2α, increasing time spent near the pheromone. The same rapid steroid modulation has not been researched in zebrafish. Studying rapid modulation of this response in zebrafish would allow researchers to take advantage of other molecular tools in zebrafish, including a fully sequenced genome.

In this project, I aimed to determine whether T or 11-ketotestosterone (11-KT), the most potent androgen-receptor (as opposed to estrogen-receptor) binding steroid in fish brains, rapidly modulates this PGF2α approach response in male zebrafish. If T rapidly modulates this response, further research will be necessary to reveal whether the response is androgen- or estrogen-mediated; T can be aromatized into estradiol, an estrogen, which may ultimately mediate the behavioral response. Since the previous work that revealed the PGF2α approach response in zebrafish tested male fish in groups of eight, another goal of my research was to confirm this response in untreated fish while controlling for following behavior. Consistent with previous work on T's rapid modulation of this response in goldfish, I expected that T would rapidly modulate this response in the closely related cyprinid, zebrafish.

Methodology Used

Subjects

Sexually mature male zebrafish (hatched December 2018 by the Thompson Lab, with adult fish from the Jackman Lab at Bowdoin College, Brunswick, ME) were housed together in 28C water and exposed to a regular 24-hour light-dark cycle, simulating natural light conditions. Fish were fed twice daily.

Steroid Priming

Fish were pre-treated with either vehicle (DMSO), T, or 11-KT. Stock solutions for T and 11-KT were prepared ($1x10^{-2}$M in DMSO). Fish were exposed in pairs for 40 minutes to either vehicle (n=7), T (n=8), or 11-KT (n=8) ($1x10^{-6}$M) in 200mL of heated and oxygenated reservoir water.

Pheromone Preparation

A stock solution of PGF2α was prepared ($1x10^{-3}$M) and then diluted immediately prior to each trial to a working concentration of $1x10^{-5}$M. A vehicle solution of equal ethanol concentration was prepared immediately prior to each trial.

Behavioral Testing

Immediately following steroid exposure, fish pairs were transferred to an experimental tank with dimensions 76.20x15.24x24.13cm. Fish were habituated for 5 minutes and then successively exposed to ethanol and PGF2α for 6 minutes each. Vehicle/pheromone solutions were administered dropwise in the middle of the tank (at one wall) at a rate of 150μl/minute (about one drop every 10 seconds). At the end of each trial, fish were sacrificed in 0.1% MS-222, bled, and gonadally sexed. Plasma from each fish was collected and stored at -80C.

Data Collection and Analysis

Trials were recorded from above the tank and videos were analyzed using EthoVision XT software. The strength of the approach response was measured by determining the time spent by at least one fish in the target zone and, to control for following behavior, adding the time spent by both fish in the target zone during each of the 6 minute test periods. The target zone was a rectangular zone of dimensions 19.00x7.62x10.48cm surrounding the point of vehicle/pheromone entry in the experimental tank (Fig. 1). Adjusted difference scores were then calculated for each fish pair to quantify the change in approach response: [(time spent in target zone by at least one fish during PGF2α exposure) + (time spent in target zone by both fish during PGF2α exposure)] – [(time spent in target zone by at least one fish during vehicle exposure) + (time spent in target zone by both fish during vehicle exposure)]. Statistical analyses were performed with GraphPad PRISM software.

Results Obtained

There were no significant differences in time spent in the middle third between treatment groups (control vs. 11-KT p=0.9755; control vs. T p=0.4805; 11-KT vs. T p=0.5867) (Fig. 2). Control fish spent an average of 5.0457 more seconds in the middle third in the PGF2α condition than the vehicle condition, 11-KT fish an average of 14.1010 more seconds, and T fish an average of 55.2273 more seconds. Thus, while there were not between-group significant differences in difference scores, mean difference scores were positive across all groups. T-treated fish tended to show the greatest increase in time spent in the middle third in the PGF2α condition and control fish the least. Within-group analyses revealed no significant differences within the control (p=0.8196), 11-KT (p=0.6710) or T (p=0.1366) treatment groups.

Significance and Interpretation of Results

There were no significant differences in adjusted difference scores between treatment groups, suggesting that neither T nor 11-KT rapidly modulates the PGF2α approach response in male zebrafish. However, it is troubling that the control group did not show a more robust response. Based on the findings of Yabuki, et al. (2016), control fish should have shown a clear approach response toward the PGF2α, but the lack of a within-group significant difference apparently refutes the claim that male zebrafish respond to PGF2α at all. This result could be a function of low steroid concentration, low pheromone concentration, or a lower response threshold in the absence of excessive following. If controlling for these possible confounds results in a more robust response among control fish, based on trends discovered in this experiment, it is likely that responses of 11-KT and T fish will become more robust as well.

Concentrations for steroid treatments were determined based on previous literature. Six hour exposure to $3.47*10^{-9}$M T solution resulted in a four-fold increase in plasma T levels in the 3-spine stickleback, a cyprinid (Maunder, et al. 2007). Zebrafish have been shown to absorb 48μg T from 20ml of water in 90 minutes (Miguel-Queralt & Hammond, 2008). The Thompson Lab previously elevated plasma T levels in zebrafish using both $1x10^{-9}$M and $1x10^{-8}$M T treatments. However, an Enzyme-Linked Immunosorbent Assay (ELISA) has not been performed with zebrafish plasma from this experiment to determine how much plasma T and 11-KT levels are actually changing based on the steroid treatments used. Similarly, the concentration of the PGF2α working solution for this experiment was decided based on doses used by Yabuki and colleagues (2016) as well as Belanger, Pachkowski and Stacey (2010), but given differing paradigms – including fewer subjects per trial, shorter habituation and a more complex dependent variable in my experiment – it is possible that a greater PGF2α concentration is necessary to achieve similar results to Yabuki, et al. (2016).

A second experiment is underway with methods similar to those of the project I discuss here, but includes ten-fold increases in both steroid and pheromone working concentrations, one subject per trial instead of two, a 10 instead of 5 minute habituation, and two 5 minute test periods instead of two 6 minute test periods. These changes were implemented to account for ineffectual steroid dosing and undetectable pheromone, following behavior, novelty of experimental tank and associated isolation stress, and any habituation to the vehicle/pheromone stimulus itself, respectively. I also plan to perform two ELISAs with plasma from the first experiment, one for T and one for 11-KT, to determine how 40 minutes of $1x10^{-6}$ M steroid treatment alters plasma T and 11-KT levels in male zebrafish. These next steps will help refine the current data and advise further research, in this lab and others.

Figures

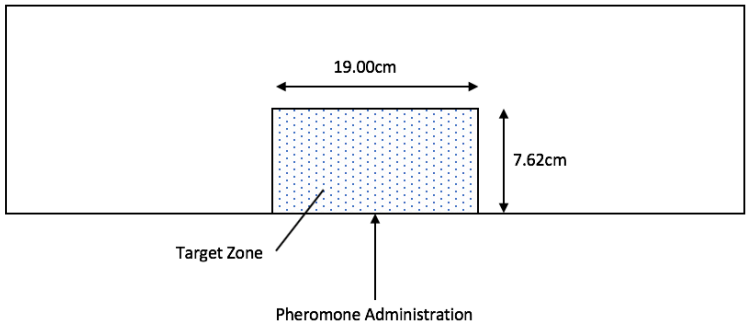

Figure 1 **Top view of the experimental tank with target zone placement and dimensions.** The target zone extended through the water depth as well (not shown due to top view).

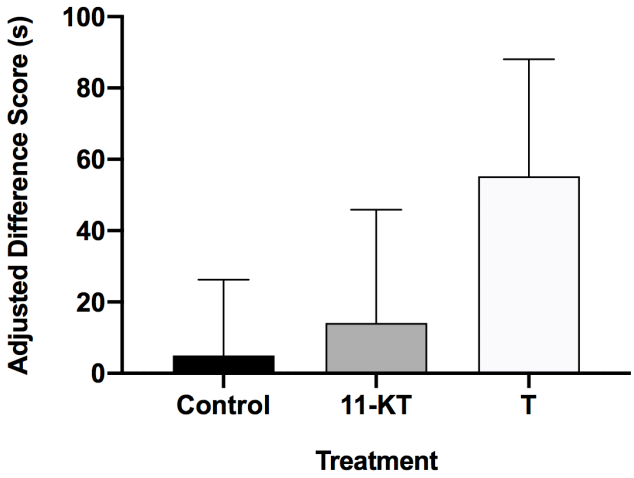

Figure 2 **Change in time spent "in zone" by steroid-treated male zebrafish pairs.** Columns represent mean scores for fish pairs in each group. There were no significant differences in difference scores between control and 11-KT fish (p=0.9755), control and T fish (p=0.4805) or 11-KT and T fish (p=0.5867). Error bars represent SEM.

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