Rapid effects of sex steroids in goldfish (*Carassius auratus)*: behavioral and anatomical approaches.

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Abstract

Sex steroid hormones, such as testosterone (T) and estrogen, have been shown to influence social behavior among many different species. By increasing the likelihood of certain behaviors, there exists a higher chance of the maintenance of group dynamics, as well as reproductive success, in goldfish (Thompson and Mangiamele, 2018). Sex steroids might also rapidly affect responses to pheromone cues that enhance reproductive success. In particular, 15-keto prostaglandin $F_{2\alpha}$ (15K-$PGF_{2\alpha}$) is released by female goldfish when they ovulate, and it elicits courtship responses from males (Sorenson et al. 1988, 1995a; Applet et al. 1995). Understanding how sex steroids rapidly affect responses to social cues, including those that elicit reproductive behaviors, will help us better understand how these molecules affect neural circuits in ways that influence goldfish behavior. In turn, further analysis of their effects can be applied to numerous other species, where these mechanisms have been highly conserved during vertebrate evolution. The effects of T in *Carassius aurtus* were tested behaviorally in a competitive behavior experiment and anatomically with immunohistochemistry runs targeting neuronal activity. While the competition behavior experiment did not show any significant effects of T on the mating behavior of male goldfish, male fish that ate T-pellets had increased following behavior relative to fish that ate control pellets. A PS6 and Anti-HuC immunohistochemistry protocol was also developed in order to determine differences between the brain sections of fish that were exposed to 15-keto prostaglandin and fish that were exposed to ethanol. The development of a competitive behavior paradigm and a neuronal visualization protocol will allow for further testing of the rapid effects of sex steroids on olfactory mechanisms.

Project Objectives

The objective of the research was to investigate how testosterone affected the olfactory system. This objective was addressed in two specific approaches: behaviorally and anatomically. Behaviorally, we wanted to determine how T rapidly modulates goldfish behavior. In a competition experiment, we tested if feeding a T-laced pellet to a male goldfish rapidly increases behavioral responses to females emitting pheromone cues relative to animals given a control pellet. In our anatomical approach, we wanted to identify where T acts rapidly within the goldfish olfactory pathway. In order to visualize differences between treatments, we needed to quantify the neuronal activity within the brain. We wanted to determine a ratio of neurons that were active per total number of neurons in an area and compare them between treatments. To do this, we developed an immunohistochemistry protocol using PS6, a protein that becomes phosphorylated when cells are activated, and Anti-HuC, an antibody that labels the nuclei of all neuronal cells. Utilizing the PS6 and Anti-HuC antibodies, we could visualize neurons activated by visual cues, so that we would be able to determine if and where in the brain T increases activated neurons.

Methods

A. Behavioral approach

Preparation of Pellets: 2 g of dehydrated fish food (Repashy Superfoods, Super Gold) was dissolved in 10 mL of 2.5% aqueous DMSO, which resulted in a 10 g food disk. The disk was allowed to dry in the fridge for 30-60 minutes, then a 4 mm biopsy punch was used to cut out control + DMSO pellets. This process was repeated with 6.25 mg of T dissolved in DMSO for the T pellets.

Experimental Design: Two milting fish (ranging ±5 g from each other) from a 160gal tank were selected and weighed. The fish were put into the experimental tank (30.48 cm x 60.96 cm x 45.72 cm), fed control pellets without DMSO, and left in the experimental tanks overnight to allow for habituation to the experimental settings. On the second day, the fish were fed control pellets without DMSO in the morning and afternoon. Colored fluorescent dots were glued onto the fish heads after the second feeding. On the third day, one male fish was fed a control pellet with DMSO (55.95±5.08 g) and the other fish was fed a T pellet (57.85±5.81 g). The female fish was primed with 50 µL of lutalyse and placed into the experimental tank. After habituating all three fish for 20 minutes, a camera situated overhead began to record behavioral data for 1 hour. The milt of the male fish were then collected in capillary tubes. The male fish's proximity and mean distance to the female fish was then tracked on the program Ethovision (Noldus, Ver. 14.1).

B. Anatomical approach

First, 10 milting fish were selected and injected with 25 µL of Ovaprim to increase their T production. They were left overnight in 6-liter tanks with no visual stimulus. The next day, a bolus of ethanol (530 µL of 9% ethanol) was injected into the tanks of 5 fish as a control and a bolus of 15-keto prostaglandin and prostaglandin (265 µL each, final concentration=$1.3x10^{-7}$ µL) into the tanks of 5 other fish ("pheromone-exposed fish"). The fish were removed 30 minutes after the bolus was injected and euthanized in MS-222. The brains were then removed, put into 4% PFA for 1.5 hours, embedded, and cut into 15 µm brain sections with a cryotome.

Different series of brain sections went through varying immunohistochemistry runs in order to determine the best protocol to clearly visualize the PS6 and Anti-HuC cell signals. Different dilutions (1:500, 1:1000, 1:2000) of anti-HuC were ran to determine the best dilution for visualization of signal. Images of brain sections that underwent the double-labeling immunohistochemistry run with PS6 and Anti-HuC were captured on the program Jenoptik. All pictures were taken at the same microscope light settings and contrast. To count the number of cell signals, the images were run through the program ImageJ.

Results

In the competitive behavior trials, there were some following behaviors, such as closer nudging of the females, expressed in the male fish that ate T pellets. However, preliminary analysis of the data indicate that fish fed T pellets did not significantly increase their average distance (Fig. 1) or time in proximity (Fig. 2) to females releasing ovulatory pheromones than fish fed control pellets.

In the brain images, all dilutions showed promising signal but the 1:2000 dilution of Anti-HuC appears to be the best signal (Fig. 3c). The signal surrounding the midline in the 1:2000 dilution showed the sharpest contrast and brightness compared to the other dilutions. We proceeded with the 1:2000 dilution in later immunohistochemistry runs with double-labeling. The double-labeling image (Fig. 4) showed all the neurons within the brain in a green signal (Alexa 488) and the activated PS6 in cells in a red signal (Alexa 594). The overlap of colors illustrated a neuron that was activated by the injected bolus of pheromone.

Discussion

These results suggest that rapid elevations of T may not result in increased responsiveness to female sexual stimuli, including ovulatory pheromones, that lead to increased competitive mating success. However, we have not yet measured the elevations of T that resulted from our manipulations. It is possible that the control fish in contact with females had rapid elevations of T that masked the influences of the exogenous T we administered. Our sample size was small, and the fish may have

deviated from their natural behavior due to the stress caused by gluing on fluorescent hats and other handling in the experiment. The behavioral paradigm developed will allow for future experiments, perhaps with larger sample sizes, to track and observe the competitive behavior of male goldfish.

Determining an effective double-labeling immunohistochemistry protocol with PS6 and Anti-HuC will allow for an accurate method of counting the double-labeled colored cells per green cell for the purpose of obtaining a measure of activated neurons per total neurons in an area. With this protocol developed, we will be in position to identify whether testosterone induced more cell activity in response to sex pheromones and if so, where in the brain.

Figures

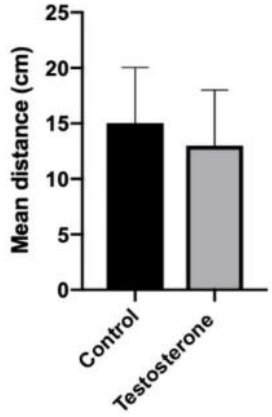


Figure 1*:* The mean distance from the male fish to the female fish in male subjects that ate a control pellet (n=11) and subjects that ate a T pellet (n=11). Distance was measured from the fluorescent hat on the male fish to the fluorescent hat on the female fish. Mean distance did not significantly decrease in the T pellet group (t-test, $p>0.05$).

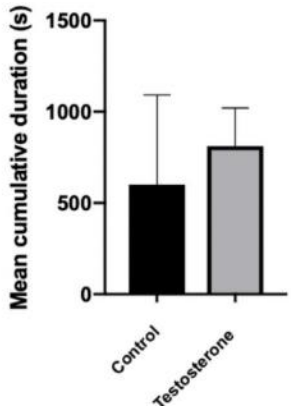
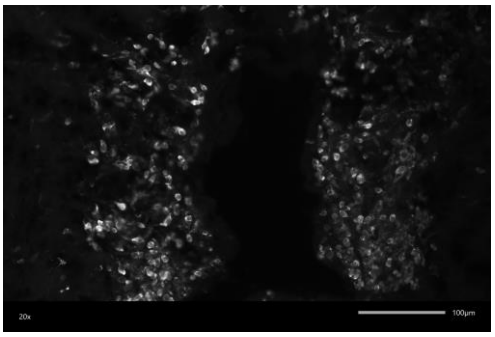
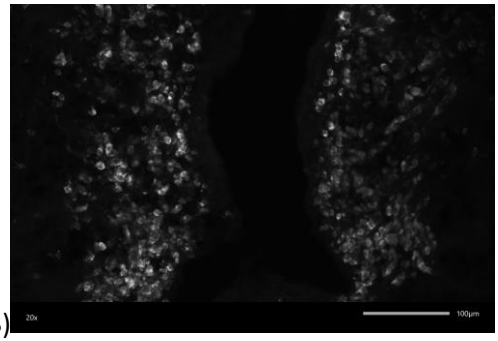


Figure 2: The mean cumulative duration that the male fish was within proximity (5 cm) of the female fish (n=11). The cumulative duration of fish that had eaten a testosteron pellet did increase but was not significant (t-test, $p>0.05$).

(A)  (B) 

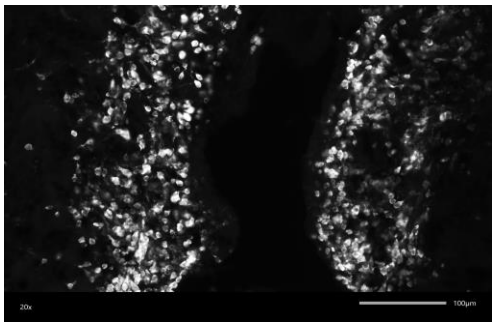
(C) 

Figure 3: Brain sections (20x magnification) at the preoptic area with an immunohistochemistry run with varying Anti-HuC dilutions. Midline of the brain is middle of image with no signal. (A) 1:500 dilution of anti-HuC. (B) 1:1000 dilution of anti-HuC. (C) 1:2000 dilution of anti-HuC.

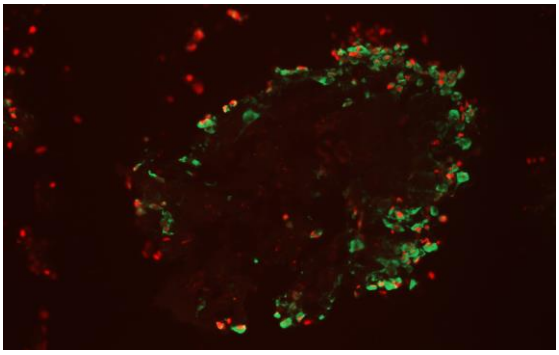


Figure 4: Multifluorescence of midbrain at 20x magnification. Red signal (Alexa 594) is PS6 and green signal (Alexa 488) is Anti-HuC. An overlap indicates a neuron activated by sensory stimuli.

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