Estradiol increases visual habituation learning independently of the canonical estrogen receptors

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Abstract

Habituating to the constant stimuli in the environment is a critical adaptive learning process conserved across species. We use the larval zebrafish visual response to sudden darkness as a model for studying habituation learning, where zebrafish reduce their responses to repeated stimulations. In this paradigm, treatment with Estradiol strongly increases learning rate, resulting in reduced responses. In an attempt to identify the receptor(s) mediating these effects we used established mutant lines with expected null alleles for the known estrogen receptors (*esr1*, *esr2a*, *esr2b*, *gper1*). Our experiments failed to identify a receptor required for the effects of Estradiol on habituation learning. Surprisingly, nuclear-receptor mutants showed increased habituation relative to sibling controls when treated with estradiol, indicating that activation of these receptors has paradoxical inhibitory effects on habituation learning. These experiments confirm that Estradiol is a potent modulator of learning in the vertebrate brain, but suggest that these effects occur independently of the classical estrogen receptor-mediated signaling pathways, which may in fact act to inhibit learning performance in this paradigm.

Introduction

A primary task of the brain is to learn from ongoing experiences and adjust behavior accordingly. This often involves sharpening attention and behavioural resources toward salient cues while tuning out irrelevant background stimuli. For instance, it may be critical to recognize the alarm calls of a nearby animal, whereas continually registering a steady hum from distant traffic is far less important. The capacity to reduce responses to repetitive, non-essential stimuli is known as habituation, a phenomenon widely considered one of the simplest forms of learning and memory (***Rankin et al., 2009***).

We have been studying a paradigm for long-term habituation where larval zebrafish reduce their responsiveness to sudden pulses of whole-field darkness, or dark flashes (DFs) (***Wolman et al., 2011***; ***Randlett et al., 2019***; ***Lamiré et al., 2023***). In this analysis, we emphasize long-term habituation as a practical model for examining the fundamental processes that shape neural circuit plasticity. We recently reported that multiple hormonal signaling pathways show strong modulation of habituation learning performance, including Melatonin, progesterone, and estrogen (***Lamiré et al., 2023***). The ability of these signaling pathways to modulate learning is consistent with previous results in other systems and paradigms (***Nilsson and Gustafsson, 2002***; ***Naderi et al., 2020***; ***Dillon et al., 2013***; ***Rawashdeh***

et al., 2007; *Jilg et al., 2019*; *El-Sherif et al., 2003*; *Barros et al., 2015*), and may be an important mechanism to shift learning and memory performance or strategies based on biological rhythms or external fluctuations like seasons, weather or the day/night cycle.

In this project we have focused on estrogens, where

Estrogen signaling in learning and memory, any data about receptors.

In this project we aimed to identify the relevant estrogen receptor mediating the effects of estradiol analogs on habituation learning by undertaking a classical analysis of genetic knockout alleles. Not only did we fail to identify a mutant (or combination of mutants) that lead to estradiol instensitivity, but our results indicat that esrX receptors act to inhibit habituation learning – effects which are usually masked by an unidentified estradiol-responsive pathway that promotes learning, resulting in estrogne receptor mutants that show increased sensitivity to estradiol in the habituation paradigm.

Materials and Methods

Animal Ethics Statement

Adult zebrafish used to generate larvae were housed in accordance with PRCI facility approved by the animal welfare committee (comité d'éthique en expérimentation animale de la Région Rhône-Alpes: CECCAPP, Agreement # C693870602). Behaviour experiments were performed at the 5dpf stage, and are thus not subject to ethical review, but these procedures do not harm the larvae.

Animals

All experiments were performed on larval zebrafish at 5 days post fertilization (dpf), raised at a density of ≈1 larvae/mL of E3 media supplemented with 0.02% HEPES pH 7.2. Larvae were raised in a 14:10h light/dark cycle at 28-29°C. Adult zebrafish were housed, cared for, and bred at the Lyon PRECI zebrafish facility. Mutant lines were obtained from D. Gorelick's lab, and were of the following alleles:

esr1^{uab118} is a 4bp deletion (ZDB-ALT-180420-2), yielding a predicted null frameshift/stop mutation, confirmed by a lack of estradiol responsiveness in the heart as assayed by *Tg(5xERE:GFP)*^{c262} expression (***Romano et al., 2017***).

esr2a^{uab134} is a 2bp deletion (ZDB-ALT-180420-3), yielding a predicted null frameshift/stop mutation (***Romano et al., 2017***)

esr2b^{uab127} is a 4bp deletion (ZDB-ALT-180420-4), yielding a predicted null frameshift/stop mutation, confirmed by a lack of estradiol responsiveness in the liver as assayed by *Tg(5xERE:GFP)*^{c262} expression (***Romano et al., 2017***).

gper1^{uab102} is a 133bp deletion (ZDB-ALT-180420-1), yielding a predicted null frameshift/stop mutation, confirmed by a lack of estradiol responsiveness in heart beating rate in maternal-zygotic mutants (***Romano et al., 2017***).

Genotyping

esr1^{uab118} was genotyped by PCR using the forward/reverse primer pair:
GCTGGTCACCTTGAATGCTT/TGAGATGTGAGAGATGACTAGGA with a T_M of 58°C yielding a 381 bp PCR product that was digested with the restriction enzyme ApeKI. The mutant product is not digested, and the wild type has two bands at 177 and 204 bp.

esr2a^{uab134} was genotypes by PCR using the forward/reverse primer pair:
CTTCAGCTGCAGGAAGTGGA/AAAGTCGGGCTTAGCGACTG with a T_M of 58°C yielding a 236 bp PCR product that was digested with the restriction enzyme MboI. The mutant product is not digested, and the wild type has two bands at 180 and 56 bp

esr2b^{uab127} was genotypes by PCR using the forward/reverse primer pair:
TGGGCCTGAGATGCAGTAGT/GTGTGTGTCTTGGCCTCCTC with a T_M of 60°C yielding a 431 bp PCR product that was digested with the restriction enzyme MbiI. The mutant product is digested into two bands of 150 and 281 bp and the wild type into 3 bands of 78, 150 and 198 bp.

gper1^{uab102} was genotypes by PCR using the forward/reverse primer pair:
ATGGAGGAGCAGACTACCAATGTG/CCATCCAGATGAGGCTGCAA with a T_M of 60°C yielding a mutant product of 372bp and a wild type product of 505 bp.

Pharmacology

β-Estradiol (Sigma E2758, here referred to as "estradiol") was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. Larvae were treated with estradiol immediately before the behavioural assay by pipetting 10-30uL of 10x solution directly into the behavioural wells, always with a final concentration of 0.1% DMSO in E3.

Results and Discussion

Nuclear Estrogen receptors are not required for the effects of estradiol on habituation learning

Conclusion

Here I described our system for tracking the tail of the larval zebrafish during microscopy. Many of the practical considerations of this setup may be specific to our application, and therefore may need modification for use in other experiments in other labs. However, I feel that the core and simple idea of using an IR-sensitive Raspberry Pi Camera, a simple Python script, coupled with IR LEDs and and IR filter, provides an approachable and flexible solution that may be widely useful for observing and tracking the behaviour of zebrafish (or perhaps other animals) while performing imaging experiments. This system's attributes may also make it an ideal tool for community engagement activities such as school outreach programs. It could serve as a platform for learning about microelectronics, behavioural analyses, machine vision, and hardware design and construction.

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Data Availability

Software and analysis code is available here: https://github.com/owenrandlett/2025_HabEstrogen. Datasets are available here: .

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