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 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, *esr1*, *esr2a*, and *gper1* mutants showed weak but consistent increases in habituation relative to sibling controls, indicating that activation of these receptors may have paradoxical inhibitory effects on habituation learning. These experiments confirm that estradiol is a potent modulator of learning processes in the vertebrate brain, but in the context of visual habituation learning in larval zebrafish, these effects do not occur via the classical estrogen receptor-mediated signaling pathways, which instead act in competition to subtly inhibit habituation learning. Therefore, our data indicate that the positive effects of estradiol on habituation learning occur via an as-yet unidentified receptor, or via allosteric modulation of a parallel signaling pathway.

Introduction

A primary function of the brain is to learn from experiences and adjust behavior in response. One aspect of learned behaviour involves sharpening attention and behavioural resources toward salient cues by ignoring irrelevant background stimuli. For instance, it may be critical to recognize the alarm calls of a nearby animal, whereas continually registering the steady hum from distant traffic is far less important. The capacity to reduce responses to repetitive, non-essential stimuli is known as habituation, which is considered the simplest form of learning and memory (***Rankin et al., 2009***).

We study 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***). 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, 2002a; ***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 estrogen signaling. We identified multiple estradiol analogs which strongly increased habituation learning when added at 5-10μM doses in the water (ethinyl estradiol, estradiol valerate, and hexestrol, ***Lamiré et al., 2023***). 17β-estradiol (here referred to as estradiol) is the most potent and biologically active form of estrogen, and is used in a variety of clinical contexts including contraception, hormone replacement therapy and feminizing hormone therapy. Our discovery of a role for estradiol in promoting habituation learning is not surprising, as it has well-documented effects on other learning and memory processes. This has been most extensively characterized in the hippocampus, where estradiol promotes behavioural performance and the cellular/circuit hallmarks of hippocampal plasticity, including Long-term potentiation (LTP) and modulation of dendritic spine density (***Iqbal et al., 2024***; ***Luine, 2014***; ***Finney et al., 2020***; ***Nilsson and Gustafsson, 2002b***). While the role of estradiol in habituation is less well explored, it has previously been shown to increase memory retention for olfactory habituation in mice (***Dillon et al., 2013***), indicating it plays broad roles in plasticity regulation.

Estradiol signals via two classes of Estrogen Receptors (ERs): the ligand-activated transcription factors ERα, and ERβ, and the seven-transmembrane G-protein coupled receptor GPER1. ERα/β are thought to mediate the long-term genomic effects of estrogens through transcriptional activation of target genes, and thus are typically termed nuclear ERs. Gper1 is thought to mediate acute Estrogen-dependent signaling – often called "non-genomic", or "membrane-initiated", and does so via multiple G-proteins, and potentially epidermal growth factor (EGF) receptor transactivation (***Prossnitz and Barton, 2023***; ***Revankar et al., 2005***; ***Filardo et al., 2000***). In this way, Gper1 signalling impacts multiple core second-messenger systems, including: adenylyl cyclase, ERK, PI3K-Akt, and nitric oxide synthase. There is evidence from receptor-specific pharmacology and genetic knockout experiments for a role of all of these receptors in hippocampal plasticity, although ERα appears to have the most prominent role. EXPAND A BIT AND REFs

In this project we aimed to identify the relevant ER(s) mediating the effects of estradiol on habituation using genetic knockout alleles. Zebrafish have single gene encoding ERα (*esr1*) and GPER1 (*gper1*), and two homologs of ERβ : ERβ1 (*esr2a*) and ERβ2 (*esr2a*) (***Romano et al., 2017***; ***Menuet et al., 2002***). We found that none of these mutants were insensitive to estradiol's effects, indicating that estradiol acts in this context via an alternative receptor or pathway. Surprisingly, our experiments found that mutants for *esr1*, *esr2a*, and *gper1* actually habituate more than their sibling controls. While the effect size is small and behavioural-genetic experiments can be variable, these data indicate that these receptors actually act to inhibit habituation learning, rather than mediating the habituation-promoting effects of estradiol that we discovered pharmacologically.

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.

Habituation behaviour testing

Larval behavior was evaluated in 300-well plates using an updated version of the experimental setup previously described (***Randlett et al., 2019***; ***Lamiré et al., 2023***). Briefly, 300-well plates were custom made using laser-cut acrylic sheets where each well measures 8 mm in diameter and 6 mm in depth, corresponding to an approximate water volume of 300 µL. These plates are suspended under a water bath held at 31°C, acting as a heated lid to minimize condensation and maintain a 29°C water temperature within each well. Behavioral recordings were made using a Mikrotron CXP-4 camera running at 444hz in conjunction with a Silicon Software frame grabber (Marathon ACX-QP, Basler), illuminated by IR LEDs (TSHF5410, digikey.com). Visual stimuli were presented using a rectangular array of 155 WS2813 RGB LEDs (144LED/M, aliexpress.com). For the DF stimulus, the LEDs were briefly switched off (1 s), then linearly returned to the original brightness over a 20 s interval. Acoustic tap stimuli were administered using a solenoid (ROB-10391, Sparkfun). This behavioral paradigm was designed to be symmetrical: each 1 hr block of stimulation was followed by 1 hr of rest. During these rest periods, the camera was moves using a stepper motor controlled linear actuator (Hanpose HPV4, 500cm), which moved the camera between two plates, allowing us to screen up to 600 fish per experiment across two 300-well plates.

Control of the apparatus (RGB LEDs, solenoid, camera linear actuator) was implemented via a Raspberry Pi Pico microcontroller running CircuitPython (https://circuitpython.org/) (code : code.py) and custom Python software which handled the camera acquisition via the Python wrapper of the Silicon Software Framgrabber SDK, triggered stimuli via the Raspberry Pi Pico, and tracked the head and tail coordinates of the larvae across the 300-wells at a baseline framerate of between 20-30hz (code : Run_BigRig2.py). When a stimulus is delivered (DF or Acoustic Tap), a 1-second "Burst" video is recorded at the full frame rate as a Tiff file, from which the head and tail coordinates are subsequently tracked offline (code : TrackBurst_BigRig.py). Larval zebrafish tracking was done via background subtraction and morphological operations implemented using multiple open-source packages, including: OpenCV (***Bradski, 2000***), scikit-image (***Van der Walt et al., 2014***), NumPy (***Harris et al., 2020***), SciPy (***Virtanen et al., 2020***), and Numba (***Lam et al., 2015***).

Data analysis

Data was analyzed in Python using custom written analysis scripts (code : Analyze_EsrHab.py). Responses to DFs and acoustic taps were identified as movement events that had a cumulative tail bend angle greater than 3 rad and 1 rad, respectively. Data was analyzed using multiple open-source packages, including: NumPy (***Harris et al., 2020***), SciPy (***Virtanen et al., 2020***),Numba (***Lam et al., 2015***) and Pandas (***Wes McKinney, 2010***). Data was plotted using Matplotlib (***Hunter, 2007***) and seaborn (***Waskom, 2021***). Statistical "significance" between the distributions was tested using the Mann-Whitney U test implemented in Scipy (***Virtanen et al., 2020***).

The cumulative difference plots to assess changes in habituation performance for the treatments were calculated as previously (***Randlett et al., 2019***), where we first calculated the average response across larvae for each group for each DF. This generated a mean vector for each group. These two vectors were normalized by dividing them by the naive response (mean response to the first 5 DFs), and then the treatment group was subtracted from the control group, yielding a mean difference vector between stimulus and controls at each flash. From this mean difference vector we calculated the cumulative mean distribution using Numpy's nancumsum function. To generate statistical confidence in these distributions, we bootstrapped 2000 replicates, and calculated the 99.5% confidence intervals using SciPy's stats.norm.interval funciton. The assumption of this analysis is that if the two groups are habituating similarly, then the difference vectors will also have a mean of 0, and thus the cumulative mean distribution would remain near 0. Treatments that affect habituation will show strong increasing or decreasing cumulative mean distributions, reflecting increased or decreased habituation performance throughout training, respectively.

Results and Discussion

Estradiol increases habituation learning

In response to a sudden global dimming event, which we refer to as a dark flash (DF), larval zebrafish execute an "O-bend" maneuver, characterized by a deep "O"-shaped bend and a high-amplitude turn (***Burgess and Granato, 2007***). Habituation learning manifests as a progressive reduction in response to repeated stimuli, and this learning can be retained for seconds/minutes or hours/days for short- and long-term habituation, respectively (***Rankin et al., 2009***). We use high-speed cameras, machine-vision analysis, and 300-well plates to quantify habituation across large populations of larvae to identify molecular/genetic mechanisms of long-term habituation(***Figure 1***A,B, ***Randlett et al., 2019***; ***Lamiré et al., 2023***). When stimulated with DFs repeated at 1-minute intervals in repeated blocks of 60 stimuli, larval zebrafish exhibit long-term habituation, reducing not only the probability of executing a response, but also modulating the latency and other kinematic aspects of the response (***Randlett et al., 2019***).

Our previous small-molecule screening experiments identified multiple synthetic Estrogen Receptor agonists as positive modulators of dark-flash habituation learning, including ethinyl estradiol, estradiol valerate, and hexestrol (***Lamiré et al., 2023***). The major effect we observed was a stronger decrease in the probability of executing a O-bend response during the training/learning blocks. We have confirmed and extended these results using β-Estradiol (estradiol), which is the major natural estrogen in humans.

An acute dose of 10μM estradiol potently increases habituation learning, which is observable when the response probability of the population of estradiol-treated larvae is compared with DMSO-treated vehicle controls across stimuli (***Figure 1***C-H). Consistent with our previous experiments (***Lamiré et al., 2023***), there is a modest reduction in the initial responsiveness of the estradiol-treated larvae to the first DF stimulus (***Figure 1***D), but the major effect is observed during the training phase (***Figure 1***C,E,F), as is revealed by the consistent positive deviation in the cumulative mean difference plot (***Figure 1***H, ***Randlett et al., 2019***). Importantly, the responsiveness of the larvae to acoustic stimuli delivered after the DF training is indistinguishable from controls (***Figure 1***Ci, G), indicating that estradiol does not affect global arousal levels but rather has specific effects on habituation learning.

Membrane-initiated estradiol signaling via Gper1 does not increase habituation learning

The effects of estradiol we have observed occur very rapidly – larvae are only pretreated with estradiol for ≈25min-1hr before the first DF, as this is the time necessary to set the apparatus and begin the experiment. Since classical nuclear hormone receptors (like the Esr1 and Esr2) exert their effects via transcriptional adaptation, this necessitates a delay in signaling response. Therefore, we hypothesized that membrane-initiated signaling through Gper1 was the more likely mechanism. To test this we obtained and tested the presumed null frameshift/early stop allele

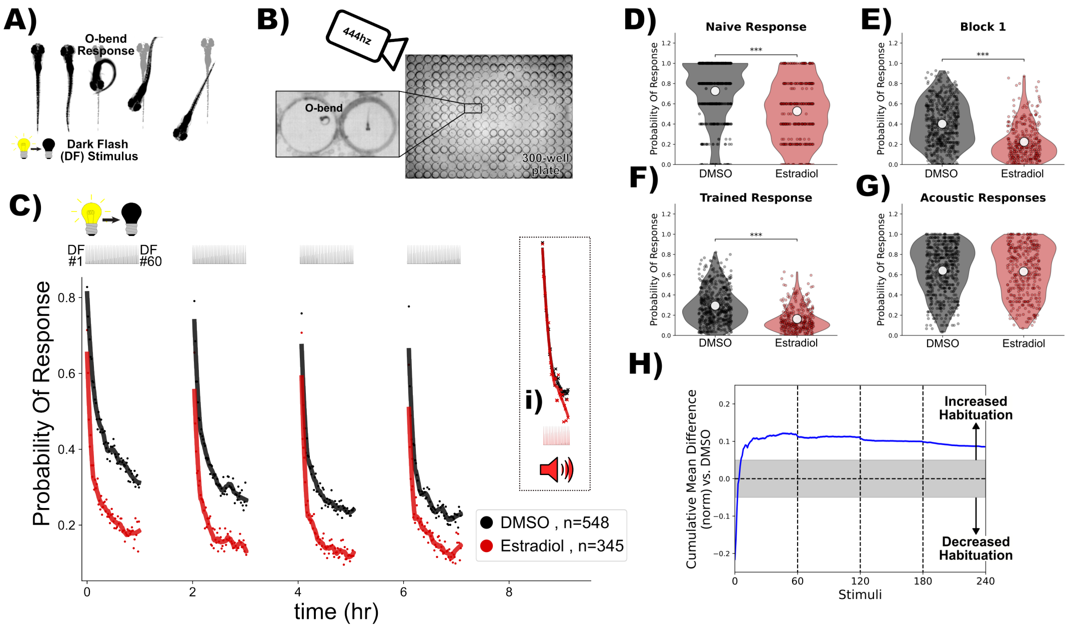

Figure 1. Estradiol increases habituation learning.
A) In response to a dark flash (DF), larval zebrafish perform a large turning maneuver termed an O-bend response.
B) High-throughput setup for recording and quantify O-bend responsiveness using a high-speed camera recording at 444hz observing larvae in 300-well plates.
C) Treatment with estradiol (red) results in more rapid and profound decreases in the probability of response to DF stimuli during habituation training relative to DMSO vehicle controls (black). DF stimuli are delivered at 1-minute intervals, in 4 blocks of 60 stimuli, separated by 1hr of rest (from 0:00-7:00). 1.5 hours later a block of 30 acoustic stimuli are delivered at 1-minute intervals (**i**). Each dot is the probability of response to one DF. Lines are smoothed in time with a Savitzky–Golay filter (window = 15 stimuli, order = 2).
D-G) Distributions responsiveness for different epochs of the experiment. Each dot is the per-fish average of the epoch. Statistical significance was calculated using Mann-Whitney U test, *** = $p < 0.001$. **D)** the naive response to the first 5 DF stimuli; **E)** the mean response to the remaining DF stimuli in the Block 1 (DFs 6:60); **F)** the trained response to the last 45 DFs in all four training blocks (DFs 16:60,76:120,136:180,196:240); **G)** the 30 acoustic stimuli delivered with a tap from a solenoid on the 300-well plate platform.
H) Cumulative mean difference plot quantifying relative habituation performance after estradiol treatment. These plots display the cumulative average differences in the mean response across larvae of the treatment group (estradiol) relative to the control group (DMSO). Difference from 0 reflect a divergence in the change in responsivness across the 240 DF stimuli in the 4 training blocks, with positive values reflecting increased habituation. The widths of the line is a bootstrapped 99.5% confidence intervals. The gray boxed region reflects the expected non-significant effect size (***Randlett et al., 2019***).
Treatment groups are: Estradiol = 10μM estradiol treatment (n = 345 fish); DMSO = 0.1% DMSO vehicle controls (n = 548 fish)

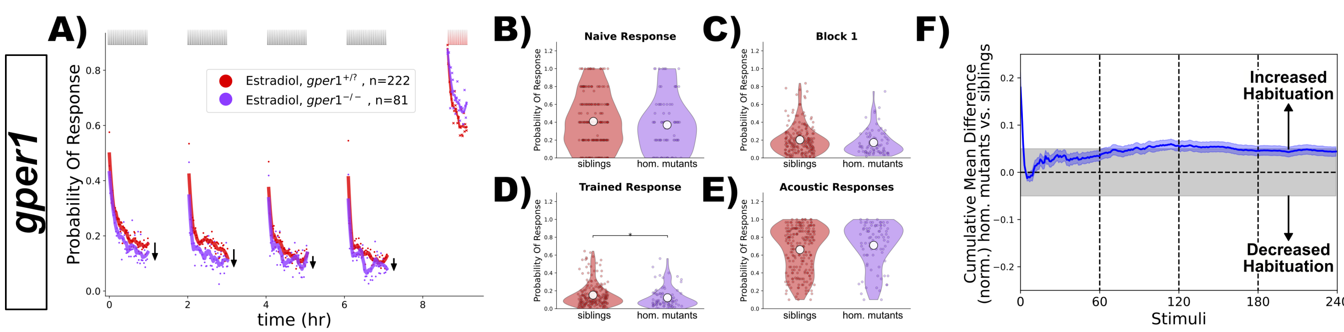

Figure 2. *gper1* mutants do not show habituation deficits after treatment with estradiol.
A) Homozygous *gper1*$^{(-/-)}$ mutants do not show impaired habituation relative to sibling controls (*gper1*$^{(+/-)}$ and *gper1*$^{(+/+)}$). Rather, there is a slight suppression of responsiveness in the mutant group (arrows), indicating weakly increased habituation.
B-E) No significant differences are observed in the responsiveness distributions for the naive response to the first 5 DF stimuli (**B**), during the first training block (**C**), or the acoustic response (**E**), while a subtle but statistically significant decrease in responsiveness is observed in the trained response (**D**). Statistical significance was calculated using Mann-Whitney U test, * = $p < 0.05$.
F) Cumulative mean difference plot quantifying relative habituation performance in after estradiol treatment, consistent with slightly increased habituation rate in mutant larvae.

gper1^{uab102}, which was previously shown to be insensitive to estradiol for heart-rate modulation (***Romano et al., 2017***).

To test if *gper1* is required for the effects of estradiol on habituation we generated larvae from *gper1^{uab102}* heterozygous or homo/hererozygous crosses to generate clutches of larvae of mixed genotypes. Larvae were treated with estradiol during habituation, and were subsequently genotyped. We reasoned that if *gper1* is required for the effect of estradiol on habituation, mutants would habituate significantly less than controls. Contrary to this hypothesis, we found that *gper1* mutants showed no deficits in habituation (***Figure 2***). Remarkably, rather than observing the anticipated inhibition of habituation, *gper1* mutants appeared to habituate slightly more, with the responsiveness trendline running slightly but consistently below the sibling controls (***Figure 2***A). This is further supported by a weak but statistically significant decrease in the responsiveness of the larvae during the training period (***Figure 2***D), and a trend towards positive values in the Cumulative Mean DIfference plot (***Figure 2***F).

From these experiments we conclude that:

1) Gper1 does not mediate the positive effects of estradiol on habituation learning, and therefore these effects are mediated by a different receptor.

2) Gper1 appears to have a paradoxical inhibitory effect on habituation learning, although this effect size is relatively minor and does not rise substantially above biological noise levels.

3) The observed paradoxical inhibitory effect confirms that these mutants do have a phenotype in our assay, despite it not being of the predicted sign. This provides compelling evidence that our negative results do not simply result from an issue or mischaracterization of this predicted null-allele (***Romano et al., 2017***).

Nuclear-mediated estradiol signaling via Esr1, Esr2a or Esr2b does not increase habituation learning

Since we found the *gper* was unnecessary for the positive effects of estradiol on habituation learning, we next focused on the three nuclear receptors in the zebrafish genome: *esr1*, *esr2a* and *esr2a*. Using the same strategy as for *gper*, we analyzed previously established and presumed null mutants (*esr2a^{uab134}*, *esr2a^{uab134}*, and *esr2b^{uab127}*), looking for a mutant with habituation deficits after estradiol treatment. However, we again failed to identify any decreases in habituatuin (***Figure 3***), and to our surpise we again found that both *esr1* and *esr2a* mutants showed sublte **increases** in habituation (***Figure 3***Avi,Bvi), similar in magnitude to what we had seen for *gper1* mutants (***Figure 2***F).

Membrane-initiated and nuclear-mediated signaling are simultaneously dispensable for estradiol-induced enhancements in habituation learning

Finally, since it is formally possible that nuclear and membrane-initated estradiol-signaling could act in a redundant fashion, we analyzed combinations of double and triple mutants, but again failed to identify suppressions in habituation.

Conclusions

Other ERs have been identified, such as ERX and an STX-sensitive Gq-membrane ER, but are less well characterized (Srivastava et al., 2013)

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Data Availability

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

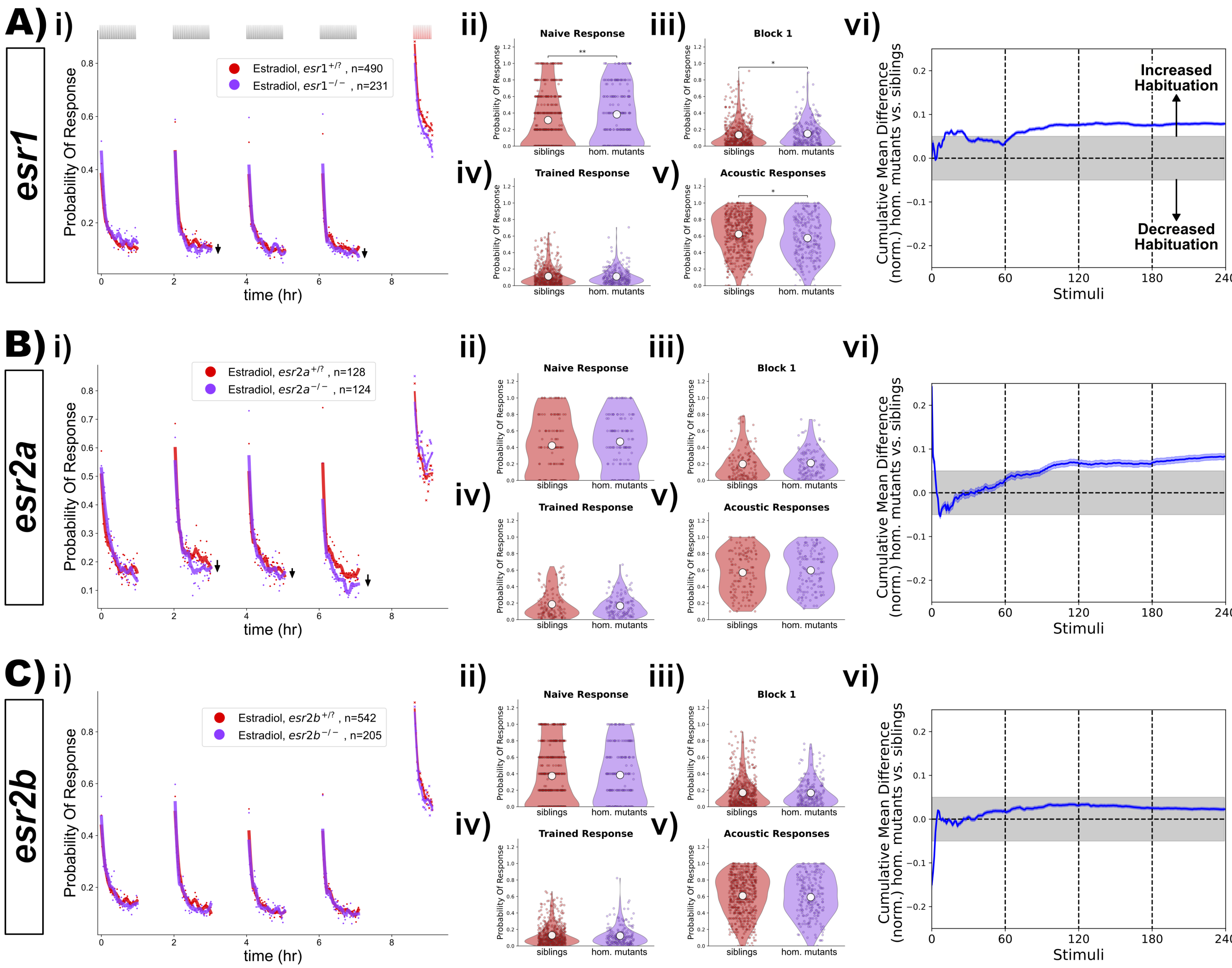

Figure 3. *esr1*, *esr2a* and *esr2a* mutants do not show habituation deficits after treatment with estradiol.
A) Homozygous *esr1*$^{(-/-)}$ mutants do not show impaired habituation relative to sibling controls (*esr1*$^{(+/-)}$ and *esr1*$^{(+/+)}$).
B) Homozygous *esr2a*$^{(-/-)}$ mutants do not show impaired habituation relative to sibling controls (*esr2a*$^{(+/-)}$ and *esr2a*$^{(+/+)}$).
C) Homozygous *esr2b*$^{(-/-)}$ mutants do not show impaired habituation relative to sibling controls (*esr2b*$^{(+/-)}$ and *esr2b*$^{(+/+)}$).

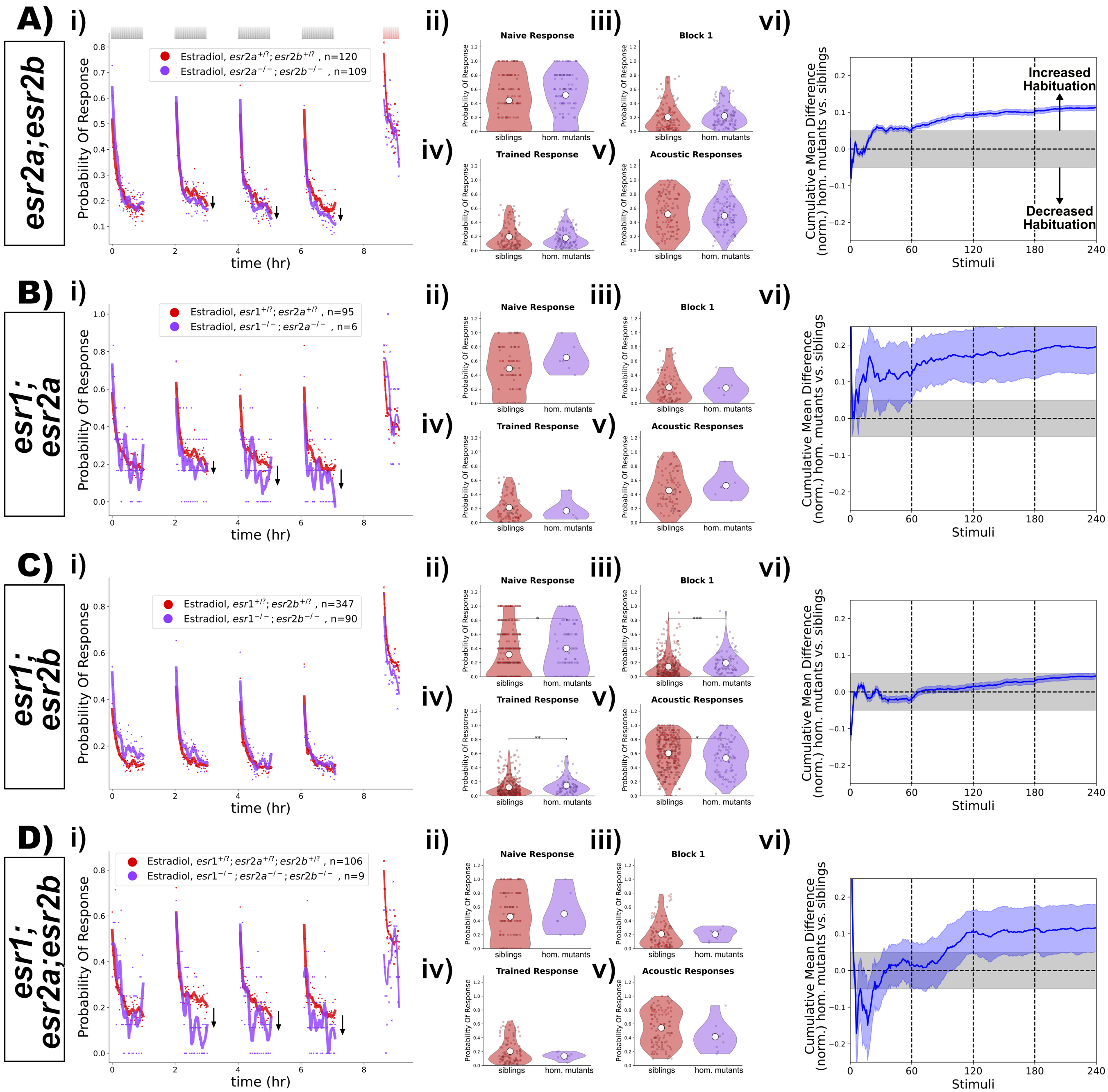

Figure 4. Double and triple mutant combinations of *esr1*, *esr2a* and *esr2a* do not show habituation deficits after treatment with estradiol.

A) Homozygous *esr1*$^{(-/-)}$ mutants do not show impaired habituation relative to sibling het/wt controls.

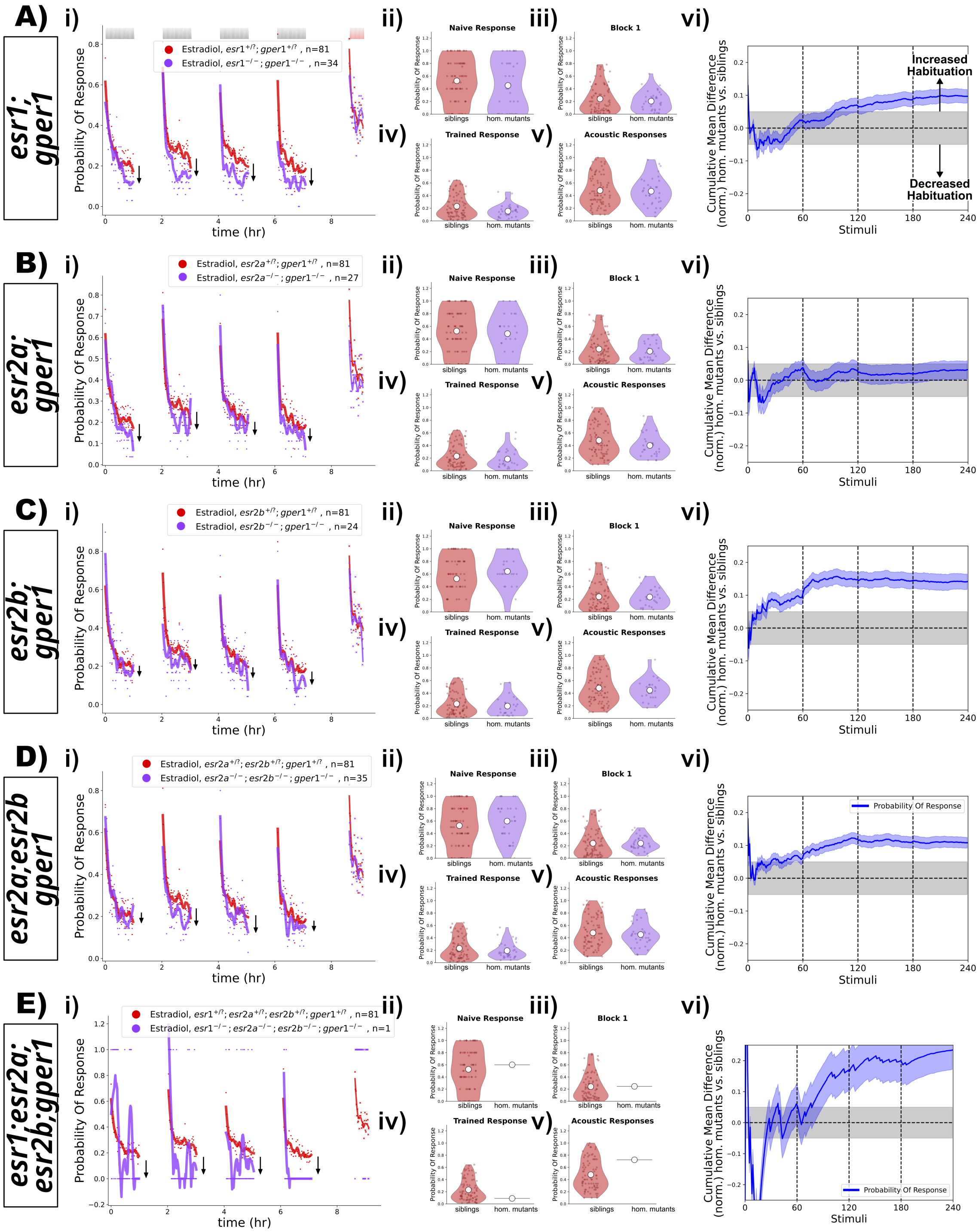

Figure 5. Double and triple and quaruple mutant combinations of *esr1*, *esr2a*, *esr2a*, and *gper1* do not show habituation deficits after treatment with estradiol.

A) Homozygous *esr1*$^{(-/-)}$ mutants do not show impaired habituation relative to sibling het/wt controls.

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