

¹ Estradiol promotes habituation learning via an unidentified target, bypassing the suppressive effects of established Estrogen Receptors

⁵ Andrew Hsiao ^{!,1,2}, Isabelle Darvaux-Hubert ^{!,1}, Dominique Hicks ^{!,1,3}, Emilie Joux ^{1,2},
⁶ Sarah De Freitas ^{1,2}, Emeline Dracos ^{1,2}, Jeanne Lizé ^{1,2}, Julien Perrichet¹, Dominique
⁷ Baas ^{*,1}, Owen Randlett ^{*,1,@}

¹ Laboratoire MeLiS, Université Claude Bernard Lyon 1 - CNRS UMR5284 - Inserm U1314, Institut NeuroMyoGène, Faculté de Médecine et de Pharmacie, 8 avenue Rockefeller, 69008 Lyon, France

² International Master in Life Sciences, Université Claude Bernard Lyon 1, France

³ Master of Biology Program, École normale supérieure de Lyon, France

[!]equal contribution

^{*}equal contribution

⁸ @correspondence: owen.randlett@univ-lyon1.fr, ORCID: 0000-0003-0181-5239

⁹ Disclosures

¹⁰ The authors have nothing to disclose.

¹¹ Keywords

¹² Estrogens, estradiol, learning, zebrafish, behaviour, habituation, Gper1, ER α , ER β , unidentified target

¹³

¹⁴ Abstract

¹⁵ Habituating to the constant stimuli in the environment is a critical learning process conserved across species.
¹⁶ We use a larval zebrafish visual response to sudden darkness as a model for studying habituation learning,
¹⁷ where zebrafish reduce their responses to repeated stimulations. In this paradigm, treatment with estradiol
¹⁸ strongly increases learning rate, resulting in more strongly suppressed responses. We used mutant lines for the
¹⁹ Estrogen Receptors (*esr1*, *esr2a*, *esr2b*, *gper1*) in an attempt to identify the receptor(s) mediating these effects.
²⁰ These experiments failed to identify a necessary receptor (or combination of receptors). Surprisingly, *esr1*, *esr2a*,
²¹ and *gper1* mutants showed weak but consistent increases in habituation, indicating these receptors suppress
²² habituation learning. These experiments demonstrate that estradiol is a complex modulator of learning in our
²³ model, where the learning-promoting effects are mediated by an unidentified estradiol target, and the classical
²⁴ Estrogen Receptors act in competition to subtly suppress learning.

²⁵

26 Introduction

27 A primary function of the brain is to learn from experiences and adjust behavior in response. One aspect of
28 learned behaviour involves sharpening attention and behavioural resources toward salient cues by ignoring
29 irrelevant background stimuli. For instance, it may be critical to recognize the alarm calls of a nearby animal,
30 whereas continually registering the steady hum from distant traffic is far less important. The capacity to reduce
31 responses to repetitive, non-essential stimuli is known as habituation, which is considered the simplest form
32 of learning and memory [1].

33 We study a paradigm for long-term habituation where larval zebrafish reduce their responsiveness
34 to sudden pulses of whole-field darkness, or dark flashes (DFs) [2, 3, 4]. We recently reported that
35 multiple hormonal signaling pathways show strong modulation of habituation learning performance, including
36 melatonin, progesterone, and estrogen [4]. The ability of these signaling pathways to modulate learning is
37 consistent with previous results in other systems and paradigms [5, 6, 7, 8, 9, 10, 11], and may be an important
38 mechanism to shift learning and memory performance or strategies based on biological rhythms or external
39 fluctuations like seasons, weather or the day/night cycle.

40 In this project we have focused on estrogen signaling. We identified multiple estradiol analogs which
41 strongly increased habituation learning when bath applied at 5-10 μ M doses (ethinyl estradiol, estradiol valerate,
42 and hexestrol, 4). 17 β -estradiol (here referred to as estradiol) is the most potent and biologically active form of
43 estrogen, and is used in a variety of clinical contexts including contraception, hormone replacement therapy,
44 and feminizing hormone therapy [12, 13, 14].

45 Our discovery of a role for estradiol in promoting habituation learning is not surprising, as it has
46 well documented effects on other learning and memory processes [15]. This has been most extensively
47 characterized in the hippocampus, where estradiol promotes behavioural performance and the cellular/circuit
48 hallmarks of hippocampal plasticity, including Long-term potentiation (LTP) and modulation of dendritic spine
49 density [16, 17, 18, 5]. While the role of estradiol in habituation is less well explored, it has previously been
50 shown to increase memory retention for olfactory habituation in mice [7], indicating it plays conserved roles in
51 plasticity regulation across paradigms.

52 Estradiol signals via two established classes of Estrogen Receptors (ERs): the ligand-activated transcription
53 factors ER α , and ER β , and the seven-transmembrane G-protein coupled receptor Gper1. ER α and ER β mediate
54 long-term, genomic effects of estrogens through transcriptional regulation of target genes. However, both ER α
55 and ER β are also present at the cell membrane, where they contribute to rapid, non-genomic, or membrane-
56 initiated estrogen signaling. Gper1 also mediates rapid estrogen-induced physiological responses via
57 membrane-initiated signaling pathways involving various G-proteins, and potentially through transactivation
58 of the epidermal growth factor receptor (EGFR) [19, 20, 21]. In this way, Gper1 signalling impacts multiple
59 core second-messenger systems, including: adenylyl cyclase, ERK, PI3K-Akt, and nitric oxide synthase. There
60 is evidence from receptor-specific pharmacology and genetic/mutant experiments in mice for a role of all of
61 these receptors in hippocampal plasticity [18, 22, 23].

62 Pharmacological experiments in adult zebrafish indicate that ER α and ER β are involved in the consolidation
63 of object recognition memory, while Gper1 is involved in the consolidation of object placement memory [6].
64 Therefore, both classes of receptors are good candidates for plasticity regulation in zebrafish. However, we
65 are unaware of any previous studies using mutant lines to test for the function of zebrafish ERs in learning or
66 memory.

67 In this project we aimed to identify the relevant ER(s) mediating the effects of estradiol on habituation using
68 mutant alleles. Zebrafish have single gene encoding ER α (ER1, *esr1*) and Gper1 (*gper1*), and two homologs of
69 ER β : ER2a (*esr2a*) and ER2b (*esr2b*) [24, 25]. We found that none of these mutants were insensitive to estradiol's
70 effects, indicating that estradiol acts in this context via an alternative receptor or pathway. Surprisingly, our

71 experiments found that mutants for *esr1*, *esr2a*, and *gper1* actually habituate more than their sibling controls.
72 While the effect size is small and behavioural-genetic experiments can be variable, these data indicate that
73 these ERs actually act to inhibit habituation learning, rather than mediating the habituation-promoting effects
74 of estradiol that we observe pharmacologically.

75 Materials and Methods

76 Animals

77 All experiments were performed on larval zebrafish at 5 days post fertilization (dpf), raised at a density of \approx 1
78 larvae/mL of E3 media supplemented with 0.02% HEPES pH 7.2. Larvae were raised in a 14:10h light/dark
79 cycle at 28-29°C. Adult zebrafish were housed, cared for, and bred at the following zebrafish facilities: Plateau
80 de Recherche Expérimentale en Criblage In Vivo (PRECI, SFR Biosciences, Lyon) and the Animalerie Zebrafish
81 Rockefeller (AZR, SFR Santé Lyon Est, Lyon). Adult zebrafish used to generate larvae were housed in accordance
82 with regulations of the PRECI and AZR facilities, which are regulated by an internal animal wellbeing committee,
83 and were approved by the animal welfare committee (comité d'éthique en expérimentation animale de la
84 Région Rhône-Alpes: CECCAPP, Agreement # C693870602). Behaviour experiments were performed at the
85 5dpf stage, and are thus not subject to ethical review, but these procedures do not harm the larvae.

86 Mutant lines were obtained from D. Gorelick's lab, and were of the following alleles:

87 *esr1uab118* is a 4bp deletion (ZDB-ALT-180420-2), yielding a predicted null frameshift/stop mutation,
88 confirmed by a lack of estradiol responsiveness in the heart as assayed by *Tg(5xERE:GFP)^{c262}* expression [24].

89 *esr2a^{uab134}* is a 2bp deletion (ZDB-ALT-180420-3), yielding a predicted null frameshift/stop mutation [24]

90 *esr2b^{uab127}* is a 4bp deletion (ZDB-ALT-180420-4), yielding a predicted null frameshift/stop mutation,
91 confirmed by a lack of estradiol responsiveness in the liver as assayed by *Tg(5xERE:GFP)^{c262}* expression [24].

92 *gper1uab102* is a 133bp deletion (ZDB-ALT-180420-1), yielding a predicted null frameshift/stop mutation,
93 confirmed by a lack of estradiol responsiveness in heart beating rate in maternal-zygotic mutants [24].

94 Genotyping

95 *esr1uab118* was genotyped by PCR using the forward/reverse primer pair:

96 GCTGGTCACCTTGAATGCTT/TGAGATGTGAGAGATGACTAGGA with a T_m of 58°C yielding a 381 bp PCR product
97 that was digested with the restriction enzyme ApeKI. The mutant product is not digested, and the wild type has
98 two bands at 177 and 204 bp.

99 *esr2a^{uab134}* was genotypes by PCR using the forward/reverse primer pair:

100 CTTCAGCTGCAGGAAGTGGAAAGTCGGCTTAGCGACTG with a T_m of 58°C yielding a 236 bp PCR product that
101 was digested with the restriction enzyme MboI. The mutant product is not digested, and the wild type has two
102 bands at 180 and 56 bp

103 *esr2b^{uab127}* was genotypes by PCR using the forward/reverse primer pair:

104 TGGGCCTGAGATGCAGTAGT/GTGTGTCTTGGCCTC with a T_m of 60°C yielding a 431 bp PCR product that
105 was digested with the restriction enzyme Mbil. The mutant product is digested into two bands of 150 and 281
106 bp and the wild type into 3 bands of 78, 150 and 198 bp.

107 *gper1uab102* was genotypes by PCR using the forward/reverse primer pair:

108 ATGGAGGAGCAGACTACCAATGTG/CCATCCAGATGAGGCTGCAA with a T_m of 60°C yielding a mutant product of
109 372bp and a wild type product of 505 bp.

110 Pharmacology

111 β -Estradiol (Sigma E2758, here referred to as "estradiol") was dissolved in dimethyl sulfoxide (DMSO) and stored

112 at -20°C. Larvae were treated with estradiol immediately before the behavioural assay by pipetting 10-30 μ L of
113 10x solution directly into the behavioural wells, always with a final concentration of 0.1% DMSO in E3.

114 **Habituation behaviour testing**

115 Larval behavior was evaluated in 300-well plates using an updated version of the experimental setup previously
116 described [3, 4]. Briefly, 300-well plates were custom made using laser-cut acrylic sheets where each well
117 measures 8 mm in diameter and 6 mm in depth, corresponding to an approximate water volume of 300 μ L.
118 These plates are suspended under a water bath held at 31°C, acting as a heated lid to minimize condensation
119 and maintain a 29°C water temperature within each well. Behavioral recordings were made using a Mikrotron
120 CXP-4 camera running at 444hz in conjunction with a Silicon Software frame grabber (Marathon ACX-QP, Basler),
121 illuminated by IR LEDs (TSHF5410, digikey.com). Visual stimuli were presented using a rectangular array of 155
122 WS2813 RGB LEDs (144LED/M, aliexpress.com). For the DF stimulus, the LEDs were briefly switched off (1 s),
123 then linearly returned to the original brightness over a 20 s interval. Vibration/Tap stimuli were administered
124 using a solenoid (ROB-10391, Sparkfun). This behavioral paradigm was designed to be symmetrical: each 1
125 hr block of stimulation was followed by 1 hr of rest. During these rest periods, the camera was moves using
126 a stepper motor controlled linear actuator (Hanpose HPV4, 500cm), which moved the camera between two
127 plates, allowing us to screen up to 600 fish per experiment across two 300-well plates.

128 Control of the apparatus (RGB LEDs, solenoid, camera linear actuator) was implemented via a Raspberry
129 Pi Pico microcontroller running CircuitPython (<https://circuitpython.org/>) (code : [code.py](#)) and custom Python
130 software which handled the camera acquisition via the [Python wrapper of the Silicon Software Framegrabber](#)
131 [SDK](#), triggered stimuli via the Raspberry Pi Pico, and tracked the head and tail coordinates of the larvae across
132 the 300-wells at a baseline framerate of between 20-30hz (code : [Run_BigRig2.py](#)). When a stimulus is delivered
133 (DF or Vibration/Tap), a 1-second "Burst" video is recorded at the full frame rate as a Tiff file, from which the head
134 and tail coordinates are subsequently tracked offline (code : [TrackBurst_BigRig.py](#)). Larval zebrafish tracking
135 was done via background subtraction and morphological operations implemented using multiple open-source
136 packages, including: OpenCV [26], scikit-image [27], NumPy [28], SciPy [29], and Numba [30].

137 **Data analysis**

138 Data was analyzed in Python using custom written analysis scripts (code : [Analyze_EsrHab.py](#)). Responses to
139 DFs and vibration/taps were identified as movement events that had a cumulative tail bend angle greater than
140 3 radian (O-bend) and 1 radian (C-bend), respectively. Data was analyzed using multiple open-source packages,
141 including: NumPy [28], SciPy [29], Numba [30] and Pandas [31]. Data was plotted using Matplotlib [32] and
142 seaborn [33]. Statistical "significance" between the distributions was tested using the Mann-Whitney U test
143 implemented in Scipy [29].

144 The cumulative difference plots to assess changes in habituation performance for the treatments were
145 calculated as previously [3], where we first calculated the average response across larvae for each group for
146 each DF. This generated a mean vector for each group. These two vectors were normalized by dividing them
147 by the naive response (mean response to the first 5 DFs), and then the treatment group was subtracted from
148 the control group, yielding a "mean difference" vector between stimulus and controls at each flash. From this
149 mean difference vector we calculated the cumulative mean distribution using Numpy's 'nancumsum' function
150 divided by the number of stimuli experienced, or the index of the vector. To generate statistical confidence
151 in these distributions, we bootstrapped 2000 replicates, and calculated the 99.5% confidence intervals using
152 SciPy's 'stats.norm.interval' function. The assumption of this analysis is that if the two groups are habituating
153 similarly, then the "mean difference" vector will exhibit a noise distribution centered at a mean of 0, and
154 thus the cumulative mean distribution would remain near 0. Treatments that affect habituation will show
155 strong increasing or decreasing cumulative mean distributions, reflecting increased or decreased habituation

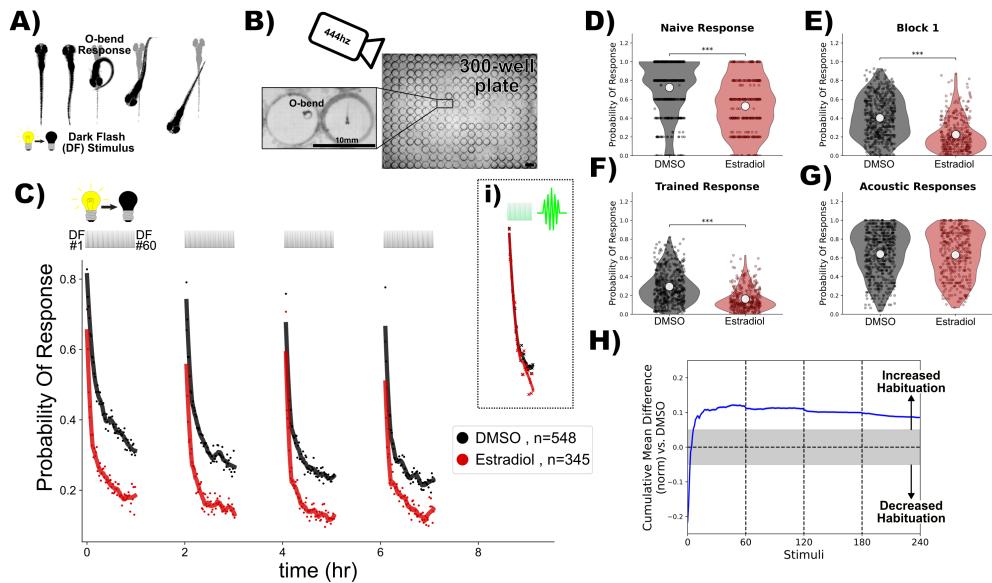


Figure 1. Estradiol increases habituation learning.

A) In response to a dark flash (DF), larval zebrafish perform a large turning manoeuvre termed an "O-bend" response.
 B) High-throughput setup for recording and quantifying responsiveness using a high-speed camera recording at 444hz observing larvae in 300-well plates. Scale bar = 10mm.
 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 vibration 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 vibration stimuli delivered with a tap from a solenoid on the 300-well plate platform.
 H) Cumulative mean difference (CMD) 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 responsiveness 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 [3].
 Treatment groups are: Estradiol = 10 μ M estradiol treatment ($n = 345$ fish); DMSO = 0.1% DMSO vehicle controls ($n = 548$ fish)

156 performance throughout training, respectively. We use an empirically defined threshold of ± 0.05 as the
 157 statistically meaningful effect size in this analysis, as is reflected in the shaded gray regions in the plots [3].

Results

159 Estradiol increases visual habituation learning

160 In response to a sudden global darkening stimulus, which we refer to as a dark flash (DF), larval zebrafish
 161 execute an "O-bend" maneuver, characterized by a deep "O"-shaped bend and a high-amplitude turn (34,
 162 **Figure 1a**). Habituation learning manifests as a progressive reduction in response to repeated stimuli, and this
 163 learning can be retained for seconds/minutes, or hours/days for short-, and long-term habituation, respectively
 164 [1]. We use high-speed cameras, machine-vision analysis, and 300-well plates to quantify habituation across
 165 large populations of larvae to identify molecular/genetic mechanisms of long-term habituation (**Figure 1A,B,**
 166 **3, 4**). When stimulated with DFs repeated at 1-minute intervals in blocks of 60 stimuli, larval zebrafish exhibit
 167 long-term habituation, reducing not only the probability of executing a response, but also modulating the
 168 latency and other kinematic aspects of the response [3].

169 Our previous small-molecule screening experiments identified multiple synthetic Estrogen Receptor
 170 agonists as positive modulators of DF habituation learning at 5-10 μ M doses, including ethinyl estradiol,
 171 estradiol valerate, and hexestrol [4]. The major effect we observed was a stronger decrease in the probability

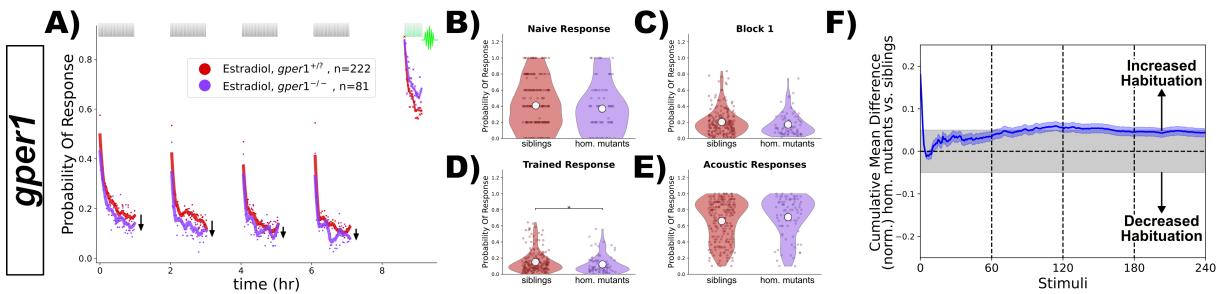


Figure 2. *gper1* mutants do not show habituation deficits after treatment with estradiol.

A) Homozygous *gper1*^{-/-} mutants (n = 81 fish, purple) do not show impaired habituation relative to sibling controls (*gper1*^{+/+}) and *gper1*^(+/+), n = 222 fish, red). Rather, there is a slight suppression of responsiveness in the mutant group (arrows), indicating weakly increased habituation. Each dot is the probability of response to one stimulus. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2).

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 vibration 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 (CMD) plot quantifying habituation performance of mutants relative to sibling controls, consistent with slightly increased habituation rate in mutant larvae.

172 of executing a O-bend response during the training/learning blocks. We have confirmed and extended these
173 results using estradiol, which is the major natural estrogen in vertebrates.

174 An acute dose of 10 μ M estradiol potently increases habituation learning, which is observable when the
175 response probability of the population of estradiol-treated larvae is compared with DMSO-treated vehicle
176 controls (Figure 1C-H). Consistent with our previous experiments [4], there is a reduction in the naive
177 responsiveness of the estradiol-treated larvae to the first DF stimuli (Figure 1D), but the major effect is observed
178 during the training phase (Figure 1C,E,F), as is revealed by the consistent positive deviation in the cumulative
179 mean difference (CMD) plots that are normalized to the naive response level in order to quantify response
180 suppression indicative of habituation performance (Figure 1H, 3). Importantly, the responsiveness of the larvae
181 to vibration stimuli delivered after the DF stimulation (Figure 1Ci), which elicit c-bend escape responses [35], is
182 indistinguishable from controls (Figure 1G). From this we conclude that estradiol does not affect global arousal
183 levels but rather has specific effects on habituation learning.

184 **Gper1 is dispensable for estradiol-promoted habituation learning**

185 The effects of estradiol that we have observed occur very rapidly – larvae are only pretreated with estradiol
186 for \approx 25min-1hr before the first DF. This is the time necessary to set the apparatus and begin the experiment.
187 Since ER α and ER β are thought to primarily exert their effects via transcriptional alterations, this necessitates a
188 delay in this aspect of their signaling. For this reason, we first hypothesized that membrane-initiated signaling
189 through Gper1 was the most likely mechanism.

190 To test this we used the *gper1*^{uab102} mutant [24], and generated larvae from *gper1*^{uab102} heterozygous or
191 homo/heterozygous crosses to generate clutches of larvae of mixed genotypes. Larvae were treated with
192 estradiol during habituation, and were subsequently genotyped. We reasoned that if *gper1* is required for the
193 effect of estradiol on habituation, mutants would be insensitive to estradiol and habituate significantly less than
194 sibling controls. Contrary to this hypothesis, we found that *gper1* mutants showed no deficits in habituation
195 (Figure 2). Remarkably, rather than observing the anticipated inhibition of habituation, *gper1* mutants appeared
196 to habituate slightly more than controls, with the responsiveness level slightly but consistently below the sibling
197 controls across stimuli (Figure 2A). This is further supported by a weak but statistically significant decrease
198 in the responsiveness of the larvae during the training period (Figure 2D), and a deviation towards positive
199 values in the CMD plot (Figure 2F). From these experiments we conclude that Gper1 agonism does not promote
200 habituation learning, but rather may act to suppress it.

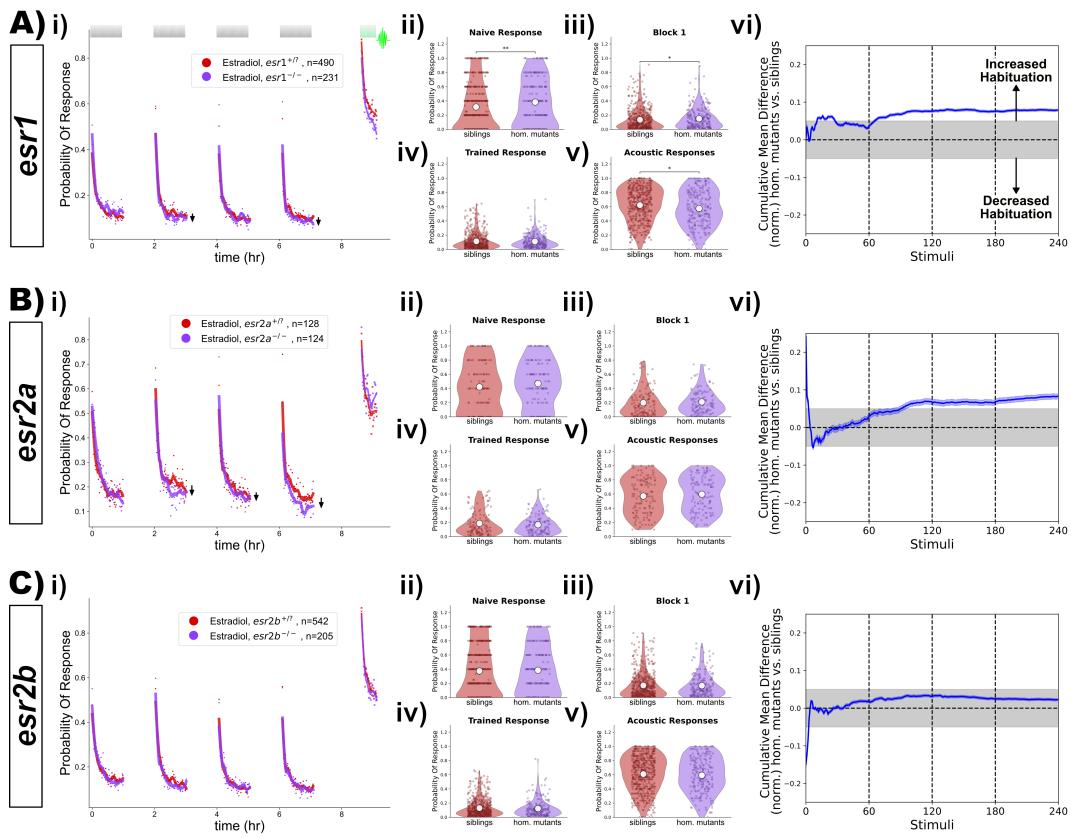


Figure 3. *esr1*, *esr2a* and *esr2b* mutants do not show habituation deficits after treatment with estradiol.

A) Homozygous *esr1*^(-/-) mutants (n = 231 fish) do not show impaired habituation relative to sibling controls (*esr1*^(+/+) and *esr1*^(+/+), n = 490 fish).

B) Homozygous *esr2a*^(-/-) mutants (n = 124 fish) do not show impaired habituation relative to sibling controls (*esr2a*^(+/+) and *esr2a*^(+/+), n = 128 fish).

C) Homozygous *esr2b*^(-/-) mutants (n = 205 fish) do not show impaired habituation relative to sibling controls (*esr2b*^(+/+) and *esr2b*^(+/+), n = 542 fish).

i-vi) For each lettered section:

i) Responsiveness to stimuli comparing homozygous mutants to sibling controls (heterozygous or wild-type). Each dot is the probability of response to one stimulus. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2). Suppression of responsiveness is indicated by arrows, potentially reflecting increased habituation.

ii-v) 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 < 0.05, ** = p < 0.01. ii) the naive response to the first 5 DF stimuli; iii) the mean response to the remaining DF stimuli in the Block 1 (DFs 6:60); iv) the trained response to the last 45 DFs in all four training blocks (DFs 16:60,76:120,136:180,196:240); v) the 30 vibration stimuli delivered with a tap from a solenoid on the 300-well plate platform.

vi) Cumulative mean difference (CMD) plot quantifying habituation performance of mutants relative to sibling controls

ER α and ER β are dispensable for estradiol-promoted habituation learning

Since we found that *gper1* was unnecessary for the habituating-promoting effects of estradiol, we next focused on the three other ERs in the zebrafish genome: *esr1*, *esr2a* and *esr2b*. While we initially prioritized Gper1 due to its rapid signaling properties that aligned with the rapid actions of estradiol on habituation, it is equally possible that the rapid membrane-initiated actions of ER α and/or ER β could underlie these effects. Using the same strategy as for *gper1*, we analyzed previously established mutants (*esr2a*^{uab134}, *esr2a*^{uab134}, and *esr2b*^{uab127}), looking for a mutant with insensitivity to estradiol. However, we failed to identify any deficits in habituation (**Figure 3**). To our surprise, we again found that both *esr1* and *esr2a* mutants showed subtle increases in habituation (**Figure 3Avi,Bvi**), similar in magnitude to what we had seen for *gper1* mutants (**Figure 2F**). From these data we conclude that none of the identified ERs are required for the effects of estradiol on promoting habituation. As for Gper1, ER1 and ER2a show a weak inhibitory effect on habituation, indicating that they also act to suppress habituation learning.

213 **ER α and ER β are simultaneously dispensable for estradiol-promoted habituation
214 learning**

215 While our experiments demonstrated that *esr1*, *esr2a* and *esr2b* mutants remain sensitive to estradiol, it is
216 plausible that they could act in a redundant fashion to mediate the effects of estradiol on habituation, perhaps
217 via co-expression in a critical cell type, or via genetic compensation [36]. To test for this possibility, we generated
218 combinations of mutants by crossing individual lines together (*Figure 4*). A likely scenario could be that the two
219 ER β paralogs, ER2a and ER2b, act redundantly. However, we found that double mutants for *esr2a;esr2b* did not
220 show habituation deficits (*Figure 4A*). Neither did we observe suppression of habituation in double mutants
221 for *esr1;esr2a* (*Figure 4B*), or *esr1;esr2b* (*Figure 4C*). Finally, we tested triple mutants (*esr1;esr2a;esr2b*), but again
222 failed to identify suppressions in habituation (*Figure 4D*).

223 We note that we did see a statistically "significant" increase in DF responsiveness in the *esr1;esr2b* double
224 mutants (*Figure 4Cii-iv*). This is attributable to a small increase in naive responsiveness, and did not result
225 from habituation deficits according to our normalized CMD analysis (*Figure 4Cvi*). Considering that we did
226 not observe any increased responsiveness in the triple mutants (*Figure 4D*), we conclude that the observed
227 increased responsiveness in the double mutants is likely a result of biological noise, that only rose to statistical
228 "significance" due to the very large number of larvae tested in our high-throughput experiments.

229 Consistent with the paradoxical effect of increased habituation in *esr1* and *esr2a* single mutants (*Figure 3A,B*),
230 we again observed that double and triple mutants containing these genes also showed a slight increase in
231 habituation (with the exception of the *esr1;esr2b* double mutants). This adds further support to the model in
232 which ER1 and ER2b act to suppress learning in this context, rather than promote it.

233 **Gper1, ER α , and ER β are simultaneously dispensable for estradiol-promoted
234 habituation learning**

235 While it is unclear to us how Gper1 might act redundantly with ER α/β , we nevertheless decided to test this
236 possibility by combining the *gper1* and the *esr1*, *esr2a*, and *esr2b* mutations (*Figure 5*). As with the previous
237 iterations of this experiment, we did not find combinations of mutants with suppressed habituation (*Figure 5*).
238 Consistent with our model of (*gper1*, *esr1* and *esr2a*) having inhibitory affects on learning, we again found
239 that most of these combinations of mutants showed evidence of increased habituation (*Figure 5i,vi*). While
240 the responsiveness distributions did not show significant differences (*Figure 5ii-iv*), the normalized CMD plots
241 consistently showed positive deviations, which reflect increased habituation (*Figure 5vi*).

242 Despite the fact that we analyzed the behaviour of 1152 larvae and successfully genotyping all 4 ER genes
243 in 373 individuals (after >4600 genotyping PCRs), we were only able to identify a single quadruple mutant
244 larva lacking all known ERs (*Figure 5E*). This is likely simply due to the limitations of combinatorial Mendelian
245 inheritance, and the fact that *esr1* and *esr2a* are linked on chromosome 20. While it is dubious to conclude much
246 from an n = 1 experiment, we find it remarkable that this larva exhibits the strongest increased habituation of
247 all of our experiments, with suppression of responses (*Figure 5Ei*), and strong positive deviation in the CMD
248 plot (*Figure 5Ev*).

249

Discussion

250 **The promotion of habituation learning by estradiol is mediated by an unidentified
251 target**

252 Our experiments indicate that Gper1, ER1, ER2a and ER2b do not mediate the positive effects of estradiol on
253 habituation learning. As this is fundamentally a negative result, it is difficult to conclusively demonstrate this
254 beyond any doubt. One major caveat relates to the actual functional nature of the mutant alleles that we have

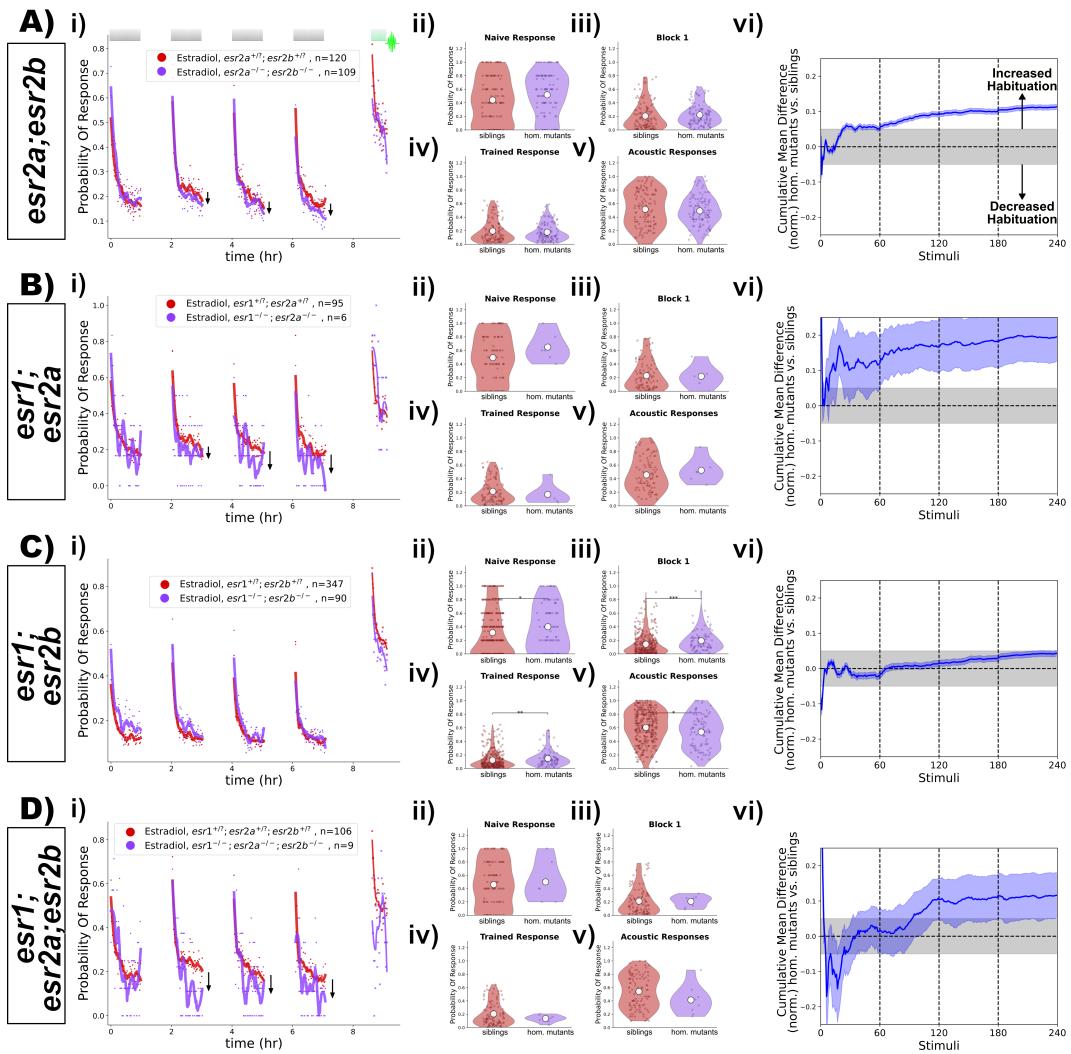


Figure 4. Double and triple mutant combinations of *esr1*, *esr2a* and *esr2a* do not show habituation deficits after treatment with estradiol.

A) Homozygous *esr2a^(-/-);esr2b^(-/-)* double mutants ($n = 109$ fish) do not show impaired habituation relative to double heterozygous or homozygous sibling controls ($(^{+/-})$, $(^{+/-})$, $n = 120$ fish).

B) Homozygous *esr1^(-/-);esr2a^(-/-)* double mutants ($n = 6$ fish) do not show impaired habituation relative to double heterozygous or homozygous sibling controls ($(^{+/-})$, $(^{+/-})$, $n = 95$ fish).

C) Homozygous *esr1^(-/-);esr2b^(-/-)* double mutants ($n = 90$ fish) do not show impaired habituation relative to double heterozygous or homozygous sibling controls ($(^{+/-})$, $(^{+/-})$, $n = 347$ fish).

D) Homozygous *esr1^(-/-);esr2a^(-/-);esr2b^(-/-)* triple mutants ($n = 9$ fish) do not show impaired habituation relative to triple heterozygous or homozygous sibling controls ($(^{+/-})$, $(^{+/-})$, $(^{+/-})$, $n = 106$ fish).

i-vi) For each lettered section:

i) Responsiveness to stimuli comparing homozygous mutants to sibling controls (heterozygous or wild-type). Each dot is the probability of response to one stimulus. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2).

ii-v) 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, * = $p < 0.05$, ** = $p < 0.01$. **ii)** the naive response to the first 5 DF stimuli; **iii)** the mean response to the remaining DF stimuli in the Block 1 (DFs 6:60); **iv)** the trained response to the last 45 DFs in all four training blocks (DFs 16:60, 76:120, 136:180, 196:240); **v)** the 30 vibration stimuli delivered with a tap from a solenoid on the 300-well plate platform.

vi) Cumulative mean difference (CMD) plot quantifying habituation performance of mutants relative to sibling controls.

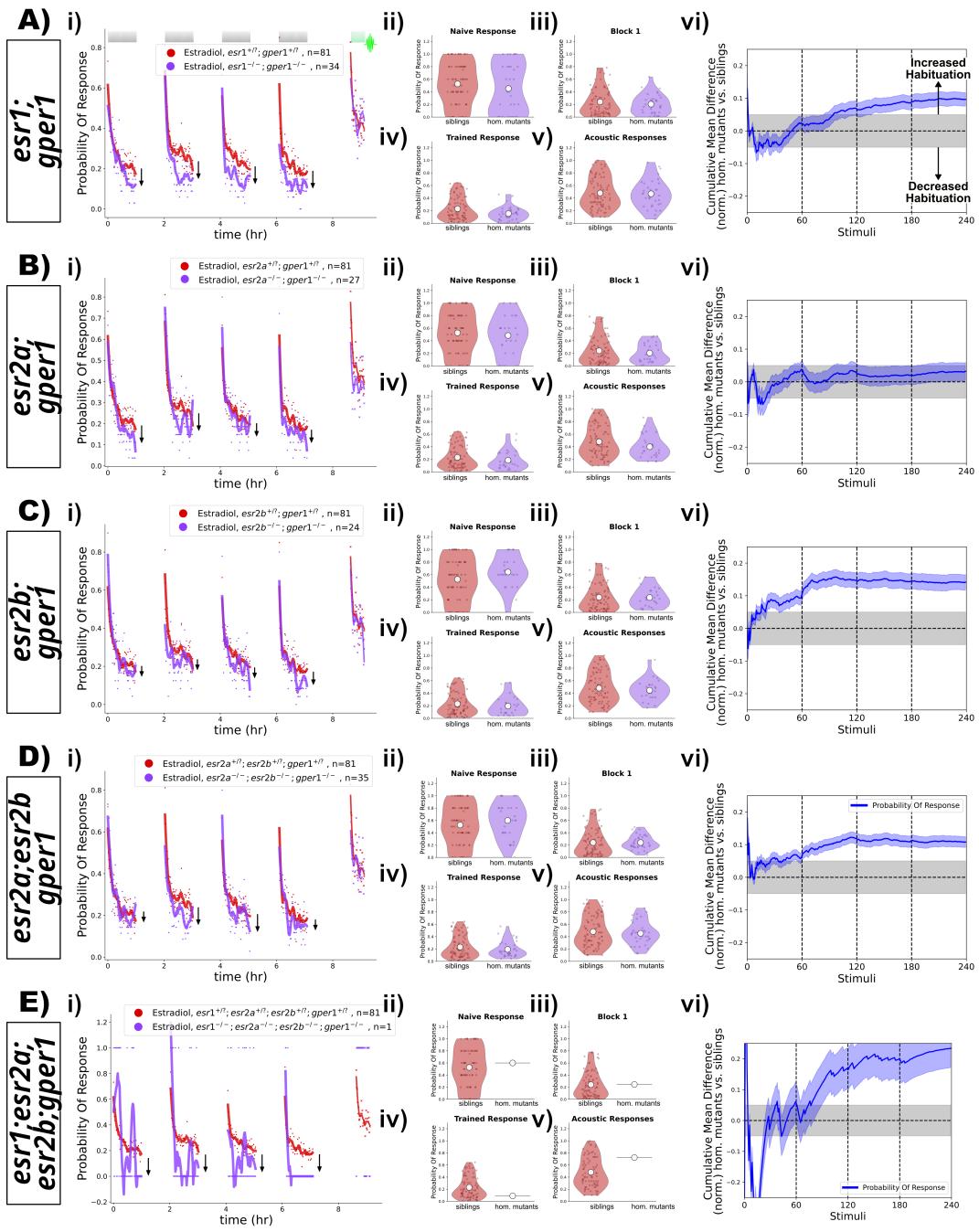


Figure 5. Double, triple and quadruple mutant combinations of *esr1*, *esr2a*, *esr2b*, and *gper1* do not show habituation deficits after treatment with estradiol.

A) Homozygous *esr1^(-/-);gper1^(-/-)* double mutants (n = 34 fish, purple) do not show impaired habituation relative to double heterozygous or homozygous sibling controls (n = 81 fish, red). **B)** Homozygous *esr2a^(-/-);gper1^(-/-)* double mutants (n = 27 fish, purple) do not show impaired habituation relative to double heterozygous or homozygous sibling controls (n = 81 fish, red). **C)** Homozygous *esr2b^(-/-);gper1^(-/-)* double mutants (n = 24 fish, purple) do not show impaired habituation relative to double heterozygous or homozygous sibling controls (n = 81 fish, red). **D)** Homozygous *esr2a^(-/-);esr2b^(-/-);gper1^(-/-)* triple mutants (n = 35 fish, purple) do not show impaired habituation relative to heterozygous or homozygous sibling controls (n = 81 fish, red). **E)** A single homozygous *esr1^(-/-);esr2a^(-/-);esr2b^(-/-);gper1^(-/-)* quadruple mutant (n = 1 fish, purple) does not appear to show impaired habituation relative to heterozygous or homozygous sibling controls (n = 81 fish, red).

i)-vi) For each lettered section: **i)** Responsiveness to stimuli comparing homozygous mutants to sibling controls (heterozygous or wild-type). Each dot is the probability of response to one stimulus. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order = 2). **ii)-v)** 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 < 0.05, ** = p < 0.01. **ii)** the naive response to the first 5 DF stimuli; **iii)** the mean response to the remaining DF stimuli in the Block 1 (DFs 6:60); **iv)** the trained response to the last 45 DFs in all four training blocks (DFs 16:60,76:120,136:180,196:240); **v)** the 30 vibration stimuli delivered with a tap from a solenoid on the 300-well plate platform. **vi)** Cumulative mean difference (CMD) plot quantifying habituation performance of mutants relative to sibling controls. As mutant larvae were all derived from the same experiments, the sibling control data (red) is the same in **A-E**.

used. These are all Cas9-generated small deletions resulting in frameshift mutations that lead to early stop codons, and are thus predicted null/knockout lines. Despite this genetic confidence, it is always possible that residual activity could still remain, perhaps via alternate splicing or alternate start codons. This could be further complicated by genetic/transcriptional compensation, where frameshift alleles can lead to the upregulation of paralogs in some circumstances [36]. As with all negative results, it is not possible to rule out all possible alternative explanations. However, we recognize that this bias against publishing negative results is bad for science. Negative results from well-designed and executed experiments are of value for the community and making this knowledge public is our duty as responsible scientists [37].

While the possibility of "residual activity" in our mutants is a clear limitation of our approach, we argue that this alternative interpretation is very unlikely. The *esr1^{uab118}* and *esr2b^{uab127}* alleles both exhibited a lack of estradiol responsiveness in other tissues [24], and *esr2b^{uab127}* mutants are female sterile/subfertile (D. Gorelick, personal communication), indicating a non-functional receptor. Similarly, *gper1^{uab102}* mutants show a lack of estradiol responsiveness in heart rate modulation [24]. Interestingly this was only observed in maternal-zygotic mutants. While it seems unlikely that sufficient maternal mRNA/protein for Gper1 could persist in 5dpf larvae, we can formally rule this out with our current datasets. The *esr2a^{uab134}* mutants have no previously published phenotype, and so we do not have an independent positive control for the nature of this allele. However, the best evidence we have against the "residual activity" hypothesis is that we actually found phenotypes in our assays for *esr1^{uab118}*, *esr2a^{uab134}*, and *gper1^{uab102}* mutants. These phenotypes are just of the unexpected sign, where mutants show increased habituation (discussed below).

Candidate estradiol targets that could promote habituation learning

We have concluded that the lack of habituation deficits in our mutants is due to the presence of an alternative receptor or pathway that mediates the learning-promoting effects of estradiol. In support of this model, a very recent study of estradiol-induced thrombosis came to the same conclusion, namely that an unidentified target mediates this process in larval zebrafish [38]. Importantly, these thrombosis experiments were performed on different knockout alleles which are full genetic deletions, in which "residual activity" is not plausible. Together with our observations, these data suggest that a novel estradiol target exists which has multiple critical functions (at least in zebrafish).

What might this unidentified target be? Various leads exist in the literature. One hypothesis posits the existence of an unidentified "Gq-mER" (Gq-coupled membrane estrogen receptor) [39, 40], and therefore estradiol may signal via additional GPCRs beyond Gper1. Another possibility is an interaction between estradiol and other membrane receptors, for example: the Voltage-Gated Sodium Channel Nav1.2 [41, 42], transient receptor potential (TRP) channels [43, 44], or various other ion channels [45]. The robust nature of our "non-canonical" but clearly estradiol-dependent phenotype, combined with the high-throughput nature of our behavioural assays, could be an ideal assay for future screening efforts to attempt to identify novel estradiol target(s).

Multiple Estrogen Receptors act to suppress habituation learning.

While we were surprised to find that the classical ERs do not promote habituation, we were shocked to find evidence of the opposite! We found that *esr1^{uab118}*, *esr2a^{uab134}*, and *gper1^{uab102}* mutants habituate more than their sibling controls (**Figure 1**, **Figure 2**), consistent with a role for these ERs in inhibiting habituation. While effect sizes of these magnitudes border on those that are easily dismissible as "noise", they were not only observed in the single mutants experiments, but also in the double and triple mutant combinations of these alleles (**Figure 3-Figure 5**), providing good evidence that they are biologically meaningful effects. In fact, these multi-mutants generally exhibited larger effect sizes, consistent with an additive interaction.

Untangling the mechanisms of ER1-, ER2a- and Gper1-dependent suppression of habituation will require

299 considerable further work. The additive interaction we observed genetically indicates that the ERs act
300 cooperatively to suppress habituation learning. All three receptors are expressed in the larval zebrafish brain
301 [46, 24], but whether they are acting in the same or different cell types awaits characterization. The study of this
302 inhibitory pathway may be challenging since it opposes the major learning-promoting effect of estradiol, and
303 therefore may be more straightforward to study after the identification and deletion of the estradiol target that
304 promotes habituation. One attractive hypothesis relates to the observation that estradiol exposure increases
305 aromatase expression in the zebrafish brain [47, 48]. Since aromatase catalyzes the conversion of androgens
306 into estrogens, ER mutants may have reduced levels of endogenous endogenous estrogens in the brain, which
307 could lead to inhibited habituation. Future experiments aimed at manipulating the aromatase system in wild-
308 type and ER mutant backgrounds could be used to test this hypothesis.

309 Conclusion

310 What began as a straightforward study to identify the receptor(s) that mediate the habituation-promoting
311 effects of estradiol has instead led us to a surprising and paradoxical result; canonical ERs do regulate
312 habituation, but are suppressive and act in opposition to the habituation-promoting effects of estradiol. This
313 fits with the general theme of our studies of this habituation paradigm – we find increasing complexity
314 and contradiction within this "simple" learning process the deeper we look. This began with our detailed
315 observations of behaviour, leading us to conclude that habituation results from a distributed plasticity process
316 that adapts different aspects of behavior independently [3]. We believe that this property underlies our
317 subsequent discoveries of pharmacological and genetic manipulations that can result in either specific changes
318 in specific aspects of habituation (but not others), or even opposing effects, where a single manipulation can
319 simultaneously increase and decrease habituation, depending on which component of behavior is measured
320 [3, 4]. This complexity appears to be a fundamental property of habituation [49], and that the study of
321 habituation will likely continue to surprise us, hopefully leading to unexpected insights into the nature of
322 plasticity underlying learning and memory.

323 Data Availability Statement

324 Software and analysis code is available here: https://github.com/owenrandlett/2025_HabEstrogen. All datasets
325 used in these analyses are available here: [HabEstrogen_Datasets](#).

326 Acknowledgments

327 We thank the Gorelick lab for sharing the ER-mutant zebrafish lines, and for sharing genotyping protocols and
328 expertise, and the Randlett group members for helpful advice regarding the manuscript and work. We are
329 grateful to the staff of the PRECI and AZR zebrafish facilities, including Laure Benard, Robert Renard, Annie
330 Desenfant, Olivier Lohez, for the expert care provided to the zebrafish. We also gratefully acknowledge the
331 communities that develop and maintain the numerous open-source software packages we rely on, most of
332 which could not be cited here.

333 Funding

334 This work was supported by funding from the ATIP-Avenir program of the CNRS and Inserm, a Fondation Fyssen
335 research grant, and the IDEX-Impulsion initiative of the University of Lyon.

References

- [1] Catharine H Rankin, Thomas Abrams, Robert J Barry, Seema Bhatnagar, David F Clayton, John Colombo, Gianluca Coppola, Mark A Geyer, David L Glanzman, Stephen Marsland, Frances K McSweeney, Donald A Wilson, Chun-Fang Wu, and Richard F Thompson. Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. *Neurobiol. Learn. Mem.*, 92(2):135–138, September 2009.
- [2] Marc A. Wolman, Roshan A. Jain, Laura Liss, and Michael Granato. Chemical modulation of memory formation in larval zebrafish. *Proceedings of the National Academy of Sciences*, 108(37):15468–15473, September 2011.
- [3] Owen Randlett, Martin Haesemeyer, Greg Forkin, Hannah Shoenthal, Alexander F Schier, Florian Engert, and Michael Granato. Distributed plasticity drives visual habituation learning in larval zebrafish. *Curr. Biol.*, 29(8):1337–1345.e4, April 2019.
- [4] Laurie Anne Lamiré, Martin Haesemeyer, Florian Engert, Michael Granato, and Owen Randlett. Functional and pharmacological analyses of visual habituation learning in larval zebrafish. *Elife*, 12, December 2023.
- [5] Stefan Nilsson and Jan-Ake Gustafsson. Biological role of estrogen and estrogen receptors. *Crit. Rev. Biochem. Mol. Biol.*, 37(1):1–28, 2002.
- [6] Mohammad Naderi, Arash Salahinejad, Anoosha Attaran, Som Niyogi, and Douglas P Chivers. Rapid effects of estradiol and its receptor agonists on object recognition and object placement in adult male zebrafish. *Behav. Brain Res.*, 384(112514):112514, April 2020.
- [7] T Samuel Dillon, Laura C Fox, Crystal Han, and Christiane Linster. 17β -estradiol enhances memory duration in the main olfactory bulb in CD-1 mice. *Behav. Neurosci.*, 127(6):923–931, December 2013.
- [8] Oliver Rawashdeh, Nancy Hernandez de Borsetti, Gregg Roman, and Gregory M Cahill. Melatonin suppresses nighttime memory formation in zebrafish. *Science*, 318(5853):1144–1146, November 2007.
- [9] Antje Jilg, Philipp Bechstein, Anastasia Saade, Moritz Dick, Tian Xiao Li, Gianluca Tosini, Abdelhaq Rami, Ajmal Zemmar, and Jörg H Stehle. Melatonin modulates daytime-dependent synaptic plasticity and learning efficiency. *J. Pineal Res.*, 66(3):e12553, April 2019.
- [10] Y El-Sherif, J Tesoriero, M V Hogan, and A Wierszko. Melatonin regulates neuronal plasticity in the hippocampus. *J. Neurosci. Res.*, 72(4):454–460, May 2003.
- [11] L A Barros, S Tufik, and M L Andersen. The role of progesterone in memory: an overview of three decades. *Neurosci. Biobehav. Rev.*, 49:193–204, February 2015.
- [12] H Kuhl. Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric*, 8 Suppl 1(sup1):3–63, August 2005.
- [13] Cécile A Unger. Hormone therapy for transgender patients. *Transl. Androl. Urol.*, 5(6):877–884, December 2016.
- [14] Szidónia Farkas, Adrienn Szabó, Anita Emőke Hegyi, Bibiána Török, Csilla Lea Fazekas, Dávid Ernszt, Tamás Kovács, and Dóra Zelena. Estradiol and estrogen-like alternative therapies in use: The importance of the selective and non-classical actions. *Biomedicines*, 10(4):861, April 2022.
- [15] Karyn M Frick. Molecular mechanisms underlying the memory-enhancing effects of estradiol. *Horm. Behav.*, 74:4–18, August 2015.
- [16] Javed Iqbal, Geng-Di Huang, Yan-Xue Xue, Mei Yang, and Xiao-Jian Jia. Role of estrogen in sex differences in memory, emotion and neuropsychiatric disorders. *Mol. Biol. Rep.*, 51(1):415, March 2024.
- [17] Victoria N Luine. Estradiol and cognitive function: past, present and future. *Horm. Behav.*, 66(4):602–618, September 2014.

- 376 [18] C A Finney, A Shvetcov, R F Westbrook, N M Jones, and M J Morris. The role of hippocampal estradiol in synaptic
377 plasticity and memory: A systematic review. *Front. Neuroendocrinol.*, 56(100818):100818, January 2020.
- 378 [19] Eric R Prossnitz and Matthias Barton. The G protein-coupled oestrogen receptor GPER in health and disease: an update.
379 *Nat. Rev. Endocrinol.*, 19(7):407–424, July 2023.
- 380 [20] Chetana M Revankar, Daniel F Cimino, Larry A Sklar, Jeffrey B Arterburn, and Eric R Prossnitz. A transmembrane
381 intracellular estrogen receptor mediates rapid cell signaling. *Science*, 307(5715):1625–1630, March 2005.
- 382 [21] E J Filardo, J A Quinn, K I Bland, and A R Frackelton, Jr. Estrogen-induced activation of erk-1 and erk-2 requires the
383 G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor
384 through release of HB-EGF. *Mol. Endocrinol.*, 14(10):1649–1660, October 2000.
- 385 [22] Aune Koitmäe, Yannik Karsten, Xiaoyu Li, Fabio Morellini, Gabriele M Rune, and Roland A Bender. GPER1 deficiency
386 causes sex-specific dysregulation of hippocampal plasticity and cognitive function. *J. Endocrinol.*, 258(3), September
387 2023.
- 388 [23] Victor Briz, Yan Liu, Guoqi Zhu, Xiaoning Bi, and Michel Baudry. A novel form of synaptic plasticity in field CA3 of
389 hippocampus requires GPER1 activation and BDNF release. *J. Cell Biol.*, 210(7):1225–1237, September 2015.
- 390 [24] Shannon N Romano, Hailey E Edwards, Jaclyn Paige Souder, Kevin J Ryan, Xiangqin Cui, and Daniel A Gorelick. G
391 protein-coupled estrogen receptor regulates embryonic heart rate in zebrafish. *PLoS Genet.*, 13(10):e1007069, October
392 2017.
- 393 [25] Arnaud Menuet, Elisabeth Pellegrini, Isabelle Anglade, Odile Blaise, Vincent Laudet, Olivier Kah, and Farzad Pakdel.
394 Molecular characterization of three estrogen receptor forms in zebrafish: Binding characteristics, transactivation
395 properties, and tissue distributions1. *Biol. Reprod.*, 66(6):1881–1892, June 2002.
- 396 [26] Gary Bradski. The openCV library. *Dr. Dobb's Journal: Software Tools for the Professional Programmer*, 25(11):120–123,
397 2000.
- 398 [27] Stefan Van der Walt, Johannes L Schönberger, Juan Nunez-Iglesias, François Boulogne, Joshua D Warner, Neil Yager,
399 Emmanuelle Gouillart, and Tony Yu. scikit-image: image processing in python. *PeerJ*, 2:e453, 2014.
- 400 [28] Charles R Harris, K Jarrod Millman, Stéfan J van der Walt, Ralf Gommers, Pauli Virtanen, David Cournapeau, Eric Wieser,
401 Julian Taylor, Sebastian Berg, Nathaniel J Smith, Robert Kern, Matti Picus, Stephan Hoyer, Marten H van Kerkwijk,
402 Matthew Brett, Allan Haldane, Jaime Fernández Del Río, Mark Wiebe, Pearu Peterson, Pierre Gérard-Marchant,
403 Kevin Sheppard, Tyler Reddy, Warren Weckesser, Hameer Abbasi, Christoph Gohlke, and Travis E Oliphant. Array
404 programming with NumPy. *Nature*, 585(7825):357–362, September 2020.
- 405 [29] Pauli Virtanen, Ralf Gommers, Travis E Oliphant, Matt Haberland, Tyler Reddy, David Cournapeau, Evgeni Burovski,
406 Pearu Peterson, Warren Weckesser, Jonathan Bright, Stéfan J van der Walt, Matthew Brett, Joshua Wilson, K Jarrod
407 Millman, Nikolay Mayorov, Andrew R J Nelson, Eric Jones, Robert Kern, Eric Larson, C J Carey, İlhan Polat, Yu Feng,
408 Eric W Moore, Jake VanderPlas, Denis Laxalde, Josef Perktold, Robert Cimrman, Ian Henriksen, E A Quintero, Charles R
409 Harris, Anne M Archibald, Antônio H Ribeiro, Fabian Pedregosa, Paul van Mulbregt, and SciPy 1.0 Contributors. SciPy
410 1.0: fundamental algorithms for scientific computing in python. *Nat. Methods*, 17(3):261–272, March 2020.
- 411 [30] Siu Kwan Lam, Antoine Pitrou, and Stanley Seibert. Numba: A llvm-based python jit compiler. In *Proceedings of the
412 Second Workshop on the LLVM Compiler Infrastructure in HPC*, pages 1–6, 2015.
- 413 [31] Wes McKinney. Data Structures for Statistical Computing in Python. In Stéfan van der Walt and Jarrod Millman, editors,
414 *Proceedings of the 9th Python in Science Conference*, pages 56 – 61, 2010.
- 415 [32] J. D. Hunter. Matplotlib: A 2d graphics environment. *Computing in Science & Engineering*, 9(3):90–95, 2007.
- 416 [33] Michael Waskom. seaborn: statistical data visualization. *J. Open Source Softw.*, 6(60):3021, April 2021.

- 417 [34] Harold A. Burgess and Michael Granato. Sensorimotor gating in larval zebrafish. *The Journal of Neuroscience*,
418 27(18):4984–4994, May 2007.
- 419 [35] C B Kimmel, J Patterson, and R O Kimmel. The development and behavioral characteristics of the startle response in
420 the zebra fish. *Dev. Psychobiol.*, 7(1):47–60, January 1974.
- 421 [36] Mohamed A El-Brolosy, Zacharias Kontarakis, Andrea Rossi, Carsten Kuenne, Stefan Günther, Nana Fukuda, Khrievono
422 Kikhi, Giulia L M Boezio, Carter M Takacs, Shih-Lei Lai, Ryuichi Fukuda, Claudia Gerri, Antonio J Giraldez, and Didier Y R
423 Stainier. Genetic compensation triggered by mutant mRNA degradation. *Nature*, 568(7751):193–197, April 2019.
- 424 [37] Ana Mlinarić, Martina Horvat, and Vesna Šupak Smolčić. Dealing with the positive publication bias: Why you should
425 really publish your negative results. *Biochem. Med. (Zagreb)*, 27(3):030201, October 2017.
- 426 [38] Xinge Yu, Queena Y Zhao, Murat Yaman, Sylvia M Emly, Jacqueline K Lee, Hongyu Su, Allison C Ferguson,
427 Chandrasekaran Nagaswami, Saireudee Chaturantabut, Wolfram Goessling, John W Weisel, Richard J Auchus, and
428 Jordan A Shavit. Hormone-induced thrombosis is mediated through non-canonical fibrin(ogen) aggregation and a
429 novel estrogen target in zebrafish. *bioRxiv*, November 2024.
- 430 [39] Jian Qiu, Martha A Bosch, Sandra C Tobias, Andree Krust, Sharon M Graham, Stephanie J Murphy, Kenneth S Korach,
431 Pierre Chambon, Thomas S Scanlan, Oline K Rønneklev, and Martin J Kelly. A g-protein-coupled estrogen receptor is
432 involved in hypothalamic control of energy homeostasis. *J. Neurosci.*, 26(21):5649–5655, May 2006.
- 433 [40] Gwyndolin Vail and Troy A Roepke. Membrane-initiated estrogen signaling via gq-coupled GPCR in the central nervous
434 system. *Steroids*, 142:77–83, February 2019.
- 435 [41] Altin Sula, David Hollingworth, Leo C T Ng, Megan Larmore, Paul G DeCaen, and B A Wallace. A tamoxifen receptor
436 within a voltage-gated sodium channel. *Mol. Cell*, 81(6):1160–1169.e5, March 2021.
- 437 [42] Lindsey S Treviño and Daniel A Gorelick. The interface of nuclear and membrane steroid signaling. *Endocrinology*,
438 162(8), August 2021.
- 439 [43] Maja Payrits, Éva Sághy, Kata Csekő, Krisztina Pohóczky, Kata Bölcskei, Dávid Ernszt, Klaudia Barabás, János Szolcsányi,
440 István M Ábrahám, Zsuzsanna Helyes, and Éva Szőke. Estradiol sensitizes the transient receptor potential vanilloid 1
441 receptor in pain responses. *Endocrinology*, 158(10):3249–3258, October 2017.
- 442 [44] Ricardo Ramírez-Barrantes, Karina Carvajal-Zamorano, Belen Rodriguez, Claudio Cordova, Carlo Lozano, Felipe Simon,
443 Paula Díaz, Pablo Muñoz, Ivanny Marchant, Ramón Latorre, Karen Castillo, and Pablo Olivero. TRPV1-estradiol
444 stereospecific relationship underlies cell survival in oxidative cell death. *Front. Physiol.*, 11:444, May 2020.
- 445 [45] Lee-Ming Kow and Donald W Pfaff. Rapid estrogen actions on ion channels: A survey in search for mechanisms. *Steroids*,
446 111:46–53, July 2016.
- 447 [46] Christine Thisse and Bernard Thisse. High-resolution *in situ* hybridization to whole-mount zebrafish embryos. *Nat. Protoc.*, 3(1):59–69, 2008.
- 449 [47] Arnaud Menuet, Elisabeth Pellegrini, François Brion, Marie-Madeleine Gueguen, Isabelle Anglade, Farzad Pakdel, and
450 Olivier Kah. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. *J. Comp. Neurol.*,
451 485(4):304–320, May 2005.
- 452 [48] Ruixin Hao, Maria Bondesson, Amar V Singh, Anne Riu, Catherine W McCollum, Thomas B Knudsen, Daniel A Gorelick,
453 and Jan-Åke Gustafsson. Identification of estrogen target genes during zebrafish embryonic development through
454 transcriptomic analysis. *PLoS One*, 8(11):e79020, November 2013.
- 455 [49] Troy A McDiarmid, Alex J Yu, and Catharine H Rankin. Habituation is more than learning to ignore: Multiple mechanisms
456 serve to facilitate shifts in behavioral strategy. *Bioessays*, 41(9):e1900077, September 2019.