

Estradiol promotes habituation learning via an unidentified target, bypassing the suppressive effects of established ERs

Andrew Hsiao^{1,1,2}, Isabelle Darvaux-Hubert^{1,1}, Dominique Hicks^{1,1,3}, Emilie Joux^{1,2}, Sarah De Freitas^{1,2}, Emeline Dracos^{1,2}, Jeanne Lizé^{1,2}, Julien Perrichet¹, Dominique 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

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

Disclosures

The authors have nothing to disclose.

Keywords

Estrogens, Habituation, Learning, Zebrafish, Estrogen Receptors, Unidentified Target

Corresponding Author

Owen Randlett

Team ZeNeB: Zebrafish Neurogenetics and Behaviour

Laboratoire MeLiS, UCBL - CNRS UMR5284 - Inserm U1314, Institut NeuroMyoGène,

Faculté de Médecine et de Pharmacie, 8 avenue Rockefeller, 69008 Lyon

email: owen.randlett@univ-lyon1.fr

ORCID: 0000-0003-0181-5239

Abstract

Habituating to the constant stimuli in the environment is a critical learning process conserved across species. We use a 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 more strongly suppressed responses. We used mutant lines for the Estrogen Receptors (*esr1*, *esr2a*, *esr2b*, *gper1*) in an attempt to identify the receptor(s) mediating these effects. These experiments failed to identify a necessary receptor (or combination of receptors). Surprisingly, *esr1*, *esr2a*, and *gper1* mutants showed weak but consistent increases in habituation, indicating these receptors suppress habituation learning. These experiments demonstrate that estradiol is a complex modulator of learning in our model, where the learning-promoting effects are mediated by an unidentified estradiol target, and the classical Estrogen Receptors act in competition to subtly suppress learning.

31

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 [1].

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) [2, 3, 4]. We recently reported that multiple hormonal signaling pathways show strong modulation of habituation learning performance, including melatonin, progesterone, and estrogen [4]. The ability of these signaling pathways to modulate learning is consistent with previous results in other systems and paradigms [5, 6, 7, 8, 9, 10, 11], 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 bath applied at 5-10 μ M doses (ethinyl estradiol, estradiol valerate, and hexestrol, [4]). 17 β -estradiol (here referred to as estradiol) is the most potent and biologically active form

49 of estrogen, and is used in a variety of clinical contexts including contraception, hormone replacement therapy,
50 and feminizing hormone therapy [12, 13, 14].

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

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

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

73 In this project we aimed to identify the relevant ER(s) mediating the effects of estradiol on habituation using
74 mutant alleles. Zebrafish have single gene encoding ER α (ER1, *esr1*) and Gper1 (*gper1*), and two homologs of
75 ER β : ER2a (*esr2a*) and ER2b (*esr2a*) [24, 25]. We found that none of these mutants were insensitive to estradiol's
76 effects, indicating that estradiol acts in this context via an alternative receptor or pathway. Surprisingly, our
77 experiments found that mutants for *esr1*, *esr2a*, and *gper1* actually habituate more than their sibling controls.
78 While the effect size is small and behavioural-genetic experiments can be variable, these data indicate that
79 these ERs actually act to inhibit habituation learning, rather than mediating the habituation-promoting effects
80 of estradiol that we observe pharmacologically.

Materials and Methods

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 following zebrafish facilities: Plateau de Recherche Expérimentale en Criblage In Vivo (PRECI, SFR Biosciences, Lyon) and the Animalerie Zebrafish Rockefeller (AZR, SFR Santé Lyon Est, Lyon). Adult zebrafish used to generate larvae were housed in accordance with regulations of the PRECI and AZR facilities, which are regulated by an internal animal wellbeing committee, and were 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.

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 [24].

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

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 [24].

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 [24].

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:

CTTCAGCTGCAGGAAGTGG/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 MboI. The mutant product is digested into two bands of 150 and 281

112 bp and the wild type into 3 bands of 78, 150 and 198 bp.

113 *gper1^{uab102}* was genotypes by PCR using the forward/reverse primer pair:

114 ATGGAGGAGCAGACTACCAATGTG/CCATCCAGATGAGGCTGCAA with a T_M of 60°C yielding a mutant product of
115 372bp and a wild type product of 505 bp.

116 Pharmacology

117 β -Estradiol (Sigma E2758, here referred to as "estradiol") was dissolved in dimethyl sulfoxide (DMSO) and stored
118 at -20°C. Larvae were treated with estradiol immediately before the behavioural assay by pipetting 10-30 μ L of
119 10x solution directly into the behavioural wells, always with a final concentration of 0.1% DMSO in E3.

120 Habituation behaviour testing

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

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

Data analysis

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

The cumulative difference plots to assess changes in habituation performance for the treatments were calculated as previously [3], 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 divided by the number of stimuli experienced, or the index of the vector. To generate statistical confidence in these distributions, we bootstrapped 2000 replicates, and calculated the 99.5% confidence intervals using SciPy's 'stats.norm.interval' function. The assumption of this analysis is that if the two groups are habituating similarly, then the "mean difference" vector will exhibit a noise distribution centered at 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. We use an empirically defined threshold of ± 0.05 as the statistically meaningful effect size in this analysis, as is reflected in the shaded gray regions in the plots [3].

Software and Datasets

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

Results

Estradiol increases visual habituation learning

In response to a sudden global darkening stimulus, 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 ([34], **Figure 1a**). 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

[1]. 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 1A,B**, [3, 4]). When stimulated with DFs repeated at 1-minute intervals in 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 [3].

Our previous small-molecule screening experiments identified multiple synthetic Estrogen Receptor agonists as positive modulators of DF habituation learning at 5-10 μ M doses, including ethinyl estradiol, estradiol valerate, and hexestrol [4]. 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 estradiol, which is the major natural estrogen in vertebrates.

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

Gper1 is dispensable for estradiol-promoted habituation learning

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

To test this we used the *gper1^{uab102}* mutant [24], and generated larvae from *gper1^{uab102}* heterozygous or homo/heterozygous 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 be insensitive to estradiol and habituate significantly less than sibling 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 than controls, with the responsiveness level slightly but consistently below the sibling

controls across stimuli (**Figure 2A**). This is further supported by a weak but statistically significant decrease in the responsiveness of the larvae during the training period (**Figure 2D**), and a deviation towards positive values in the CMD plot (**Figure 2F**). From these experiments we conclude that *Gper1* agonism does not promote habituation learning, but rather may act to suppress it.

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 3A**_{vi},**B**_{vi}), 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.

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

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

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

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

242 **Gper1, ER α , and ER β are simultaneously dispensable for estradiol-promoted** 243 **habituation learning**

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

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

258 **Discussion**

259 **The promotion of habituation learning by estradiol is mediated by an unidentified** 260 **target**

261 Our experiments indicate that Gper1, ER1, ER2a and ER2b do not mediate the positive effects of estradiol on
262 habituation learning. As this is fundamentally a negative result, it is difficult to conclusively demonstrate this
263 beyond any doubt. One major caveat relates to the actual functional nature of the mutant alleles that we have
264 used. These are all Cas9-generated small deletions resulting in frameshift mutations that lead to early stop
265 codons, and are thus predicted null/knockout lines. Despite this genetic confidence, it is always possible that
266 residual activity could still remain, perhaps via alternate splicing or alternate start codons. This could be further
267 complicated by genetic/transcriptional compensation, where frameshift alleles can lead to the upregulation of

268 paralogs in some circumstances [36]. As with all negative results, it is not possible to rule out all possible
269 alternative explanations. However, we recognize that this bias against publishing negative results is bad for
270 science. Negative results from well-designed and executed experiments are of value for the community and
271 making this knowledge public is our duty as responsible scientists [37].

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

283 **Candidate estradiol targets that could promote habituation learning**

284 We have concluded that the lack of habituation deficits in our mutants is due to the presence of an alternative
285 receptor or pathway that mediates the learning-promoting effects of estradiol. What might this unidentified
286 target be? Various leads exist in the literature. One hypothesis posits the existence of an unidentified "Gq-
287 mER" (Gq-coupled membrane estrogen receptor) [38, 39], and therefore estradiol may signal via additional
288 GPCRs beyond Gper1. Another possibility is an interaction between estradiol and other membrane receptors,
289 for example: the Voltage-Gated Sodium Channel Nav1.2 [40, 41], transient receptor potential (TRP) channels
290 [42, 43], or various other ion channels [44]. The robust nature of our "non-canonical" but clearly estradiol-
291 dependent phenotype, combined with the high-throughput nature of our behavioural assays, could be an ideal
292 assay for future screening efforts to attempt to identify novel estradiol target(s).

293 **Multiple Estrogen Receptors act to suppress habituation learning.**

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

300 multi-mutants generally exhibited larger effect sizes, consistent with an additive interaction.

301 Untangling the mechanisms of ER1-, ER2a- and Gper1-dependent suppression of habituation will require
302 considerable further work. The additive interaction we observed genetically indicates that the ERs act
303 cooperatively to suppress habituation learning. All three receptors are expressed in the larval zebrafish brain
304 [45, 24], but whether they are acting in the same or different cell types awaits characterization. The study of this
305 inhibitory pathway may be challenging since it opposes the major learning-promoting effect of estradiol, and
306 therefore may be more straightforward to study after the identification and deletion of the estradiol target that
307 promotes habituation. One attractive hypothesis relates to the observation that estradiol exposure increases
308 aromatase expression in the zebrafish brain [46, 47]. Since aromatase catalyzes the conversion of androgens
309 into estrogens, ER mutants may have reduced levels of endogenous endogenous estrogens in the brain, which
310 could lead to inhibited habituation. Future experiments aimed at manipulating the aromatase system in wild-
311 type and ER mutant backgrounds could be used to test this hypothesis.

312 **Conclusion**

313 What began as a straightforward study to identify the receptor(s) that mediate the habituation-promoting
314 effects of estradiol has instead led us to a surprising and paradoxical result; canonical ERs do regulate
315 habituation, but are suppressive and act in opposition to the habituation-promoting effects of estradiol. This
316 fits with the general theme of our studies of this habituation paradigm – we find increasing complexity
317 and contradiction within this "simple" learning process the deeper we look. This began with our detailed
318 observations of behaviour, leading us to conclude that habituation results from a distributed plasticity process
319 that adapts different aspects of behavior independently [3]. We believe that this property underlies our
320 subsequent discoveries of pharmacological and genetic manipulations that can result in either specific changes
321 in specific aspects of habituation (but not others), or even opposing effects, where a single manipulation can
322 simultaneously increase and decrease habituation, depending on which component of behavior is measured
323 [3, 4]. This complexity appears to be a fundamental property of habituation [48], and that the study of
324 habituation will likely continue to surprise us, hopefully leading to unexpected insights into the nature of
325 plasticity underlying learning and memory.

326 **Data Availability Statement**

327 Original data generated and analyzed during this study are included in this published article or in the data
328 repositories listed in References.

Acknowledgments

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

Funding

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

References

- [1] Rankin CH, Abrams T, Barry RJ, Bhatnagar S, Clayton DF, Colombo J, et al. Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. *Neurobiol Learn Mem*. 2009 Sep;92(2):135-8.
- [2] Wolman MA, Jain RA, Liss L, Granato M. Chemical modulation of memory formation in larval zebrafish. *Proceedings of the National Academy of Sciences*. 2011 Sep;108(37):15468-73.
- [3] Randlett O, Haesemeyer M, Forkin G, Shoenhard H, Schier AF, Engert F, et al. Distributed plasticity drives visual habituation learning in larval zebrafish. *Curr Biol*. 2019 Apr;29(8):1337-45.e4.
- [4] Lamiré LA, Haesemeyer M, Engert F, Granato M, Randlett O. Functional and pharmacological analyses of visual habituation learning in larval zebrafish. *Elife*. 2023 Dec;12.
- [5] Nilsson S, Gustafsson JA. Biological role of estrogen and estrogen receptors. *Crit Rev Biochem Mol Biol*. 2002;37(1):1-28.
- [6] Naderi M, Salahinejad A, Attaran A, Niyogi S, Chivers DP. Rapid effects of estradiol and its receptor agonists on object recognition and object placement in adult male zebrafish. *Behav Brain Res*. 2020 Apr;384(112514):112514.
- [7] Dillon TS, Fox LC, Han C, Linster C. 17β -estradiol enhances memory duration in the main olfactory bulb in CD-1 mice. *Behav Neurosci*. 2013 Dec;127(6):923-31.
- [8] Rawashdeh O, de Borsetti NH, Roman G, Cahill GM. Melatonin suppresses nighttime memory formation in zebrafish. *Science*. 2007 Nov;318(5853):1144-6.

- [9] Jilg A, Bechstein P, Saade A, Dick M, Li TX, Tosini G, et al. Melatonin modulates daytime-dependent synaptic plasticity and learning efficiency. *J Pineal Res.* 2019 Apr;66(3):e12553.
- [10] El-Sherif Y, Tesoriero J, Hogan MV, Wieraszko A. Melatonin regulates neuronal plasticity in the hippocampus. *J Neurosci Res.* 2003 May;72(4):454-60.
- [11] Barros LA, Tufik S, Andersen ML. The role of progesterone in memory: an overview of three decades. *Neurosci Biobehav Rev.* 2015 Feb;49:193-204.
- [12] Kuhl H. Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric.* 2005 Aug;8 Suppl 1(sup1):3-63.
- [13] Unger CA. Hormone therapy for transgender patients. *Transl Androl Urol.* 2016 Dec;5(6):877-84.
- [14] Farkas S, Szabó A, Hegyi AE, Török B, Fazekas CL, Ernszt D, et al. Estradiol and estrogen-like alternative therapies in use: The importance of the selective and non-classical actions. *Biomedicines.* 2022 Apr;10(4):861.
- [15] Frick KM. Molecular mechanisms underlying the memory-enhancing effects of estradiol. *Horm Behav.* 2015 Aug;74:4-18.
- [16] Iqbal J, Huang GD, Xue YX, Yang M, Jia XJ. Role of estrogen in sex differences in memory, emotion and neuropsychiatric disorders. *Mol Biol Rep.* 2024 Mar;51(1):415.
- [17] Luine VN. Estradiol and cognitive function: past, present and future. *Horm Behav.* 2014 Sep;66(4):602-18.
- [18] Finney CA, Shvetsov A, Westbrook RF, Jones NM, Morris MJ. The role of hippocampal estradiol in synaptic plasticity and memory: A systematic review. *Front Neuroendocrinol.* 2020 Jan;56(100818):100818.
- [19] Prossnitz ER, Barton M. The G protein-coupled oestrogen receptor GPER in health and disease: an update. *Nat Rev Endocrinol.* 2023 Jul;19(7):407-24.
- [20] Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science.* 2005 Mar;307(5715):1625-30.
- [21] Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol.* 2000 Oct;14(10):1649-60.
- [22] Koitmäe A, Karsten Y, Li X, Morellini F, Rune GM, Bender RA. GPER1 deficiency causes sex-specific dysregulation of hippocampal plasticity and cognitive function. *J Endocrinol.* 2023 Sep;258(3).
- [23] Briz V, Liu Y, Zhu G, Bi X, Baudry M. A novel form of synaptic plasticity in field CA3 of hippocampus requires GPER1 activation and BDNF release. *J Cell Biol.* 2015 Sep;210(7):1225-37.
- [24] Romano SN, Edwards HE, Souder JP, Ryan KJ, Cui X, Gorelick DA. G protein-coupled estrogen receptor regulates embryonic heart rate in zebrafish. *PLoS Genet.* 2017 Oct;13(10):e1007069.

- 386 [25] Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O, et al. Molecular characterization of three estrogen receptor
387 forms in zebrafish: Binding characteristics, transactivation properties, and tissue Distributions¹. *Biol Reprod*. 2002
388 Jun;66(6):1881-92.
- 389 [26] Bradski G. The openCV library. *Dr Dobb's Journal: Software Tools for the Professional Programmer*. 2000;25(11):120-3.
- 390 [27] Van der Walt S, Schönberger JL, Nunez-Iglesias J, Boulogne F, Warner JD, Yager N, et al. scikit-image: image processing
391 in Python. *PeerJ*. 2014;2:e453.
- 392 [28] Harris CR, Millman KJ, van der Walt SJ, Gommers R, Virtanen P, Cournapeau D, et al. Array programming with NumPy.
393 *Nature*. 2020 Sep;585(7825):357-62.
- 394 [29] Virtanen P, Gommers R, Oliphant TE, Haberland M, Reddy T, Cournapeau D, et al. SciPy 1.0: fundamental algorithms
395 for scientific computing in Python. *Nat Methods*. 2020 Mar;17(3):261-72.
- 396 [30] Lam SK, Pitrou A, Seibert S. Numba: A llvm-based python jit compiler. In: Proceedings of the Second Workshop on the
397 LLVM Compiler Infrastructure in HPC; 2015. p. 1-6.
- 398 [31] Wes McKinney. Data Structures for Statistical Computing in Python. In: Stéfan van der Walt, Jarrod Millman, editors.
399 Proceedings of the 9th Python in Science Conference; 2010. p. 56 61.
- 400 [32] Hunter JD. Matplotlib: A 2D graphics environment. *Computing in Science & Engineering*. 2007;9(3):90-5.
- 401 [33] Waskom M. seaborn: statistical data visualization. *J Open Source Softw*. 2021 Apr;6(60):3021.
- 402 [34] Burgess HA, Granato M. Sensorimotor Gating in Larval Zebrafish. *The Journal of Neuroscience*. 2007 May;27(18):4984-94.
- 403 [35] Kimmel CB, Patterson J, Kimmel RO. The development and behavioral characteristics of the startle response in the
404 zebra fish. *Dev Psychobiol*. 1974 Jan;7(1):47-60.
- 405 [36] El-Brolosy MA, Kontarakis Z, Rossi A, Kuenne C, Günther S, Fukuda N, et al. Genetic compensation triggered by mutant
406 mRNA degradation. *Nature*. 2019 Apr;568(7751):193-7.
- 407 [37] Mlinarić A, Horvat M, Šupak Smolčić V. Dealing with the positive publication bias: Why you should really publish your
408 negative results. *Biochem Med (Zagreb)*. 2017 Oct;27(3):030201.
- 409 [38] Qiu J, Bosch MA, Tobias SC, Krust A, Graham SM, Murphy SJ, et al. A G-protein-coupled estrogen receptor is involved
410 in hypothalamic control of energy homeostasis. *J Neurosci*. 2006 May;26(21):5649-55.
- 411 [39] Vail G, Roepke TA. Membrane-initiated estrogen signaling via Gq-coupled GPCR in the central nervous system. *Steroids*.
412 2019 Feb;142:77-83.
- 413 [40] Sula A, Hollingworth D, Ng LCT, Larmore M, DeCaen PG, Wallace BA. A tamoxifen receptor within a voltage-gated
414 sodium channel. *Mol Cell*. 2021 Mar;81(6):1160-9.e5.
- 415 [41] Treviño LS, Gorelick DA. The interface of nuclear and membrane steroid signaling. *Endocrinology*. 2021 Aug;162(8).

- 416 [42] Payrits M, Sághy É, Csekő K, Pohóczky K, Bölcskei K, Ernszt D, et al. Estradiol sensitizes the transient receptor potential
417 vanilloid 1 receptor in pain responses. *Endocrinology*. 2017 Oct;158(10):3249-58.
- 418 [43] Ramírez-Barrantes R, Carvajal-Zamorano K, Rodriguez B, Cordova C, Lozano C, Simon F, et al. TRPV1-estradiol
419 stereospecific relationship underlies cell survival in oxidative cell death. *Front Physiol*. 2020 May;11:444.
- 420 [44] Kow LM, Pfaff DW. Rapid estrogen actions on ion channels: A survey in search for mechanisms. *Steroids*. 2016
421 Jul;111:46-53.
- 422 [45] Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc*. 2008;3(1):59-69.
- 423 [46] Menuet A, Pellegrini E, Brion F, Gueguen MM, Anglade I, Pakdel F, et al. Expression and estrogen-dependent regulation
424 of the zebrafish brain aromatase gene. *J Comp Neurol*. 2005 May;485(4):304-20.
- 425 [47] Hao R, Bondesson M, Singh AV, Riu A, McCollum CW, Knudsen TB, et al. Identification of estrogen target genes during
426 zebrafish embryonic development through transcriptomic analysis. *PLoS One*. 2013 Nov;8(11):e79020.
- 427 [48] McDiarmid TA, Yu AJ, Rankin CH. Habituation is more than learning to ignore: Multiple mechanisms serve to facilitate
428 shifts in behavioral strategy. *Bioessays*. 2019 Sep;41(9):e1900077.

Figure Legends

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 1 hr 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)

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.

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

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

469 **B)** Homozygous *esr2a*^(-/-) mutants (n = 214 fish) do not show impaired habituation relative to sibling controls (*esr2a*^(+/+) and
470 *esr2a*^(+/+), n = 128 fish).

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

473 **i)-vi)** For each lettered section:

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

477 **ii)-v)** Distributions responsiveness for different epochs of the experiment. Each dot is the per-fish average of the epoch.
478 Statistical significance was calculated using Mann-Whitney U test, * = p < 0.05, ** = p < 0.01. **ii)** the naive response to the
479 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
480 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
481 tap from a solenoid on the 300-well plate platform.

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

484

485 **Figure 4. Double and triple mutant combinations of *esr1*, *esr2a* and *esr2b* do not show habituation deficits after**
486 **treatment with estradiol.**

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

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

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

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

495 **i)-vi)** For each lettered section:

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

499 **ii)-v)** Distributions responsiveness for different epochs of the experiment. Each dot is the per-fish average of the epoch.
500 Statistical significance was calculated using Mann-Whitney U test, * = p < 0.05, ** = p < 0.01. **ii)** the naive response to the
501 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
502 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
503 tap from a solenoid on the 300-well plate platform.

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

506

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

509 **A)** Homozygous *esr1*^(-/-);*gper1*^(-/-) double mutants (n = 34 fish, purple) do not show impaired habituation relative to double
510 heterozygous or homozygous sibling controls (n = 81 fish, red). **B)** Homozygous *esr2a*^(-/-);*gper1*^(-/-) double mutants (n = 27
511 fish, purple) do not show impaired habituation relative to double heterozygous or homozygous sibling controls (n = 81
512 fish, red). **C)** Homozygous *esr2b*^(-/-);*gper1*^(-/-) double mutants (n = 24 fish, purple) do not show impaired habituation relative
513 to double heterozygous or homozygous sibling controls (n = 81 fish, red). **D)** Homozygous *esr2a*^(-/-);*esr2b*^(-/-);*gper1*^(-/-) triple
514 mutants (n = 35 fish, purple) do not show impaired habituation relative to heterozygous or homozygous sibling controls (n
515 = 81 fish, red). **E)** A single homozygous *esr1*^(-/-);*esr2a*^(-/-);*esr2b*^(-/-);*gper1*^(-/-) quadruple mutant (n = 1 fish, purple) does not
516 appear to show impaired habituation relative to heterozygous or homozygous sibling controls (n = 81 fish, red).

517 **i)-vi)** For each lettered section: **i)** Responsiveness to stimuli comparing homozygous mutants to sibling controls
518 (heterozygous or wild-type). Each dot is the probability of response to one stimulus. Lines are smoothed in time with a
519 Savitzky–Golay filter (window = 15 stimuli, order = 2). **ii)-v)** Distributions responsiveness for different epochs of the
520 experiment. Each dot is the per-fish average of the epoch. Statistical significance was calculated using Mann-Whitney U
521 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
522 stimuli in the Block 1 (DFs 6:60); **iv)** the trained response to the last 45 DFs in all four training blocks (DFs
523 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
524 platform. **vi)** Cumulative mean difference (CMD) plot quantifying habituation performance of mutants relative to sibling
525 controls. As mutant larvae were all derived from the same experiments, the sibling control data (red) is the same in **A-E**.
526
527