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Functional and pharmacological analyses of visual habituation learning in larval zebrafish

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Abstract

Habituation allows animals to learn to ignore persistent but inconsequential stimuli. Despite being the most basic form of learning, a consensus model on the underlying mechanisms has yet to emerge. To probe relevant mechanisms we took advantage of a visual habituation paradigm in larval zebrafish, where larvae reduce their reactions to abrupt global dimming (a dark flash). We used Ca^{2+} imaging during repeated dark flashes and identified 12 functional classes of neurons that differ based on their rate of adaptation, stimulus response shape, and anatomical location. While most classes of neurons depressed their responses to repeated stimuli, we identified populations that did not adapt, or that potentiated their response. These neurons were distributed across brain areas, consistent with a distributed learning process. Using a small molecule-screening approach, we confirmed that habituation manifests from multiple distinct molecular mechanisms, and we have implicated molecular pathways in habituation, including: Melatonin, Estrogen and GABA signaling. However, by combining anatomical analyses and pharmacological manipulations with Ca^{2+} imaging, we failed to identify a simple relationship between pharmacology, altered activity patterns, and habituation behaviour. Collectively, our work indicates that habituation occurs via a complex and distributed plasticity processes that cannot be captured by a simple model. Therefore, untangling the mechanisms of habituation will likely require dedicated approaches aimed at sub-component mechanisms underlying this multidimensional learning process.

eLife assessment

This **valuable** manuscript attempts to identify the brain regions and cell types involved in habituation to dark flash stimuli in larval zebrafish. Habituation being a form of learning widespread in the animal kingdom, the investigation of neural mechanisms underlying it is a worthwhile endeavor. The authors use a combination of behavioral analysis, neural activity imaging, and pharmacological manipulation to investigate brain-wide mechanisms of habituation. While the data presented are **solid**, the authors conclude that there is no simple relationship between pharmacological intervention, neural activity patterns, and behavioral outcomes, and a robust causative link can therefore not be established.

Introduction

A central function of the brain is to learn and change with experience. These adaptations can reflect attempts to identify and attend preferentially to salient stimuli. For example, identifying the smell of a predator or prey may be crucial, while identifying that my home still smells like my kin is not. This ability to suppress responses to continuous non-salient stimuli is known as habituation, a process generally considered to be the simplest form of learning and memory (***Rankin et al., 2009***). Habituation is conserved across all animals, and like other forms of plasticity, exists in at least two mechanistically distinct forms: transient short-term habituation, and protein-synthesis dependent long-term habituation. Here we focus on long-term habituation, which serves as a pragmatic model to dissect plasticity processes in neural circuits.

Work on long-term habituation in various species and paradigms has led to significant insights into the adaptations underlying this process (***Cooke and Ramaswami, 2020***; ***McDiarmid et al., 2019b***), nonetheless a consensus model on the general principles underlying habituation is yet to emerge. Physiological and genetic work in *Aplysia*, and *C. elegans* were consistent with a model in which homosynaptic depression of excitatory synapses drives habit-uation (***Bailey and Chen, 1983***; ***Rose et al., 2003***) (although see (***Glanzman, 2009***)). In contrast, work in the *Drosophila* olfactory and gustatory systems indicate that the potentiation of inhibitory neurons drives habituation rather than depression of excitatory connections (***Das et al., 2011***; ***Paranjpe et al., 2012***; ***Trisal et al., 2022***), and habituation to specific orientations of visual cues in mice is associated with the potentiation of neuronal activity and synapses in the visual cortex (***Cooke et al., 2015***), which requires GABAergic interneurons (***Kaplan et al., 2016***; ***Hayden et al., 2021***). These studies are more consistent with a model in which the potentiation of inhibition, rather than depression of excitation, drives habituation (***Cooke and Ramaswami, 2020***).

Recently, we found that long-term habituation of the response of larval zebrafish to sudden pulses of whole-field darkness, or dark flashes (DFs), involves multiple molecularly independent plasticity processes that act to suppress different components of the behavioural response (***Randlett et al., 2019***). Similar behavioural, pharmacological, and genetic experiments have led to comparable conclusions in acoustic short-term habituation (***Nelson et al., 2023***), and habituation in *C. elegans* (***McDiarmid et al., 2019a,b***), indicating that habituation generally acts via multiple modular plasticity processes. These modules act to mute or shift behavioural responses to repeated stimuli. How and where these processes are implemented in the circuit, and how conserved or derived these processes are across species or paradigms remains to be determined. Here we have used a combination of high-throughput behavioural analyses, pharmacology and Ca^{2+} imaging to dissect DF habituation. Our results are consistent with a model in which habituation results from a multidimensional and distributed plasticity process, involving

multiple independent molecular mechanisms. We propose that GABAergic inhibition is central to DF habituation, but how individual cell types and molecular events lead to behavioural adaptations during habituation will require targeted genetic and cellular approaches.

Results

Volumetric 2-photon Ca^{2+} imaging of habituation learning

When stimulated with a dark flash (DF), larval zebrafish execute an O-bend response (***Figure 1A***). The O-bend is characterised by a strong body bend and a large turn that forms part of the phototactic strategy of larval zebrafish, helping them navigate towards lit environments (***Burgess and Granato, 2007***; ***Chen and Engert, 2014***). When presented with repeated DFs, larvae habituate and reduce their responsiveness, remaining hypo-responsive for multiple hours (***Figure 1B***), (***Randlett et al., 2019***).

To explore the circuit mechanisms leading to this form of habituation, we asked how individual neurons within the DF responsive circuit adapt to repeated dark flashes. We used a head-fixed paradigm to perform 2-photon Ca^{2+} imaging in larvae expressing nuclear-targeted GCaMP7f pan-neuronally. Imaging was performed with a resonant scanner and piezo objective, enabling us to cover a volume of ≈ 600 × 300 × 120 μm (x,y,z) sampled at 0.6 × 0.6 × 10 μm resolution, leading to the detection of 30890±3235 ROIs per larvae (±SD, ***Figure 1C-E***). ROIs were aligned to the Z-Brain atlas coordinates (***Randlett et al., 2015***), demonstrating that this volume spans the majority of the midbrain, hindbrain, pretectum and thalamus (***Figure 1C-E***).

We focused on a single training block of 60 DFs to identify neuronal adaptations that occur during the initial phase of learning (***Figure 1Bi***). This paradigm induced strong Ca^{2+} activity in neurons (***Figure 1F***), some of which were clearly associated with the DF stimuli. Ca^{2+} transients in response to DFs generally decreased across the 60 stimuli, though this pattern was not seen in all neurons, and substantial heterogeneity in their adaptations were observed. Strong correlated patterns were also seen in large groupings of neurons, predominantly in the hindbrain, which were associated with movement events through their correlation with motion artifacts in the imaging data (***Figure 1-figure Supplement 1***).

To explore the spatial patterns in these data we used a 2-dimensional lookup table to visualize tuning with regressors representing either DF stimuli or movement (***Figure 1G, H***). This revealed segregated populations of neurons coding for the DFs (pink) and movement (green/teal). As expected, DF-tuned neurons were located predominantly in visual sensory areas of the midbrain (tectum) and the diencephalon (pretectum and thalamus). Motor-coding neurons dominated in the hindbrain, with the exception of the cerebellum and inferior olive, which was predominantly tuned to the sensory stimulus. Some neurons did show approximately equal correlation values to both stimuli, as evidenced by the blue-ish hues. Finally, some areas of the brain appeared to contain mixtures of neurons with different coding properties, including the ventral diencephalon and midbrain.

To determine if there was any spatial logic to how different neurons adapt their responsiveness to DFs during imaging, we plotted the ROIs using a lookup table highlighting the preference of for either the first three DFs (pink, naive response), or last three DFs (green, trained response). Strong preferences for the naive stimuli reflects a depressing response profile (***Figure 1I, J***). While most neurons did show tuning consistent with strong depression, there were neurons that showed an equal preference for naive and trained stimuli, or even stronger preference for the latter, indicating stable or potentiating response profiles. These non-depressing neurons were mostly contained in the dorsal regions of the brain, including the torus longitudinalis, cerebellum and

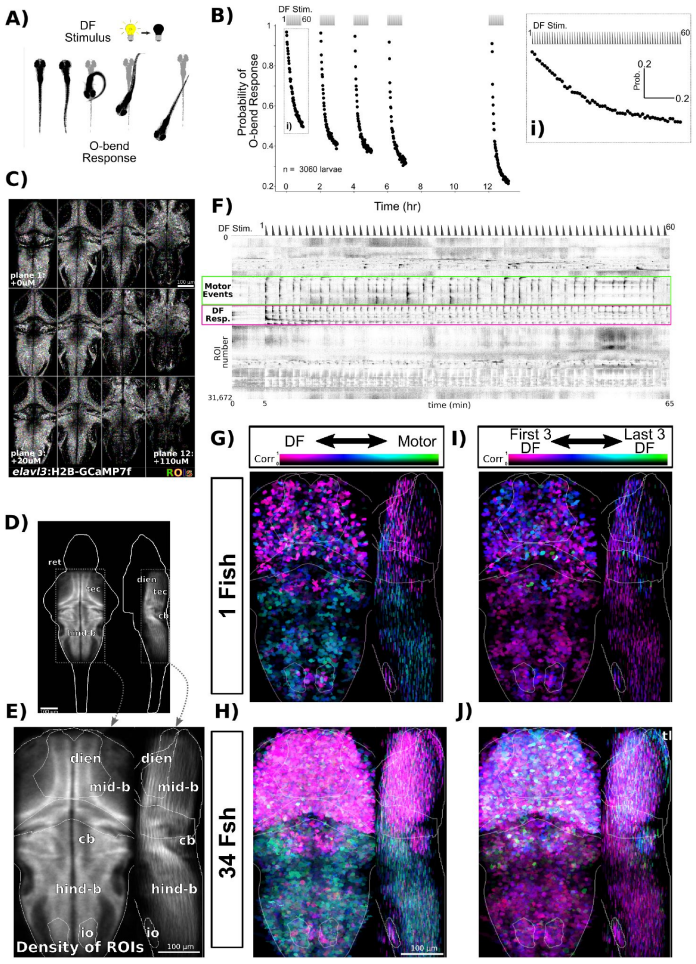

Figure 1—figure supplement 1. Validation of motion analysis based on image artifacts during 2-photon imaging

Figure 1.

Volumetric 2-photon Ca^{2+} imaging of dark flash habituation.

A) In response to a dark flash (DF), larval zebrafish execute a high-amplitude turn called an O-bend response.
B) Habituation results in a progressive decrease in response probability to dark flashes repeated at 1-minute intervals, delivered in 4 blocks of 60 stimuli, separated by 1hr of rest (from 0:00-7:00), and after a 5hr retention period (12:00-). Inset **i)** shows and expanded view of the first training block.
C) *Tg(elavl3:H2B-GCaMP7f)* larvae were imaged across 12 z-planes at 10μm steps. ROIs are overlaid in random colors.
D) Density of detected ROIs registered and plotted in the Z-Brain coordinate space. n=1,050,273 ROIs across 34 larvae.
E) Cropped field of view used for plotting and analyzing Ca^{2+} imaging data and approximate anatomical localizations of major brain areas: dien=diencephalon, mid-b = midbrain, cb = cerebellum, hind-b = hindbrain, io = inferior olive, ret = retina, tec = tectum
F) Functional responses of neurons to 60 dark flashes at 1-minute intervals, plotted as a clustered heatmap ("rastermap" (***Pachitariu et al., 2017***), *github.com/MouseLand/rastermap*), where rows represent individual neurons ordered by the similarities in their activity. Darker shades reflect increased activity. This clustering reveals neurons that are tuned to the DF stimuli (pink box) or motor events (green box). Dashed trace above the heatmap depicts the DF stimulus convolved with a kernel approximating H2B-GCaMP7f kinetics.
G) ROIs in an individual fish plotted based on their correlation and tuning to regressors defining either Motor or DF stimulus events, highlighting the spatial distributions of these tunings across the imaged population. Plotted as a maximum intensity projection.
H) Same analysis as G, but across the entire population of 34 larvae.
I) ROIs in an individual fish plotted based on their correlation and tuning to regressors defining either the first or last three DF stimuli.
J) Same analysis as I, but across the entire population of 34 larvae. tl = torus longitudinalis

dorsal hindbrain. These results demonstrate that while the majority of neurons across the brain depress their responsiveness during habituation, a smaller population of neurons exists that show the opposite pattern.

Functional classification and anatomical localization of neuronal types observed during habituation learning

To explore the functional heterogeneity within the DF-tuned neurons we used affinity propagation clustering. This method has the advantage that cluster numbers do not need to be defined beforehand, and instead attempts to identify the most representative response profiles (***Förster et al., 2020***). This identified 12 clusters that differed both in their adaptation to repeated DFs, as well as the shape of their response to the DF (***Figure 2A,B***).

We therefore use these two aspects of the response to label the clusters:

Adaptation Profile

No Adaptation = ^{noA} : Cluster 1, 9, 10

Weak Depression = ^{weakD} : Cluster 5, 6, 11

Medium Depression = $^{med\ D}$: Cluster 2, 3, 7

Strong Depression = ^{strgD} : Cluster 4, 8

Potentiation = ^{Pot} : Cluster 12

Response Shape

On-response = $_{On}$: Cluster 1, 2

Long/sustained response = $_{L}$: Cluster 3, 4

Medium-length response = $_{M}$: Cluster 5, 6, 9

Short/transient response = $_{S}$: Cluster 7, 8, 10, 11

Yielding clusters: 1^{noA}_{On}, 2^{medD}_{On}, 3^{medD}_{L}, 4^{strgD}_{L}, 5^{weakD}_{M}, 6^{weakD}_{M}, 7^{medD}_{S}, 8^{strgD}_{S}, 9^{noA}_{M}, 10^{noA}_{S}, 11^{weakD}_{S}, and 12^{Pot}_{M}

While these results indicate the presence of a dozen functionally distinct neuron types, such clustering analyses will force categories upon the data irrespective of if such categories actually exist. To determine if our cluster analyses identified genuine neuron types, we analyzed their anatomical localization (***Figure 2C-E***). Since our clustering was based purely on functional responses, we reasoned that anatomical segregation of these clusters would be consistent with the presence of truly distinct types of neurons. Indeed, we observed considerable heterogeneity both within and across brain regions. For example: 11^{weakD}_{S} was mostly restricted to medial positions within the optic tectum; 3^{medD}_{L} and 4^{strgD}_{L} were more prevalent within motor-related regions of the brain including the tegmentum and hindbrain rhombomeres; 9^{noA}_{M} was the most prominent cluster in the torus longitudinalis, consistent with the presence of non-depressing signals in the area (***Figure 1I,J***).

We then quantified the similarity in the spatial relationships among the clusters by looking at the correlations in the positions of the ROIs in the Z-Brain (***Figure 2E***). This revealed similar hierarchical relationships to those identified functionally (***Figure 2B***), especially with respect to

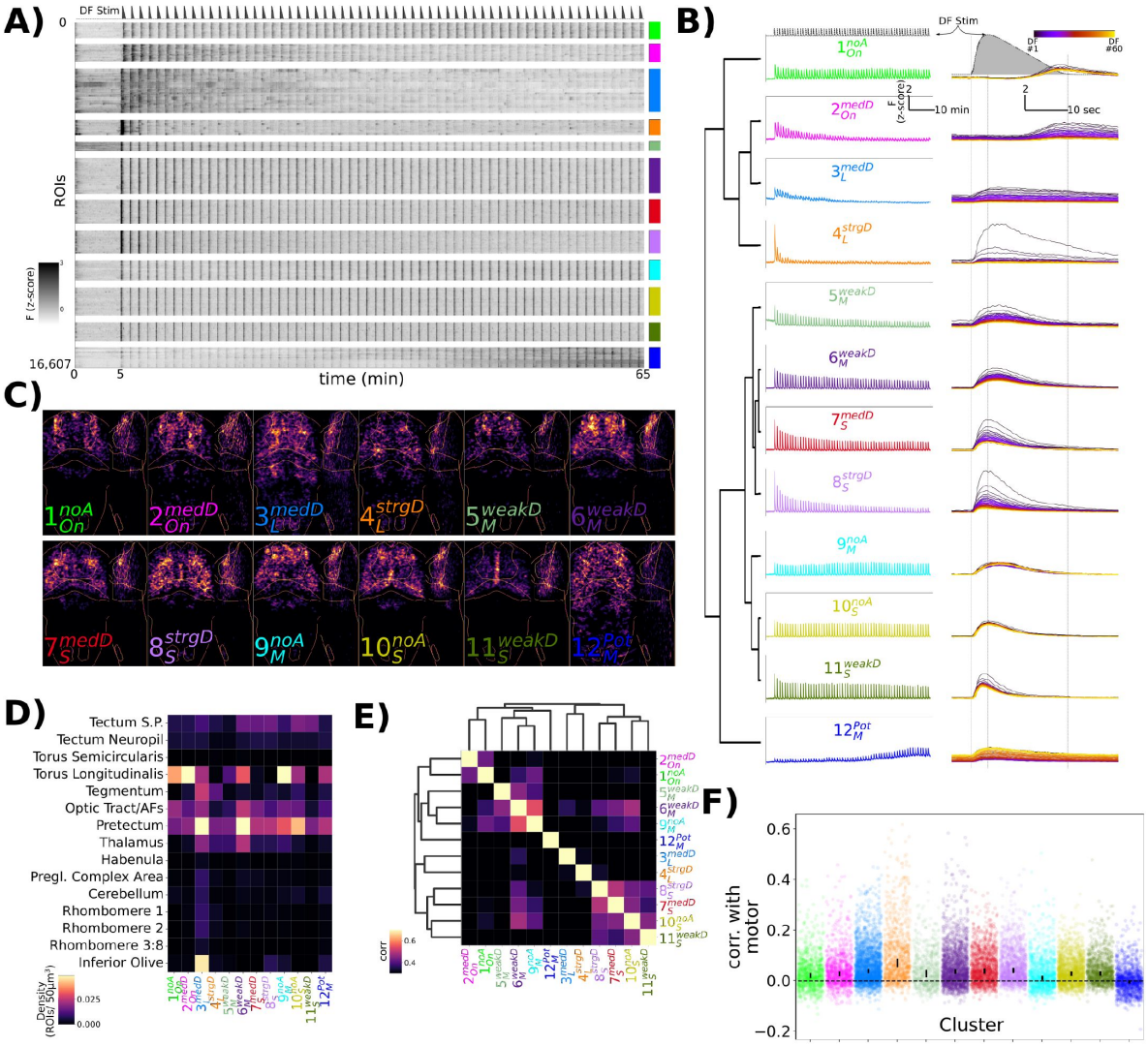

Figure 2.

Characterization of functional response types during habituation learning.

A) Heatmap of the response profiles of ROIs categorized into 12 functional clusters. n=16,607 ROIs from 34 larvae.

B)Average z-scored fluorescence of each functional cluster plotted for the whole experiment (left column), and centered on each DF stimulus (right column), demonstrating the differences in both *Adaptation Profiles* and *Response Shape* for each cluster. Clusters were identified using Affinity Propagation clustering (affinity = Pearson correlation, damping = 0.9, preference =-9), and organized using Hierarchical clustering, distance = complete, correlation. Dashed lines in top panels are the DF stimulus convolved with a kernel approximating H2B-GCaMP7f kinetics, used as the regressor in the analysis.

C)Summed intensity projection of the ROIs belonging to each functional cluster in Z-Brain coordinate space depicting their physical locations in the brain. Projection images are normalized to the maximum value.

D)Heatmap depicting the density of each cluster that is found within different Z-Brain regions.

E)Correlogram calculated from the Pearson correlation in downsampled volumes for the ROI centroid positions for each cluster (see Methods). Hierarchical clustering, distance = complete, correlation.

F)Correlation between motor events and the Ca^{2+} traces for each ROI assigned to the functional clusters. dots = individual ROIs, bar height = 99.99999% confidence interval around the median value.

Response Shape, indicating that physical location is associated with functional response type.

Finally, since our functional analysis was performed purely based on correlations with the DF stimuli, we asked to what extent neurons belonging to each cluster were correlated with motor output (***Figure 2F***). This identified 4_L^{strgD} as the most strongly correlated to motor output, consistent with its strong habituation profile and its localization within motor-regions of the hindbrain. This indicates that 4_L^{strgD} neurons likely occupy the most downstream positions within the sensory-motor network.

These results highlight a diversity of functional neuronal classes active during DF habituation. Whether there are indeed 12 classes of neurons, or if this is an over- or under-estimate, awaits a full molecular characterization. Independent of the precise number of neuronal classes, we proceed under the hypothesis that these clusters define neurons that play distinct roles in the DF response and/or its modulation during habituation learning.

Pharmacological screening to identify habituation modulators

We next used a pharmacological screening approach to both identify molecular mechanisms of habituation and to further probe the habituating circuit. For this we screened 1953 small molecule compounds with known targets (***Figure 3-source data 1***)), in conjunction with the high-throughput assay we previously established, which has a maximum throughput of 600 larvae/day (***Figure 3A***, (***Randlett et al., 2019***)). As we aimed to identify modulators specific for habituation, we included additional behavioural assays as controls, including the response to acoustic stimuli, the optomotor response, and the spontaneous swimming behaviour of the fish in the absence of stimulation (***Figure 3B,C***). In each 300-well plate, 40 groups of 6 larvae were treated in individual wells, and compared to 60 vehicle treated controls (***Figure 3A***). We chose these numbers based on a sub-sampling analysis that determined these numbers were sufficient to identify the effect of a known modulator of habituation (haloperidol (***Randlett et al., 2019***)) at a false-negative rate of less than 0.05 (not shown), while allowing us to screen 80 compounds per experiment across 2 plates.

We were able to collect the full behavioural record of 1761 compounds (***Figure 3D***, ***Figure 3-source data 2***)), indicating that the fish survived the treatment and maintained their ability to swim. Behavioural records for fish treated with each compound were compressed into a fingerprint (***Rihel et al., 2010***) – a vector representing the strictly standardised mean difference (SSMD) across 47 aspects of behaviour (see Methods). For measurements related to dark-flash habituation behaviour, responses were time-averaged across three epochs chosen to highlight changes in habituation: the naive response (first 5 dark flashes), the response during the remaining training flashes, and the re-test block 5 hrs after training (***Figure 3B***). This was done across 10 different components of the dark flash response (Probability of Response, Latency, Displacement, etc.).

We found that 176 compounds significantly altered at least one aspect of measured behaviour, yielding a 9% hit rate (hit threshold of $|SSMD| \geq 2$). While the average effect was to suppress behavioural output $(\overline{SSMD} = -0.20)$, which could reflect non-specific toxicity or a generalized inhibition of motor output, most small molecules induced both positive and negative changes in behavioural output, indicating that toxicity is not the primary phenotypic driver. While the false negative rate is difficult to determine since so little is known about the pharmacology of the system, we note that of the three small molecules we previously established to alter dark flash habituation that were included in the screen, Clozapine, Haloperidol and Pimozide (***Randlett et al., 2019***), the first two were identified among our hits while Pimozide was lethal at the 10μM screening concentration.

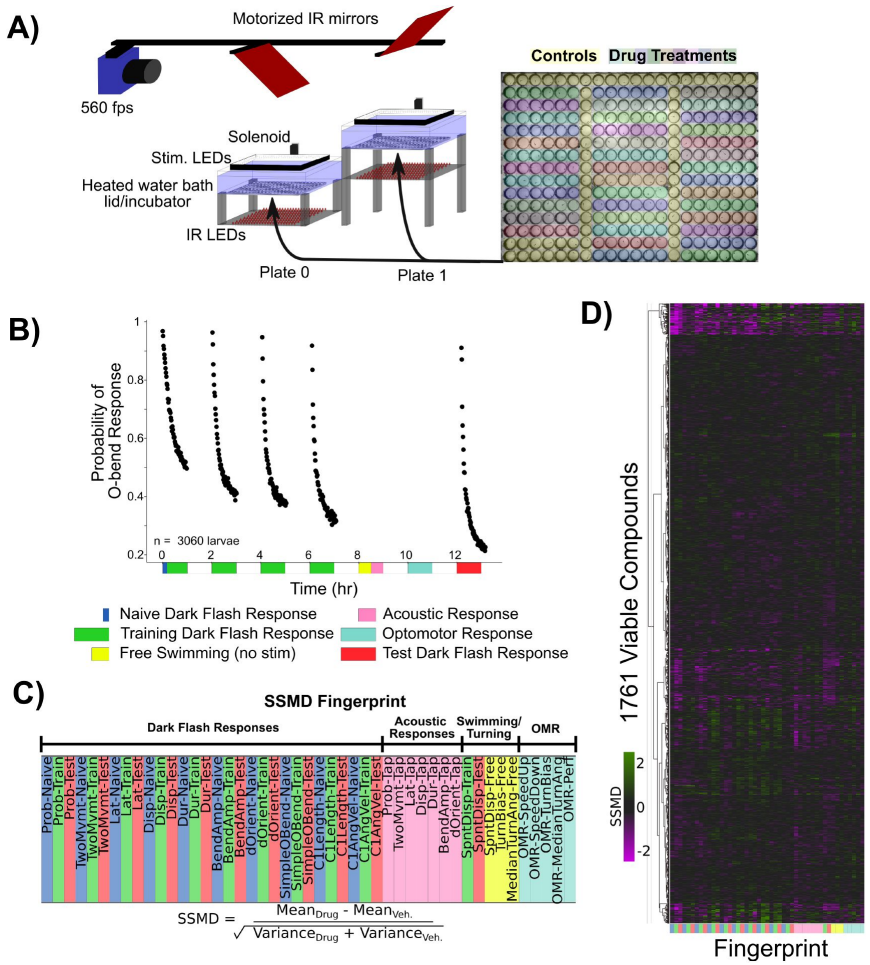

Figure 3.

Pharmacological screening for dark flash habituation modulators.

A) Screening setup to record larval zebrafish behaviour in 300-well plates, which are placed below a 31°C water bath that acts as a heated lid for the behaviour plates. Two 300-well plates are imaged in alternation using mirrors mounted on stepper motors. Fish are illuminated with infra-red LEDs and imaged with a high-speed camera recording at 560 frames per second (fps). Visual stimuli are delivered by a rectangular ring of RGB LEDs, and acoustic stimuli are delivered via a solenoid mounted on the back of the water tank. Colors overlaid on the 300-well plate indicate the arrangement of small molecule treatments and controls (yellow).

B)Habituation results in a progressive decrease in responsiveness to dark flashes repeated at 1-minute intervals, delivered in 4 training blocks of 60 stimuli, separated by 1hr of rest (from 0:00-7:00). This epoch is separated into periods reflective of the Naive response (first 5 stimuli, blue), and the remaining 235 stimuli during Training (green). From 8:00-8:30, no stimuli are delivered and fish are monitored for spontaneous behaviour (yellow). From 8:30-9:00 fish are given acoustic stimuli via the solenoid tapping on the water bath (pink). From 10:00 - 11:00 fish are stimulated with alternating leftward and rightward motion using the RGB LEDs to induce the optomotor response and turning towards the direction of motion (light blue). Finally, at 12:00-13:00, larvae are given 60 additional dark flashes during the test period (red). Same data as ***Figure 1B***.

C) The strictly standardized mean difference (SSMD) is calculated across these different time periods, behaviours and the different components of O-Bend behavioural habituation (Randlett et al., 2019). All compounds were dosed at 10 μM in 0.1% DMSO (n = 6 larvae), relative to 0.1% DMSO vehicle controls (n = 60 larvae).

D)These vectors are assembled across all screened compounds that were viable and did not cause death or paralysis of the larvae. Displayed as a hierarchically clustered heatmap of behavioural Fingerprints (vectors of SSMD values). Clustering distance = ward, standardized euclidean.

Figure 3—source data 1. Small molecule library, Selleckchem Bioactive: FDA-approved/FDA-like small molecules

Figure 3—source data 2. Behavioural fingerprint parameter descriptions

Figure 3—source data 3. Behavioural fingerprints for viable compounds

Correlational structure in the pharmaco-behavioural space

To explore the pharmaco-behavioural space in our dataset we clustered the hits based on their behavioural phenotypes (***Figure 4*A**). This strategy can identify compounds that share common pharmacological targets, or perhaps distinct pharmacological targets that result in convergent behavioural effects (***Bruni et al., 2016***; ***Rihel et al., 2010***). Indeed, compounds known to target the same molecular pathways often showed similar behavioural fingerprints lying proximal on the linkage tree, indicating that our dataset contains sufficient signal-to-noise to recover consistent pharmaco-behaviour relationships.

Alternatively, compounds can be considered as tools to manipulate different aspects of brain function agnostic to their molecular mechanisms. Consequently, using similarities and differences among the induced alterations should uncover molecular and neural linkages among different behavioural outputs. Following this logic, the ability of a compound to co-modify different aspects of behaviour would reflect molecular and/or circuit-level dependencies. For example, visual behaviours that all depend upon photoreceptors should be similarly affected by any compounds that modulate phototransduction in these photoreceptors. We quantified these relationships by calculating the correlated effects on our different behavioural measurements across compounds (***Figure 4*B**).

Consistent with our previous results highlighting uncorrelated learning across the behavioural components of the O-bend response during habituation (***Randlett et al., 2019***), we found that different aspects of the response were independently affected pharmacologically, resulting in distinctive correlated groupings within the correlogram. While we previously found that O-Bend response Probability and Latency habituate independently in individual fish (***Randlett et al., 2019***), in our small molecule screen data these appear to be tightly coupled (***Figure 4*B**). The performance of the animals in the OMR assay under different treatments was also associated with O-bend Probability and Latency, suggesting that pharmacological modulation of vision or arousal could drive these correlations within the small molecule screen dataset.

These analyses confirm habituation behaviour manifests from multiple distinct molecular mechanisms that independently modulate different behavioural outputs.

Modulation of habituation by GABA, Melatonin and Estrogen signaling

For the remainder of the analyses we decided to focus on the mechanisms leading to the habituation of response probability, as this is the criterion for which it is easiest to identify the link between neural activity and behavior, providing the best entry point for studying the circuit mechanisms of long-term habituation. To identify the most promising hits, we sought to identify compounds that:

1. Have minimal effects on the naive response to DFs, but strong effects during the training and/or memory-retention periods. This would prioritize pathways that affect habituation, rather than simply DF responsiveness.
2. Have minimal effects on other aspects of behaviour, in order to exclude compounds that would alter generalized arousal, movement ability/paralysis, or visual impairment. Such compounds would strongly influence DF responsiveness, but likely independently of pathways related to habituation.
3. Show similar behavioural effects to other compounds tested that target the same molecular pathway. Such relationships can be used to cross validate, yet we note that our library choice was very broad, and target coverage is non-uniform. Therefore a lack of multiple hits targeting the same pathway should not be taken as strong evidence of a false positive.

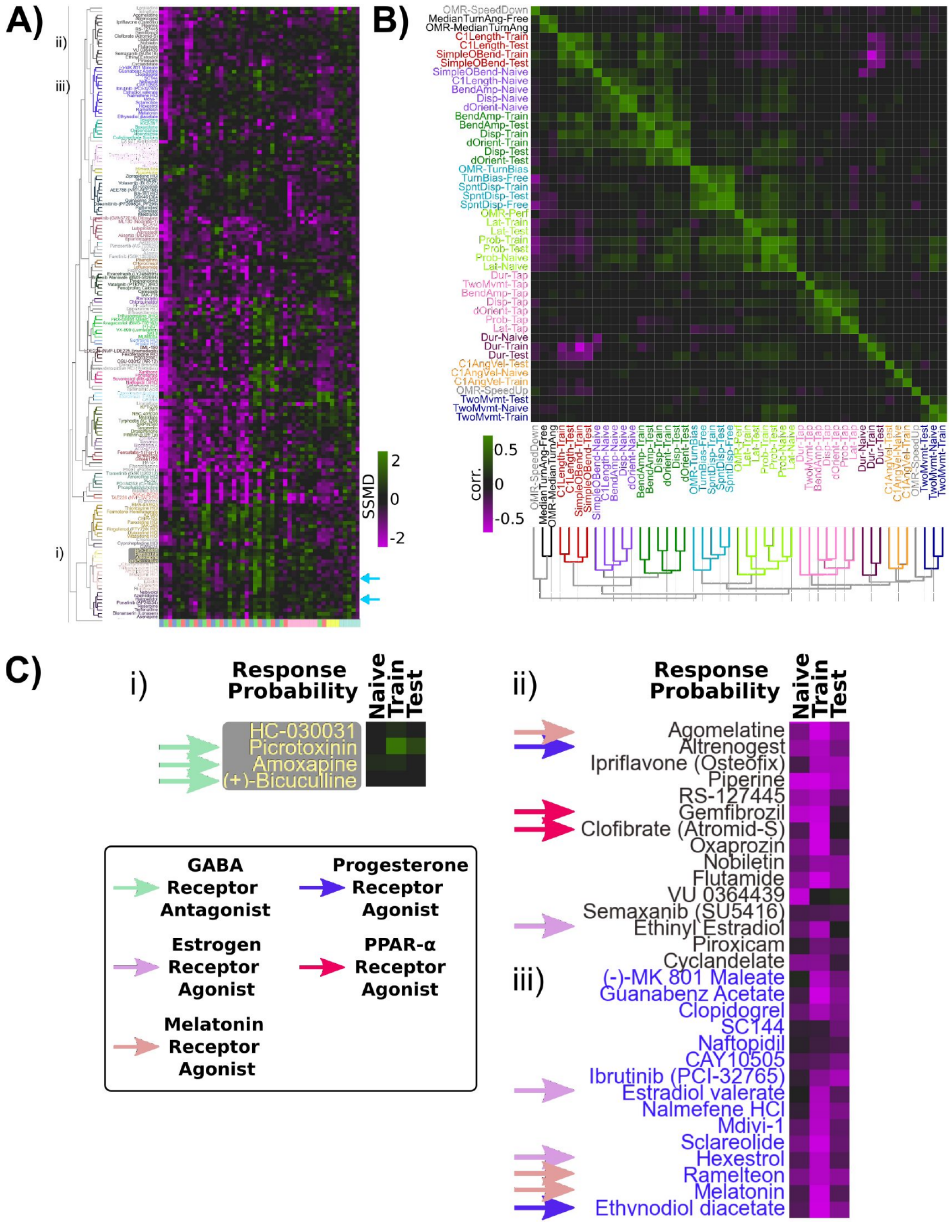

Figure 4.

Pharmaco-behavioural analyses of behaviour-modifying compounds.

A) Clustered heatmap of the behavioural Fingerprints for the 176 hits of the screen, showing at least one behaviour measure with $|SSMD| \geq 2$. Clustering distance = ward, standardized euclidean, colour/cluster threshold = 9.5. This led to the re-identification of Haloperidol and Clozapine as habituation modifiers (light blue arrows).

B) Clustered correlogram of the Pearson correlation coefficients for the different measured components of behaviour across hits (same data as (A)) revealing the independence or co-modulation of behaviours. Clustering distance = average, correlation, colour/cluster threshold = 1.5.

C) Subsets of clustered heatmap from (A), highlighting the similar phenotypes exhibited by i) GABA Receptor antagonists and ii), iii) Melatonin receptor agonists, Estrogen receptor agonists, Progesterone receptor agonists and peroxisome proliferator-activated receptor alpha (PPAR*α*) agonists. Heatmap is cropped to the first three columns of (A), depicting the SSMD of response Probability relative to vehicle controls.

This manual prioritization led to the identification of the $GABA_{A/C}$ Receptor antagonists Bicuculline, Amoxapine, and Picrotoxinin (PTX). PTX treatment had the strongest effects, with increased responsiveness to DFs during the training and test periods, indicative of defects in habituation (***Figure 4*Ci**). Dose-response experiments confirmed a strong effect of PTX on inhibiting the progressive decrease in responsiveness during habituation learning at 1-10μM doses (***Figure 5*A**). Importantly, like the naive dark-flash response, the probability of responding to an acoustic stimulus and the optomotor response were not inhibited (***Figure 5-figure Supplement 1*A**). While strong $GABA_{A/C}R$ inhibition results in epileptic activity in larval zebrafish, we did not observe evidence of seizure-like behaviour at these doses, consistent with a partial $GABA_{A/C}R$ in our experiments and previous results (***Bandara et al., 2020***). Therefore, we conclude that partial antagonism of $GABA_AR$ and/or $GABA_CR$ is sufficient to strongly suppress habituation but not generalized behavioural excitability, indicating that GABA plays a very prominent role in habituation. This is consistent with a model in which the potentiation of inhibition actively silences sensory-induced activity during habituation to suppress motor output (***Cooke and Ramaswami, 2020***; ***Ramaswami, 2014***).

We next turned our attention to the upper portion of the clustered behavioural fingerprint graph (***Figure 4*A**), where strong and relatively specific inhibition of responsiveness during training and testing were observed, indicative of enhanced habituation (***Figure 4*Cii, iii**). Among the hits observed here were multiple agonists of both Melatonin and Estrogen receptors, indicating that hormonal signaling may play a prominent role in habituation. Dose response studies with Melatonin confirmed strong potentiation of habituation (***Figure 5*B**). Melatonin did cause a decrease in spontaneous movement behaviour, consistent with its role in arousal/sleep regulation in zebrafish and other vertebrates (***Gandhi et al., 2015***; ***Zhdanova et al., 2001***), yet Melatonin did not inhibit the naive response to dark flashes, the responsiveness to acoustic stimuli or OMR performance (***Figure 5*B**, ***Figure 5-figure Supplement 1*B**). Melatonin's effect on habituation was also most prominent for the Probability of response, and did not strongly alter habituation for Displacement ***Figure 5-figure Supplement 1*F**, indicating it does not cause generalized sedation but modulates specific aspects of behaviour at these doses, including increasing habituation of the Probability of response.

We similarly validated that the Estrogen Receptor agonists Ethinyl Estradiol and Hexestrol, potentiated habituation at 5-100μM and 1-10μM doses, respectively (***Figure 5*C,D**). Ethinyl Estradiol strongly suppressed movement rates at these doses, and both treatments suppressed acoustic responsiveness and OMR performance at doses >=10μM (***Figure 5* - *figure Supplement 1*C,D**). Thus, it is less clear how specific or generalized Estrogen Receptor agonism is on behaviour, although the effective doses of Hexestrol for influencing habituation (1-5uM) were lower than those that significantly affected other behaviours (10uM). Nevertheless we decided to focus on PTX and Melatonin for the remaining experiments.

Our screening approach identified both expected (GABA) and unexpected (Melatonin, Estrogen) pathways that strongly modulate habituation of responsiveness. We also implicated other pathways in habituation, including Progesterone and PPARα (***Figure 4*C**), and identified compounds that strongly modify other aspects of behaviour (OMR, acoustic and spontaneous behaviour). These hits can be mined for future projects investigating the molecular basis of behaviour.

Pharmacological manipulations of functional circuit properties during habituation

Our Ca^{2+} imaging experiments identified 12 distinct functional classes of neurons during habituation learning, but it is unclear how these might be organized in a circuit. Based on the diversity of functional response profiles identified, it is clear that solving this circuit will take considerable further work. As as starting point in this long-term effort, we used the

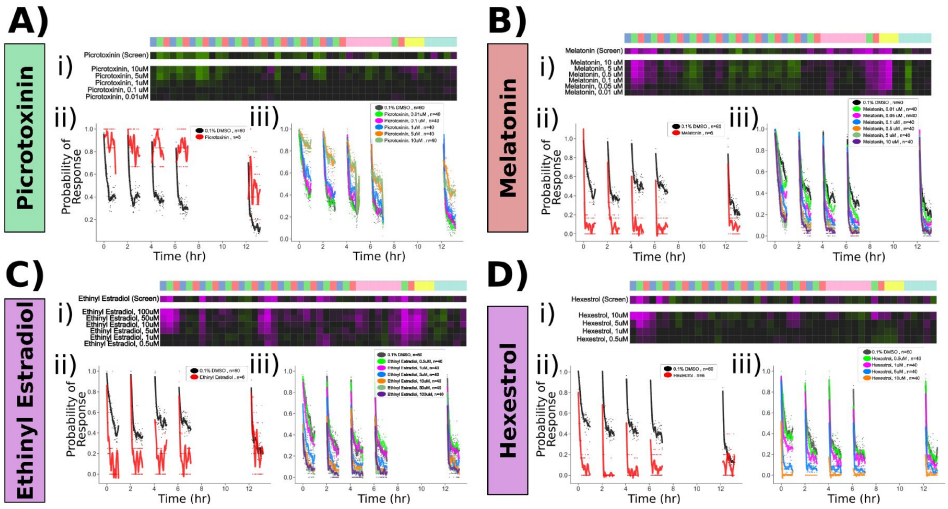

Figure 5.

Confirmed pharmacological modulators of habituation.
Dose response studies for **A)** Picrotoxinin, **B)** Melatonin, **C)** Ethinyl Estradiol and **D)** Hexestrol.
Displayed for each treatment are: i) Behavioural fingerprint for the original screen data (10 uM), and the dose response data. ii) Original screen data for the probability of response to DF stimuli. Each dot is the probability of response to one flash. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order=2). iii) Dose response data for the probability of response, plotted as in ii)

Figure 5—figure supplement 1. Pharmacological manipulation of control behaviours and response displacement during habituation

pharmacological manipulations as these treatments provide us with tools to ask how treatments that potently alter habituation behaviour also alter the functional properties of neurons. We compared the Ca^{2+} activity patterns after treatment with vehicle (0.1% DMSO), PTX, or Melatonin (***Figure 6***). At the behavioural level, we found a trend indicating that we were able to manipulate habituation pharmacologically in our tethered imaging assay, though this was very subtle (***Figure 6*****A**). This discrepancy relative to the very strong behavioural effects in freely-swimming animals (***Figure 5***) likely result from the head-restrained protocol, which itself strongly inhibits behavioural output. Yet, since we did observe a trend in behavioural data, we proceeded under the assumption that the compounds were having the desired effects.

As PTX and Melatonin have opposing effects on habituation behaviour, we reasoned that these two treatments should have opposite effects in the circuit, with PTX inhibiting depression and Melatonin promoting depression. Indeed Melatonin has been found to increase the effects of GABA, and so such a relationship could be direct (***Cheng et al., 2012***; ***Niles et al., 1987***). In contrast to this straightforward hypothesis, what we observed was considerably more complex. We did not observe alterations of the average response profiles of individual neuronal classes, which remained indistinguishable after the treatments (***Figure 6-figure Supplement 1*****C-K**). Instead, the proportion of neurons that belonged to the different classes was altered (***Figure 6*****B-D**). Therefore, the pharmacological manipulations did not alter the activity of neurons in such a way as to alter the average activity states of populations, but instead the proportion of neurons belonging to different populations changed. This may point to fixed and relatively inflexible processing strategies that the brain is using in the context of dark-flash habituation which constrain the possible functional response types.

The effect of PTX on cluster reassignment generally tended towards weaker depression, increasing the proportion of cells falling into the weaker depressing classes at the expense of strongly depressing classes for a given response shape (***Figure 6*****D**). This pattern was most clear in the classes with “Short” and “Long” *Response Shapes*, which are those that included the most strongly depressed classes of neurons.

Based on the hypothesis that Melatonin and GABA cooperate during habituation, we expected PTX and Mela-tonin to have opposite effects. This clearly does not fit with our observations: for example, the size of the 12^{Pot}_{M} neuron population was increased by both PTX and Melatonin (***Figure 5*****C**). While habituation of the Probability of response is oppositely modulated by PTX and Melatonin, this is not true of behaviour globally – the behavioural fingerprints of Melatonin and GABA are not opposites (***Figure 5*****A,B**) and opposing effects are not seen for the habituation of Displacement (***Figure 5-figure Supplement 1*****E,F**). Therefore, a lack of coherent shifts across the entire neural population when applying these treatments is expected. However, opposite effects of PTX and Melatonin were observed for 4^{sPeD}_{L} neurons (***Figure 6*****C**), which we found to be most strongly correlated with motor output (***Figure 2*****F**). Therefore, this class might be most critical for habituation of response Probability.

Combined, these experiments reveal that pharmacological manipulations that affect habituation behaviour manifest in complex functional alterations in the circuit. These effects can not be captured by a simple model, and considerable additional knowledge of the circuit, including the connectivity and signalling capacity of different neurons will be necessary to understand these dynamics.

Identification of GABAergic neurons classes in the habituating circuit

Since our pharmacological experiments point to the importance of GABAergic inhibition in habituation, we asked which functional classes of neurons are GABAergic? An obvious model for habituation would assign a GABAergic identity to the 12^{Pot}_{M} neurons that potentiate their responses,

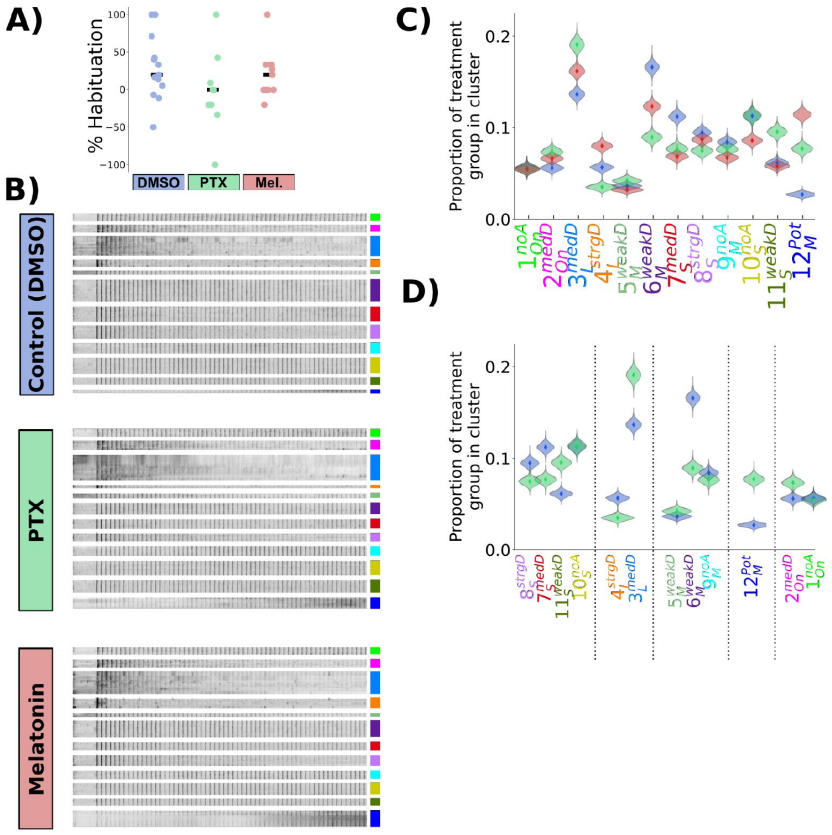

Figure 6.

Picrotoxinin and Melatonin alter the proportions of functionally identified neurons

A) Percent habituation for larvae during Ca^{2+} imaging, calculated as: $\%Habituation = 100 \times (1 - \frac{P(Resp)_{11\to60}}{0.5\times(P(Resp)_{1\to10}+P(Resp)_{51\to60})})$

B) Heatmap of response profiles of ROIs categorized into the 12 functional clusters from larvae treated with DMSO (vehicle control, n = 428,720 total ROIs in 14 larvae), Picrotoxinin (PTX, 10uM, n = 271,037 total ROIs in 9 larvae), or Melatonin (1uM, n = 350,516 total ROIs in 11 larvae).

C) Proportion of neurons belonging to each functional cluster across treatment groups. Distributions for violin plots are bootstrapped from 5000 replicates.

D) Same data as C, only showing the data for PTX vs DMSO vehicle control, re-ordered to reflect the cluster Adaptation Profiles grouped by cluster Response Shape.

Figure 6—figure supplement 1. Mean response of functionally identified clusters after different pharmacological treatments

and thus could act to progressively depress the responses of other neuronal classes. We began with virtual co-localization analyses with 3D atlases to identify candidate molecular markers for functionally identified neurons. Such a strategy can be powerful to generate hypotheses from brain-wide imaging data, provided sufficient stereotypy exists in the positioning of neurons, and the relevant marker exists in the atlas (***Dunn et al., 2016***; ***Randlett et al., 2015***). Therefore, we analyzed the spatial correlations for markers contained in the Z-Brain (***Randlett et al., 2015***), Zebrafish Brain Browser (***Gupta et al., 2018***; ***Marquart et al., 2017***; ***Tabor et al., 2018***), and mapZebrain atlases (***Kunst et al., 2018***; ***Shainer et al., 2022***). We identified markers showing the highest spatial correlations with any of our functional clusters (corr. > 0.15, n=89 of 752 markers), and organized these hierarchically (***Figure 7A***). GABAergic reporter lines based on the *gad1b* promoter were located in a region of the hierarchy showing greatest spatial similarity with 10_S^{noA} and 11_S^{weakD} (***Figure 7B-E***). An enrichment along the medial tectum is common to markers in this region of the hierarchy, where the highest density of GABAergic neurons within the tectum reside.

To confirm that 10_S^{noA} and 11_S^{weakD} classes are GABAergic, we imaged the response of neurons in *Tg(Gad1b:DsRed); Tg(elavl3:H2B-GCaMP6s)* double transgenic larvae, and classified neurons as *gad1b*-positive or -negative based on DsRed/GCaMP levels (***Figure 7F,G***). Indeed we saw a heterogeneous distribution of *gad1b*-positive neurons across functional clusters, including a significant enrichment in not only 10_S^{noA} and 11_S^{weakD}, but also the other two clusters with the "Short" *Response Shape* (7_S^{medD} and 8_S^{strgD}). The remaining clusters either showed no significant bias, indicating that they contain mixed populations, or a significant depletion of *gad1b*-positive cells, suggesting that they comprise mostly of excitatory or neuromodulatory neurons (3_L^{medD} and 12_M^{Pot}).

These experiments indicate that GABAergic neurons in the habituating circuit are not characterized by their *Adaptation Profile* (other than non-potentiating), and instead have a characteristic "Short" *Response Shape*, perhaps reflecting a transient bursting style of activity relative to other neuronal types that exhibit more sustained firing patterns. This lack of coherence in adaptation profile may explain why global manipulations of GABAergic signaling through PTX have complex manifestations in the functional properties of neurons (***Figure 6D***).

Discussion

Molecular mechanisms of DF habituation

To explore the molecular mechanisms of habituation, we performed a small molecule screen testing for effects on DF habituation behaviour. Analyses of the correlated effects of drugs across different aspects of behaviour (***Figure 4***) are consistent with our previous results indicating that habituation results from multiple molecularly independent plasticity processes which act to adapt different aspects of the DF response during habituation (***Randlett et al., 2019***). Here we focused our analysis on those pharmacological agents and pathways that strongly and relatively specifically modulated habituation when measuring response Probability. We found that inhibition of $GABA_{A/C}$ Receptors using PTX reduced habituation learning. GABA is the main inhibitory neurotransmitter in the zebrafish brain, and deficits in GABA signaling lead to epileptic phenotypes (***Baraban et al., 2005***). We were fortunate that our screening concentration (10μM) did not cause seizures, but was still sufficient to inhibit habituation. This implies that the habituation circuit is exquisitely sensitive to changes in GABA signaling at levels well below the threshold required to drastically change excitatory-inhibitory balances. We cannot rule out the possibility that off-targets of PTX, or subtle non-specific changes in excitatory/inhibitory balance alter habituation behaviour. However, the lack of strong modulation of other behaviours, including the response to acoustic stimuli or the optomotor response (***Figure 5-figure Supplement 1A***), suggests that GABAergic inhibition plays a crucial role in the process of DF habituation.

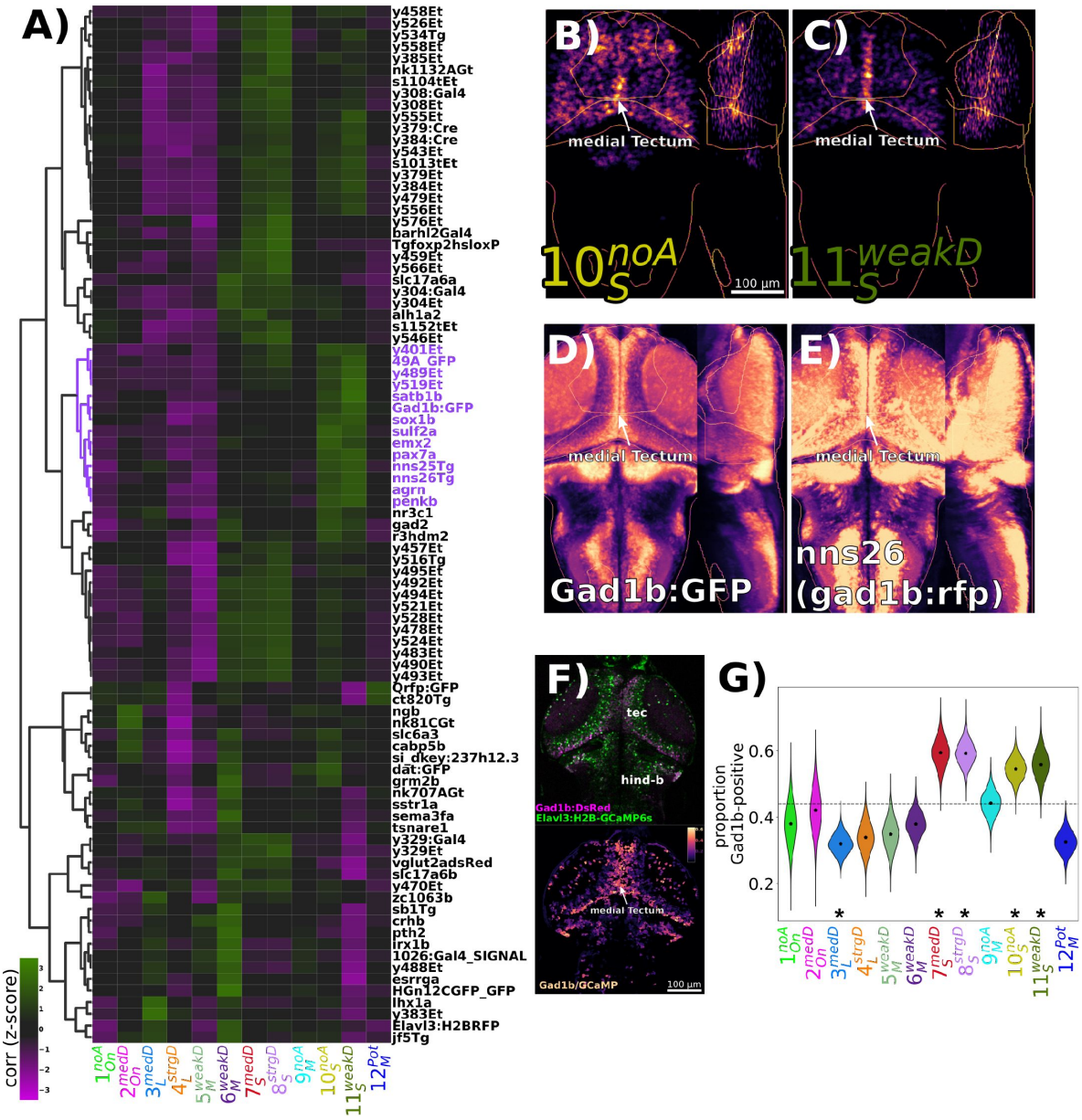

Figure 7.

Identification of GABAergic neuronal classes

A) Hierarchically clustered heatmap depicting the correlation of markers aligned to the Z-Brain atlas with the spatial arrangement of the 12 functional clusters (distance = complete, correlation). Correlation values are z-scored by rows to highlight the cluster(s) most strongly correlated or anti-correlated with a given marker. The subset of the hierarchy containing the *gad1b*-reporters is coloured in purple.

B-D) Normalized summed intensity projections of **B)** 10_S^{noA}, and **C)** 11_S^{weakD}

,, D) *TgBAC(gad1b:GFP)* (**Satou et al., 2013**), Z-Brain Atlas, and **E)** *nns26*, aka *TgBAC(gad1b:LOXP-RFP-LOXP-GFP)* (**Satou et al., 2013**), mapZebrain Atlas

F) 2-photon imaging of *Tg(Gad1b:DsRed);Tg(elavl3:H2B-GCaMP6s)* larvae depicting the raw data for each channel (top), and the ratio of Gad1b/GCaMP6s fluorescence in each ROI functionally identified using suite2p.

G) ROIs imaged in double transgenic larvae are assigned a cluster identity based on their correlation to the cluster mean trace, and classified as Gad1b-positive based on a DsRed/GCaMP6s ratio of greater than 0.25. Dotted line = expected proportion based on total number of cells classified as Gad1b-positive. *=p<0.05, Chi Square test with Bonferroni correction. Distributions for violin plots calculated by bootstrapping 5000 replicates. n = 1835 ROIs in 6 larvae.

A critical role for GABA in habituation is also consistent with data from *Drosophila*, where both olfactory and gustatory habituation have been linked to GABAergic interneurons (**Das et al., 2011**; **Paranjpe et al., 2012**; **Trisal et al., 2022**). Therefore, this circuit motif of increasing inhibition to drive habituation may be a conserved feature of habituation, which would allow for a straightforward mechanism for habituation override during dis-habituation via dis-inhibition (**Cooke and Ramaswami, 2020**; **Trisal et al., 2022**).

Our screen also identified that neuro-hormonal signaling is critical for habituation, where Melatonin and Estrogen receptor agonists potently increase habituation learning rate. The role of Estrogens in learning and memory is well established (**Luine et al., 1998**; **Nilsson and Gustafsson, 2002**). Though its role in habituation is less well explored, it has previously been shown to increase memory retention for olfactory habituation in mice (**Dillon et al., 2013**). To our knowledge, Melatonin has not previously been implicated in habituation, though it has been implicated in other learning paradigms (**El-Sherif et al., 2003**; **Jilg et al., 2019**). Notably, Melatonin was shown to block operant learning at night in adult zebrafish (**Rawashdeh et al., 2007**), and therefore Melatonin appears to be able to both promote or inhibit plasticity in zebrafish, depending on the paradigm.

While Melatonin and Estrogen were not strong candidates for involvement in DF habituation plasticity before our screen, their previous associations with learning and memory reinforce the idea that these molecules play critical roles in plasticity processes. In support of this idea, we have previously shown that habituation is regulated in a circadian-dependent manner (**Randlett et al., 2019**), and both Melatonin and Estrogen levels fluctuate across the circadian cycle (**Alvord et al., 2022**; **Gandhi et al., 2015**; **Zhdanova et al., 2001**), suggesting that either or both of these pathways may act to couple the circadian rhythm with learning performance.

Finally, approximately 2% of the US population use Melatonin as a sleep-aid (**Li et al., 2022**), and a substantial proportion of US women take Estrogen as part of either oral contraceptives or hormone replacement therapy. Therefore, understanding the roles these molecules play in neuroplasticity is a clear public health concern.

Circuit mechanisms of DF habituation

Based on behavioural experiments, we previously postulated that multiple plasticity loci cooperate in the habituating dark-flash circuit, arranged in both parallel and series within the circuit (**Randlett et al., 2019**). Here, our Ca^{2+} imaging experiments identified a diverse range of neuronal *Adaptation Profiles*, including non-adapting and potentiating neurons spread throughout sensory- and motor-related areas of the brain. Thus, non-habituated signals are transmitted throughout the brain, consistent with a distributed learning process. Such a model is further supported with brain-wide imaging data for short-term habituation to looming stimuli, where distributed neurons were identified that showed differential rates of habituation (**Marquez-Legorreta et al., 2022**). It is important to point out that Marquez-Legorreta et al. did not observe non-adapting or potentiating neurons in their experiments. This may be due to differences in analysis methods, or could highlight a difference between short- and long-term habituation circuit mechanisms, the latter of which may rely on more complex circuit mechanisms involving both potentiation and suppression of neuronal responses.

We also observed classes exhibiting an On-response profile (1^{noA}_{On} and 2^{medD}_{On}). These neurons fire at the ramping increase in luminance after the DF, making it unlikely that they play a role in aspects of acute DF behaviour we measured here. These neurons exist in both non-adapting and depressing forms suggesting a yet unidentified role in behavioural adaptation to repeated DFs.

While we have insufficient anatomical data to constrain circuit connectivity models that drive DF habituation, here we demonstrate the use of pharmacology, functional imaging and neurotransmitter classifications to constrain our models. Specifically, pharmacology indicated a

role for GABA and Melatonin in habituation, and our functional imaging identified distinct classes of neuronal types in the DF circuit, including potentiating neurons (12_M^{Pot}). These results point to a model where 12_M^{Pot} neurons are GABAergic and thus progressively inhibit the other neuronal classes, and that perhaps this effect is bolstered by Melatonin. However, in silico co-localization analyses and double transgenic Ca^{2+} imaging identified 12_M^{Pot} neurons as predominantly non-GABAergic, inconsistent with this simple model. Instead, we found that the GABAergic neurons in the circuit are characterized by their short burst of activity to the stimulus onset. If the GABAergic neurons are not increasing in their firing rates but do drive habituation, then per-haps it is the potentiation of GABAergic synapses that drives habituation. This is a somewhat unexpected model, as studies of long-term synaptic plasticity (e.g. LTP and LTD) have overwhelmingly focused on plasticity at excitatory synapses. Although a functional link to behaviour is less well established, long-term inhibitory synaptic plasticity has been well documented, including inhibitory (i)-LTP and i-LTD (***Castillo et al., 2011***). Alternatively, there may be a key minority subset of 12_M^{Pot} neurons that are GABAergic and exert a strong influence over the rest of the circuit driving depression and habituation.

We also found that the same pharmacological treatments that result in strong alterations to habituation behaviour in freely swimming larvae (***Figure 5***), resulted in relatively subtle and complex functional alterations in the circuit (***Figure 6***). Making direct comparisons between freely-swimming behaviour and head-fixed Ca^{2+} imaging is always challenging due to the differences in behaviour observed in the two contexts, and therefore our failure to identify a clear logic in these experiments may have technical explanations that will require approaches to measure neural activity from unrestrained and freely-behaving animals to resolve (***Kim et al., 2017***). Alternatively, these results are again consistent with the idea that habituation is a multidimensional and perhaps highly non-linear phenomenon in the circuit, which cannot be captured by a simple model.

Circuit loci of DF habituation

Where in the brain does habituation take place? As discussed above and previously, our data is inconsistent with a single-locus of plasticity (***Randlett et al., 2019***). Instead, we propose that plasticity is distributed throughout the circuit. Since PTX inhibits most aspects of habituation learning (***Figure 5*Ai**), these all may all involve GABAergic motifs. Moreover, the different functional classes of neurons are distributed through sensory- and motor-related areas of the brain, consistent with the notion that habituation plasticity occurs in a very distributed manner. While distributed, there are clear associations between anatomical location and functional neuron type (***Figure 2*A-E**), indicating that there is some degree of regional logic to the localization of *Adaptation Profiles*. For example, 5_M^{weakD} and 6_M^{weakD} are the most prevalent in the pretectum, and mostly absent from the tegmentum and posterior hindbrain, whereas 3_L^{medD} and 4_L^{strgD} are numerous in tegmentum and posterior hindbrain, and thus likely occupy more downstream positions in the sensori-motor circuit.

The tectum is one of the largest brain areas in larval zebrafish, and is directly innervated by nearly all retinal ganglion cells (***Robles et al., 2014***). Therefore, the tectum is a prime candidate for implementing DF habituation for anatomical reasons. In further support of this notion, the neurons we have identified as GABAergic and propose to be driving habituation (7_S^{medD}, 8_S^{strgD}, 10_S^{noA} and 11_S^{weakD}) are concentrated in the tectum (***Figure 2*C,D**). The tectum contains multiple anatomically distinct types of GABAergic neurons, most of which are locally projecting interneu-rons (SINs, ITNs, PVINs), although GABAergic projection neurons have been observed with axons projecting to the anterior hindbrain (***Gebhardt et al., 2019***; ***Martin et al., 2022***; ***Nevin et al., 2010***; ***Robles et al., 2011***). Therefore, we expect that our GABAergic classes correspond to subsets of these GABAergic tectal neurons, which is testable using genetic approaches based on marker co-expression and/or single cell morphometric and transcriptomic analyses.

Beyond the tectum, conspicuous neuronal clustering was observed in the inferior olive and cerebellum, which have been implicated in motor-related learning behaviours in larval zebrafish (***Ahrens et al., 2012***; ***Lin et al., 2020***; ***Markov et al., 2021***). Both structures contained many stimulus-tuned neurons (***Figure 1I***), and non-adapting (1^{noA}_{On}, 9^{noA}_{M} and 10^{noA}_{S}), and potentiating (12^{Pot}_{M}) neurons were among the most concentrated in the cerebellum (***Figure 2C,D***). Non-adapting 9^{noA}_{M} neurons were also prominent in the torus longitudinalis. The torus longitudinalis has recently been implicated in the binocular integration of luminance cues (***Tesmer et al., 2022***), and therefore is ideally placed to influence habituation to whole-field stimuli like DFs.

Collectively, our brain-wide imaging data indicate that the adaptations underling habituation span many regions of the brain, and therefore a comprehensive model will need to span many regions of the brain in order to explain the neural and behavioural dynamics underlying habituation learning

Conclusion

Habituation is the simplest form of learning, yet despite its presumed simplicity a model of how this process is regulated in the vertebrate brain is still emerging. Here we have combined two methods offered by the larval zebrafish model: whole brain functional imaging and high-throughput behavioural screening. By applying these methods to long-term habituation, we identified and validated pharmacological agents that strongly modulate habituation learning, and distinct classes of neurons that are activated by DFs and adapt their activity during learning. The systematic datasets we generated contain large amounts of additional information that await future validation and integration into our understanding of DF habituation. Nonetheless, the diversity of molecular pathways and functional neuronal types we have identified here indicate that considerable biological complexity exists that awaits discovery within the “simplest” form of learning.

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 in a 14:10h light/dark cycle at 28-29°C. Wild type zebrafish were of the TLF strain (ZDB-GENO-990623-2). Transgenic larvae used were of the following genotypes: *Tg(elavl3:H2B-GCaMP7f)*^{jf90} (***Yang et al., 2021***), *Tg(elavl3:H2B-GCaMP6s)*^{jf5} (***Freeman et al., 2014***), and *Tg(gad1b:DsRed)*^{nns26} (***Satou et al., 2013***). Zebrafish were housed, cared for, and bred at the Harvard MCB, UPenn CDB, and Lyon PRECI zebrafish facilities. All experiments were done in accordance with relevant approval from local ethical committees at Harvard University, the University of Pennsylvania, and the University of Lyon.

High-throughput screening setup and protocol

Larvae were assayed for behaviour in 300-well plates using the apparatus described previously (***Randlett et al., 2019***). Briefly, each well is 8mm in diameter and 6mm deep, yielding a water volume of ≈300uL. Behaviour plates are suspended below a water bath kept at 31°C, which acts as a heated lid to prevent condensation and maintains the water temperature in the well at 29°C. Behaviour was tracked using a Mikrotron CXP-4 camera, Bitflow CTN-CX4 frame grabber, illuminated with IR LEDs (TSHF5410, *digikey.com*). Visual stimuli were delivered via a ring of 155 WS2812B RGB LEDs (144LED/M, *aliexpress.com*). For a dark flash stimulus, the LEDs were turned off for 1s, and then the light intensity was increased linearly to the original brightness over 20s. The optomotor response was induced by illuminating every 8th LED along the top and bottom of the plate, and progressively shifting the illuminated

LED down the strip resulting in an approximately sinusoidal stimulus, 5.5 cm peak to peak, translating at 5.5 cm per second. Direction of motion was switched every 30 s, for a total testing period of 1 hour, and performance was scored as the average change in heading direction towards the direction of motion during these 30s epocs. Acoustic tap stimuli were delivered using a Solenoid (ROB-10391, Sparkfun). The behavioural paradigm was designed to be symmetrical such that 1hr worth of stimulation was followed by 1hr worth of rest (***Figure 1B***), allowing us to alternate the view of the camera between two plates using 45-degree incidence hot mirrors (43-958, Edmund Optics) mounted on stepper motors (***Figure 1A***, ROB-09238, Sparkfun), driven by an EasyDriver (ROB-12779, Sparkfun).

Apparatus were controlled using arduino microcontrollers (Teensy 2.0 and 3.2, PJRC) interfaced with custom written software (Multi-Fish-Tracker), available here: *github.com/haesemeyer/MultiTracker*.

The protocol for assessing behaviour (***Figure 1B***, ***Figure 3B***) consisted of dark flashes repeated at 1-minute intervals, delivered in 4 training blocks of 60 stimuli, separated by 1hr of rest (from 0:00-8:00, hr:min of the protocol). For analyses, this epoch is separated into periods reflective of the Naive response (first 5 stimuli), and the remaining 235 stimuli during training. From 8:00-8:30, no stimuli are delivered and fish are monitored for spontaneous behaviour. From 8:30-9:00 fish are given acoustic stimuli, and from 10:00 - 11:00 fish are assayed for the optomotor response and turning towards the direction of motion (light blue). Finally, at 12:00-13:00, larvae are given 60 additional dark flashes during the test period (red).

Behavioural analyses

The behaviour of the fish was tracked online at 28 hz, and 1-second long videos at 560 hz were recorded in response to DF and Acoustic Tap stimuli. Offline tracking on recorded videos was performed in MATLAB (Mathworks) using the script "TrackMultiTrackerTiffStacks_ParallelOnFrames.m", as described previously, to track larval posture (***Randlett et al., 2019***). Tracks were then analyzed using Python. Analysis code available here: *github.com/owenrandlett/lamire_2022*.

Responses to DFs and to taps were identified as movement events that had a bend amplitude greater than 3*rad* and 1*rad*, respectively. Behavioural fingerprints were created by first calculating the average value for each fish reflecting either the DF response during the specified time period (Naive = DFs 1-5, Training = DFs 6-240, Test = DFs 241-300), or the average response during the entire stimulus period (Acoustic Taps, OMR, Free Swimming). Periods where the tracking data was incomplete were excluded from the analysis. DFs where larvae did not respond were excluded from the behavioural components other than the Probability of Response. The Strictly Standardized Mean Difference was then calculated for each of these average fish values for the compound-treated larvae relative to the vehicle (DMSO) control (***Figure 3C***). The threshold for determining hit compounds was set at $|SSMD| \geq 2$. These analyses were performed using: Analyze_MultiTracker_TwoMeasures.py.

Hierarchical clustering (***Figure 3D***, ***Figure 4A-C***) was performed using SciPy (***Virtanen et al., 2020***). Correlations across different behavioural measures (***Figure 4B***) was calculated computing all pairwise comparisons for each behavioural measure in the SSMD fingerprint across the 176 hit compounds.

Further details and code for the analyses used to create the figure panels are in the following notebook: 2022_LamireEtAl_BehavFigs.ipynb. Analyses made use of open-source Python packages, including: NumPy (***Harris et al., 2020***), SciPy (***Virtanen et al., 2020***), matplotlib (***Hunter, 2007***), seaborn (***Waskom, 2021***), and open-cv (***Bradski, 2000***).

Pharmacology

Compounds were prepared as 1000x frozen stock solutions in DMSO. Stock solutions were initially diluted 1:100 in E3, yielding a 10x solution. 30uL of this solution was then pipetted into the wells, yielding a 1x compound solution in 0.1% DMSO (Sigma). Vehicle treatment followed the same protocol, using pure DMSO. Larvae were incubated in compound solution for between 30 to 90 minutes prior to behavioural testing.

The small molecule compound library (Selleckchem Bioactive: FDA-approved/FDA-like small molecules, ***Figure 1*** ***-source data 1)*** was obtained from the UPenn High-Throughput Screening Core. The library concentration was 10mM, and thus all compounds were screened at approximately 10μM. For subsequent pharmacological experiments chemicals were obtained from: Picrotoxinin: Sigma, P-8390; Melatonin: Cayman, 14427; Sigma, M5250; Ethinyl Estradiol: Cayman, 10006486; Hexestrol: Sigma, H7753

Microscopy

Imaging was performed on 5dpf larvae, mounted tail-free in 2% LMP agarose (Sigma A9414) in E3, using a 20x 1.0NA water dipping objective (Olympus). Volumetric Imaging (***Figure 1*** ,***Figure 2*** , ***Figure 6***) was performed at 930 nm on a Bruker Ultima microscope at the CIQLE imaging platform (Lyon, LYMIC), using a resonant scanner resonant scanner over a rectangular region of 1024×512 pixels (0.6μm x/y resolution) and piezo objective mount for fast z-scanning. Imaging sessions began by taking an "Anatomy Stack" consisting of 150 slices at 1µm z-steps, summed over 128 repeats (imaging time ≈11 minutes). This served as the reference stack used for alignment to the Z-Brain atlas, and to detect Z-drift in the imaging session (see below). The functional stack consisted of 12 slices separated at 10μm steps, thus covering 120μm in the brain acquired at 1.98 hz). To image *Tg(elavl3:H2B-GCaMP6s);Tg(gad1b:DsRed)* double transgenic larvae (***Figure 7***), we used a custom built 2-photon microscope (***Haesemeyer et al., 2018***), imaging 512×512 images at (0.98 μm x/y resolution) at 1.05 hz. The anatomy stack was taken at 2 μm step sizes for both the green and red channels in the dark. Functional imaging was performed only on the green/GCaMP channel since the red stimulus LED was incompatible with DsRed imaging.

When developing this protocol we determined that substantial shifts of more than a cell-body diameter (5uM) in the Z-plane are common during the ≈1.2 hrs of imaging. We determined this by comparing the sum of the functional image planes during 5 equally sized time epochs (1540 frames per epoch), aligned to the "Anatomy Stack", using "phase_cross_correlation" in the scikit-image library (***van der Walt et al., 2014***). This allowed us to quantify shifts in the imaging plane as shifts in this alignment. These tended to occur within the first hour of imaging, therefore we performed an hour of imaging of this functional stack before beginning the DF stimulation protocol to allow the preparation to settle under imaging conditions. Dark flashes were delivered using a 3mm red LED mounted above the fish, controlled by an Arduino Nano connected to the microscope GPIO board and the Prairie View software to deliver pulses of darkness consisting of 1 sec light off, 20 sec linear ramp back to light on, delivered at 60 second intervals.

Even with this pre-imaging protocol, z-shifts were still observed in a considerable number of fish. Since our habituation-based analysis is focused on how individual neurons change their responses over time, shifts in the z-plane are extremely problematic as they are not correctable post-acquisition and can result in different neurons being imaged at individual voxels. This could easily be confused for changes in functional responses over time during habituation. Therefore, any fish showing a z-drift of greater than 3μm was excluded from our analysis. Stable z-positioning was further confirmed by manual inspection of the eigan images in the imaging timecourse using "View registration metrics" in suite2 to confirm that these do not reflect z-drift. Of 56 larvae imagined total, 22 were excluded, leaving 34 included. Larvae were treated with 0.1% DMSO,

Picrotoxinin (PTX, 10uM), or Melatonin (1uM), from approximately 1hr before imaging. These fish were analyzed as a single population (***Figure 1***,***Figure 2***) and separately to determine the effects of the treatments (***Figure 6***).

To quantify responses to the dark flash stimuli we used motion artifacts in the imaging data to identify frames associated with movements (***Figure 1*-*figure Supplement 1***). Motion artifact was quantified using the "corrXY" parameter from suite2p, which reflects the peak of phase correlation comparing each acquired frame and reference image used for motion correction. The "motion power" was quantified as the standard deviation of a 3-frame rolling window, which was smoothed in time using a Savitzky-Golay filter (window length = 15 frames, polyorder = 2). A response to a dark flash was defined as a "motion power" signal greater than 3 (z-score) occurring within 10-seconds of the dark-flash onset, and was used to quantify habituation in the head-embedded preparation (***Figure 6*A**).

Ca^{2+} imaging analysis

ROIs were identified using suite2p (***Pachitariu et al., 2017***) using the parameters outlined in *RunSuite2p_BrukerData_ScreenPaper.py* and *RunSuite2p_MartinPhotonData_ScreenPaper.py* scripts for the data from the Bruker Ultima microscope (***Figure 5*-*Figure 7***), and custom built 2-photon microscope (***Figure 7*F,G**), respectively. These ROIs mostly reflected individual neuronal nuclei/soma. The clustered heatmap image of neural activity ((***Figure 3*F**) was generated using the suite2p GUI using the "Visualize selected cells" function, and sorting the neurons using the rastermap algorithm (***Pachitariu et al., 2017***), *github.com/MouseLand/rastermap*). The imaging planes were then aligned to the anatomical stack taken before functional imaging using "phase_cross_correlation" in the scikit-image library (***van der Walt et al., 2014***). For the volumetric data, the anatomical stack was then aligned to the Z-Brain atlas coordinates using CMTK, and ROI coordinates were transformed into Z-Brain coordinates using streamxform in CMTK. These steps were performed using *Bruker2p_AnalyzePlanesAndRegister.py*.

To identify ROIs that were correlated with the stimulus we use a regression-based approach (***Miri et al., 2011***), where we identified ROIs that were correlated with vectors representing the time course of the DF stimuli convolved with a kernel approximating the slowed H2B-GCaMP time course with respect to neuronal activity. These regressors reflected either the entire 21 second dark flash stimulus, or only the onset of the flash, and either the first 3, last 3, or all 60 flashes (6 regressors in total). To identify neurons correlated to motor output, we took advantage of the plane-based registration statistics calculated by suite2p. Specifically, the "ops['corrXY']" metric, which reflects the correlation of each registered image frame with the reference image. We reasoned that movements would cause image artifacts and distortions that would be reflected as a transient drop in these correlations. Indeed, we confirmed this association by imaging the tail using an infrared camera, and compared the motion index calculated through tail tracking, and that which we calculated based on the motion artifacts, which showed good overall agreement in predicted movement events and average correlation of 0.4, demonstrating that these image-based artifacts can be used as reliable proxies of tail movements (***Figure 1*-*figure Supplement 1***). Therefore, regressors based on these motion indices were used to identify neurons correlated with motor output.

Images for the functional tuning of individual neurons (***Figure 1*G-J**) were computed using the the Hue Saturation Value (HSV) colorscheme, with the maximal correlation value to either regressor mapped to saturation, and the hue value reflecting the linear preference for either regressor. Clustering of functional response types (***Figure 2***) was done by first selecting all those ROIs that showed a correlation of 0.25 or greater with any of the 6 stimulus regressors across all imaged fish. Then among these ROIs we removed any ROIs that did not show a correlation of 0.3 or greater with at least 5 ROIs imaged in a different larvae. This filtered out ROIs that were unique in

any individual fish, allowing us to focus on those neuron types that were most consistent across individuals. We then used the Affinity Propagation clustering from scikit-learn (***Pedregosa et al., 2011***), with “affinity” computed as the Pearson product-moment correlation coefficients (corrcoef in NumPy (***Harris et al., 2020***)), preference=-9, and damping=0.9, and clustered using Hierarchical clustering (cluster.hierarchy in SciPy (***Virtanen et al., 2020***)). Cluster number was assigned based on the ordering of the hierarchical clustering tree.

To generate the final cluster assignments we re-scanned all the ROIs calculating their correlation with the mean-response vectors for each of the identified 12 functional clusters, selecting those with a correlation value of 0.3 or greater, which were then assigned to the cluster with which they had the highest correlation. To determine the cluster assignments for the data from *Tg(Gad1b:DsRed);Tg(elavl3:H2B-GCaMP6s)* double transgenic larvae (***Figure 7*****F,G**) data were realigned and interpolated to match the frame rate of the clustered data, and assigned to the 12 clusters as above.

To compare the spatial relationships between the neuronal positions of different functional clusters (***Figure 2*****E**), and between the functional clusters and reference brain labels (***Figure 7*****A-E**), image volumes were cropped to the imaged coordinates (***Figure 1*****E**), downsampled to isometric 10 um^3 voxels, and linearized to calculate the Pearson’s correlation coefficient between the image sub-volumes.

Analyses made use of multiple open-source Python packages, including: suite2p (***Pachitariu et al., 2017***) NumPy (***Harris et al., 2020***), SciPy (***Virtanen et al., 2020***), scikit-learn (***Pedregosa et al., 2011***), scikit-image (***van der Walt et al., 2014***), numba (***Lam et al., 2015***), matplotlib (***Hunter, 2007***), seaborn (***Waskom, 2021***), and open-cv (***Bradski, 2000***). Details of the analyses used to create the figure panels are in the following notebook: *2022_LamireEtAl_FunctionalFigs.ipynb*

Data and Code Availability

Code for data analysis and for generating the figure panels is available here: *github.com/owenrandlett/lamire_2022* Data are available here: *doi.org/10.5061/dryad.jdfn2z3fc*

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Writing – Original Draft Preparation: OR

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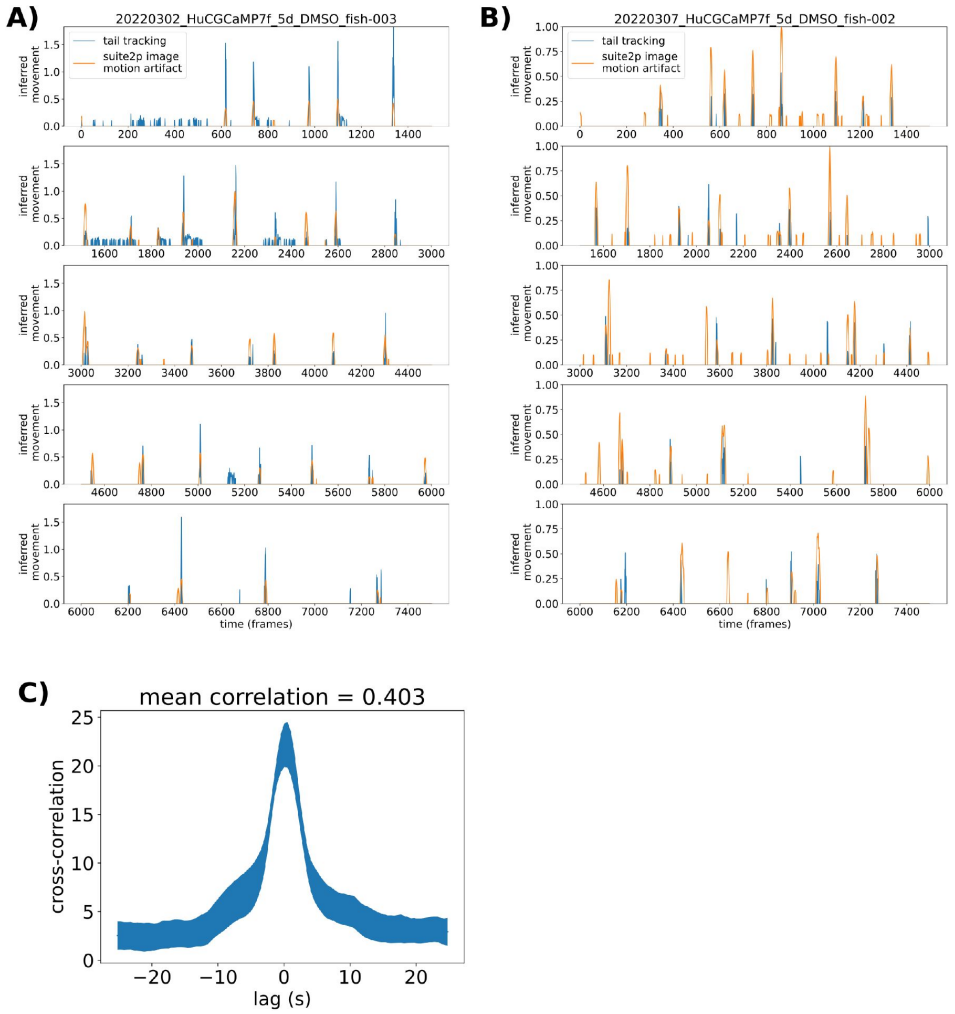

Figure 1—figure supplement 1.

Validation of motion analysis based on image artifacts during 2-photon imaging.

A) Motion indexes as calculated based on tail tracking (blue) and based on decreases in the correlation between individual frames and the reference frame used for motion alignment (orange) across the entire imaging experiment (65 minutes).

B)Same analysis as (A), for a different larva.

C)Cross-correlation plot comparing the two motion index vectors. Mean across 6 larvae, and line thickness = standard error.

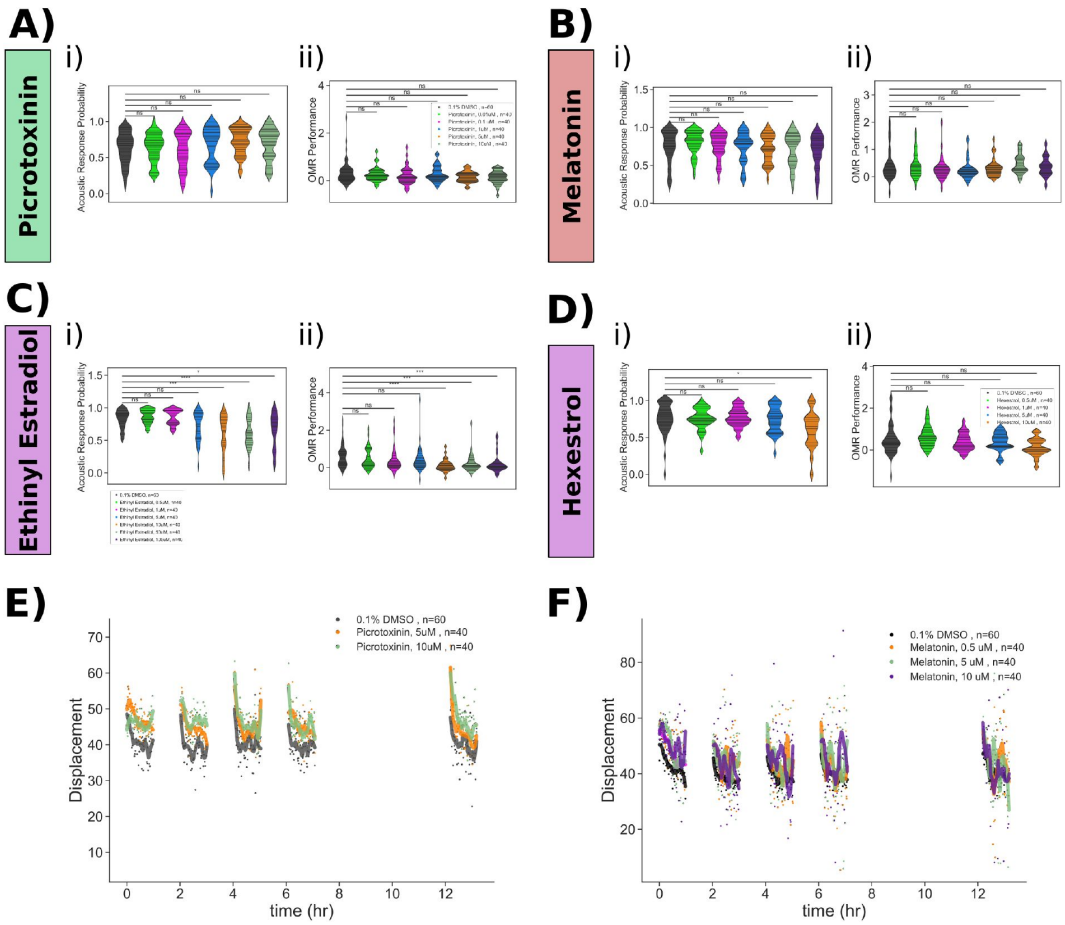

Figure 5—figure supplement 1.

Pharmacological manipulation of control behaviours and response displacement during habituation.
Dose response studies for **A)** Picrotoxinin, **B)** Melatonin, **C)** Ethinyl Estradiol and **D)** Hexestrol. Displayed for each treatment are: i) Violin plots for the dose response data, showing the probability of response to 30 acoustic tap stimuli. Horizontal lines = individual fish. ii) Violin plots for the dose response data OMR performance. Horizontal lines = individual fish. Statistical tests: Mann Whitney with bonferroni correction, ns=not significant; $p \leq$****= $1\mathscr{D}10^{-4}$; ***= $1\mathscr{D}10^{-3}$; **= $1\mathscr{D}10^{-2}$; *= 0.05.
E)Treatment with Picrotoxinin inhibits the decreases in movement displacement during habituation training.
F)Treatment with Melatonin inhibits the decreases in movement displacement during habituation training. Each dot is the mean response of the population to one flash. Lines are smoothed in time with a Savitzky-Golay filter (window = 15 stimuli, order=2).

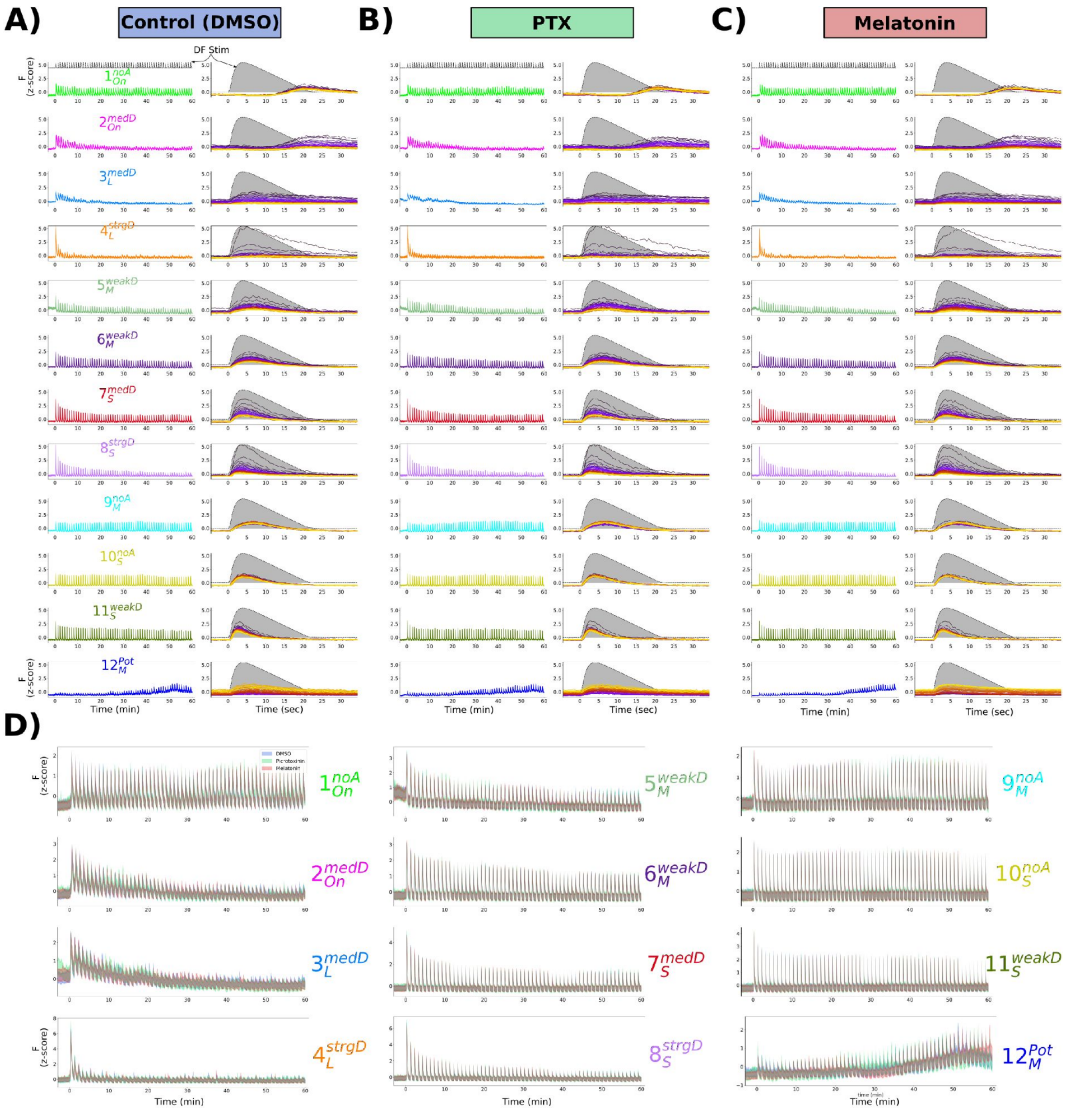

Figure 6—figure supplement 1.

Mean response of functionally identified clusters after different pharmacological treatments. **A-C)** Average z-scored fluorescence each functional cluster plotted for the whole experiment (left column), and centered on each DF stimulus (right column), demonstrating the differences in both adaptation and *Response Shape* for each cluster after treatment with **(A)** 0.1% DMSO vehicle control, **(B)** Picrotoxinin (10uM), or **(C)** Melatonin (1uM). **D)** Same data as A-C, plotted together for each treatment group.

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Reviewer #1 (Public Review):

This manuscript addresses the important and understudied issue of circuit-level mechanisms supporting habituation, particularly in pursuit of the possible role of increases in the activity of inhibitory neurons in suppressing behavioral output during long-term habituation. The authors make use of many of the striking advantages of the larval zebrafish to perform whole brain, single neuronal calcium imaging during repeated sensory exposure, and high throughput screening of pharmacological agents in freely moving, habituating larvae. Notably, several blockers/antagonists of GABAA(C) receptors completely suppress habituation of the O-bend escape response to dark flashes, suggesting a key role for GABAergic transmission in this form of habituation. Other substances are identified that strikingly enhance habituation, including melatonin, although here the suggested mechanistic insight is less specific. To add to these findings, a number of functional clusters of neurons are identified in the larval brain that have divergent activity through habituation, with many clusters exhibiting suppression of different degrees, in line with adaptive filtration during habituation, and a single cluster that potentiates during habituation. Further assessment reveals that all of these clusters include GABAergic inhibitory neurons and excitatory neurons, so we cannot take away the simple interpretation that the potentiating cluster of neurons is inhibitory and therefore exerts an influence on the other adapting (depressing) clusters to produce habituation. Rather, a variety of interpretations remain in play.

Overall, there is great potential in the approach that has been used here to gain insight into circuit-level mechanisms of habituation. There are many experiments performed by the authors that cannot be achieved currently in other vertebrate systems, so the manuscript serves as a potential methodological platform that can be used to support a rich array of future work. While there are several key observations that one can take away from this manuscript, a clear interpretation of the role of GABAergic inhibitory neurons in habituation has not been established. This potential feature of habituation is emphasized throughout, particularly in the introduction and discussion sections, meaning that one is obliged as a reader to interrogate whether the results as they currently stand really do demonstrate a role for GABAergic inhibition in habituation. Currently, the key piece of evidence that may support this conclusion is that picrotoxin, which acts to block some classes of GABA receptors, prevents habituation. However, there are interpretations of this finding that do not specifically require a role for modified GABAergic inhibition. For instance, by lowering GABAergic inhibition, an overall increase in neural activity will occur within the brain, in this case below a level that could cause a seizure. That increase in activity may simply prevent learning by massively increasing neural noise and therefore either preventing synaptic plasticity or, more likely, causing indiscriminate synaptic strengthening and weakening that occludes information storage. Sensory processing itself could also be disrupted, for instance by altering the selectivity of receptive fields. Alternatively, it could be that the increase in neural activity produced by the blockade of inhibition simply drives more behavioral output, meaning that more excitatory synaptic adaptation is required to suppress that output. The authors propose two specific working models of the ways in which GABAergic inhibition

could be implemented in habituation. An alternative model, in which GABAergic neurons are not themselves modified but act as a key intermediary between Hebbian assemblies of excitatory neurons that are modified to support memory and output neurons, is not explored. As yet, these or other models in which inhibition is not required for habituation, have not been fully tested.

This manuscript describes a really substantial body of work that provides evidence of functional clusters of neurons with divergent responses to repeated sensory input and an array of pharmacological agents that can influence the rate of a fundamentally important form of learning.

- https://doi.org/10.7554/eLife.84926.2.sa2

Reviewer #2 (Public Review):

In this study, Lamire et al. use a calcium imaging approach, behavioural tests, and pharmacological manipulations to identify the molecular mechanisms behind visual habituation. They show a valuable drug screen paradigm to assess the impact of pharmacological compounds on the behaviour of larval zebrafish.

The pharmacological screen identifies an expected suppression of habituation by GABA receptor antagonists. More interestingly, it identifies potentially new contributions of melatonin receptor agonists, and oestrogen receptor agonists to habituation, as they seem to increase the rate of habituation.

The volumetric calcium imaging of habituation to dark flashes is valuable, but the mix of responses to visual cues that are not relevant to the dark flash escape, such as the slow increase back to baseline luminosity, lowers the clarity of the results. The link between the calcium imaging results and free-swimming behaviour is not especially convincing, however, that is a common issue of head-restrained imaging with larval zebrafish. The identification of a cluster of neurons with potentiating responses, which could drive the habituation is intriguing, but more characterizations of these neurons would be needed to fully understand their function in habituation. The pharmacological manipulation of the habituation circuits mapped in the first part does not arrive at any satisfying conclusion, which is acknowledged by the authors.

Overall, the authors did identify interesting new molecular pathways that may be involved in habituation to dark flashes. Their screening approach, while not novel, will be a powerful way to interrogate other behavioural profiles. The authors identified circuit loci apparently involved in habituation to dark flashes, and the potentiation and no adaptation clusters have not been previously observed and are interesting targets for future work. This work suggests that the circuits and mechanisms underlying habituation are likely more complex than anticipated. The data will be useful to guide follow-up experiments by the community on the new pathway candidates that this screen has uncovered, including behaviours beyond dark flash habituation.

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Reviewer #3 (Public Review):

To analyze the circuit mechanisms leading to the habituation of the O-bed responses upon repeated dark flashes (DFs), the authors performed 2-photon Ca2+ imaging in larvae expressing nuclear-targeted GCaMP7f pan-neuronally panning the majority of the midbrain, hindbrain, pretectum, and thalamus. They found that while the majority of neurons across the brain depress their responsiveness during habituation, a smaller population of neurons

in the dorsal regions of the brain, including the torus longitudinalis, cerebellum, and dorsal hindbrain, showed the opposite pattern, suggesting that motor-related brain regions contain non-depressed signals, and therefore likely contribute to habituation plasticity.

Further analysis using affinity propagation clustering identified 12 clusters that differed both in their adaptation to repeated DFs, as well as the shape of their response to the DF.

Next by the pharmacological screening of 1953 small molecule compounds with known targets in conjunction with the high-throughput assay, they found that 176 compounds significantly altered some aspects of measured behavior. Among them, they sought to identify the compounds that 1) have minimal effects on the naive response to DFs, but strong effects during the training and/or memory retention periods, 2) have minimal effects on other aspects of behaviors, 3) show similar behavioral effects to other compounds tested in the same molecular pathway, and identified the GABAA/C Receptor antagonists Bicuculline, Amoxapine, and Picrotoxinin (PTX). As partial antagonism of GABAAR and/or GABACR is sufficient to strongly suppress habituation but not generalized behavioral excitability, they concluded that GABA plays a very prominent role in habituation. They also identified multiple agonists of both Melatonin and Estrogen receptors, indicating that hormonal signalling may also play a prominent role in habituation response.

To integrate the results of the Ca2+ imaging experiments with the pharmacological screening results, the authors compared the Ca2+ activity patterns after treatment with vehicle, PTX, or Melatonin in the tethered larvae. The behavioral effects of PTX and Melatonin were much smaller compared with the very strong behavioral effects in freely-swimming animals, but the authors assumed that the difference was significant enough to continue further experiments. Based on the hypothesis that Melatonin and GABA cooperate during habituation, they expected PTX and Melatonin to have opposite effects. This was not the case in their results: for example, the size of the 12(Pot, M) neuron population was increased by both PTX and Melatonin, suggesting that pharmacological manipulations that affect habituation behavior manifest in complex functional alterations in the circuit, making capturing these effects by a simple difficult.

Since the 12(*Pot*, *M*) neurons potentiate their responses and thus could act to progressively depress the responses of other neuronal classes, they examined the identity of these neurons with GABA neurons. However, GABAergic neurons in the habituating circuit are not characterized by their Adaptation Profile, suggesting that global manipulations of GABAergic signalling through PTX have complex manifestations in the functional properties of neurons.

Overall, the authors have performed an admirably large amount of work both in whole-brain neural activity imaging and pharmacological screening.

- https://doi.org/10.7554/eLife.84926.2.sa0

Author Response

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *This valuable manuscript attempts to identify the brain regions and cell types involved in habituation to dark flash stimuli in larval zebrafish. Habituation being a form of learning widespread in the animal kingdom, the investigation of neural mechanisms underlying it is an important endeavor. The authors use a combination of behavioral analysis, neural activity imaging, and pharmacological manipulation to investigate brain-wide mechanisms of habituation. However, the data presented are incomplete and do not*

> *show a convincing causative link between pharmacological manipulations, neural activity patterns, and behavioral outcomes.*

We thank the reviewers and editors for their careful reading and reviews of our work. We are grateful that they appreciate the value in our experimental approach and results. We acknowledge what we interpret as the major criticism, that in our original manuscript we focused too heavily on the hypothesized role of GABAergic neurons in driving habituation. This hypothesis will remain only indirectly supported until we can identify a GABAergic population of neurons that drives habituation. Therefore, we have revised our manuscript, decreasing the focus on GABA, and rather emphasizing the following three points:

1. By performing the first Ca2+ imaging experiments during dark flash habituation, we identify multiple distinct functional classes of neurons which have different adaptation profiles, including non-adapting and potentiating classes. These neurons are spread throughout the brain, indicating that habituation is a complex and distributed process.

2. By performing a pharmacological screen for dark flash habituation modifiers, we confirm habituation behaviour manifests from multiple distinct molecular mechanisms that independently modulate different behavioural outputs. We also implicate multiple novel pathways in habituation plasticity, some of which we have validated through dose-response studies.

3. By combining pharmacology and Ca2+ imaging, we did not observe a simple relationship between the behavioural effects of a drug treatment and functional alterations in neurons. This observation further supports our model that habituation is a multidimensional process, for which a simple circuit model will be insufficient.

We would like to point out that, in our opinion, there appears to be a factual error in the final sentence of the eLife assessment:

“However, the data presented are incomplete and do not show a convincing causative link between pharmacological manipulations, neural activity patterns, and behavioral outcomes”.

We believe that a “convincing causative link” between pharmacological manipulations and behavioural outcomes has been clearly demonstrated for PTX, Melatonin, Estradiol and Hexestrol through our dose response experiments. Similarly a link between pharmacology and neural activity patterns has also been directly demonstrated. As mentioned in (3), we acknowledge that our data linking neural activity and behaviour is more tenuous, as will be more explicitly reflected in our revised manuscript.

Nevertheless, we maintain that one of the primary strengths of our study is our attempt to integrate analyses that span the behavioural, pharmacological, and neural activity-levels.

In our revised manuscript, we have substantially altered the Abstract and Discussion, removed the Model figure (previously Figure 8), and changed the title from :

“Inhibition drives habituation of a larval zebrafish visual response”

to:

“Functional and pharmacological analyses of visual habituation learning in larval zebrafish”

Text changes from the initial version are visible as track changes in the word document: “LamireEtAl_2022_eLifeRevisions.docx”

Reviewer #1 (Public Review):

This manuscript addresses the important and understudied issue of circuit-level mechanisms supporting habituation, particularly in pursuit of the possible role of increases in the activity of inhibitory neurons in suppressing behavioral output during long-term habituation. The authors make use of many of the striking advantages of the larval zebrafish to perform whole brain, single neuronal calcium imaging during repeated sensory exposure, and high throughput screening of pharmacological agents in freely moving, habituating larvae. Notably, several blockers/antagonists of GABAA(C) receptors completely suppress habituation of the O-bend escape response to dark flashes, suggesting a key role for GABAergic transmission in this form of habituation. Other substances are identified that strikingly enhance habituation, including melatonin, although here the suggested mechanistic insight is less specific. To add to these findings, a number of functional clusters of neurons are identified in the larval brain that has divergent activity through habituation, with many clusters exhibiting suppression of different degrees, in line with adaptive filtration during habituation, and a single cluster that potentiates during habituation. Further assessment reveals that all of these clusters include GABAergic inhibitory neurons and excitatory neurons, so we cannot take away the simple interpretation that the potentiating cluster of neurons is inhibitory and therefore exerts an influence on the other adapting (depressing) clusters to produce habituation. Rather, a variety of interpretations remain in play.

Overall, there is great potential in the approach that has been used here to gain insight into circuit-level mechanisms of habituation. There are many experiments performed by the authors that cannot be achieved currently in other vertebrate systems, so the manuscript serves as a potential methodological platform that can be used to support a rich array of future work. While there are several key observations that one can take away from this manuscript, a clear interpretation of the role of GABAergic inhibitory neurons in habituation has not been established. This potential feature of habituation is emphasized throughout, particularly in the introduction and discussion sections, meaning that one is obliged as a reader to interrogate whether the results as they currently stand really do demonstrate a role for GABAergic inhibition in habituation. Currently, the key piece of evidence that may support this conclusion is that picrotoxin, which acts to block some classes of GABA receptors, prevents habituation. However, there are interpretations of this finding that do not specifically require a role for modified GABAergic inhibition. For instance, by lowering GABAergic inhibition, an overall increase in neural activity will occur within the brain, in this case below a level that could cause a seizure. That increase in activity may simply prevent learning by massively increasing neural noise and therefore either preventing synaptic plasticity or, more likely, causing indiscriminate synaptic strengthening and weakening that occludes information storage. Sensory processing itself could also be disrupted, for instance by altering the selectivity of receptive fields. Alternatively, it could be that the increase in neural activity produced by the blockade of inhibition simply drives more behavioral output, meaning that more excitatory synaptic adaptation is required to suppress that output. The authors propose two specific working models of the ways in which GABAergic inhibition could be implemented in habituation. An alternative model, in which GABAergic neurons are not themselves modified but act as a key intermediary between Hebbian assemblies of excitatory neurons that are modified to support memory and output neurons, is not explored. As yet, these or other models in which inhibition is not required for habituation, have not been fully tested.

This manuscript describes a really substantial body of work that provides evidence of functional clusters of neurons with divergent responses to repeated sensory input and an

> *array of pharmacological agents that can influence the rate of a fundamentally important form of learning.*

We thank the reviewer for their careful consideration of our work, and we agree that multiple models of how habituation occurs remain plausible. As discussed above and below in more detail, we have revised our manuscript to better reflect this. We hope the reviewer will agree that this has improved the manuscript.

> ***Reviewer #2 (Public Review):***
>
> *In this study, Lamire et al. use a calcium imaging approach, behavioural tests, and pharmacological manipulations to identify the molecular mechanisms behind visual habituation. Overall, the manuscript is well-written but difficult to follow at times. They show a valuable new drug screen paradigm to assess the impact of pharmacological compounds on the behaviour of larval zebrafish, the results are convincing, but the description of the work is sometimes confusing and lacking details.*

We thank the reviewer for identifying areas where our description lacked details. We apologize for these omissions and have attempted to add relevant details as described below. We note that all of the analysis code is available online, though we appreciate that navigating and extracting data from these files is not straightforward.

> *The volumetric calcium imaging of habituation to dark flashes is valuable, but the mix of responses to visual cues that are not relevant to the dark flash escape, such as the slow increase back to baseline luminosity, lowers the clarity of the results. The link between the calcium imaging results and free-swimming behaviour is not especially convincing, however, that is a common issue of head-restrained imaging with larval zebrafish.*

We agree with the reviewer that the design of our stimulus, and specifically the slow increase back to baseline luminosity, is perhaps confusing for the interpretation of some of the response profiles of neurons. We originally chose this stimulus type (rather than a square wave of 1s of darkness, for example) in order to better highlight the responses of the larvae to the onset of darkness (rather than the response to abruptly returning to full brightness). We therefore believe that the slow return to baseline is an important feature of the stimulus,, which better separates activity related to the fast offset from activity related to light onset. And since all of the foundational behavioural data (Randlett et al., Current Biology 2019), and pharmacological data, used this stimulus type, we did not change it for the Ca2+ imaging experiments. Our use of relatively slow nuclear-targeted GCaMP indicators also means that the temporal resolution of our imaging experiments is relatively poor, and therefore we felt that using a stimulus that highlighted light offset might be best.

We also fully acknowledge in the Results section that the behaviour of the head embedded fish is not the same as that of free-swimming fish, and that therefore establishing a direct link between these types of experiments is complicated. This is an unavoidable caveat in the head-embedded style experiments. To further emphasize this, we have also added a paragraph to the discussion where this is acknowledged explicitly.

“We also found that the same pharmacological treatments that result in strong alterations to habituation behaviour in freely swimming larvae ([fig:5]), resulted in relatively subtle and complex functional alterations in the circuit ([fig:6]). Making direct comparisons between freely-swimming behaviour and head-fixed Ca2+ imaging is always challenging due to the differences in behaviour observed in the two contexts, and therefore our failure to identify a clear logic in these experiments may have technical explanations that will require approaches to measure neural activity from unrestrained and freely-behaving animals to resolve . Alternatively, these results are again consistent with the idea that habituation is a

multidimensional and perhaps highly non-linear phenomenon in the circuit, which cannot be captured by a simple model."

> *The strong focus on GABA seems unwarranted based on the pharmacological results, as only Picrotoxinin gives clear results, but the other antagonists do not give a consistent results. On the other hand, the melatonin receptor agonists, and oestrogen receptor agonists give more consistent results, including more convincing dose effects.*

We agree that our manuscript focused too strongly on GABA and have toned this down. We are currently performing genetic experiments aimed at identifying the Melatonin, Estrogen and GABA receptors that function during habituation, which we think will be necessary to move beyond pharmacology and the necessary caveats that such experiments bring.

> *The pharmacological manipulation of the habituation circuits mapped in the first part does not arrive at any satisfying conclusion, which is acknowledged by the authors. These results do reinforce the disconnect between the calcium imaging and the behavioural experiments and undercut somewhat the proposed circuit-level model.*

We agree with this criticism and have toned down the focus on GABA specifically in the circuit, and have removed the speculative model previously in Figure 8.

> *Overall, the authors did identify interesting new molecular pathways that may be involved in habituation to dark flashes. Their screening approach, while not novel, will be a powerful way to interrogate other behavioural profiles. The authors identified circuit loci apparently involved in habituation to dark flashes, and the potentiation and no adaptation clusters have not been previously observed as far as I know.*
>
> *The data will be useful to guide follow-up experiments by the community on the new pathway candidates that this screen has uncovered, including behaviours beyond dark flash habituation.*

We again thank the reviewer for both their support of our approach, and in pointing out where our conclusions were not well supported by our data.

> ***Reviewer #3 (Public Review):***
>
> *To analyze the circuit mechanisms leading to the habituation of the O-bed responses upon repeated dark flashes (DFs), the authors performed 2-photon Ca2+ imaging in larvae expressing nuclear-targeted GCaMP7f pan-neuronally panning the majority of the midbrain, hindbrain, pretectum, and thalamus. They found that while the majority of neurons across the brain depress their responsiveness during habituation, a smaller population of neurons in the dorsal regions of the brain, including the torus longitudinalis, cerebellum, and dorsal hindbrain, showed the opposite pattern, suggesting that motor-related brain regions contain non-depressed signals, and therefore likely contribute to habituation plasticity.*
>
> *Further analysis using affinity propagation clustering identified 12 clusters that differed both in their adaptation to repeated DFs, as well as the shape of their response to the DF.*
>
> *Next by the pharmacological screening of 1953 small molecule compounds with known targets in conjunction with the high-throughput assay, they found that 176 compounds significantly altered some aspects of measured behavior. Among them, they sought to identify the compounds that 1) have minimal effects on the naive response to DFs, but strong effects during the training and/or memory retention periods, 2) have minimal effects on other aspects of behaviors, 3) show similar behavioral effects to other*

compounds tested in the same molecular pathway, and identified the GABAA/C Receptor antagonists Bicuculline, Amoxapine, and Picrotoxinin (PTX). As partial antagonism of GABAAR and/or GABACR is sufficient to strongly suppress habituation but not generalized behavioral excitability, they concluded that GABA plays a very prominent role in habituation. They also identified multiple agonists of both Melatonin and Estrogen receptors, indicating that hormonal signaling may also play a prominent role in habituation response.

To integrate the results of the Ca2+ imaging experiments with the pharmacological screening results, the authors compared the Ca2+ activity patterns after treatment with vehicle, PTX, or Melatonin in the tethered larvae. The behavioral effects of PTX and Melatonin were much smaller compared with the very strong behavioral effects in freely-swimming animals, but the authors assumed that the difference was significant enough to continue further experiments. Based on the hypothesis that Melatonin and GABA cooperate during habituation, they expected PTX and Melatonin to have opposite effects. This was not the case in their results: for example, the size of the 12(Pot, M) neuron population was increased by both PTX and Melatonin, suggesting that pharmacological manipulations that affect habituation behavior manifest in complex functional alterations in the circuit, making capturing these effects by a simple difficult.

Since the 12(Pot, M) neurons potentiate their responses and thus could act to progressively depress the responses of other neuronal classes, they examined the identity of these neurons with GABA neurons. However, GABAergic neurons in the habituating circuit are not characterized by their Adaptation Profile, suggesting that global manipulations of GABAergic signaling through PTX have complex manifestations in the functional properties of neurons.

Overall, the authors have performed an admirably large amount of work both in whole-brain neural activity imaging and pharmacological screening. However, they are not successful in integrating the results of both experiments into an acceptably consistent interpretation due to the incongruency of the results of different experiments. Although the authors present some models for interpretation, it is not easy for me to believe that this model would help the readers of this journal to deepen the understanding of the mechanisms for habituation in DF responses at the neural circuit level.

This reviewer would rather recommend the authors divide this manuscript into two and publish two papers by adding some more strengthening data for each part such as cellular manipulations, e.g. ablation to prove the critical involvement of 12(Pot, M) neurons in habituation.

We thank the reviewer for their careful consideration of our manuscript, and we agree that our emphasis on a particular model of DF habituation, namely the potentiation of GABAergic synapses, was overly speculative. We hope they will agree that our revised manuscript better reflect the results from our experiments, and we have tried to more specifically emphasize the incongruency in our behavioural and Ca2+ imaging data after pharmacological treatment, which we agree shows that a simple model is insufficient to capture both of these sets of observations.

We have opted not to split the paper into two, since we feel that the collective message of this paper and approach combining molecular and functional analysis will be of interest. Moreover, we feel that the molecular and functional analyses feed off of each other and provide a level of complementarity that would be lost if the manuscript would be split, even if the message in this particular case is rather complex

Reviewer #1 (Recommendations For The Authors):

There is much to commend about this manuscript. The advantages of studying habituation in the zebrafish larva are very clearly demonstrated, including the wonderful calcium imaging across the brain and the relatively high throughput screening of large numbers of different pharmacological agents. The habituation to dark flashes in freely moving larvae is also striking and the very large effect size serves the screening beautifully. Thus, if we take the really substantial amount of work of a very high standard that has been done here, there is clearly potential for an important new contribution to the literature. However, as you will see from my public review, I am of the opinion that a specific role for the modification of GABAergic inhibitory systems has not yet been established through this work. While the potential role for GABAergic inhibitory neurons in habituation, either as the key modifiable element or as an intermediary between memory and motor output, is an attractive theory with many strengths, your study as it currently stands does not categorically demonstrate that one of those two options holds. For instance, the more traditional view, that adaptive filtration is mediated by weakened synaptic connectivity between excitatory sensory systems and excitatory motor output or reduced intrinsic excitability in those same neurons, could still be in operation here. By lowering GABAergic influence over post-synaptic targets with picrotoxin, it is possible that motor output remains highly active, and even lower activity or synaptic drive from those excitatory sensory systems that feed into the output may still reliably produce behavioral output. Alternatively, it could be the formation of a memory of the familiar stimulus is disrupted by reduced inhibition that alters sensory coding either by introducing noise or reducing the selectivity of receptive fields. I believe that there are several options to address these concerns:

1. *You could change the emphasis of the manuscript so that it is less focused on inhibition and instead emphasizes the categorization of clusters of neurons that have divergent responses during habituation, including either strong suppression to potentiation. To this, you add a high throughput screening system with a wide range of different agents being tested, several of which produce a significant effect on habituation in either direction. These observations in themselves provide powerful building blocks for future work.*

1. *If GABAergic neurons play a key role in habituation in this paradigm, then picrotoxin is having its effect by blocking receptors on excitatory neurons. Thus, it seems that selectively imaging GABAergic neurons before and after the application of these drugs is not likely to reveal the contribution of GABAergic synaptic influence on excitatory targets. More important is to get a stronger sense of how the GABAergic neurons change their activity throughout habituation and then influence the downstream target neurons of those GABAergic neurons (some of which may themselves be inhibitory and participating in disinhibition). For instance, you could interrogate whether anti-correlations in activity levels exist between presynaptic inhibitory neurons and putative post-synaptic targets. This analysis could be further bolstered by removing that relationship in the presence of Picrotoxin, thereby demonstrating a direct influence of inhibition from a GABAergic presynaptic partner on a postsynaptic target. While this would constitute a lot more work, it is likely to yield greater insight into a specific role for GABAergic neurons in habituation, and I suspect much of that information is in the existing datasets.*

> 1. *To really reveal causal roles for inhibition in this form of habituation, it seems to me that there needs to be some selective intervention in GABAergic neuronal activity, ideally bidirectionally, to transiently interrupt or enhance habituation. Optogenetic or chemogenetic stimulation/inactivation is one option in this regard, which I imagine would be challenging to implement and certainly involves a lot of further work, particularly if you are then going to target specific subpopulations of GABAergic neurons. I appreciate that this option seems way beyond the scope of a review process and would probably constitute a follow-up study.*

We agree with the reviewer that we have not "categorically demonstrated" that GABAergic inhibitory neurons drive habituation by increasing their influence on the circuit, and appreciate the suggestions for how to reformulate our manuscript to better reflect this. We have opted to follow suggestion (1), and have considerably changed the focus of the manuscript.

The additional analysis suggested in (2) is very interesting, but since we can not identify which cells are inhibitory in our imaging experiments with picrotoxinin treatment, nor which are pre- or post-synaptic, we feel that this analysis will be very unconstrained. Also, if GABA is acting as an inhibitory neurotransmitter, it therefore is expected to act to drive anticorrelations among pre and postsynaptic neurons through inhibition. Therefore, blockage of GABA through PTX would be expected to result in increased correlations, regardless of our hypothesized role of neurons during habituation. Our current efforts are aimed at identifying critical neurons driving habituation plasticity, and we will perform such analysis once we have mechanisms for identifying these neurons.

Finally, we agree that (3) is the obvious and only way to demonstrate causation here, and this is where we are working towards. However, since we currently have no means of genetically targeting these neurons, we are not able to perform these suggested experiments today.

> *I have some additional concerns that I would really appreciate you addressing:*
>
> 1. *The behavioral habituation is striking in the freely moving larvae, but very hard to monitor in the larvae that are immobilized for calcium imaging. Are there steps that could be taken in the long run to improve direct observation of the habituation effect in these semi-stationary fish? For instance, is it possible to observe eye movements or some more subtle behavioral readout than the O-bend reflex? I apologize if this is a naïve question, but I am not entirely familiar with this specific experimental paradigm.*

In the Dark Flash paradigm, we do not have readouts beyond the "O-bend" response itself, which is characterized by a large-angle bend of the tail and turning maneuver. We have not observed other, more subtle behavioural responses, such as eye or fin movements, for example. If we would be able to identify alternative behavioural outputs that were more robustly performed during head-embedded preparations, this would indeed be an advantage allowing us to more directly interpret the Ca2+ imaging results with respect to behaviour.

> 1. *The dark flash as a stimulus to which the larvae habituate is obviously used as a powerful and ethologically relevant stimulus. However, it does leave an element of traditional habituation paradigms out, which is a novel stimulus that can be used to immediately re-instate the habituated response (otherwise known as dishabituation). Is there a way that you can imagine implementing that with zebrafish larvae, for instance through systematically altering a visual feature, such as spatial frequency or orientation? This would be a powerful development in my view as it would not only allow you to rule out motor or sensory fatigue as an underlying cause of reduced behavior but also it would provide an extra feature that strengthens your assessment of neuronal response profiles in candidate populations of inhibitory and excitatory neurons.*

We agree that identifying a dishabituating stimulus would be very powerful for our experiments. For short-term habituation of the acoustic startle response, Wolman et al demonstrated that dishabituation occurs after a touch stimulus (Wolman et al., PNAS, 2011; https://doi.org/10.1073/pnas.1107156108). We attempted to dishabituate the O-Bend response with tap and touch stimuli, and this unfortunately did not occur. Our understanding of dishabituation is that this generally requires a second stimulus that elicits the same behaviour as the habituated stimulus (e.g. both acoustic and touch-stimuli elicit the Mauthner-dependent C-bend response). In zebrafish the only stimulus that has been identified that elicits the O-bend is a dark-flash. This lack of an appropriate alternative stimulus is perhaps why we have been unsuccessful in identifying a dishabituating stimulus.

> 1. *You have written about the concept of 'short' and 'long' response shapes when using calcium imaging as a proxy for neural activity, surmising that the short response shape may reflect transient bursting. Although calcium imaging obviously has many advantages, this feature reveals one notable limitation of calcium imaging in contrast to electrophysiology, in that the time course of the signal is considerably longer and does not allow you with confidence to fully detect the response profile of neurons. Is there some kind of further deconvolution process that you could implement to improve the fidelity of your calcium imaging to the occurrence of action potentials? The burstiness of neurons is obviously important as it can indicate a particular type of neuron (for instance fast-spiking inhibitory neurons) or it might reveal a changing influence on post-synaptic neurons. For instance, bursting can be a response to inhibition due to the triggering of T-type calcium channels in response to hyperpolarization.*

One of the major limitations to Ca2+ imaging is the lack of temporal resolution. In our particular approach, using nuclear-targeted H2B-GCaMP indicators, further reduces our temporal resolution. Deconvolution approaches can be used in some instances to approximate spike rate, since the rise-time of Ca2+ indicators can be relatively fast. However, in our imaging we chose to image larger volumes at the expense of scan rate, where our imaging is performed at only 2hz. Therefore, deconvolution and spike-rate estimation is not appropriate. Considering these limitations, we would argue that the fact that we can observe differences in kinetics of the 'short' and 'long' response shapes indicates that they likely show very different response kinetics, which we hope to confirm by electrophysiology once we have established ways of targeting these neurons for recordings.

> *1. I note that among the many substances you screened with is MK801. An obvious candidate mechanism in habituation is the NMDA receptor, given the importance of this receptor for so many forms of learning and bidirectional synaptic plasticity. If I am to understand correctly, this NMDA receptor blocker actually enhances habituation in the zebrafish larvae, similar to melatonin. That is a very surprising observation, which is worth looking into further or at least discussed in the manuscript. The finding would, at least, be consistent with the idea that plasticity is not occurring at excitatory synapses and could potentially bolster the argument that plasticity of inhibitory synapses is at play in this particular form of habituation.*

This is a very important point. We were also particularly interested in MK801, which has been shown to inhibit other forms of habituation, like short-term acoustic habituation (Wolman et al., PNAS, 2011; https://doi.org/10.1073/pnas.1107156108). In our experiments we did see that fish become even less responsive to dark flashes when treated with MK-801 (SSMD fingerprint data: Prob-Train = -0.39, Prob-Test = -1.58) which would indicate that MK-801 promotes dark flash habituation, similar to Melatonin. However, we also observed that MK-801 caused a decrease in the performance in the other visual assay we tested: the optomotor response (OMR-Perf = -0.93), indicating that MK-801 causes a generalized decrease in visual responses, perhaps by acting on circuits within the retina. Therefore, based on these experiments with global drug applications, we cannot determine if MK-801 influences the plasticity process in dark-flash habituation, and this is why we did not pursue it further in this project.

> *Anyway, I hope that you take these suggestions as constructive and, in the spirit that they are intended, as possible routes for improving an already very interesting manuscript.*

We are very grateful for your suggestions, which we feel has helped us to improve our manuscript substantially.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Overall, the manuscript is well-written, but confusing at times. The results are not always presented in a consistent way, and I found myself having to dig in the raw data or code to find answers. There is a certain disconnect between the free-swimming results, and the calcium imaging, which is somewhat inevitable based on other published work. But I am unsure of what they each bring to the other, as the results from Fig.6 do not match at all the changes observed in the behavioural assays, it almost feels like two separate studies and the inconsistencies make the model appear unlikely.*

We agree that there is a disconnect at the behavioural level in our free-swimming and head-embedded imaging experiments. However, this does not necessarily mean that the activity we observe during the imaging experiments cannot be informative about processes that are also occurring in freely-swimming fish. For example, it is possible that the dark-flash circuit is responding and habitating similarly in the head-embedded and freely-swimming preparations, but that in the latter context there is an additional blockade on motor output that massively decreases the propensity of the fish to initiate any movements. In such a case, the “disconnect between the free-swimming results, and the calcium imaging” would indicate that the relationship between neural activity and habituation behaviour is rather complex.

Without a method to record activity from freely swimming fish at our disposal, we can not determine this, one way or the other.

We hope that we now acknowledge these concerns appropriately in the discussion:

“We also found that the same pharmacological treatments that result in strong alterations to habituation behaviour in freely swimming larvae ([fig:5]), resulted in relatively subtle and complex functional alterations in the circuit ([fig:6]). Making direct comparisons between freely-swimming behaviour and head-fixed Ca2+ imaging is always challenging due to the differences in behaviour observed in the two contexts, and therefore our failure to identify a clear logic in these experiments may have technical explanations that will require approaches to measure neural activity from unrestrained and freely-behaving animals to resolve . Alternatively, these results are again consistent with the idea that habituation is a multidimensional and perhaps highly non-linear phenomenon in the circuit, which cannot be captured by a simple model. “

> *I am not convinced by the results surrounding GABA, from the inconsistent GABA receptor antagonist profile to the post hoc identification of GABAergic neurons as it is currently done in the manuscript. I think that the current focus on GABA does a disservice to the manuscript. However, the novel findings surrounding the potential role of Melatonin, and Estrogen, in habituation are quite interesting.*

We agree that we focused too heavily on our hypothesized role for GABA in our original manuscript, and we hope that the reviewer agrees that our updated manuscript is an improvement. We also thank the reviewer for their interest in our Melatonin and Estrogen results, for which follow up studies are ongoing to characterize the effects of these hormones and their receptors on habituation.

> *There is an assumption that all the adaptation profiles are related to the DF (although that is somewhat alleviated in the discussions of the ON responses) and not to the luminosity changes. But there is no easy way to deconvolve those two in the current experiments. I would like the timing of the fluorescence rise to be quantified compared to the dark flash stimulus onset, potentially spike inference methods could help with giving a better idea of the timing of those responses. Based on the behavioural responses that were <500ms in Randlet O et al, eLife, 2019; we would expect only the fastest DF responses to be linked to the behaviour.*

We agree that we are unable to disambiguate responses to the dark flash that initiate the O-bend response, and those that are related to only changes in luminosity. As discussed above, our Ca2+ imaging approach is severely limited in temporal resolution and therefore spike inference methods are not appropriate.

> ***Major comments***
>
> *Fig.1: There seems to be a very variable lag between the motor events and DF responses, furthermore, it does not seem that the motor responses follow a similar habituation rate as in 1Bi. Although this only shows the smoothed 'movement cluster' from the rastermap, it could hide individual variability. It would be important to know what the 'escape' rate was in the embedded experiment, as*
>
> *Fig.1 sup.1 seems to indicate there was little to no habituation. It would also be needed to know which motor events are considered linked to the DF stimulus, and how that was decided. Was there a movement intensity threshold and lag limit in the response?*

We interpret this concern as relating to the data presented in Figure 6A, where we quantify the habituation rate in the head-embedded experiments. As we have discussed, both above and in the manuscript, we saw very strongly muted responses to DFs in the head-embedded preparation, but we neglected to describe our method of quantifying the responses. We have added the following description to the methods:

"To quantify responses to the dark flash stimuli we used motion artifacts in the imaging data to identify frames associated with movements ([fig:1]-[fig:S1]). Motion artifact was quantified using the "corrXY" parameter from suite2p, which reflects the peak of phase correlation comparing each acquired frame and reference image used for motion correction. The "motion power" was quantified as the standard deviation of a 3-frame rolling window, which was smoothed in time using a Savitzky-Golay filter (window length = 15 frames, polyorder = 2). A response to a dark flash was defined as a "motion power" signal greater than 3 (z-score) occurring within 10-seconds of the dark-flash onset, and was used to quantify habituation in the head-embedded preparation ([fig:6]A)."

> *Line 94: This seems to be a strong claim based on the sparse presence of non-habituating, or potentiating, neurons in downstream regions. However, these neurons appear to be extremely rare, and as mentioned in my comment above, the behavioural habituation appears minimal. These neurons could encode the luminosity and be part of other responses, such as light-seeking in Karpenko S et al, eLife, 2020 or escape directionality in Heap et al, Neuron, 2018. Furthermore, dimming information has been shown to have parallel processing pathways in Robles E et al, JCN, 2020; so it would make sense that not all the observed responses in this manuscript would be involved in behavioural habituation to dark flashes.*

We agree that without functional interventions, we do not know which of the neurons we have categorized are specifically involved in the dark flash response habituation. It is possible that the non-adapting and potentiating neurons are involved in other behaviours. We have therefore removed this statement.

> *Line 103: It appears that several of those responses are to the changes in luminosity and not the DF itself, especially the ON and sustained responses. Based on the previous DF habituation study from Randlet O et al, eLife, 2019; the latency of the response is below 0.5s. So the behaviour-relevant responses must only include the shortest latency one, as discussed above.*

We appreciate the point that the reviewer is making here, but we are less clear about what the difference between "changes in luminosity" and a "dark flash" response are, since a dark flash consists of a change in luminosity. We take it that the reviewer means the difference between a luminance stimulus that elicits an O-bend, from one that does not. In order to disambiguate the two, one would likely need to use stimuli where the luminosity changes, but do not elicit O-bends.

Perhaps due to the limited temporal resolution of our Ca2+ imaging data, we do not see a clear difference in the onset of the stimulus response for any of the functional clusters that would help us to determine which neurons are more relevant to the acute DF response.

> *Fig.2B. It is very difficult to make out the actual average z-scored fluorescence, a supplementary figure would help by making these bigger. A plot to quantify the maximum response would also be useful to judge how it changes between the first few and few last DF. Another plot to give the time between the onset of the responses and the onset of the DF stimulus is also needed to judge which cluster may be relevant to the DF escapes observed in the free-swimming experiments.*

We agree with the reviewer that interpreting these datasets are challenging. We did include the actual average z-scored fluorescence in Figure 6—figure supplement 1, panel D. This figure also includes a comparison between the predicted Ca2+ response to the dark flash (the stimulus convolved with the approximate GCaMP response kernel), which shows that all OFF-responding neuronal classes show very similar rise time response kinetics, and thus this

analysis does not help to judge whether a cluster is more or less relevant to O-bend responses in the free-swimming experiments. We appreciate that there are differences in opinion about the best way to present the data, but we have opted to leave our original presentation.

> *Line 130: Is a correlation below 0.1 meaningful or significant? It does not seem like this cluster would be a motor or decision cluster.*

Our goal with this correlational analysis to motor signals was to identify if certain clusters of DF responsive neurons were more associated with motor output, and therefore may be more downstream in the sensori-motor cascade. Cluster 4 showed the highest median correlation across the population of cells. Whether a median correlation of ~0.1 is “meaningful” is impossible for us to answer, but it is highly “significant” in the statistical sense, as is evident by the 99.99999% confidence intervals plotted. We note that these cells were not selected based on their correlation to the motor stimulus, but only to the dark flash stimulus. There are “motor” clusters that show much higher correlations to the motors signals, as is evident in Figure 1G.

> *Line 165: Did the changes observed for Pimozide fall below the significance threshold, were lethal, or were the results not repeated? It does not appear in source data 2.*

Pimozide was lethal in our screen and therefore does not appear in the source data file. Indeed, in our previous experiments with Pimozide we had already established that a 10uM dose is lethal, and that the maximal effective dose we tried was 1uM as reported in (Randlett et al., Current Biology, 2019).

We have clarified this in the text:

“While the false negative rate is difficult to determine since so little is known about the pharmacology of the system, we note that of the three small molecules we previously established to alter dark flash habituation that were included in the screen, Clozapine, Haloperidol and Pimozide , the first two were identified among our hits while Pimozide was lethal at the 10\muM screening concentration.”

> *Fig.1B and Fig.3B are the same data, which is awkward and should be explicitly stated. But the legends do not match in terms of the rest period. Which is correct? It is also important to note the other behavioural assays in the 'rest' period.*

We thank the reviewer for pointing out this discrepancy in the legend. We have corrected the typo in the figure legend of Figure 3B :

“Habituation results in a progressive decrease in responsiveness to dark flashes repeated at 1-minute intervals, delivered in 4 training blocks of 60 stimuli, separated by 1hr of rest (from 0:00-7:00).”

We have also added a statement that the data is the same as that in Figure 1B.

> *Figure 3-4: SSMD fingerprint, there is no description of the different behavioural parameters. What they represent is left to the reader's inference. There is no mention of SpontDisp in the GitHub for example, so it is hard to know how these different parameters were measured. Even referring to the previous manuscript on habituation (Randlet O et al, eLife, 2019) does not shed light on most of them, for example, I suppose TwoMvmt represents the 'double responses' from the previous manuscript. Furthermore, there are inconsistencies between 3C and 4B, some minor (SpontDisp becomes SpntDisp), but Curve-Tap has disappeared for example, and I suspect became BendAmp-Tap. A*

> *more thorough description of these measures, and making the naming scheme consistent, are essential for readers to know what they are looking at.*

We again thank the reviewer for their careful assessment of our data, and we apologize for this sloppiness. We have gone through and made the naming of these parameters consistent in both figures, and have added another supplementary table that describes in more detail what each parameter is, and how it relates to the analysis code (Figure3_sourcedata3_SSMDFingerprintParameters.xls). This was an essential missing piece of information from our original manuscript.

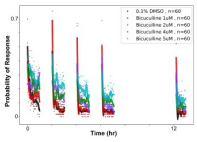
> *Line 206: While this prioritization makes sense, how was it implemented, how was the threshold decided and which were they? A table, or supplementary figure, would help to clarify the reason behind the choices. Fig.4C being cropped only around the response probability makes it impossible to judge if the criteria were respected, as the main heatmap is too small. For example, the choice of GABA receptor antagonists is somewhat puzzling, as besides PTX it does not seem that the other compounds had strong effects, with Amoxapine for example having seemingly as much effect on Naive and Train, with little in Test. And Bicuculline gave negative SSMD for prob in the three cases. The dose-response for PTX does lend credence to its effect, but I would have liked the other compounds, especially bicuculline. The melatonin results, for example, are much more convincing and interesting in our opinion.*

While in hindsight it may have been possible to do the hit prioritization in a systematic way using thresholding and ranking, we did this manually by inspecting the clustered fingerprints. We have clarified this in the text: “This manual prioritization led to the identification of the GABAA/C Receptor antagonists…”

While we agree that it is not possible to judge how well we performed this prioritization based on the images presented, we note that we do provide the full fingerprint data in the supplementary data, for which the reader is welcome to draw their own conclusions.

We have not performed further experiments with amoxapine, so we can not comment further on this. We did perform additional experiments with bicuculline, for which we did see effects similar to those of PTX, were habituation was inhibited. However, the effects are weaker and more variable than what we observe with PTX, and bicuculline also inhibits the initial responses of the larvae, causing their Naive response to be lower. Therefore we did not include it in our manuscript. We include these data here in Author response image 1 to reassure the Reviewer that picrotoxinin is not the only GABA Receptor antagonist for which we see inhibitory effects on habituation.

Author response image 1.



> *Fig.6: Why was the melatonin concentration used only 1um instead of 10um on the screen?*

Based on dose response experiments (Figure 5B, and others not shown), we found that the effect of Melatonin on habituation saturates at about 1uM, and therefore we used this dose.

> *Line 277: As the correlation with motor output is marginal at best, and the authors recognize the lack of behaviour in tethered animals, I would be careful about such speculation. Especially since the other changes are complex and go in all directions.*

While we appreciate the reviewer's caution, we feel that our statement is appropriately hedged using “might be”. We have also removed the statement “and thus is most closely associated with behavioural initiation”.

We now state:

“However, opposite effects of PTX and Melatonin were observed for 4_L^{strgD} neurons ([fig:6]C), which we found to be most strongly correlated with motor output ([fig:2]F). Therefore, this class might be most critical for habituation of response Probability.”

> *Fig.7: I am not sure how convincing these results are. 7F may have been more convincing, but to be thorough the authors would need to register the Gad1b identity to the calcium imaging and use their outline to extract the neuron's fluorescence. As it is, in the tectum, it is hard to be sure that all the identified neurons are indeed Gad1b positive, as that population is intermingled with other neuronal populations. The authors should consider the approach of Lovett-Barron M et al, Nat Neuro, 2020. Alternatively, the authors can tone down the language used in this section to match the confidence level of the association they propose.*

Figure 7A-E are what can be considered “virtual colocalization” analyses, where we are comparing the localization of data acquired in different experiments using image registration to common atlas coordinates. We agree that these results alone will never be very strong evidence for the identification of individual cells. The MultiMAP approach of Lovett-Barron is a powerful approach, though it makes the assumption that registration accuracy will be subcellular, which in practice may often not be the case. We believe that a better approach is to label the cells of interest during the Ca2+ imaging experiment itself, as we did 7F and G. The challenge in this experiment is binarizing the ROIs and thus deciding what is and is not a Gad1b-positive cell. In our opinion, the fact that these two independent experiments came to the same conclusion regarding Cluster 10 and 11 is good evidence that these cell types are likely predominantly GABAergic.

As discussed above, we have re-written the manuscript to tone down our claims about the role of GABA and GABAergic neurons in habituation, which we hope the reviewer will agree better reflects the limitations of the data in Figure 6 and 7.

> *Line 317: Based on the somewhat inconsistent results of the other GABA antagonists, I would be careful. Picrotoxin has been reported to antagonize other receptors besides GABA, see Das P et al, Neuropharma, 2003. So the results may be explained by a complex set of effects on multiple pathways with PTX.*

Off target effects are an important concern with any pharmacological experiment, and perhaps especially in zebrafish where receptors and targets can be quite divergent from those in mammals where most drug targets have been characterized. We have added this sentiment to the discussion:

“We cannot rule out the possibility that off-targets of PTX, or subtle non-specific changes in excitatory/inhibitory balance alter habituation behaviour.”

> *Line 400-403, 430: There are some conflicting statements regarding the potential role of clusters 1 and 2 in DF habituation. Do the authors think they play a role in the behaviour*

> *measured in this manuscript? Could they clarify what they mean?*

We see how our original statement in line 429 about the presence of cluster 1 and 2 neurons in the TL implied a role in dark flash habituation. This was not our intent, and we have removed "which also contains high concentrations of on-responding $_{1^{noA}_{On}, 2^{medD}_{On}}$ neurons".

Our thoughts on these neurons are now stated in the discussion as:

"We also observed classes exhibiting an On-response profile ($_{1^{noA}_{On}}$ and $_{2^{medD}_{On}}$). These neurons fire at the ramping increase in luminance after the DF, making it unlikely that they play a role in aspects of acute DF behaviour we measured here. These neurons exist in both non-adapting and depressing forms suggesting a yet unidentified role in behavioural adaptation to repeated DFs."

> *Minor comments*
>
> *Line 73 (and elsewhere): Why use adaptation instead of habituation (also in the adaptation profile)? Do you suspect your observations do not reflect habituation, but a sensory adaptation mechanism?*

We have used the convention that "habituation" refers to observations at the behavioural level, while "depression" and "potentiation" refer to observations at the neuronal level. We use the term "adaptation" to refer to neuronal adaptations of either sign (depression or potentiation), as in line 73.

We believe that our observations reflect neuronal adaptations that underlie habituation behaviour.

> *Line 71: It is debatable that the strongest learning happens in the first block, the difference between the first and last response seems to grow larger with each successive block. What do the authors mean by 'strongest'*

We agree that "strongest" was ambiguous. We have changed this to "initial":

"We focused on a single training block of 60 DFs to identify neuronal adaptations that occur during the initial phase of learning "

> *Fig.1F: there is no rastermap call in the GitHub repository, was the embedding done in the GUI? If so, it should also be shared for reproducibility's sake.*

Yes, Fig.1F was created using the suite2p GUI, as we have now clarified in the methods:

"The clustered heatmap image of neural activity (([fig:3]F) was generated using the suite2p GUI using the "Visualize selected cells" function, and sorting the neurons using the rastermap algorithm "

The image is available in the "Figure1 - Ca2Imaging.svg" file available here: https://github.com/owenrandlett/lamire_2022/tree/main/LamireEtAl_2022

> *Line 101: while true that AffinityPropagation does not require input on the number of clusters, preference can influence the number of clusters. It seems that at least two values were tested in the search for the clusters, can the authors comment on how many clusters the other preference value converged (or failed to converge) on?*

Indeed, as with any clustering approach, the resultant clusters are highly dependent on the input parameters, in this case the “preference”, as well as “damping” and the choice of affinity metric. By varying these parameters one can arrive at anywhere between 2 and hundreds of clusters.

It is for this reason that we feel that the anatomical analyses of these clusters is very important, making the assumption that neurons of differing functional types will have different localizations in the brain, as we explained in the Results:

“While these results indicate the presence of a dozen functionally distinct neuron types, such clustering analyses will force categories upon the data irrespective of if such categories actually exist. To determine if our cluster analyses identified genuine neuron types, we analyzed their anatomical localization ([fig:2]C-E). Since our clustering was based purely on functional responses, we reasoned that anatomical segregation of these clusters would be consistent with the presence of truly distinct types of neurons.”

We also acknowledge in the Results that the clustering approach has limitations:

“These results highlight a diversity of functional neuronal classes active during DF habituation. Whether there are indeed 12 classes of neurons, or if this is an over- or under-estimate, awaits a full molecular characterization. Independent of the precise number of neuronal classes, we proceed under the hypothesis that these clusters define neurons that play distinct roles in the DF response and/or its modulation during habituation learning“

> *Fig.2. My understanding is that the cluster numbers are arbitrary unless there is a meaning to them, which then should be explained. I would recommend grouping the clusters per functional category as in Fig.6 to make it easier for the reader.*

Cluster number reflects the ordering in the hierarchical clustering tree shown in Figure 2B. We feel that this is the most logical representation of their functional similarity. We have clarified this in the Methods:

“ We then used the Affinity Propagation clustering from scikit-learn , with “affinity” computed as the Pearson product-moment correlation coefficients (corrcoef in NumPy), preference=-9, and damping=0.9, and clustered using Hierarchical clustering (cluster.hierarchy in SciPy). Cluster number was assigned based on the ordering of the hierarchical clustering tree. ”

> *Fig.3 SSMD fingerprint, it would be much easier for the readers if the list of parameters was clearer and rotated 90 degrees. Maybe in a supplementary figure to show what each represents.*

We agree that the SSMD fingerprint is very difficult to interpret. As discussed above, we have now included a supplementary table (Figure3_sourcedata2_SSMDFingerprintParameters.xlsx) where we have clarified what each parameter represents.

> *Fig.4: The use of the same colours across the clustering methods is confusing, especially after the use of colours for the SSMD fingerprint in Fig.3. and at the bottom of 4A. Fig.4A for example could have been colour coded according to the most affected behaviour in the fingerprint at the bottom.*
>
> *Fig.4B the coloured text is difficult to read, especially for the lighter colours.*

We agree that our use of color is not perfect, but we have attempted to use them consistently: for example when referring to a functional cluster, or a drug manipulation. We don't think that there is a sufficient number of distinguishable colors for us to never use the same color twice.

> *Fig.4C if the goal is to show similarity, the relevant drugs could be placed adjacent to each other. One could also report the Euclidean distance, or compute how correlated the different fingerprints are within one pharmacological target space.*

The goal of Fig 4C is to highlight where Bicuculline, Amoxapine, Picrotoxinin, Melatonin, Ethinyl Estradiol and Hexestrol lie within the clustered heatmap of the behavioural fingerprints (Fig 4A), and
demonstrate how the probability of response to dark flashes is modulated by these drugs. In our analyses, "similarity" is a function of the clustering distance.

> *Fig.6D 'Same data as M, ...' I assume should be 'Same data as C,...'*

Indeed, thank you for pointing out this error that we have corrected.

> *Fig. 7 How many GCaMP6s double transgenic larvae were imaged?*

6 fish were imaged, as is stated in the legend to Fig 7G

> *Line 407: all is repeated.*

We apologize, but we do not see what is repeated at line 407. Can you please clarify?

> *Line 481: Would testing spontaneous activity after training for 7h be unbiased, could there be fatigue effects?*

We tested for fatigue effects in our previous study, comparing larvae that received the training for 7hrs and those that did not, and we saw no deficits in spontaneous activity, tap response, or OMR performance (Figure S1, Randlett et al., Current Biology, 2019).

> *Line 610: There are some inconsistencies between the authors' contributions in the manuscript and the one provided to eLife.*

Thank you, we will double check this in the resubmission forms. The authors' contributions in the manuscript are correct.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *I would rather recommend the authors divide this manuscript into two and publish two papers by adding some more strengthening data for each part such as cellular manipulations, e.g. ablation to prove the critical involvement of 12(Pot, M) neurons in habituation.*

We thank the reviewer for their suggestion, but have opted not to split the paper into two. We feel that the collective message of this paper and approach combining molecular and functional analysis will be of interest, and we believe the incongruencies in our results reflects the complexity inherent within the system.