

# Dorsal raphe dopamine neurons signal motivational salience dependent on internal and external states

Jounhong Ryan Cho<sup>1,2</sup>, Xinhong Chen<sup>1</sup>, Anat Kahan<sup>1</sup>, J. Elliott Robinson<sup>1,3</sup>,  
Daniel A. Wagenaar<sup>1</sup>, Viviana Gradinaru<sup>1,\*</sup>

<sup>1</sup> Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, United States

<sup>2</sup> Present Address: Princeton Neuroscience Institute, Princeton University, Princeton, NJ, United States

<sup>3</sup> Present Address: Cincinnati Children's Hospital Medical Center, Cincinnati, OH, United States

\* Corresponding author: [viviana@caltech.edu](mailto:viviana@caltech.edu)

## Abstract

The ability to recognize motivationally salient events and respond to them adaptively is critical for survival. Here we tested whether dopamine (DA) neurons in the dorsal raphe nucleus (DRN) contribute to this process. Population recordings of DRN<sup>DA</sup> neurons during associative learning tasks showed that their activity dynamically tracks salience, developing excitation to both reward- and punishment-paired cues. The DRN<sup>DA</sup> response to reward-predicting cues was diminished after satiety, suggesting modulation by internal states. DRN<sup>DA</sup> activity was also greater for unexpected outcomes than for expected outcomes. Two-photon imaging of DRN<sup>DA</sup> neurons demonstrated that the majority of individual neurons developed activation to reward-predicting cues but not to punishment-predicting cues, which was surprising and qualitatively distinct from the population results. Head-fixation during fear learning abolished the neural response to aversive cues, indicating modulation by behavioral context. Overall, these results suggest that DRN<sup>DA</sup> neurons encode motivational salience, dependent on internal and external factors.

## Introduction

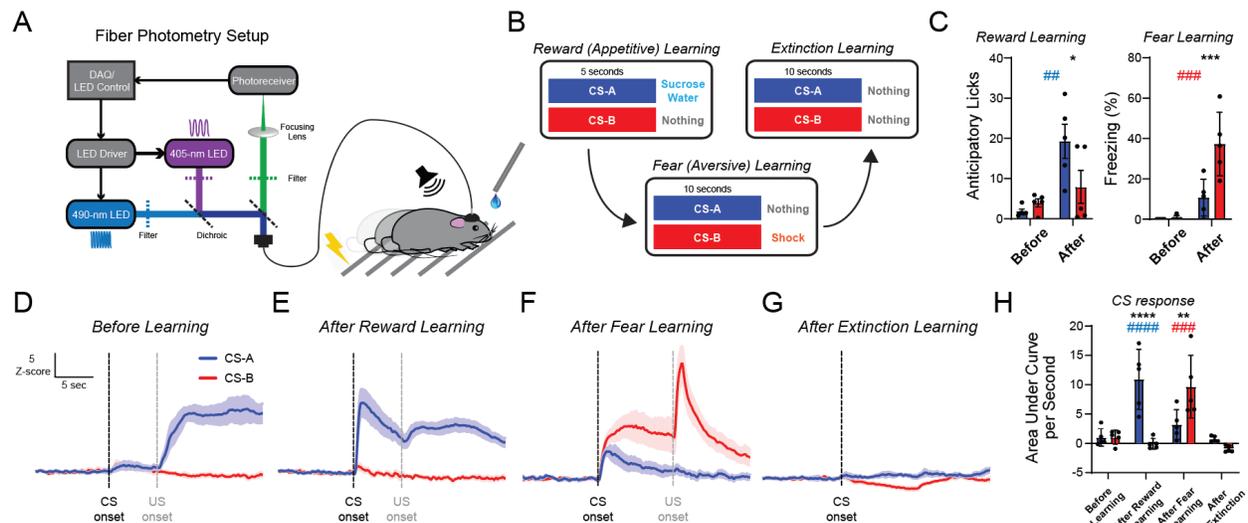
Dopamine (DA) is implicated in reward-seeking behavior and reward prediction error (RPE) encoding (Schultz et al., 1997; Bromberg-Martin et al., 2010). Increasing evidence suggests that DA also mediates non-reward functions, showing diverse responses to surprising, novel, or aversive events (Menegas et al., 2017; de Jong et al., 2019; Robinson et al., 2020; Lutas et al., 2020). These observations lead to the hypothesis that DA supports motivational control via at least two functional cell-types: one that encodes motivational value and another that signals motivational salience, defined as the absolute of motivational value (Bromberg-Martin et al., 2010). DA neurons in the lateral ventral tegmental area (VTA) or medial substantia nigra pars compacta (SNc) and those projecting to the lateral nucleus accumbens (NAc) are activated by rewarding events/cues and inhibited by aversive ones, supporting motivational value encoding (Matsumoto and Hikosaka, 2009; de Jong et al., 2019). By contrast, DA neurons in the lateral SNc and amygdala-projecting VTA cells are activated by both rewarding and aversive events/cues, consistent with salience encoding (Matsumoto and Hikosaka, 2009; Menegas et al., 2017; Lutas et al., 2020). We and others have characterized DRN<sup>DA</sup> neurons, demonstrating that their population activity reflects salience rather than value (Cho et al., 2017; Groessl., 2018; Lin et al., 2020). Here, we further examine the hypothesis that DRN<sup>DA</sup> neurons encode motivational salience, at both population and single-cell levels, using associative learning tasks in which the motivational salience and value of innately neutral cues were dynamically modulated by pairing them with positive, neutral, or negative outcomes (Figure 1 – figure

49 supplement 1). We also investigated whether DRN<sup>DA</sup> responses to the same motivationally  
 50 salient stimuli are modulated by internal state, expectation, and/or external behavioral context.  
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52

## Results and Discussion

53 To explore the encoding properties of DRN<sup>DA</sup> neurons, bulk fluorescence from DRN<sup>DA</sup> cells  
 54 expressing jGCaMP7f (Dana et al., 2019) was recorded with fiber photometry as a proxy for  
 55 population neural activity (Figure 1A; Figure 1 – figure supplement 2). Mice underwent three  
 56 stages of associative learning (Figure 1B). First, mice were trained in reward learning, in which  
 57 one auditory conditioned stimulus (CS-A) was paired with a sucrose reward (unconditioned  
 58 stimulus, US) and a second stimulus (CS-B) was paired with no reward. Subsequently, mice  
 59 underwent fear training, in which the previously rewarded CS-A predicted no outcome and the  
 60 previously unrewarded CS-B was paired with foot-shock. Finally, mice underwent extinction  
 61 training, in which both CSs were paired with no outcome. Mice discriminated the reward-  
 62 predicting CS-A from the neutral CS-B, showing increased anticipatory licks after training  
 63 (Figure 1C). They also learned the contingency shifts with fear training and responded  
 64 appropriately, displaying increased freezing to shock-predicting CS-B (Figure 1C).  
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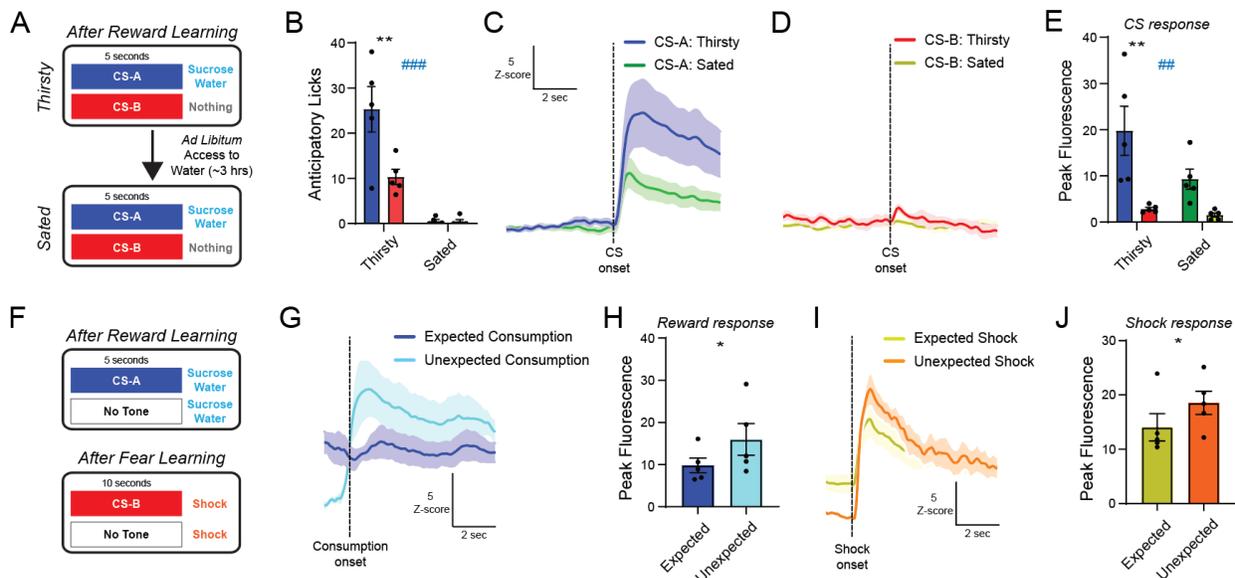


66

67 **Figure 1: DRN<sup>DA</sup> neurons dynamically track the motivational salience of conditioned stimuli.** (A) Schematic of  
 68 the fiber photometry setup used for GCaMP (490 nm) and isosbestic (405 nm) excitation and detection of emitted  
 69 signals in mice freely moving in an operant chamber, which had a speaker for presenting the CS sounds, a lickmeter  
 70 for delivering the reward, and metal grids for delivering foot-shocks. (B) Three stages of associative learning with two  
 71 cues (CS-A and CS-B). Reward learning was performed first, followed by fear learning and then extinction learning.  
 72 (C) Mice successfully discriminated the CS at each stage: they showed increased anticipatory licks to CS-A (blue)  
 73 after reward learning (n = 5 mice; 2-way repeated measures ANOVA;  $F_{1,8} = 4.583$ ,  $p_{\text{time} \times \text{CS}} = 0.0647$ ;  $F_{1,8} = 11.54$ ,  
 74  $p_{\text{time}} = 0.0094$ ;  $F_{1,8} = 2.581$ ,  $p_{\text{CS}} = 0.1468$ ; *post hoc* Sidaks test; CS-A vs CS-B after learning, \* $p = 0.0336$ ; before vs  
 75 after for CS-A, ### $p = 0.0089$ ) and increased freezing behavior to CS-B (red) after fear learning (n = 5 mice; 2-way  
 76 repeated measures ANOVA;  $F_{1,8} = 10.12$ ,  $p_{\text{time} \times \text{CS}} = 0.0130$ ;  $F_{1,8} = 33.83$ ,  $p_{\text{time}} = 0.0004$ ;  $F_{1,8} = 11.09$ ,  $p_{\text{CS}} = 0.0104$ ;  
 77 *post hoc* Sidaks test; CS-A vs CS-B after learning, \*\*\* $p = 0.0006$ ; before vs after for CS-B, #### $p = 0.0004$ ). (D)  
 78 Averaged photometry response before learning for CS-A (blue) and CS-B (red), with the CS onset (black dotted line)  
 79 and US onset (gray dotted line) indicated. Scale bar here also applies to (E-G). (E) Same as (D), but after reward  
 80 learning. (F) Same as (D), but after fear learning. (G) Same as (D), but after extinction learning. Note the absence of  
 81 a US onset. (H) DRN<sup>DA</sup> neuronal response, quantified by the area under curve during cue presentation, tracks the  
 82 change of salience in CS at each stage (n = 5 mice; 2-way repeated measures ANOVA;  $F_{3,24} = 14.98$ ,  $p_{\text{time} \times \text{CS}} <$   
 83  $0.0001$ ;  $F_{3,24} = 11.89$ ,  $p_{\text{time}} < 0.0001$ ;  $F_{1,8} = 3.305$ ,  $p_{\text{CS}} = 0.1066$ ; *post hoc* Sidaks test; CS-A vs CS-B after reward  
 84 learning, \*\*\*\* $p < 0.0001$ ; CS-A vs CS-B after fear learning, \*\* $p < 0.0048$ ; before learning vs after reward learning for  
 85 CS-A, ##### $p < 0.0001$ ; before learning vs after fear learning for CS-B, #### $p = 0.0003$ ). Data are presented as the mean  
 86  $\pm$  S.E.M.  
 87

88 Photometry data showed that before learning (day 1 of reward training), CS responses were  
 89 small for both CSs, followed by increased activity upon reward consumption (Figure 1D). After  
 90 reward learning, the reward-predicting CS-A induced excitation whereas the response to the  
 91 neutral CS-B remained small (Figure 1E). After fear learning, the CS-A response became  
 92 smaller as it no longer predicted reward, and the CS-B response became larger, reflecting its  
 93 pairing with the aversive US (Figure 1F). After extinction learning, both CS responses were  
 94 reduced to baseline (Figure 1G). Collectively, these results suggest that DRN<sup>DA</sup> population  
 95 activity dynamically tracks the motivational salience of cues through increases in activity,  
 96 regardless of the valence of the cue (Figure 1H and Figure 1 – supplement 1 ; Groessl et al.,  
 97 2018; Lin et al., 2020).

98  
 99 The motivational salience of cues may depend on the animal's internal state: for example,  
 100 water-predictive cues are highly salient to thirsty animals but are perceived as less salient and  
 101 attractive if satiated. To test this idea, after mice were fully trained in the reward-learning task,  
 102 they completed 50% of trials while thirsty and the other 50% while satiated (Figure 2A). After  
 103 satiety, mice stopped responding to the reward-predicting CS-A, as evidenced by the extinction  
 104 of anticipatory licking (Figure 2B). Neural responses to the CS-A were also diminished after  
 105 satiety (Figure 2C and 2E) while responses to the neutral CS-B remained unchanged (Figure  
 106 2D and 2E), suggesting that CS salience signals can be modulated by internal motivational  
 107 states.  
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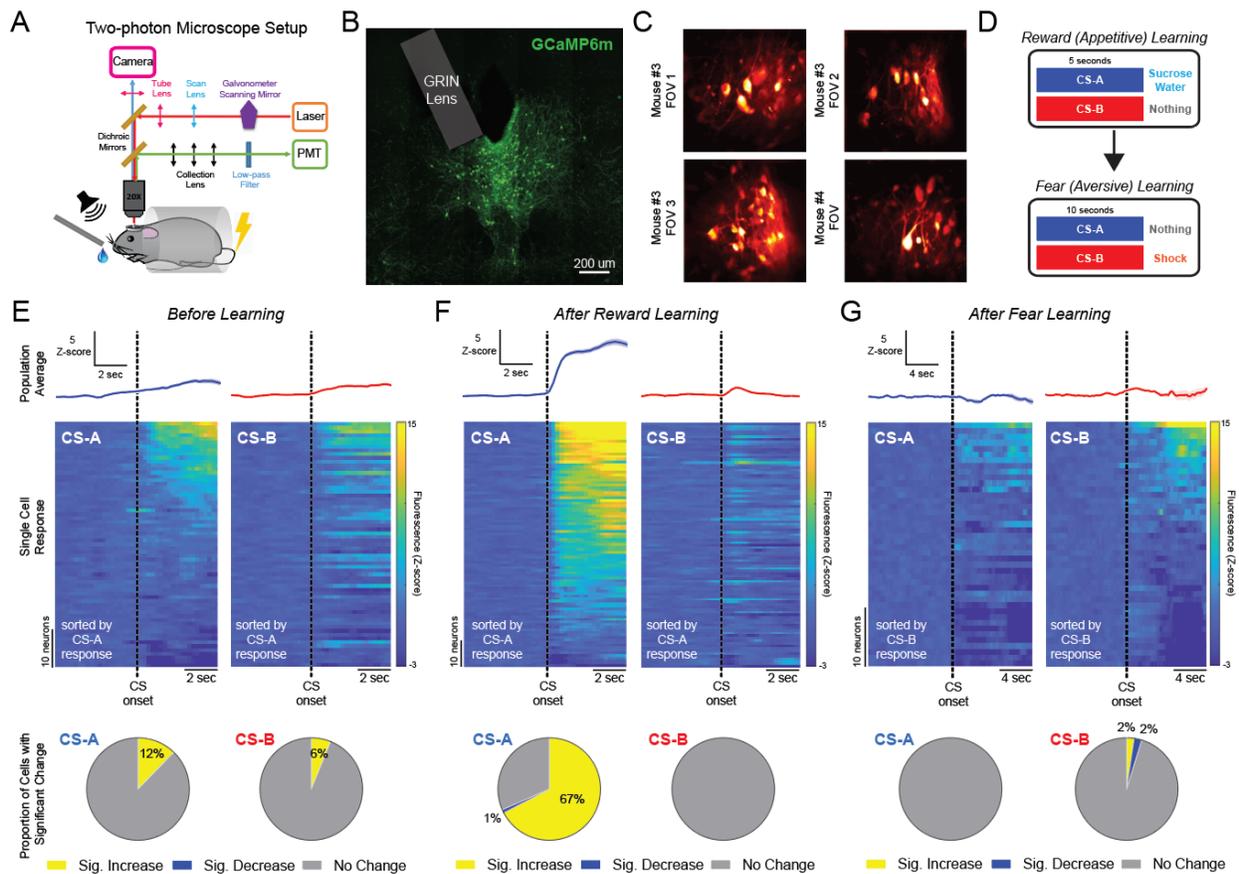


109  
 110 **Figure 2: DRN<sup>DA</sup> neuronal responses are modulated by internal state and expectation.** (A) To test whether  
 111 DRN<sup>DA</sup> CS responses were influenced by the animals' internal state, fully trained mice underwent half of a reward  
 112 learning session while thirsty and completed the other half while satiated. (B) Behavioral response to reward-paired CS-  
 113 A, quantified by anticipatory licks during CS presentation, was reduced after satiety (n = 5 mice; 2-way repeated  
 114 measures ANOVA;  $F_{1,8} = 8.093$ ,  $p_{\text{time} \times \text{CS}} = 0.0217$ ;  $F_{1,8} = 43.93$ ,  $p_{\text{time}} = 0.0002$ ;  $F_{1,8} = 7.688$ ,  $p_{\text{CS}} = 0.0242$ ; *post hoc*  
 115 Sidaks test; CS-A (blue) vs CS-B (red) when thirsty,  $**p = 0.0022$ ; thirsty vs satiated for CS-A,  $###p < 0.0003$ ). (C)  
 116 Averaged CS-A response during thirsty (blue) and satiated (green) states. Scale bar here also applies to (D). (D)  
 117 Averaged CS-B response during thirsty (red) and satiated (yellow) states. (E) The CS-A response was significantly  
 118 diminished after satiety, while the CS-B response showed no change (n = 5 mice; 2-way repeated measures ANOVA;  
 119  $F_{1,8} = 5.699$ ,  $p_{\text{state} \times \text{CS}} = 0.0440$ ;  $F_{1,8} = 9.393$ ,  $p_{\text{state}} = 0.0155$ ;  $F_{1,8} = 11.68$ ,  $p_{\text{CS}} = 0.0091$ ; *post hoc* Sidaks test; CS-A vs  
 120 CS-B during thirsty,  $**p = 0.0015$ ; thirsty vs satiated in CS-A,  $##p = 0.0097$ ). (F) To examine whether DRN<sup>DA</sup> US  
 121 responses were modulated by expectation, 5% sucrose or foot-shock were occasionally introduced in the absence of  
 122 predictive cues after reward and fear learning, respectively. (G) Averaged DRN<sup>DA</sup> response to expected (dark blue)  
 123 versus unexpected (light blue) reward consumption. Photometry traces were aligned to consumption onset. (H)  
 124 Unexpected reward consumption evoked higher neural activity than expected consumption, quantified by peak

125 fluorescence ( $n = 5$  mice; paired t-test;  $t_4 = 2.836$ ,  $^*p = 0.0470$ ). (I) Averaged DRN<sup>DA</sup> response to expected (orange)  
 126 versus unexpected (yellow) shock delivery. Photometry traces were aligned to shock onset. (J) Unexpected foot-  
 127 shock induced higher neural activity than expected shock delivery, quantified by peak fluorescence ( $n = 5$  mice;  
 128 paired t-test;  $t_4 = 3.539$ ,  $^*p = 0.0240$ ). Data are presented as the mean  $\pm$  S.E.M.

129  
 130 DA neurons can be modulated by surprise or expectation, signaling prediction error (Schultz et  
 131 al., 1997). To examine if DRN<sup>DA</sup> neurons are modulated by prediction or expectation, mice  
 132 received unexpected rewards or shocks in the absence of predictive cues, among regular CS-  
 133 US pairings (separately after reward or fear training; Figure 2F). DRN<sup>DA</sup> responses were larger  
 134 for unexpected rewards than for expected consumption (Figure 2G and 2H). Additionally,  
 135 DRN<sup>DA</sup> neurons showed larger responses to unexpected shocks than expected ones (Figure 2I  
 136 and 2J). Together, these suggest that DRN<sup>DA</sup> neurons signal unsigned prediction errors.

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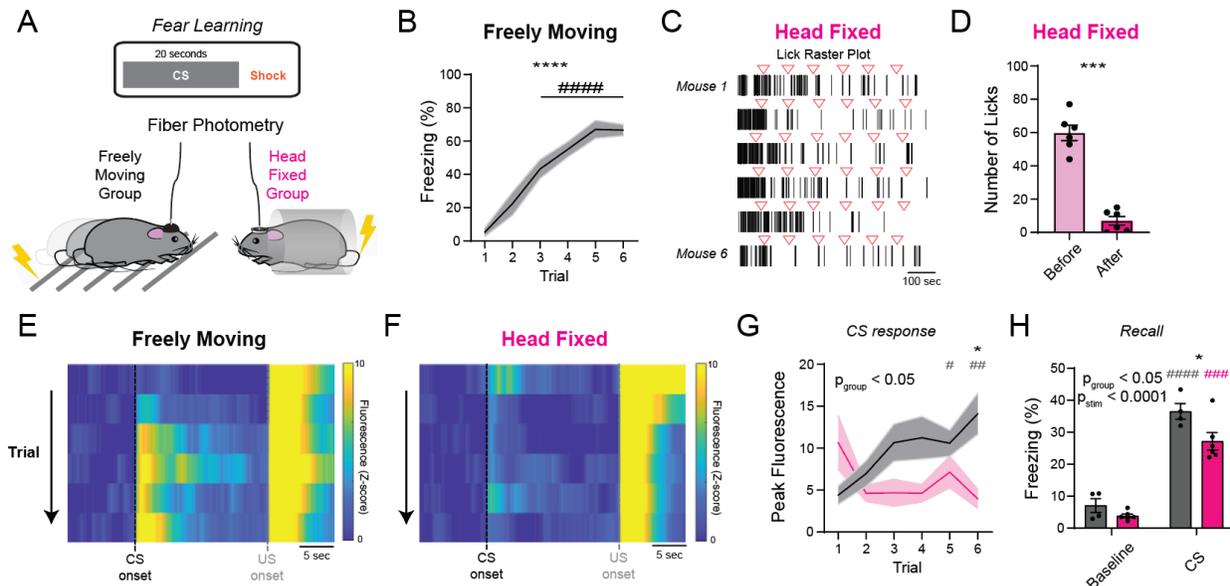
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153 neurons in total, from 8 FOVs in 4 mice. **(G)** Same as (E), but after fear learning. There were 42 neurons in total, from  
154 3 FOVs in 3 mice. Data are presented as the mean  $\pm$  S.E.M.

155  
156 DRN<sup>DA</sup> neurons track the motivational salience of CSs at the population level, as demonstrated  
157 by increases in bulk fluorescence (Figure 1), but it is unclear how individual neurons are tuned  
158 to salient cues with distinct valence. Thus, we performed two-photon imaging (Figure 3A) to  
159 visualize calcium responses in single DRN<sup>DA</sup> neurons. For this, gradient index lenses were  
160 implanted over the DRN to image GCaMP6m-expressing DRN<sup>DA</sup> neurons (Figure 3B and Figure  
161 3 – figure supplement 1), and mice were habituated to head-fixation for imaging. Mice  
162 underwent habituation for 35 minutes per day for 8–10 days. We imaged multiple fields-of-view  
163 (Figure 3C) while mice performed associative learning tasks (Figure 3D). Our analysis focused  
164 on the CS response, as US delivery (especially tail-shock) introduced uncorrectable motion from  
165 body movement. Before learning, only a small fraction of neurons showed a significantly  
166 increased CS response over baseline (Figure 3E). After reward learning, the majority of single  
167 DRN<sup>DA</sup> cells developed increased responses to the reward-predicting CS-A only (Figure 3F).  
168 Surprisingly, after fear learning, most DRN<sup>DA</sup> neurons did not show significant changes in  
169 activity from baseline, even to the shock-predicting CS-B (Figure 3G). The absence of aversive  
170 cue responses was striking, given that our and other previous results from freely-moving  
171 photometry or microendoscopic imaging showed robust increases (Figure 1F; Groessl et al.,  
172 2018; Lin et al., 2020). We reasoned that the change in behavioral context (freely-moving  
173 versus head-fixed) may have caused this unexpected variation in DRN<sup>DA</sup> responses.

174  
175 To test this hypothesis, we performed photometry in freely-moving and head-fixed mice  
176 undergoing a similar fear learning procedure (single session, 6 paired CS–US events; foot-  
177 shocks to freely-moving and tail-shocks to head-fixed mice, due to differences in experimental  
178 setups; Figure 4A and Figure 4 – figure supplement 1). All mice were water restricted and  
179 habituated for head-fixation, and then randomly assigned to one of the two groups. Freely-  
180 moving mice learned the association within these trials, showing a progressive increase of  
181 freezing in response to the CS (Figure 4B). Head-fixed mice showed a rapid decrease in licking  
182 as they received CS–US pairings (Figure 4C and 4D). CS responses in freely-moving mice  
183 gradually increased across paired trials (Figure 4E and 4G). However, head-fixed mice showed  
184 no significant change in CS responses across trials (Figure 4F and 4G). This group difference  
185 cannot be explained by the distinct shock methods, as both induced similar US responses  
186 (Figure 4 – figure supplement 2). Both groups showed learning in the form of increased freezing  
187 to the CS compared with baseline during freely-moving recall sessions, albeit with a group  
188 difference (Figure 4H). Altogether, these results indicate that salience signaling of DRN<sup>DA</sup> cells  
189 can be modulated by behavioral context, especially during stressful situations where mice are  
190 forced to be immobile and receive aversive reinforcers.

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192

193

**Figure 4: Population DRN<sup>DA</sup> responses to aversive, shock-predicting CS depend on the behavioral context during fear learning.** (A) Mice were divided into two groups (freely-moving and head-fixed) and underwent fear

194 learning experiments. Freely-moving mice received foot-shocks as the US in an operant chamber, whereas head-

195 fixed mice received tail-shocks. (B) Quantification of freezing during the shock-predicting CS showed that the mice in the

196 freely-moving group learned the CS-US association within 6 trials (n = 10 mice; 1-way repeated measures

197 ANOVA; F = 48.37; \*\*\*\*p < 0.0001; *post hoc* Sidak's test; Trial 3 to 6 vs Trial 1, #####p < 0.0001). (C) Raster plot of licks

198 from 6 head-fixed mice (each row) during fear learning. Red triangles denote the onset of shock-predictive cues. Note

199 that, before fear learning, these mice were already habituated to the head-fixation setup with occasional sucrose

200 delivery, so they licked continuously at the start. This licking behavior reduced dramatically across repeated CS-US

201 pairings. (D) The number of licks was significantly decreased after 6 trials of fear learning compared with the baseline

202 period prior to the first CS-US presentation (n = 6 mice; paired t-test;  $t_5 = 9.817$ , \*\*\*p = 0.0002). (E) Heatmap of the

203 averaged photometry responses in the freely-moving group across 6 trials. Each row is the average response of all

204 mice in the group. Note the gradual development of a time-locked CS response across CS-US pairings. (F) Same as

205 (E), but for the head-fixed group. Note the absence of time-locked CS response even across repeated CS-US

206 pairings. (G) Freely moving mice (black) showed a significant increase in the CS response during fear learning,

207 whereas head-fixed mice (magenta) showed no change (n = 10 freely-moving mice, n = 11 head-fixed mice; 2-way

208 repeated measures ANOVA;  $F_{5,95} = 6.243$ ,  $p_{\text{trial} \times \text{group}} < 0.0001$ ;  $F_{3,302,62.73} = 1.087$ ,  $p_{\text{trial}} = 0.3645$ ;  $F_{1,19} = 4.482$ ,  $p_{\text{group}} =$

209 0.0434; *post hoc* Sidak's test; freely-moving vs head-fixed group in Trial 6, \*p = 0.0150; Trial 1 vs Trial 5 in freely

210 moving group, #p = 0.0452; Trial 1 vs Trial 6 in freely moving group, ##p = 0.0042). (H) Both groups showed increased

211 freezing compared with baseline during the freely-moving recall test performed the next day (4 CS presentations in a

212 novel arena, averaged), albeit with a group difference (n = 4 freely-moving mice; n = 6 head-fixed mice; 2-way

213 repeated measures ANOVA;  $F_{1,8} = 1.639$ ,  $p_{\text{stim} \times \text{group}} = 0.2364$ ;  $F_{1,8} = 122.3$ ,  $p_{\text{stim}} < 0.0001$ ;  $F_{1,8} = 10.87$ ,  $p_{\text{group}} =$

214 0.0109; *post hoc* Sidak's test; freely-moving vs head-fixed group during CS, \*p = 0.0149; baseline vs CS in freely-

215 moving mice, #####p < 0.0001; baseline vs CS in head-fixed mice, #####p = 0.0001). Data are presented as the mean  $\pm$

216 S.E.M.

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219 The absence of neural responses to aversive cues during head-fixation (Figures 3 and 4) was

220 striking, given that our results and previous studies have shown robust responses to aversive

221 cues when animals are freely moving (Groessl et al., 2018; Lin et al., 2020), and given that our

222 animals were well habituated to the setup. Head-fixation is widely used in imaging and

223 behavioral experiments due to the need for mechanical stability or convenience, but the effects

224 on behavior or neural activity are often assumed to be negligible. However, in rodents, head-

225 fixation affects vocalization behavior (Weiner et al., 2016), and acute head restraint reduces the

226 reward and cue responses of VTA DA and DRN serotonergic neurons (Zhong et al., 2017).

227 Head-fixed mice showed higher corticosterone level over control subjects, even up to 25 days of

228 daily training (Juczewski et al., 2020). Our findings extend these observations and demonstrate

229 that neurophysiology can be affected by head-fixation even after habituation, especially in highly  
230 stressful and inescapable contexts.

231

232 This study builds upon previous findings that DRN<sup>DA</sup> activity signals the motivational salience of  
233 cues in a learning-dependent manner, increasing in response to CSs that are paired with  
234 outcomes of either valence (Groessl et al., 2018; Lin et al., 2020), and declining with extinction.  
235 We additionally demonstrated that DRN<sup>DA</sup> responses to the same CS or US can be modulated  
236 by internal state, expectation, and even external behavioral context. The dynamic nature of  
237 salience encoding by DRN<sup>DA</sup> neurons may serve as a “gain control” in downstream processing  
238 in the extended amygdala (Kash et al., 2008; Groessl et al., 2018) through both fast-acting  
239 glutamate and modulatory dopamine (Matthews et al., 2016, Li et al., 2016) to orient attention  
240 towards encountered stimuli and enable the selection of appropriate behavioral responses.

241 **Materials and Methods**

242 **Key resources table**

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	Anti-GFP (Chicken polyclonal)	Aves	Cat #: GFP-1010 RRID: AB_2307313	IHC (1:500)
Antibody	Anti-tyrosine hydroxylase (Rabbit polyclonal)	Sigma-Aldrich	Cat #: AB152 RRID: AB_390204	IHC (1:500)
Antibody	Alexa Fluor 488 AffiniPure F(ab') <sub>2</sub> Fragment Donkey Anti-Chicken IgY (IgG)	Jackson ImmunoResearch	Cat #: 703-546-155 RRID: AB_2340376	IHC (1:500)
Antibody	Alexa Fluor 647 AffiniPure Fab Fragment Donkey Anti-Rabbit IgG	Jackson ImmunoResearch	Cat #: 711-607-003 RRID: AB_2340626	IHC (1:500)
Recombinant DNA reagent	pAAV.syn.FLEX.jGCaMP7f.WPRE	Addgene	Plasmid #104492 RRID: Addgene_104492	Serotype 9; Gift from Douglas Kim & GENIE Project
Recombinant DNA reagent	pAAV.syn.Flex.GCaMP6m.WPRE.SV40	Addgene	Plasmid #100838 RRID: Addgene_100838	Serotype 9; Gift from Douglas Kim & GENIE Project
Software, Algorithm	MATLAB	Mathworks, Inc	RRID: SCR_001622	R2016a/R2018b
Software, Algorithm	ABET II for Operant Chamber Control	Lafayette Instrument Company	Model 89501	
Software, Algorithm	GraphPad Prism 8	GraphPad Software, Inc	RRID: SCR_002798	
Software, Algorithm	Fiber Photometry Processing Code	Gradinaru Lab	FP_Session_Processing2.m	<a href="http://github.com/GradinaruLab/dLigt1">http://github.com/GradinaruLab/dLigt1</a>
Other	Mono Fiberoptic Cannula	Doric Lenses, Inc	Cat #: MFC_400/430_0.48_4mm_ZF1.25_FLAT	
Other	Mono Fiberoptic Patch Cable	Doric Lenses, Inc	Cat #: MFP_400/430_0.48_2m_FC_ZF1.25_FLAT	

Other	Gradient Index (GRIN) Lens	Inscopix, Inc	Microendoscope 0584, Part# GLP-0584	
Other	Custom ferrule for holding GRIN lens	Kientec Systems, Inc	Cat #: FZI-LC-L2.5-520	

243

### 244 **Experimental animals**

245 Subjects were *Th*-ires-cre transgenic mice (*Th*: tyrosine hydroxylase, a rate-limiting enzyme for  
246 dopamine synthesis; Lindeberg et al., 2004) of both sexes, aged 2–4 months at the time of  
247 surgery. *Th*-ires-cre mice were used in this study to selectively target DRN<sub>DA</sub> neurons; the  
248 specificity of cre expression (compared to immunohistochemistry of *Th*+ neurons) in this mouse  
249 line has previously been shown to be around 60–75% in the DRN, which is comparable to an  
250 alternative line that express Cre recombinase under the dopamine transporter promoter (Li et  
251 al., 2016; Matthews et al., 2016; Cho et al., 2017). Typically, experiments lasted until the mice  
252 were 6–8 months old. Animals were originally group-housed but were later single-housed after  
253 undergoing surgery for photometry or two-photon imaging. Mice were housed in a room on a  
254 12-hour light/dark cycle (lights off at 6 AM, lights on at 6 PM). All experiments were performed  
255 during the dark phase. Mice had ad libitum access to food and water before the start of water  
256 restriction (see below for details). All animal husbandry and experimental procedures involving  
257 animal subjects were conducted in compliance with the Guide for the Care and Use of  
258 Laboratory Animals of the National Institutes of Health and approved by the Office of Laboratory  
259 Animal Resources at the California Institute of Technology (IACUC protocol number: 1730).  
260 Animals were excluded from analysis if no photometry or two-photon signals were observed 4  
261 weeks after surgery. One mouse was excluded during the associative learning experiments  
262 (Figure 1 and 2) due to health concerns related to water restriction. At the end of the  
263 experiments, the brains from all animals with fluorescence signals were histologically verified to  
264 have fibers or GRIN lenses located over the DRN.

265

### 266 **Surgical procedures**

267 Stereotaxic surgeries for viral vector injections and implantation of an optical fiber/ferrule for  
268 photometry or a GRIN lens for two-photon imaging were performed as previously described  
269 (Cho et al., 2017) with slight modifications. After anesthesia (isoflurane gas/carbogen mixture,  
270 5% for induction and 1.5–2% for maintenance), surgical preparation and exposure of the skull, a  
271 craniotomy hole was drilled in the skull (antero-posterior axis: –4.7 mm, medio-lateral axis: –1.5  
272 mm, relative to bregma). Adeno-associated viruses (AAV) encoding jGCaMP7f or GCaMP6m in  
273 a cre-dependent manner (diluted to  $1.0 \times 10^{13}$  genome copies/mL, both from Addgene) were  
274 injected to the DRN (antero-posterior axis –4.7 mm, medio-lateral axis: –1.5, dorso-ventral axis  
275 –3.2 and –2.9 mm, relative to bregma) at a 25° angle. 300 nL of AAV was infused at each site  
276 along the dorso-ventral axis, at a rate of 50 nL per minute. After injection, the needle was held in  
277 the same place for an additional 10 minutes. Finally, the needle was slowly withdrawn over  
278 about 10 minutes to prevent backflow.

279

280 For fiber photometry, an optical fiber/ferrule (fiber: 400- $\mu$ m diameter, NA 0.48, cut length: 4 mm,  
281 ferrule: 1.25-mm diameter, zirconia, glued with low-autofluorescence epoxy, Doric Lenses) was  
282 mounted to a stereotaxic cannula holder (SCH\_1.25, Doric Lenses), lowered towards the DRN  
283 at a 25° angle, stopping 0.25 mm above the site of virus injection. For two-photon imaging, a 25-  
284 gauge needle (outer diameter = 0.515 mm) was attached to the stereotaxic holder (1766AP,  
285 David Kopf Instruments) and slowly lowered up to 2 mm along the dorso-ventral axis (relative to  
286 bregma, at 25°) to make a path for the GRIN lens (GLP-0584, Inscopix; 0.5-mm diameter, 8.4-

287 mm length). Then, a small, customized zirconia ferrule (2.5-mm length, 520 $\mu$ m hole size;  
288 Kientec System) was carefully glued to surround the GRIN lens at one end. The same cannula  
289 holder was used to hold the GRIN lens, touching the surrounding zirconia ferrule rather than the  
290 fragile and sensitive lens. The GRIN lens was slowly lowered into the brain, stopping 0.25 mm  
291 above the site of virus injection. After implantation, a thin layer of adhesive cement was applied  
292 to the skull surface and around the implant for strong fixation. After the adhesive cement had  
293 completely dried, a layer of black dental cement was applied to build a head cap. For mice in  
294 the two-photon imaging experiments (Figure 3) and comparison of DRN<sup>DA</sup> dynamics in freely-  
295 moving versus head-fixed groups (Figure 4), a customized ring for head fixation (stainless steel,  
296 5-mm inner diameter, 11-mm outer diameter) was super-glued to the cement surface before the  
297 dental cement was fully dried, so that the ferrule or GRIN lens tip was located within the ring.  
298 More dental cement was applied inside the ring. To protect the GRIN lens from damage, the  
299 lens tip was covered with a small piece of parafilm and low-toxicity silicone adhesive (Kwik-sil,  
300 World Precision Instruments) was applied. After the silicone adhesive fully solidified, mice were  
301 unmounted from the stereotaxic frame and their recovery was monitored for about 2 hours.

302

### 303 ***Fiber photometry***

304 Fiber photometry was performed as previously described (Lerner et al., 2015; Cho et al., 2017;  
305 Robinson et al., 2020).

306

### 307 ***Two-photon imaging***

308 In vivo two-photon imaging was performed with a custom-built microscope. Briefly, a pulsed  
309 femtosecond laser beam from a Ti:Sapphire laser system (940 nm), coupled with OPA (Insight  
310 DS+, Spectra-Physics, CA), passed through a beam expander (75:50) and an iris (SM1D12C,  
311 Thorlabs), set to 3 mm. An XY galvanometer (6215H, Cambridge Technology) was placed  
312 before a pair of scan lenses (LSM54-1050, Thorlabs) and a tube lens (ITL200, Thorlabs). An  
313 805 nm short-pass dichroic mirror (DMSP805SPL, Thorlabs) was used to allow simultaneous  
314 near-infrared (IR) visualization along with two-photon excitation. Near-IR visualization for  
315 sample localization was achieved by a 75-mm tube lens (AC508-075, Thorlabs), directed to an  
316 HDMI-output camera (HD205-WU, AmScope). A 500–700 nm reflecting dichroic mirror  
317 (T600/200dcrb, Chroma) was used to split the two-photon excitation and emission paths. A  
318 20X/0.5 NA air objective (Olympus, UPLFLN20XP) was used, and the laser power was set to  
319 60–80 mW. Emitted photons were passed through the collective optics (AC508-100-A,  $f = 100$   
320 mm, at  $z = 100$  mm from BA, most convex side facing the sample; and a pair of LA1131,  $f = 50$   
321 mm at  $z = 150$  mm and  $z = 156$  mm from the back BA, convex sides facing each other) and a  
322 680-nm low-pass filter (et680sp-2p8, Chroma) into the photomultiplier tube (Hamamatsu  
323 R3896). Laser intensity was controlled by the rotation of a half-lambda waveplate (Thorlabs  
324 AHWP05M-980) relative to a Glan polarizer (Thorlabs GL10-B) using a motorized rotation stage  
325 (Thorlabs PRM1/Z8). Stage XY adjustment and microscope focus was controlled by motorized  
326 linear actuators (Z825B, Thorlabs). Imaging data were acquired using an FPGA DAQ board  
327 (National Instruments 7855R) and custom-written software in Labview. An electromechanical  
328 shutter (Uniblitz VS25, Vincent Associates) was used to ensure laser safety during imaging. The  
329 imaging frame size was 194 x 194 pixels with a 4-Hz frame rate. In 3 out of 4 mice, 2 or 3 fields-  
330 of-view (FOVs) were obtained (at least 100  $\mu$ m apart in the Z-direction) that showed different  
331 sets of neurons. We did not try to match or keep the same FOVs across different recording  
332 days. During each imaging session, after finding an FOV, two-photon scanning was triggered for  
333 each trial 15 seconds before the CS delivery and terminated 20 seconds after the CS delivery.

334

### 335 ***Water restriction and habituation procedures for head-fixed experiments***

336 All animals reported here underwent water restriction procedures (1.5 mL per day, provided at 4  
337 pm everyday), starting from 2–3 weeks after surgery when mice had fully recovered. The water

338 restriction was mainly to motivate the animals to engage in reward learning, but the fear-  
339 learning-only cohort (Figure 4) were also water restricted to maintain consistent experimental  
340 conditions and to facilitate habituation training (see below). Once water restriction started, mice  
341 were weighed daily and were returned to ad libitum access to water if their weight loss was  
342 >10% of their pre-restriction weight. Animals were water restricted at least for 5 days before  
343 they started freely-moving associative learning tasks or habituation training for head-fixed  
344 experiments.

345  
346 Regarding habituation procedures for head-fixation, we generally followed a previously  
347 published protocol (Guo et al., 2014), but some extra steps were introduced to ensure that the  
348 mice were slowly acclimated to the setup. On day 1, mice were familiarized with experimenter  
349 handling for about 15 minutes. After mice became calm on the experimenter's hand (exhibiting  
350 grooming behavior and spending less time looking outside the hand), they were given access to  
351 up to 0.4 mL of 5% sucrose water, delivered via a 20  $\mu$ L pipette. After reward consumption,  
352 mice were further handled for about 2 minutes before being returned to their home cages. On  
353 day 2, mice were handled in a similar fashion for 5 minutes with access to up to 0.1 mL of 5%  
354 sucrose water. We then introduced the body tube (made from plexiglass) with the other hand  
355 and let animals explore the tube freely. We performed this step up to 10 times until mice  
356 voluntarily entered the body tube. After entering the tube, the experimenter gently held the tail to  
357 prevent escape and mice were rewarded with up to 0.1 mL of 5% sucrose for another 5  
358 minutes. Next, the experimenter quickly took hold of the implanted head ring and secured it to  
359 the head fixation bar (all < 10 seconds). The body tube was also secured with a lever. Mice  
360 remained head-fixed for 5 minutes and 20  $\mu$ L of 5% sucrose was provided every 20 seconds.  
361 On day 3, mice were further acclimated to the apparatus, now with 10 minutes of head-fixation  
362 and 5% sucrose reward delivered every 30 seconds. From day 4 to day 7, mice were introduced  
363 to the lickometer used in the experiments and the duration of head-fixation was gradually  
364 increased from 15 to 30 minutes (increasing by 5 minutes every day), with reward provided  
365 every minute. We reasoned that mice showed good habituation and were ready to advance to  
366 the behavioral experiments when they consumed the reward throughout the duration of head-  
367 fixation and when they produced much less feces than on the first day of training. We trained  
368 mice in the head-fixation apparatus for up to 30 minutes, well above the duration of recordings  
369 (~25 minutes for two-photon imaging, ~15 minutes for head-fixed fear learning). Note that we  
370 extended the number of days of habituation training compared with Guo et al., 2014 to make  
371 sure that the mice were habituated to the experimental setting slowly, to minimize the level of  
372 stress as much as possible.

373

### 374 ***Associative learning tasks in freely-moving photometry recordings***

375 All behavioral experiments were programmed and executed with ABET II software (Lafayette  
376 Neuroscience). After animals were water restricted for 5 days, they were introduced to an  
377 operant chamber (Lafayette Neuroscience) and allowed to freely explore for 30 minutes with a  
378 patch cable attached. 5% sucrose was delivered to the lick port at intervals randomly drawn  
379 from a uniform distribution of 45–75 seconds, so that mice could learn the location. Licks were  
380 counted when the infrared beam at the lick port was broken. This habituation was repeated for 2  
381 days.

382

383 After habituation, the reward (appetitive) learning phase started. Mice were introduced to two  
384 types of conditioned stimuli (CS-A and CS-B; 5 kHz tone or white noise, 75 dB, 5 seconds,  
385 counterbalanced across animals). 25  $\mu$ L of 5% sucrose reward (as the unconditioned stimulus;  
386 US) was delivered only after CS-A presentation; there was no outcome after the CS-B  
387 presentation. Within a session, 20 CS-A and reward pair trials and 10 CS-B and no outcome  
388 pair trials were given. The inter-trial interval was drawn at random from a uniform distribution of

389 45–75 seconds. There was a total of 21 reward learning sessions for all animals, and  
390 photometry signals were recorded on day 1 (“before learning”) and day 21 (“after reward  
391 learning”). On day 1, video was also recorded.

392  
393 To examine whether DRN<sup>DA</sup> cue responses are influenced by internal state, mice performed half  
394 of the trials in a reward learning session (10 CS-A and reward pairs, 5 CS-B and no outcome  
395 pairs) while they were thirsty and completed the other half after satiety. In between these two  
396 separate sessions, they were given free access to water for 3 hours in their home cages. After  
397 this experiment, mice underwent regular water restriction for two days. To study whether DRN<sup>DA</sup>  
398 neurons encode positive prediction error, mice performed another experimental session in  
399 which the US was presented without the predictive CS-A. In this session, there were 10  
400 “expected” trials (CS-A paired with the US) and 5 “unexpected” trials (US only).

401  
402 Subsequently, mice underwent a fear (aversive) learning phase, in which the previously reward-  
403 predicting CS-A no longer predicted any outcome and the previously neutral CS-B was paired  
404 with an aversive foot-shock (0.5 mA for 1 second). The duration of both CSs was increased to  
405 10 seconds so that freezing behavior could be quantified. 20 CS-B and shock pairs were  
406 presented and 10 CS-A and no outcome pairs were presented with the inter-trial intervals as  
407 described above. Photometry and video recordings (to measure freezing behavior) were  
408 performed on day 2 of fear learning (“after fear learning”). On day 3, after mice were fully trained  
409 on the fear learning task, we performed a similar prediction error experiment, but now with  
410 aversive cue CS-B and foot-shock. In this experiment, 3 unpredicted shocks were presented  
411 intermixed with 10 normal CS-B and shock pairings.

412  
413 Finally, animals underwent an extinction learning phase in which both CS-A and CS-B were  
414 presented (10-second duration, 15 times each) but paired with no outcomes. This was repeated  
415 for 4 days and recording was performed on day 5 (“after extinction”).

416  
417 This series of experiments was performed in two cohorts (one mouse excluded due to health  
418 concerns). Results were replicated between those two cohorts.

#### 419 ***Associative learning tasks in head-fixed two-photon imaging***

420 Procedures for the associative learning tasks for two-photon imaging under head-fixation were  
421 similar to the procedures used in the freely-moving condition, with some small differences.  
422 ABET II software was also used to execute the associative learning tasks. Before the imaging  
423 experiments started, mice underwent habituation training (see above) for 8–10 days and were  
424 then transferred to the microscope imaging setup. Mice were further acclimated to the imaging  
425 setup for 2 days, receiving free reward (5% sucrose) every 90 seconds for 35 minutes. “Before  
426 learning” recordings were performed on day 1 of the reward learning phase: two mice with  
427 multiple FOVs performed two separate sessions in a single day, separated by a 6-hour interval.  
428 “After reward learning” recordings were obtained on days 18–20 after the mice showed clear  
429 discrimination between the reward-predicting CS-A and the neutral CS-B on the basis of their  
430 anticipatory licking behavior. One FOV was imaged per day. During training, 20 CS-A trials and  
431 10 CS-B trials (5 kHz tone or white noise, 75 dB, counterbalanced) were presented with inter-  
432 trial intervals of 45–75 seconds. On imaging days, 10 CS-A trials and 10 CS-B trials were  
433 presented per session.

434  
435 Fear learning was conducted similarly to the methods stated above, except that tail-shock was  
436 used as the US and imaging was performed on day 2. Tail-shock (0.5 mA for 1 second) was  
437 administered via two pre-gelled electrodes wrapped around the tail and connected to a stimulus  
438 isolator (Isostim A320R, World Precision Instruments), following Kim et al., 2016. Tail-shock  
439

440 was triggered by external transistor-transistor logic pulses generated by ABET II software. Due  
441 to the highly aversive nature of the US, we selected only one FOV per mouse and performed  
442 recordings once for “after fear learning”. During training, 10 CS-A trials and 20 CS-B trials were  
443 presented with inter-trial intervals of 45–75 seconds. On the imaging day, 10 CS-A trials and 10  
444 CS-B trials were presented.

445  
446 This experiment was performed in two cohorts (3 mice excluded before experiments due to  
447 absence of fluorescence signals). All individual mice showed qualitatively similar and replicable  
448 results.

#### 449 ***Fear learning tasks in freely-moving and head-fixed photometry recordings***

450 To examine whether DRN<sup>DA</sup> responses to aversive cues are affected by the external behavioral  
451 context (freely-moving versus head-fixed conditions), we performed photometry recordings in  
452 two different behavioral contexts. All mice underwent identical surgery, water restriction, and  
453 head-fixation habituation procedures before being randomly assigned to either the freely-moving  
454 or the head-fixed group. The fear learning task was slightly different from the ones described  
455 above and was adapted from previous studies (Groessl et al., 2018; Cai et al., 2020). White  
456 noise (20 seconds) was used as the CS. The duration of the CS was set to 20 seconds to better  
457 quantify freezing behavior as an index of learning. For the US, the freely moving group received  
458 foot-shocks within an operant chamber and the head-fixed group received tail-shocks. 6 CS–US  
459 pairs were presented with inter-trial intervals of 60–120 seconds. The next day, a subset of mice  
460 from both groups performed a fear recall experiment. Mice were introduced to a novel cylindrical  
461 cage and allowed to freely explore for about 5 minutes to habituate to the novel context. After  
462 the habituation period, 4 CSs were presented with no US to see if cue-induced freezing  
463 behavior was evoked.

464  
465  
466 This experiment was performed in two separate cohorts (n = 10 and 11 mice each).  
467 Qualitatively similar and replicable results were obtained from both cohorts and across  
468 individual mice.

#### 469 ***Data analysis***

470 ***Behavior:*** For reward learning, we counted the number of anticipatory licks, defined as licks  
471 during the CS presentation before the reward is delivered, as a proxy for learning. For fear  
472 learning in freely-moving conditions, freezing behavior was used as an index of associative  
473 learning and was quantified visually by an observer blind to the experimental condition. For fear  
474 learning in head-fixed conditions (Figure 4), the number of licks was counted throughout the  
475 session. As these mice received 5% sucrose during the habituation procedure, all tested mice  
476 showed continuous licking as soon as they were head-fixed (Figure 4C). We measured whether  
477 this licking behavior was affected by repeated CS–US pairings (Lovett-Barron et al., 2014). We  
478 note that this does not directly reveal whether the animals have learned the association  
479 between the CS and the aversive US *per se*. Therefore, on the next day, mice from both freely-  
480 moving and head-fixed groups performed a fear recall session. We compared their freezing  
481 behavior during the baseline (after 5 min habituation but before presentation of the first CS) with  
482 that during CS presentation to test whether mice could recall the shock-paired cues.

483  
484  
485 ***Fiber photometry:*** Acquired photometry data files were processed with custom-written MATLAB  
486 code, as in previous studies (Lerner et al., 2015; Cho et al., 2017; Robinson et al., 2020).  
487 Signals from 490- and 405-nm excitation wavelengths were low-pass filtered at 2 Hz with zero-  
488 phase distortion. To calculate  $\Delta F/F$ , a least-squares linear fit was applied to the isosbestic signal  
489 and aligned to the GCaMP signal. The fitted signal was subtracted from the 490-nm signal and  
490 subsequently divided by the fitted 405-nm signal. Fluorescence signals were then converted to

491 robust Z-scores in each trial using the median and median absolute deviation (MAD) of the  
492 baseline, defined as a 15-second epoch before the CS presentation. Neural activity was  
493 quantified either by the area under the curve (AUC) per second (Figure 1) or the peak  
494 fluorescence (Figures 2 and 4). AUC per second was used in Figure 1 because there was a  
495 possibility of inhibited or reduced activity (Figure 1 – figure supplement 1; reflected as a  
496 negative AUC value) for paired cues, and indeed our data show small but negative values for  
497 CS-B after extinction learning (Figure 1H). In other cases, neural activity was compared using  
498 the peak fluorescence, since all showed increased fluorescence from baseline upon CS or US  
499 presentation.

500  
501 **Two-photon imaging:** First, the separate imaging files (one file for each trial) were concatenated  
502 in MATLAB and saved as a tiff file. The combined movie was motion-corrected using a non-rigid  
503 registration algorithm in Suite2p (Pachitariu et al., 2017) and saved as another tiff file. This  
504 motion-corrected imaging file was loaded in ImageJ and the regions of interest (ROIs,  
505 corresponding to a single neuron) were manually drawn on the basis of the mean and standard  
506 deviation projection images (McHenry et al., 2017). Fluorescence time-series were extracted for  
507 each ROI by averaging all pixels within the ROI for each frame. To remove potential  
508 contamination from neuropil or nearby dendrites/axons, we extracted the fluorescence from a  
509 ring-shaped region (after enlarging each ROI 1.5 times and excluding the original ROI) and  
510 removing pixels in other ROIs, if any. This neuropil fluorescence was subtracted from the ROI  
511 fluorescence after being scaled by a correction factor (*cf*). Usually the correction factor is  
512 estimated as the ratio of fluorescence intensity between a blood vessel and neighboring non-  
513 ROI neuropil region; however, since we were not able to identify any blood vessels in our  
514 imaging datasets, we adopted a correction factor of 0.6, which is within the range used in  
515 previous studies (Chen et al., 2013; Cox et al., 2016). Therefore, the neuropil-corrected  
516 fluorescence ( $F_{\text{correct}}$ ) was calculated as:  $F_{\text{correct}} = F_{\text{ROI}} - cf \times F_{\text{neuropil}}$ , after  $F_{\text{ROI}}$  and  $F_{\text{neuropil}}$  were  
517 smoothed with a median filter (length: 4). We further normalized the fluorescence in each trial by  
518 calculating the robust Z-score using the median and MAD of the baseline, defined as a 10-  
519 second epoch before CS presentation. Neural activity was quantified for each cell by calculating  
520 the AUC per second during the baseline and the CS presentation.

## 521 522 **Statistical analysis**

523 Sample sizes were determined to be comparable to previous similar studies with calcium  
524 imaging (Groessl., 2018; Lin et al., 2020). Statistical analysis was performed with GraphPad  
525 Prism 8 (GraphPad Software, Inc) or MATLAB (MathWorks). All statistical tests performed and  
526 results are stated in the figure legends and provided in detail in Source Data 1. Statistical tests  
527 were chosen according to the nature of the experiments and datasets. Paired or unpaired t-tests  
528 were performed for single-value comparisons. When analysis of variance (ANOVA; one-way or  
529 two-way repeated-measures) was performed for multiple trials or groups, *post hoc* Sidak's test  
530 was used to correct for multiple comparisons. To examine if the DRN<sup>DA</sup> cue response was  
531 significantly different from baseline at the single-cell level (Figure 3), the Wilcoxon sign-rank test  
532 was used to calculate the p-value for each cell. Then, the false-discovery rate (FDR) correction  
533 was applied ( $q < 0.05$ ) to correct for multiple comparisons. When the response of a neuron was  
534 statistically significant after the FDR correction, the mean value of the cue response was  
535 compared to baseline and declared “significantly increased” if it was larger or “significantly  
536 decreased” if it was smaller. No outliers were removed from any of statistical analyses.

## 537 538 **Histology**

539 Mice were euthanized with CO<sub>2</sub> and transcardially perfused with 15 mL of ice-cold 1x PBS with  
540 heparin (10 U/mL), and then 30 mL of ice-cold 4% PFA. Mouse brains were removed from the  
541 skull and post-fixed in 4% PFA at 4°C overnight. The PFA solution was switched to 1x PBS the

542 next morning. Brains were cut into 50  $\mu\text{m}$  coronal sections with a vibratome (VT1200, Leica  
543 Biosystems). Sections were stored in 1x PBS solution at 4°C until further processing. For  
544 immunohistochemistry, brain sections were first incubated in a 1x PBS solution with 0.1%  
545 Triton-X and 10% normal donkey serum (NDS) with primary antibodies at 4°C overnight. The  
546 next day, sections were thoroughly washed four times in 1x PBS (15 minutes each). Then brain  
547 sections were transferred to 1x PBS with 0.1% Triton-X and 10% NDS with secondary  
548 antibodies and left overnight at 4°C. The next morning, sections were washed as described  
549 above and mounted on glass microscope slides (Adhesion Superfrost Plus Glass Slides, Brain  
550 Research Laboratories). After the sections were completely dry, they were cover-slipped after  
551 applying DAPI-containing mounting media (Fluoromount G with DAPI, eBioscience).  
552 Fluorescent images were obtained with either a confocal (LSM 880, Carl Zeiss) or a  
553 fluorescence microscope (BZ-X, Keyence).

554

555 ***Data and materials availability***

556 Codes for fiber photometry signal extraction and processing are available at  
557 <http://github.com/GradinaruLab/dLight1>. Source data with statistical results is available as a  
558 separate excel file.

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566

567 **Competing Interests**

568 We have no competing interest to declare.

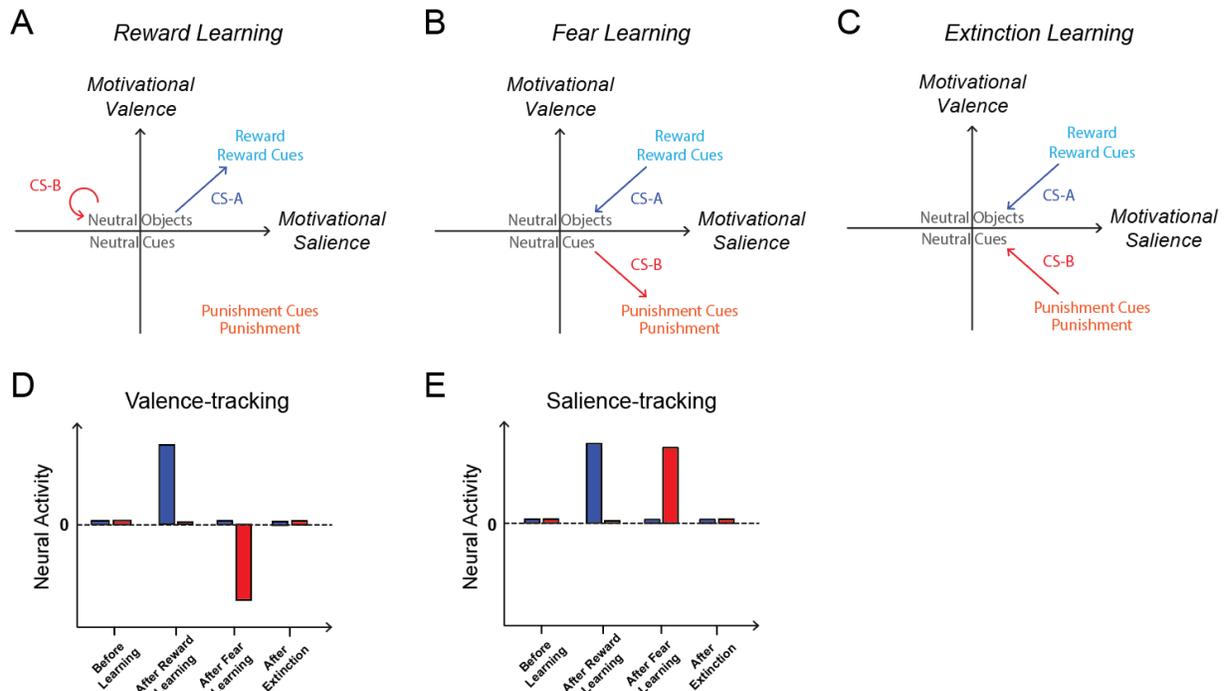
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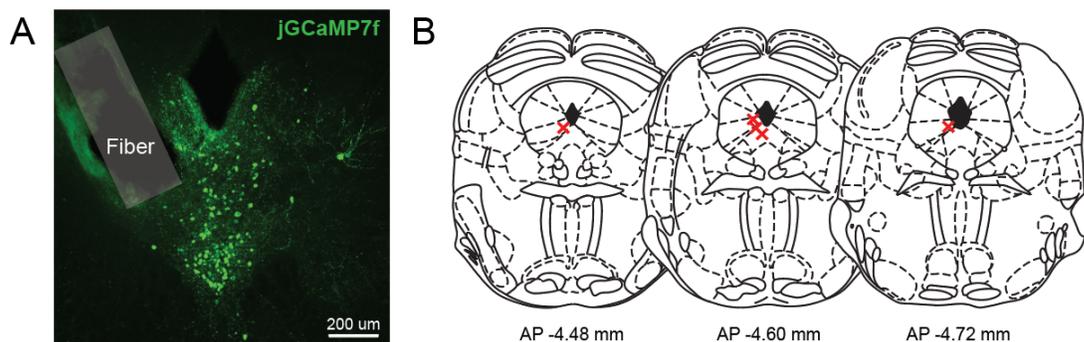
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Figure 1 - supplement 1



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 674 **Figure 1 – figure supplement 1 (A)** In reward learning, the motivational valence and saliency of conditioned  
 675 stimulus A (CS-A) becomes positive as it is paired with reward (appetitive nature). Since CS-B is not paired with an  
 676 unconditioned stimuli (US), its valence and saliency stay close to zero. **(B)** In fear learning, the motivational saliency  
 677 and valence of CS-A, which was previously paired with reward and now has no outcome, decreases back to zero. On  
 678 the other hand, as CS-B now predicts punishment (an aversive US), its valence becomes negative and its saliency  
 679 increases. **(C)** In extinction learning, the motivational valence and saliency of both CS-A and CS-B return to zero as  
 680 they no longer predict any US. **(D)** In theory, neurons that track motivational valence, such as DA neurons in the  
 681 lateral VTA or those projecting to the nucleus accumbens lateral shell (Matsumoto and Hikosaka, 2009; Martin-  
 682 Bromberg et al., 2010; de Jong et al., 2019), should show increased activity to reward-paired cues after reward  
 683 learning and decreased activity to shock-paired cues after fear learning, compared with baseline or before learning.  
 684 These changes in activity should both be reduced to close to baseline after extinction learning. **(E)** Neurons that track  
 685 motivational saliency should show increased activity to both reward-paired and shock-paired cues, after reward and  
 686 fear learning respectively, and return to close to baseline after extinction learning.  
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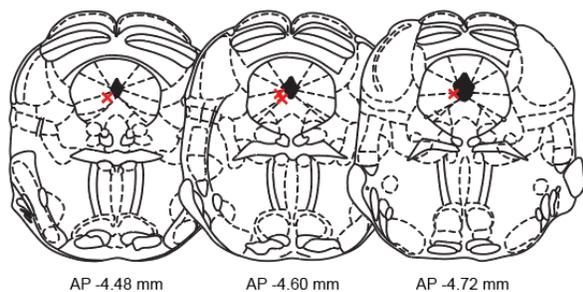
Figure 1 - supplement 2



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 689 **Figure 1 – figure supplement 2 (A)** A representative histological image of jRCaMP7f-expressing DRN<sup>DA</sup> neurons  
 690 showing the location of the photometry fiber tip. **(B)** Schematic of the anatomical locations of individual fiber implants  
 691 in the 5 mice used in the experiments shown in Figures 1 and 2.  
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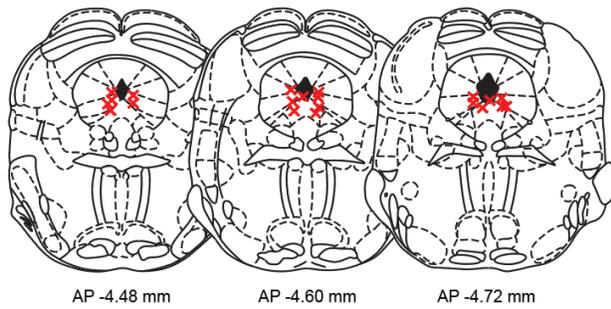
Figure 3 - supplement 1



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**Figure 3 – figure supplement 1** Schematic of the anatomical locations of GRIN lens implants in the 4 mice used in the experiments shown in Figure 3.

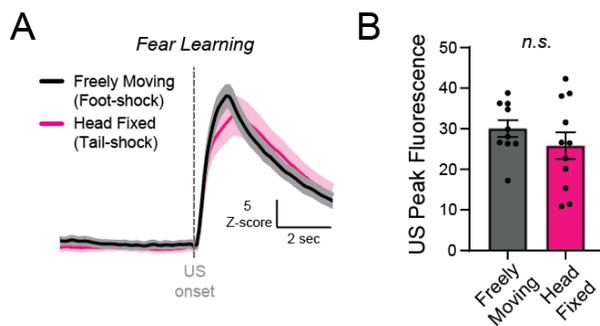
Figure 4 - supplement 1



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**Figure 4 – figure supplement 1** Schematic of the anatomical locations of the optical fiber implants in the 21 mice used in the experiments shown in Figure 4.

Figure 4 - supplement 2



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**Figure 4 – figure supplement 2 (A)** Photometry responses to foot-shocks (in freely-moving mice, black) and tail-shocks (in head-fixed mice, magenta), averaged across all trials and mice. **(B)** Responses to foot-shocks (freely-moving mice, black) and tail-shocks (head-fixed mice, magenta) were not significantly different ( $n = 10$  freely-moving mice,  $n = 11$  head-fixed mice; unpaired t-test;  $t_{19} = 1.056$ ,  $p = 0.3041$ ). Data are presented as the mean  $\pm$  S.E.M.

Figure 1, Cho et al. 2020

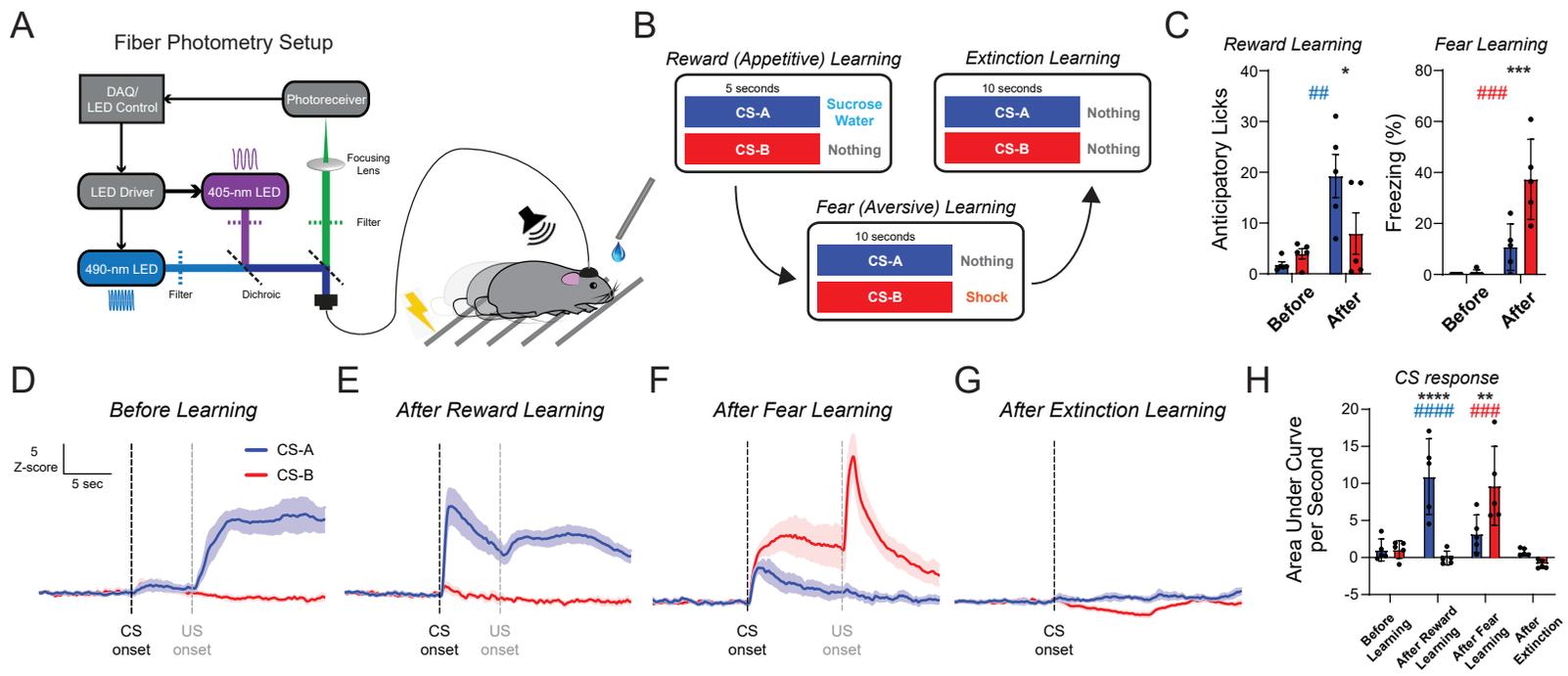


Figure 1 - supplement 1

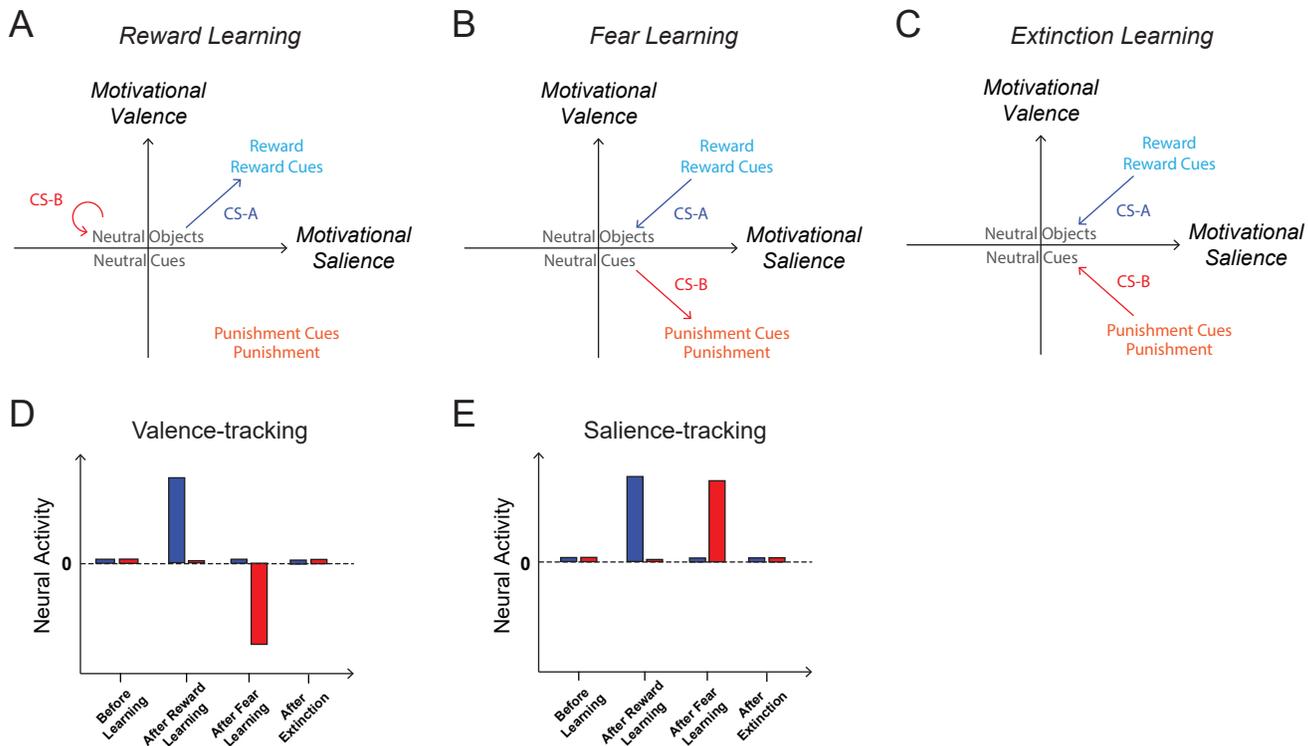
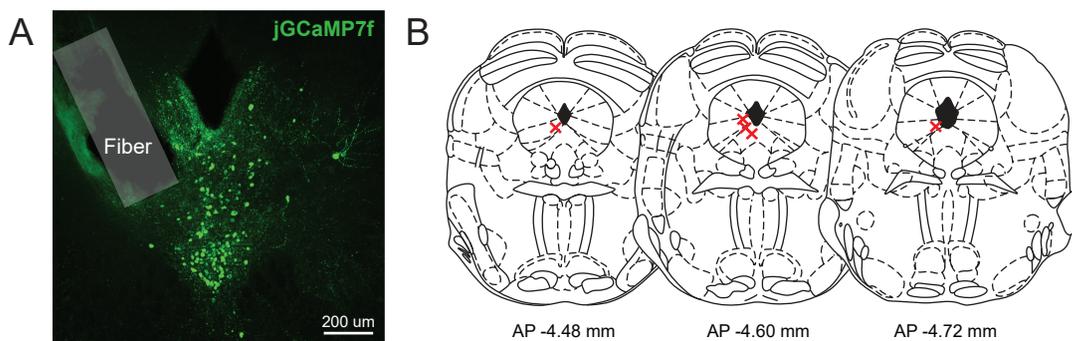
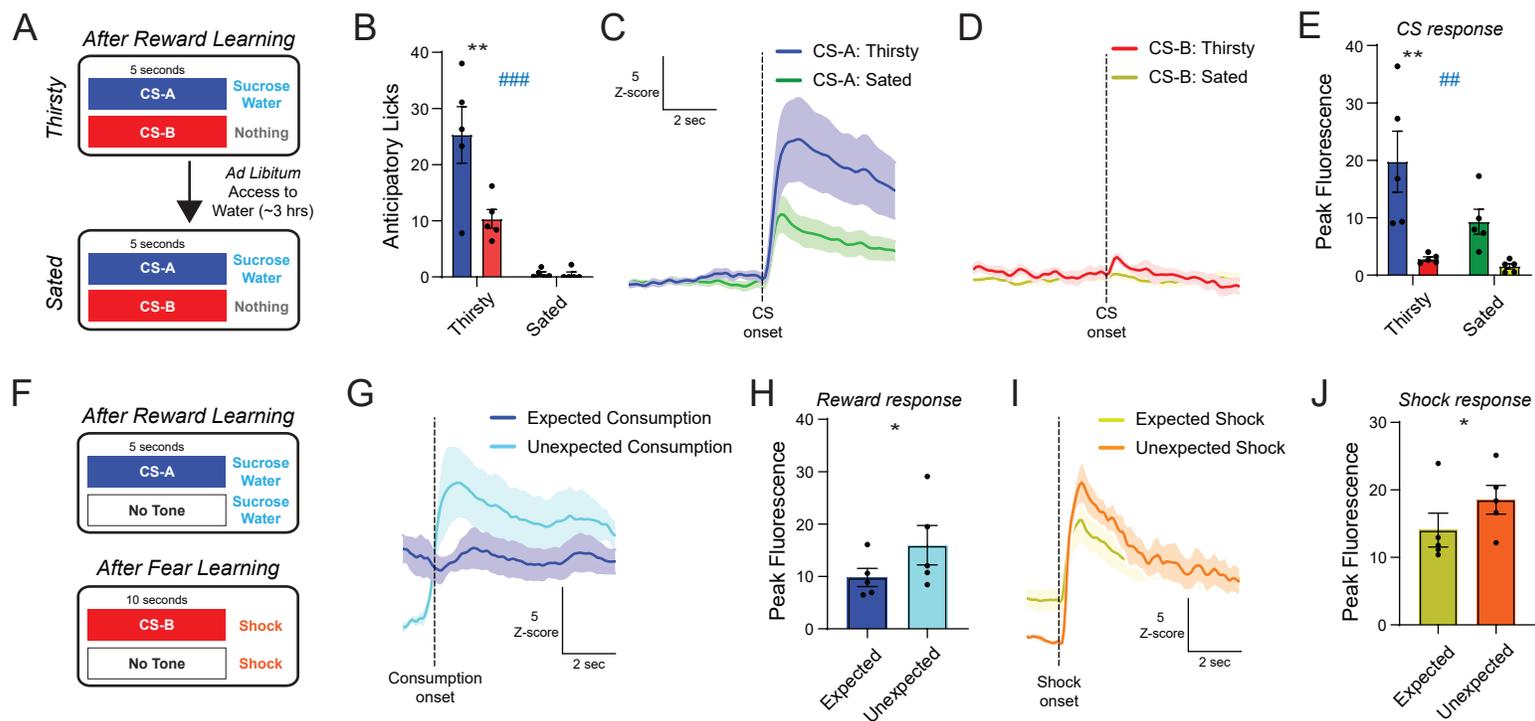


Figure 1 - supplement 2





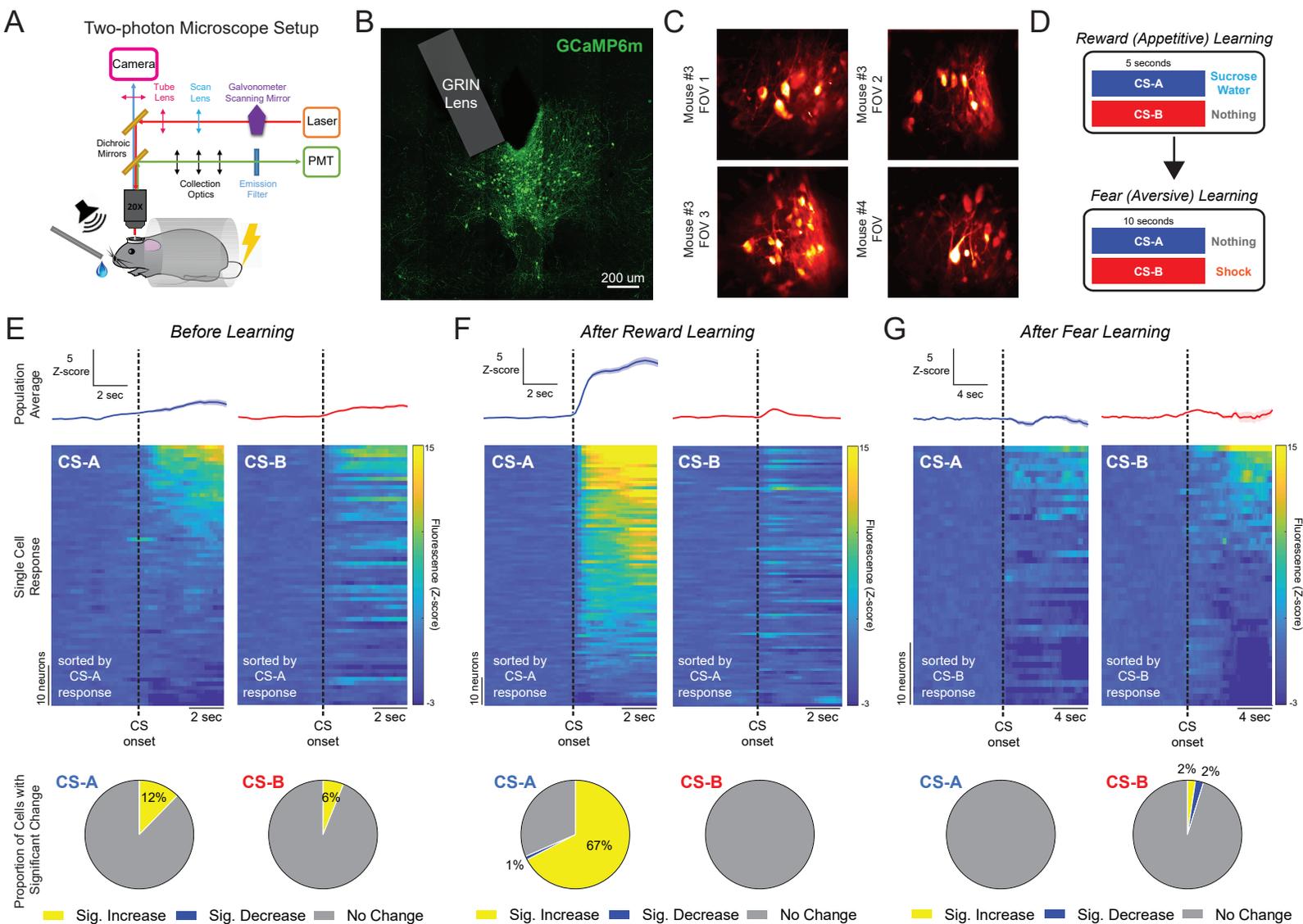
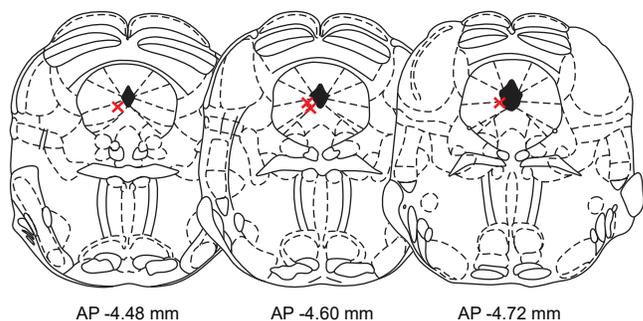


Figure 3 - supplement 1



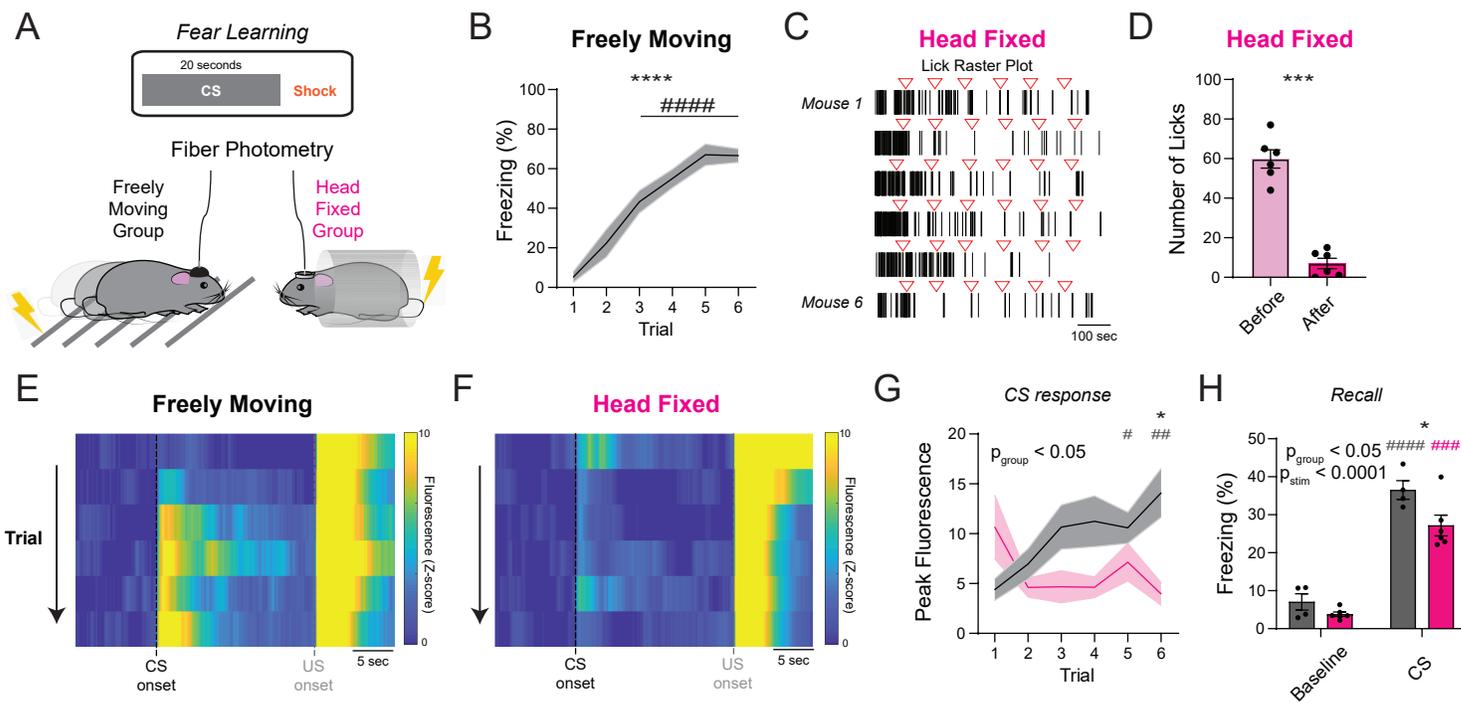


Figure 4 - supplement 1

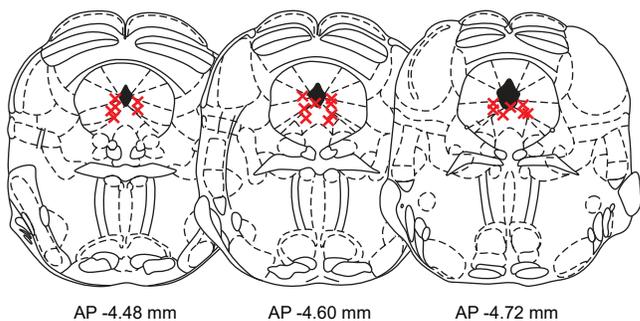


Figure 4 - supplement 2

