

1 **A function for the thalamo-habenula projection**

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22 **Abstract**

23 The thalamus mediates diverse brain functions including arousal, perception and
24 memory formation, and is characterized by widespread connectivity. One thalamus
25 projection that is evolutionarily conserved is to the habenula, a regulator of broadly
26 acting neuromodulators. The function of this projection is unknown, as the information
27 transmitted has not been identified. By two-photon calcium imaging of larval zebrafish,
28 we show that change in irradiance triggers dynamic responses in the habenula.
29 Neuroanatomical tracing shows that the dorsal left habenula neuropil, which has the
30 most prominent response to irradiance change and influences irradiance-dependent
31 behavior and raphe response, is innervated by retinorecipient nuclei in the thalamus.
32 Optogenetic stimulation of the thalamus triggers habenula activity, while lesion reduces
33 light-evoked habenula activity. These data establish that the thalamus controls habenula
34 activity following irradiance change, thereby suggesting that the thalamo-habenula
35 projection provides a pathway for visual stimuli to affect neuromodulator release and
36 behavior.

37 **Introduction**

38 The vertebrate thalamus receives information from virtually all regions of the brain
39 including sensory systems, motor systems, basal ganglia and cerebellum, and in turn
40 projects to multiple structures (Ward, 2013; Mitchell et al., 2014). It modulates the flow of
41 sensory information to the cortex (Sherman and Guillery, 2002; Lee and Dan, 2012;
42 Mitchell et al., 2014; Wimmer et al., 2015) and is considered a gateway to
43 consciousness (Newman, 1995; Crick and Koch, 2003). Connectivity with the amygdala
44 (Romanski and LeDoux, 1992) and nucleus accumbens (Cho et al., 2013) underlie roles
45 in emotion and reward processing, while connectivity with the hippocampus is essential
46 for memory formation (Aggleton et al., 2010) and navigation (Jankowski et al., 2013). A

47 less well known feature of thalamus connectivity is its innervation of the habenula
48 (Marburg, 1944; Cragg, 1961; Díaz and Puelles, 1992). The function of this projection is
49 unknown, as the information relayed from the thalamus to the habenula has not been
50 identified.

51 The habenula is an evolutionarily conserved structure (Stephenson-Jones et al.,
52 2012) that controls the release of broadly-acting neuromodulators such as serotonin,
53 dopamine, epinephrine and histamine (Wang and Aghajanian, 1977; Morley et al., 1985;
54 Jhou et al., 2009; Quina et al., 2014). As neuromodulators shape functional connectivity
55 in neural circuits (Getting, 1989; Marder, 2012; Bargmann and Marder, 2013), the
56 habenula can be viewed as a regulator of brain mode - defined as pattern of functional
57 connectivity (Getting, 1989). This role of the habenula is reflected by its involvement in
58 multiple behaviors, ranging from the control of fear (Agetsuma et al., 2010; Lee et al.,
59 2010; Zhang et al., 2016), to learning (Matsumoto and Hikosaka, 2007; 2009; Amo et al.,
60 2014), addiction (Fowler et al., 2011), sleep (references), and performance under stress
61 (Thornton and Davies, 1991) .

62 The habenula regulates neuromodulators based on inputs reflecting reward value,
63 internal state and sensory stimuli. The entopeduncular nucleus, or internal segment of
64 the globus pallidus, provides excitatory input in response to negative reward (Hong and
65 Hikosaka, 2008). Internal states such as circadian clock, may be conveyed from the
66 hypothalamus, from example by hypocretin-secreting neurons (Appelbaum et al., 2009).
67 Odor-evoked activity in the habenula, which has been documented in the zebrafish
68 (Krishnan et al., 2014), is mediated by a direct input from the olfactory bulb (Miyasaka et
69 al., 2009). Light-evoked activity has been detected in the habenula in rat (Zhao and
70 Rusak, 2005), pigeon (Semm and Demaine, 1984) and zebrafish (Dreosti et al., 2014).
71 Reduction in irradiance has also been shown to affect the habenula (Portugues et al.,

72 2014; Bianco and Engert, 2015). How changes in irradiance affect the habenula is
73 unclear.

74 The thalamus receives input from all sensory systems, except the olfactory system.
75 It is thus possible that the pathway from the thalamus to the habenula functions to
76 control habenula activity in response to change in irradiance. Here we provide evidence
77 for this hypothesis. In doing so, this paper identifies one function of the thalamus-
78 habenula projection.

79 **Results**

80 **The habenula displays dynamic response to irradiance change**

81 The zebrafish habenula consists of neurons surrounding neuropils that are
82 innervated by afferent neurons (Hendricks and Jesuthasan, 2007; Miyasaka et al., 2009;
83 Amo et al., 2014). To identify the pathways by which change in irradiance influences the
84 habenula, we first characterized habenula activity evoked by these changes. Two-
85 photon imaging was performed on a transgenic zebrafish line expressing the calcium
86 indicator GCaMP3 in the habenula (Krishnan et al., 2014) (Figure 1A). Resonant-
87 scanning, combined with piezo-driven focusing, was used to record the activity of cells at
88 multiple focal planes throughout the habenula (Figure 1B, C). With a step size of 10 μm ,
89 so that each cell would be sampled only once, most of the habenula could be covered
90 with 5 planes at a rate of 1 Hz. Habenula activity was monitored as the larva was
91 exposed to discrete pulses of blue light. Pixel-wise analysis in one fish indicates that
92 evoked activity – both transient and sustained - occurred throughout the habenula in
93 response to both increase and decrease in irradiance (Figure 1D, E). The spatio-
94 temporal pattern of activity was reproducible across several cycles, as shown by the
95 trajectory of the system through state space (Figure 1F).

96 To assess if responses were reproducible across multiple fish, we imaged the
97 habenula in 6 fish. Habenula neurons were segmented (Figure 2A-C; total of 4986 cells,
98 with an average of 831 ± 53 cells (95% CI) per fish) and their activity was clustered by *k*-
99 means. Cluster centers were classified by response type. Transient and sustained
100 responses to increase and decrease in irradiance could be reliably evoked (Figure 2D-F).
101 The percentage of responding cells per fish ($\pm 95\%$ CI) were ON: $30.98 \pm 9.94\%$; OFF:
102 $19.03 \pm 3.93\%$; Inhibitory: $7.93 \pm 3.50\%$. Correlating the cells corresponding to the
103 different response types revealed that, in general, neurons that were excited by an
104 increase of irradiance did not fire to a decrease (Figure 2G, H). Some neurons that were
105 inhibited by light did, however, show a response at light offset (Figure 2F, blue trace; see
106 also Figure 1E, red trace). These observations confirm that the activity of zebrafish
107 habenula neurons is affected by change in irradiance, and that in addition to excitation
108 there is inhibition by light, as well as excitation to loss of light.

109 **Activity occurs prominently in the dorsal left neuropil**

110 To obtain a more detailed view of light evoked activity, we used higher speed
111 widefield microscopy. With imaging at 20 Hz, responses was detected in multiple regions
112 of the habenula at the onset of the LED used to induce fluorescence of the reporter
113 (Movie 1). One region with a reproducible increase in fluorescence was the dorsal left
114 neuropil (Figure 3A-C; $n = 6$ fish). The calcium indicator R-GECO (Zhao et al., 2011),
115 which has a faster rise time than GCaMP3 (approx 10 msec half rise time (Akerboom et
116 al., 2013)) and is more sensitive (Walker et al., 2013), also showed a reproducible strong
117 rise in the dorsal left neuropil (Movie 2; Figure 3D-H; $n = 3$ fish). As the calcium indicator
118 is expressed in habenula neurons in both these lines, this neuropil response probably
119 occurs in dendrites, implying that at least some information about irradiance change is
120 provided by afferents to the dorsal left neuropil.

121 To further explore this, we performed imaging in the *Tg(elavl3:GCaMP6f)* line,
122 which has broad expression of a calcium-reporter, and thus likely also in afferent
123 neurons. A prominent increase in fluorescence was detected in neuropil of the dorsal left
124 habenula (Movie 3; Figure 3I-K). This was seen in all fish imaged ($n = 3$) and may reflect
125 activity in terminals of afferent neurons. In addition to the asymmetric habenula neuropil
126 response, bilateral increase was seen more posteriorly, within the thalamic/pretectal
127 area (Fig. 3J). This latter symmetry argues against the habenula response being a result
128 of asymmetric expression of the reporter. Rather, the pattern of increase in the habenula
129 suggests that information regarding increase in irradiance is conveyed asymmetrically to
130 the left dorsal neuropil.

131 We asked what role activation of the dorsal left neuropil might have. Larval
132 zebrafish show a strong preference for light over darkness (Steenbergen et al., 2011),
133 which appears to involve the dorsal raphe (Cheng et al., 2016). Lesioning the dorsal
134 neuropil of the left habenula with the two-photon laser (Supplemental Figure 1A-C) led to
135 a reduction in this preference (Supplemental Figure 1D-F; $p = 0.0086$ Mann-Whitney's U
136 test; effect size $r = 0.413$). This manipulation also led to an alteration of raphe response
137 to change in irradiance (Supplemental Figure 1G-K). Instead of the inhibition to increase
138 in irradiance in the anterior raphe, there was an excitation. These observations indicate
139 that the dorsal left neuropil influences irradiance-dependent neuromodulator release and
140 behavior.

141 **The thalamus provides a source of input to the dorsal left neuropil**

142 The entopeduncular nucleus (EN) is the major source of habenula afferents in
143 teleosts (Yañez and Anadon, 1994), including zebrafish (Amo et al., 2014). This nucleus
144 is labeled in the *Et(sqKR11)* line (Lee et al., 2010), providing a simple way of visualizing

145 EN afferents to the habenula. Some labeled fibers were detected in the dorsal left
146 neuropil, indicating that the EN does provide innervation to this region of the habenula
147 (Figure 4A). To test for additional input sources, the lipophilic tracer DiD was injected
148 into the dorsal left neuropil (n = 6 fish). In all cases, neurons in the dorsal left habenula
149 (which extend dendrites into the neuropil), the parapineal, and a thalamic nucleus
150 located ventrally to both habenula (Figure 4B-D) were labelled. DiD label was not
151 detected in any other regions of the brain, and only rarely in the entopeduncular nucleus
152 (Figure 4C), suggesting that the thalamus is the major source of input to the dorsal left
153 neuropil. The label in the thalamus cannot represent anterograde label from the
154 habenula, as tracing of projections from the habenula by expressing fluorescent proteins
155 specifically in the habenula does not result in a projection to the thalamus (Movie 4).
156 Moreover, the labeling of cell bodies in the thalamus (Figure 4D inset, E) indicates that
157 this is likely to be a retrograde label. The neuropil of this thalamic nucleus contains
158 terminals of retinal ganglion cells, as shown by Dil injection into the retina (Figure 4E, F).
159 Based on the location of these terminals relative to the optic tract, these terminals make
160 up AF2 and AF4 (Easter and Nicola, 1996; Robles et al., 2014). Thus, the habenula
161 neuropil with the initial response to light is innervated by thalamic nuclei that receive
162 retinal input.

163 **The habenula may receive glutamatergic and GABAergic input from the thalamus**

164 Light caused both excitation and inhibition in zebrafish habenula neurons, implying
165 that there may be excitatory and inhibitory afferents. Using an antibody to vGlut1/2,
166 glutamatergic pre-synapses were detected in all neuropils of the habenula, including the
167 dorsal left neuropil (Figure 4G), indicating the existence of excitatory afferents.
168 GAD65/67 labeled puncta could also be detected in the dorsal left neuropil (Figure 4H).
169 Label was seen also in other neuropils, while in the lateral regions, corresponding to the

170 ventral habenula, streaks were detected adjacent to cell bodies. These labels were not
171 located within habenula neurons, as they did not co-localize with cytoplasmic label
172 provided by GCaMP3, nor did they fill the cytoplasm, implying that these puncta and
173 streaks must reside in habenula afferents (i.e. axon terminals). Labeled cell bodies were
174 seen below the level of the habenula [see Movie 5]. Consistent with this, inhibitory
175 neurons could be detected in the dorsal thalamus using the transgenic line
176 *Tg(gad1b:RFP, vGlut2a:GAL4, UAS:eGFP)* (Satou et al., 2013) (Figure 4I, J; Movie 6).
177 No label was seen in the entopeduncular nucleus, which has previously been shown to
178 be glutamatergic (Amo et al., 2014). These observations confirm that the thalamus
179 contains both GABAergic and glutamatergic neurons, as described previously (Mueller,
180 2012), which may mediate light-evoked excitation and inhibition of habenula neurons.

181 **Irradiance change evokes activity in thalamus neurons**

182 If the thalamus provides afferents mediating illumination-dependent information to
183 the habenula, there should be responses to increase and decrease of illumination in
184 thalamic neurons. To test this, calcium imaging was carried out using a driver line with
185 GAL4 expression in the thalamic neurons that innervate that habenula (Figure 5A, B). A
186 response to increase and decrease in irradiance was detected in scattered cells in the
187 anterior thalamus (Figure 5C-I) in all fish imaged (n = 5, Figure 5 J,K). The response to
188 increase in irradiance was more prominent in the dorsal region of the neuropil (cyan
189 pixels; Figure 5C-E, 5J), whereas response to decrease (magenta pixels) was more
190 prominent ventrally (Figure 5K). Terminals of neurons within the habenula also showed a
191 response (Figure 5L-O). These observations indicate that change in irradiance leads to
192 activity in thalamic neurons innervating the habenula, with different subsets responding
193 to increase versus decrease.

194 **Optogenetic stimulation of the thalamus drives habenula activity**

195 To test the ability of thalamic neurons to drive activity in the habenula, we used
196 optogenetics. Channelrhodopsin-2 (ChR2) was expressed in thalamic neurons using the
197 *s1020tGAL4* driver (Figure 6A). Experiments were carried out on fish lacking eyes, to
198 prevent a response to visual stimulation. Short pulses of blue light reproducibly caused
199 an increase in fluorescence of GCaMP6f in habenula neurons of fish with expression of
200 ChR2 in the thalamus (Figure 6B-E, G). A small response was seen in fish without ChR2
201 expression (Fig. 6F), however, implying that some habenula response may be due to
202 deep brain photoreceptors (Fernandes et al., 2013). The larger response in fish with
203 ChR2 expression suggests that neural activity in thalamic neurons can elicit a response
204 in the habenula.

205 **Thalamic lesion inhibits habenula response to illumination change**

206 To test the role of the thalamus pathway in light-evoked activity in the habenula,
207 we lesioned the thalamus neuropil with a two-photon laser. The laser was targeted to the
208 region of AF4 that showed excitation to light ON (Figure 7A-C). This manipulation is
209 expected to damage fibers innervating this neuropil (Semmelhack et al., 2014).
210 Following lesion, there was a reduction of evoked activity in the habenula (Figure 7D-F,
211 I). There was some variability in the effect, possibly reflecting the limitations of this
212 technique in enabling consistent ablation of the entire neuropil. Lesioning other targets
213 of retinal ganglion cell axons did not lead to a loss of light-evoked activity (Figure 7G, I).
214 These observations support the hypothesis that a pathway involving the thalamic
215 neuropil AF4 has a role in light-evoked activity in the habenula.

216 **Discussion**

217 This paper identifies one function of the projection from the thalamus to the
218 habenula. By calcium imaging, we found that increase and decrease in irradiance

219 causes activity throughout the habenula. A strong increase in the fluorescence of
220 calcium reporters was detected in the neuropil of the dorsal left habenula. Although
221 calcium indicators are only a proxy for neural activity, i.e. reflecting the opening of
222 voltage sensitive calcium channels, and have slow dynamics, they can indicate the
223 number of spikes (Chen et al., 2013). The strong fluorescence in the dorsal left neuropil
224 may thus reflect relatively high spiking rate of afferents following change in irradiance.
225 These afferents appear to derive primarily from the thalamus. Lipophilic tracing and
226 labeling of thalamic neurons with the *GAL4s1020t* driver demonstrate that the thalamus
227 directly innervates the zebrafish habenula. Optogenetic stimulation of the thalamus led
228 to activity in the habenula, while lesion inhibited light-evoked activity. Thus, by calcium
229 imaging, anatomical tracing and manipulation, our data demonstrates that innervation
230 from the thalamus enables habenula responses to irradiance change.

231 The region of the thalamus mediating activity in the habenula can be functionally
232 separated into two domains, based on the response to light – excitation to light OFF in
233 the ventral regions and excitation to light ON more dorsally. The neuropil of the thalamus
234 contains two previously defined targets of retinal ganglion cells - AF2 and AF4 (Burrill
235 and Easter, 1994), and would thus correspond to first-order nuclei. AF4 is innervated
236 predominantly by M3 and M4 retinal ganglion cells (Robles et al., 2014), which extend
237 their dendritic tree into the proximal layer of the inner plexiform layer and are considered
238 ON neurons. AF2 is innervated by B1 retinal ganglion cells that have dendrites in the
239 distal layer (Robles et al., 2014), and these may account for the OFF responses in the
240 thalamus and habenula. The distinct domains suggest that the habenula is innervated by
241 two retino-recipient thalamic nuclei; these nuclei may also receive input from non-retinal
242 sources, but this remains to be investigated.

243 Light is a potent regulator of brain function – it can affect mood (Vandewalle et al.,

244 2010), alertness (Badia et al., 1991), cognitive ability (LeGates et al., 2012) and
245 movement (Aschoff, 1960; Burgess and Granato, 2007). These phenomena are
246 sensitive to irradiance, not image formation, and are mediated by a number of sensors
247 including intrinsically-sensitive retinal ganglion cells (Hattar et al., 2003), and deep brain
248 detectors (Fernandes et al., 2012; Kokel et al., 2013). The ability of light to affect normal
249 movement patterns (Burgess et al., 2010) or to disrupt mood and cognition (LeGates et
250 al., 2012) involves neuromodulators such as serotonin, and changing irradiance affects
251 activity in the dorsal raphe (Fite et al., 2005; Cheng et al., 2016). Based on the data
252 here, and the well-established roles of the habenula in regulating neuromodulators, we
253 suggest that some of these effects of light may be mediated by the thalamic projection to
254 the habenula.

255 **Materials and Methods**

256 **Fish lines**

257 Experiments were performed in accordance with guidelines issued by the
258 Institutional Animal Care and Use Committee of the Biological Resource Centre at
259 Biopolis, Singapore. Zebrafish (*Danio rerio*) lines used for this study were:
260 *Tg(UAS:GCaMP6s)sq202*, *SqKR11Et*, *GAL4s1011t*, *GAL4s1020t*, *Tg(UAS-R-GECO)*,
261 *Tg(UAS:GCaMP3)sq200*, *Tg(elavl3:GCaMP6f)a12200*, *Tg(UAS:ChR2-eYFP)*(Arrenberg
262 et al., 2009) and AB wildtype.

263 *Tg(elavl3:GCaMP6f)a12200* was generated by PCR amplification of the GCaMP6f
264 open reading frame (Addgene plasmid 40755 (Chen et al., 2013)) with forward primer
265 ataACTAGTgccaccATGGGTTCTCATCATCAT and reverse
266 ataCCGCGGcTCACTTCGCTGTCATCATTTGTAC (restriction site and coding
267 sequences are in upper case). This fragment was cloned into a plasmid with Tol2 arms

268 flanking an upstream attR1-R2 cassette and the insertion site using restriction enzymes
269 SpeI and SacII. Previously described *elav/3* (*HuC*) *cis*-regulatory elements (Higashijima
270 et al., 2003) were placed upstream via LR recombination (Invitrogen) with an attL
271 flanked *elav/3* entry clone. The resulting plasmid was then co-injected into 1-cell stage
272 embryos at a concentration of 30 ng/ μ L with Tol2 transposase mRNA at a concentration
273 of 30 ng/ μ L. A single founder was selected based on high and spatially broad expression.
274 Outcrossing this founder generated 50% GCaMP6f-positive embryos, which were
275 selected to establish the line.

276 **Imaging**

277 Zebrafish larvae (aged 5 - 10 dpf) were anaesthetized in mivacurium and
278 embedded in low-melting temperature agarose (1.2-2.0 % in E3) in a glass-bottom dish
279 (Mat Tek). They were imaged on a Nikon two-photon microscope (A1RMP), attached to
280 a fixed stage upright microscope, using a 25x water immersion objective (NA = 1.1). The
281 femtosecond laser (Coherent Vision II) was tuned to 920 nm for GCaMP imaging. Stacks
282 were collected in resonant-scanning mode with a 525/50 nm bandpass emission filter
283 and with 8x pixel averaging; single-plane images were collected in galvano-scanning
284 mode with 2x pixel averaging. The sample size was based on (Dreosti et al., 2014).

285 Light stimuli were generated by 5 mm blue LEDs (458 nm peak emission). They
286 were powered by a 5 V TTL signal from a control computer and synchronized with image
287 capture using a National Instruments DAQ board, controlled by the Nikon Elements
288 software. Light intensity at the sample was 0.13 mW/cm².

289 For widefield microscopy, excitation was provided by LEDs (Cairn OptoLED) at
290 470 nm. Images were captured on a Zeiss Axio Examiner with a 40x water immersion
291 objective, using an Evolve camera (Photometrics) or a Flash4 camera (Hamamatsu).

292 After background subtraction, ratio images were obtained by dividing each frame against
293 the first frame of this time-stack using Fiji. A mask, created with Fiji, was used to isolate
294 the fluorescent structures. Movie 2 was made using the ratio command in MetaMorph,
295 after background subtraction.

296 **Data analysis**

297 **Data Preprocessing:** Raw images obtained were first registered to correct for any
298 vertical/horizontal movement artifacts using cross correlation. In case of high speed data
299 using a resonant scanner, a median filter of size 3 was applied to remove noise. A
300 darker region outside the region of interest was chosen as the background and
301 subtracted from the image to remove any background noise. Non linear trends in the
302 data were detrended using polynomials of order 2-5. Data was then normalized into Z-
303 scores by subtracting the overall mean and dividing by the standard deviation. A rolling
304 window average was then used to smooth noisy traces where necessary.

305 **Analysis of Habenula and Thalamus:** The Thunder platform (Freeman et al.,
306 2014) was used for fast pixel based clustering and factorization. Principal Component
307 Analysis (PCA) was used to obtain a low dimensional representation. The number of
308 principal components was identified based on the total variance explained. *K-means*
309 clustering was performed to identify pixels with similar responses profiles. The number of
310 clusters (k) was chosen such that cluster members were highly correlated with the
311 cluster centroid and to each other after multiple iterations. Details for different data sets
312 are given below.

313 Figure 1D-E shows the temporal and spatial distribution of responses to change in
314 irradiance and is not intended for cell segmentation. Since pixel based analysis are
315 sensitive to noise, images were first run through a median filter of kernel size 5 and time

316 traces were detrended and smoothed before clustering was performed. The number of
317 clusters were chosen to reveal as many stimulus related clusters as possible, until there
318 was little change in the number and types of stimulus related clusters and increase in
319 noise related clusters. Noise clusters were then removed from the spatial and temporal
320 plots for clarity. After multiple runs, *k*-means was performed for $k=10$. 6 clusters were
321 light related and are shown in Figure 1D-E, 4 noisy clusters were removed. Similarly, for
322 Figures 5C-I, *k*-means was run on 6 focal planes obtained from the thalamus with $k=8$, 4
323 light related clusters are plotted in Figure 5I, 4 were removed.

324 For Figures 2D-F, *k*-means was performed from cells segmented by a semi
325 automated algorithm (see below). The purpose is to show heterogeneity of temporal
326 responses to changes in irradiance, accurately classify cells into ON, OFF and Inhibitory
327 responses and perform correlation between them. Analysis was done on traces from
328 4986 cells from 6 fish. Traces were detrended, smoothed and normalized to z-scores
329 using baseline as the time before the first blue light. Traces that did not reach a Z-score
330 of 2 during the period of irradiance change were classified as not having an evoked
331 response and not included in the clustering analysis. 2456 of 4986 cells were thus
332 removed. *K*-means was first run with an arbitrary $k = 60$. This generated a wide range of
333 clusters capturing the temporal heterogeneity of the responses. The clusters were then
334 divided into ON, OFF, Inhibitory and No response. Neurons belonging to each cluster
335 were correlated among each other and to the cluster centroid. Neurons with low
336 correlation (<0.6) were inspected, if they had no evoked response, they were discarded.
337 If they had an evoked response, they were correlated with other clusters and assigned to
338 one with the highest correlation. 138 such cells were reclassified.

339 For data following AF4/AF9/AF7 lesion (Figure 7), *k*-means was performed to
340 differentiate responses between the controls and the lesion. Hence, number of clusters

341 were chosen such that cluster centroids adequately differentiated responses before and
342 after lesion. Cluster centroids not responding to light were also plotted here.

343 Terminals of thalamic afferents in neuropils of the habenula were isolated pixels in
344 the image that showed increases in calcium to changes in irradiance. For Figure 5 L-O,
345 recordings from two fish were registered and averaged. The images were then
346 thresholded to find pixels with high standard deviation and those corresponding to ON
347 and OFF were plotted.

348 For images obtained with widefield microscopy, which included out-of-focus
349 information, non-negative matrix factorisation (NMF) was used for accurate source
350 separation and identifying temporal differences in regions of the habenula. NMF
351 factorizes a non-negative matrix V , into two non-negative matrices W and H (Maruyama
352 et al., 2014). For Figure 3G-H, 4 recordings from the same fish were registered (using
353 TurboReg in ImageJ) and NMF was run on a dataset formed by averaging the
354 recordings and filtering the resulting images with a median filter of window 5. The
355 algorithm was run multiple times with different number of components k , ensuring
356 convergence of at least 0.01 in H . Finally, $k=6$ was selected for its accuracy in picking
357 the different temporal profiles when compared to manual inspection.

358 The scripts used for analysis are provided at <http://dx.doi.org/10.5061/dryad.q0171>.

359 **Segmentation of Region of Interest (ROI):** Each stack was scaled 2x in imageJ,
360 then maximally projected to a single image, which was then subjected to a minimum filter
361 and unsharp mask to sharpen the boundary of cells. ROIs were identified using the “find
362 maxima...” command, as a way to localize regional darkest point as the center of each
363 ROI. The boundary of the ROI was outlined by “analyze particle...” that connects bright
364 pixels into mosaic-like tessellated plane, encircling each darkest point. Each ROI was

365 then numbered sequentially using the ImageJ ROI Manager and mapped back to the
366 original despeckled image stack. Manual segmentation was done here to delete
367 extraneous ROIs outside the habenula and to encircle cells that were not detected by
368 the algorithm (<10% of total ROIs). In the last step, “Set measurements...” and “measure”
369 in ImageJ provided the mean fluorescence value of all pixels within each ROI across the
370 entire image stack and the x-y coordinates of each ROI. Time-lapse series in which z
371 drifting occurred were excluded, as in this case ROIs could not be defined.

372 **Ratio images:** Following background subtraction, a ratio of each plane relative to
373 the first plane was obtained using FIJI. A median filter (radius = 1.0 pixels) and a mask
374 was then applied.

375 **Neural tracing**

376 DiD (Life Technologies) was dissolved in 50 µl ethanol to make a saturated
377 solution. This was heated to 55°C for 5 minutes prior to injection into the fish that had
378 been fixed in 4% paraformaldehyde. Fish were mounted in 1.2% low melting
379 temperature agarose dissolved in PBS. The dye was pressure injected into the habenula
380 under a compound microscope (Leica DM LFS), using a 20X water immersion objective.
381 For labeling the retina, a saturated solution of Dil in chloroform was used. Injections
382 were carried out under a stereo microscope (Zeiss Stemi 2000). After injections, fish
383 were stored at 4°C overnight to allow tracing, and then imaged with a 40x water
384 immersion objective on a Zeiss LSM 710 confocal microscope.

385 **Antibody label**

386 Larvae were fixed in 4% para-formaldehyde/PBS overnight at 4°C. They were then
387 rinsed in PBS. The brains were dissected out, and permeabilized using 1% BSA

388 (fraction V; Sigma), 0.1% DMSO and 0.1% Triton X-100. The antibodies used here, anti-
389 vGlut1/2 (Synaptic Systems 135503, RRID:AB_1279466; 1:100) and anti-GAD65/67
390 (Abcam ab11070, RRID:AB_297722; 1:500), have previously been used in zebrafish
391 (Wyart et al., 2009; Lee et al., 2010). The brains were incubated in the primary antibody
392 overnight, rinsed several times in PBS, then incubated in secondary antibody (Alexa 488
393 goat anti-rabbit; 1:1000). After washing, these were mounted in 1.2% agarose/PBS.
394 Imaging was carried out using a Zeiss LSM 510 laser scanning confocal microscope,
395 with a 40x water immersion objective.

396 **Optogenetic stimulation**

397 *5 dpf Tg(s1020GAL4, UAS:ChR2-eYFP, elavl3:GCaMP6f)* larvae were used. The
398 eyes were removed using fine tungsten needles in fish that were anesthetized with
399 MS222. This procedure was carried out in Ringers saline. Fish were then mounted in
400 1.2% agarose in Ringers saline, and imaged using two-photon microscopy as described
401 above, at 1 Hz. Optical stimulation was carried out using a 50 μ m fiber optic probe (Doric
402 Lenses), placed approximately 20 μ m from the thalamus. The 465 nm LED (Doric) was
403 driven with a current of 900 mA, 30 seconds after the start of imaging. 10 pulses were
404 provided, with a pulse duration of 25 milliseconds and a frequency between 1 and 8 Hz.
405 Each fish was exposed to at least 3 pulse trains. For Figure 6B-C, the average of the first
406 29 frames was used as a reference. The ratio of all frames relative to this reference was
407 obtained using FIJI. The analysis to generate Figure 6G was blind to the genotype.

408 **Laser ablation**

409 *Tg(Elavl3:GCaMP6f)* larvae were anaesthetized and then mounted in 2% low-
410 melting temperature agarose. Lesions were created with the femto-second laser tuned to
411 960 nm and fixed on a single point. Several pulses, each lasting 100 - 500 msec, were
412 used. Lesioning was monitored by time-lapse imaging before and after each pulse, and

413 was terminated when there was a localized increase in GCaMP6f fluorescence. Sample
414 size was chosen based on (Aizenberg and Schuman, 2011). Animals with bleeding in
415 the brain after lesioning, due to bursting of a blood vessel in AF4, were discarded.

416 **Behavioural Assay**

417 The chamber for the light/dark assay has been described previously (Cheng et al.,
418 2016). Control fish were mock-lesioned, i.e. subject to the same anaesthetic (MS222)
419 and mounted in agarose for the same duration as lesioned fish. Fish were observed in
420 the chamber for 10 minutes, by imaging at 15 Hz under infra-red illumination.

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426 *UAS:ChR2* transgenic line.

427 **Author contributions**

428 Experiments were designed by CRK, QL and SJ. CRK carried out two-photon
429 imaging. SK developed software and analyzed imaging data. CK performed antibody
430 label and generated the *UAS:GCaMP6s* line. MKZL performed the behavior assay, using
431 fish lesioned by QL. DGCH and IHB generated the *elav13:GCaMP6f* transgenic line. SJ
432 performed wide-field imaging, analysis, dye tracing, optogenetic manipulation and wrote
433 the manuscript.

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606 **Figure legends**

607 **Figure 1. The habenula responds to change in irradiance**

608 (A) Dorsal view of a live 7 day-old fish, with GCaMP3 expression in the habenula
609 (arrows) under the control of the *s1011tEt* GAL4 driver. (B) A single two-photon slice
610 through the habenula of the fish in panel A. (C) A yz reconstruction at the point indicated
611 by the yellow line in panel B. The dotted lines indicate imaging planes separated by 10
612 μm . The yellow line indicates the plane imaged in B. (D) Spatial distribution of major
613 responses in the habenula of one fish. 5 planes are shown here. The colors are coded
614 according to the temporal pattern of response, as indicated in (E). Images were collected
615 at a rate of 1 stack/second, and four pulses of light were delivered for 20 seconds each,
616 with variable inter-stimulus interval. (E) Centers of *k*-means clusters corresponding to
617 colors of pixels in (D). (F) Trajectory of the habenula response in (D-E) is shown in two-
618 dimensional state space, using the first two principal components (PC1 and PC2). The
619 first two principal components explain 79.7% of the total variance. Traces are color-
620 coded according to the wedges in panel E to represent direction in which change in
621 irradiance drives the neural state. In panels E and F, the bold lines correspond to light
622 onset while the dashed lines indicate offset.

623 **Figure 2. Habenula response to irradiance change is reproducible.**

624 (A-C) Semi-automated segmentation of habenula neurons. (D-F) K-means cluster
625 analysis of segmented habenula neuron responses to pulses of blue light in 6 fish. Cells
626 with excitation to light (ON, top) or darkness (OFF, middle), or inhibition to light
627 (Inhibitory, bottom) were seen. Traces show cluster centroids, with shaded
628 regions indicating standard error of the mean. (G) Correlation between activity of cells
629 belonging to ON, OFF and Inhibitory (Inh) clusters shown in panels D-F. In general, the
630 ON and OFF responding cells were uncorrelated (correlation coefficient < 0). (H) Activity

631 traces of cells in ON and OFF clusters that showed high correlation with the other
632 category (381 of 1767 cells). The traces showed that this correlation may be due to OFF
633 cells showing slow decay in fluorescence following light ON. Manual inspection of the
634 traces also did not reveal any that responded reliably to both light ON and OFF. Scale
635 bar = 25 μ m. a: anterior; p: posterior.

636 **Figure 3. High-speed imaging identifies the dorsal left neuropil as a site of prominent**
637 **response to irradiance change**

638 (A) Dorsal view of the habenula in a 5 day old *Tg(s1011t:GAL4, UAS:GCaMP3)*
639 fish. (B) Relative change in fluorescence (F/F_0) 250 msec after the start of imaging. The
640 arrowhead indicates the dorsal left neuropil. (C) F/F_0 in five different regions of the
641 habenula. (D-H) Light-evoked calcium change in the habenula of a 5 day old
642 *Tg(s1011t:GAL4, UAS: R-GECO)* fish. (D) Average projection of one 20 second time-
643 lapse, showing habenula neurons. (E) F/F_0 200 msec after light onset. (F) Traces from 8
644 different regions, indicated in panel D. (G) Averaged response from 4 recordings of the
645 same fish, imaged at 50 hz, analyzed using non-negative matrix factorization.
646 Arrowhead indicates the dorsal left neuropil. Pixels are colored according to the traces
647 (NMF components) in (H). (I-K) 200 Hz widefield imaging of an (*elav/3:GCaMP6f*) 5 day
648 old fish at the level of the dorsal habenula. (I) Average projection of the initial 2.475
649 seconds of the timelapse. Relative fluorescence change in the colored regions are
650 shown in panel I. (J) Relative change in GCaMP6f fluorescence 100 milliseconds after
651 the onset of light. Increase is seen in the left habenula (red arrowhead) and in the
652 thalamus (white arrowheads). (K) Region 1, which is the dorsal left neuropil, shows a
653 sharp increase in fluorescence. Region 3 shows gradual decrease. The inset shows a
654 zoom of the first 100 milliseconds. No rise is detected in the right habenula at this time.
655 Scale bar = 25 μ m.

656 **Figure 4. The thalamus projection to the habenula**

657 (A) Dorsal view of the forebrain of a *Et(SqKR11)* larva, in which habenula afferents
658 from the entopeduncular nucleus are fluorescently labeled. The dorsal left neuropil
659 (arrow) is weakly labeled. (B) Dorsal view of the habenula of a *Et(SqKR11)* larva,
660 following DiD injection into the dorsal neuropil of the left habenula. The parapineal
661 (arrow) has been retrogradely labeled. Habenula afferents from the entopeduncular
662 nucleus are labeled by the transgene, and shown in red here. (C) 80 μ m deeper in the
663 same fish, showing bilateral label in the thalamus (white arrows). A labeled cell in the
664 entopeduncular nucleus is also indicated (yellow arrow). (D) Lateral view of another larva,
665 in which the dorsal left neuropil had been injected with DiD. The retrogradely labeled
666 thalamic neuropil is indicated (white arrow). The entopeduncular nucleus is indicated by
667 the yellow arrow. The inset shows a higher magnification labeled thalamic neuropil. Cell
668 bodies are labeled (white arrowhead). (E) A close up view of the neuropil retrogradely
669 labeled by DiD (cyan), in a fish where retinal ganglion cells had been labeled with Dil
670 (yellow). RGC terminals intermingle with fibers from DiD-labeled cells innervating the
671 neuropil (arrow). The arrowhead indicates a DiD thalamic neuron labeled retrogradely.
672 (F) Dorsal view of another fish with Dil injection (yellow) into the right retina, and DiD
673 (cyan) into the left habenula. The thalamic neuropil is indicated (arrowhead). (G) Dorsal
674 view of a 6-day-old fish, labeled with an anti-vGlut1/2, which marks glutamatergic pre-
675 synapses. There is strong label in the dorsal left neuropil (arrowhead). (H) Dorsal view
676 showing label with an anti-GAD65/67 antibody. Labeled puncta are visible in the dorsal
677 left neuropil (arrowhead). No labeled cell bodies were detected. (I) A *Tg(gad1b:RFP,*
678 *vGlut2:GAL4, UAS:eGFP)* fish, with GABAergic cells indicated in magenta, and
679 glutamatergic cells shown in green. Both cell types can be detected in the thalamus. The
680 arrowhead indicates the region of the thalamus that is labeled in panel D. (J) RFP
681 expression in the thalamus of a *Tg(gad1b:RFP)* fish. Arrowheads indicate neurites

682 extending to the thalamic neuropil. All panels except (G) are single optical sections. EN:
683 entopeduncular nucleus; Pa: pallium; OT: optic tectum; Hb: habenula; Th: thalamus; fr:
684 fasciculus retroflexus. Scale bar = 25 μ m.

685 **Figure 5. The thalamus has non-overlapping responses to increase and decrease in**
686 **irradiance**

687 Dorsal view of the thalamus (A) and habenula (B) of a fish expressing mCherry
688 (magenta) under the control of the *GAL4s1020t* driver. Cell bodies are labeled in the
689 thalamus (A, white arrowheads). Labelled neurites are visible in the thalamic neuropil (A,
690 yellow arrowheads) and in the habenula neuropils (B, arrowheads). The puncta appear
691 to be secreted fragments of the labeled cells. GCaMP6f (green) is broadly expressed in
692 this fish. (C-H) Six different focal planes, from dorsal to ventral, of a fish expressing
693 GCaMP6s in thalamic neurons. The colors of the pixels represent clusters obtained from
694 *k-means* as shown in (I). Responses are seen in the thalamic neuropil (arrowhead) and
695 in neurons (arrows). (I) Cluster centroids obtained from running *k-means* on data in
696 panels (C-H). (J-K) Color coded activity obtained by registering and performing *k-means*
697 on 4 fish, in the dorsal (J) and ventral (K) thalamus. Colored pixels are a maximum
698 projection and superimposed on an image obtained by averaging the 4 fish. Pixels
699 showing an excitation to light are colored blue and those showing an excitation to
700 darkness are in magenta. (L-O) Thalamic afferents in neuropils in the dorsal (L) and
701 ventral (N) habenula. Pixels in cyan show a calcium increase during light ON and traces
702 are plotted as heatmap in the bottom panel of (M) and (O). Pixels in magenta show an
703 increase during light OFF and their heatmaps are shown in the top panel of (M) and (O).
704 In panels I, M and O, blue light was delivered for 20 seconds, with an inter-stimulus
705 interval of 20 seconds. Light onset is indicated by the solid line, while light offset is
706 indicated by the dashed line. a: anterior; p: posterior; Th: thalamus; rHb: right habenula;

707 IHB: left habenula. Anterior is to the top in all cases. Scale bar = 25 μ m.

708 **Figure 6. Optogenetic stimulation of the thalamus**

709 (A) Expression of ChR2-eYFP in the thalamus (arrowheads) of a 5 day old
710 *Tg(s1020t:GAL4, UAS:ChR2-eYFP, elavl3:GCaMP6f)* fish. (B, C) Activity in the
711 habenula of a ChR2-expressing fish, with (B) and without (C) blue LED stimulation of the
712 thalamus. The images show the maximum projections of F/F_0 images for a 25-second
713 period after blue LED illumination, following subtraction of maximum projections of the
714 period before illumination (i.e difference in activity before and after stimulation). (D-F)
715 Heatmaps showing temporal activity from cells segmented in fish with (D, E) and without
716 (F) ChR2. In D (n = 5 fish) and F (n = 2 fish), blue light pulse was given at the time
717 indicated by the black dashed line. No blue light stimulation was given in E (n = 4
718 fish). Z-scores were calculated by subtracting each time traces by the total mean and
719 dividing by the standard deviation. (G) Mean amplitude of z-scores before and after
720 optogenetic stimulation. Each square stands for a stimulus trial. Amplitude difference
721 before and after stimulation, mean \pm 95% CI: 1 Hz: 0.43 ± 0.56 , 2 Hz: 0.72 ± 0.35 , 4 Hz:
722 0.89 ± 0.28 and 8 Hz : 1.05 ± 0.18 . Scale bar = 25 μ m.

723 **Figure 7. The effect of AF4 lesion on habenula response to irradiance change**

724 (A, B) Dorsal view of an 8-day old *Tg(elavl3:GCaMP6f)* fish, before (A) and after
725 (B) lesion of AF4 (arrowheads in panel A). Pixels are colored according to their activity,
726 as indicated by traces in (C). The prominent AF4 sustained response to blue light (cyan
727 pixels) is reduced after lesion, although some response is still detected in the brain
728 (brown pixels). (D, E) Habenula activity before (D) and after (E) lesion of AF4. Pixels are
729 colored according to the traces in (F). There is a reduction in the sustained response to
730 light, but some activity that was not stimulus-locked was still detected. (G) The habenula
731 after lesion of AF9, with pixels colored according to the traces in panel (H). (I) Number of

732 cells in one plane of the dorsal left habenula that are excited by blue light, following
733 lesion of AF4 (n = 12), or AF7 (n = 2) or AF9 (n = 3), or before lesion (n = 5). Error bars
734 represent 95% CI. a: anterior; p: posterior; Pa: pallium; rHb: right habenula. Images are
735 all single optical sections. Scale bar = 25 μ m.

736 **Supplemental Figure 1. Dorsal left habenula lesion affects light-dependent behavior**
737 **and raphe activity.**

738 (A-C) A sequence of frames taken during lesioning of the dorsal left neuropil in a
739 *elavl3:GCaMP6f* fish. The laser caused an increase in intracellular calcium in the left
740 habenula, and eventually led to the formation of a bubble at the lesion site (arrow). (D,
741 E) A minimal projection of a time-lapse of control (D) and lesioned (E) fish, imaged at 15
742 Hz using infra-red illumination. The lesioned fish crossed into the dark side, whereas the
743 control did not. (F) Latency to cross into the dark side (n = 25 fish for each group). The
744 bar indicates median. As the assay was run for 10 minutes, a value of 10 indicates a
745 failure to cross. (G-J) Activity in dorsal raphe in an *elavl3:GCaMP6f* fish, before (G) and
746 after (H) lesioning of the dorsal left neuropil. Pixels are color-coded according to the
747 traces in panel (I). Instead of inhibition (magenta), cells show excitation (green) to
748 increase in irradiance. (J) The effect of lesioning the left dorsal habenula neuropil on
749 raphe response in three different fish. Scale bar = 25 μ m.

750 **Movie 1. Response in the habenula to increase in irradiance**

751 Widefield imaging of three different focal planes, 10 μ m apart, of a fish expressing
752 GCaMP3 in the habenula. Red represents an increase, whereas blue represents a
753 decrease. Responses can be detected throughout the habenula.

754 **Movie 2. Response dynamics in the habenula, as assessed using R-GECO**

755 Time-lapse of R-GECO fluorescence (F/F₀) in the habenula of a 5 day old fish,
756 following onset of illumination. Red represents an increase, whereas blue represents a

757 decrease. An increase in fluorescence was detected first in the neuropil of the left
758 habenula, and then in the cell bodies of habenula neurons. This is a *GAL4s1011t*,
759 *UAS:R-GECO* fish. Anterior is to the left.

760 **Movie 3. Widefield imaging of response to blue light onset**

761 200 Hz recording of a 5 day old fish expressing GCaMP6f under the *elav13*
762 promoter. Dorsal view; anterior is to the left.

763 **Movie 4. Habenula neurons do not project to the thalamus**

764 3D rendition of habenula projection in a zebrafish larva, visualized by expression of
765 RFP under the *narp* promoter (red) and eGFP under the *brn3a* promoter (green). There
766 is a clear projection to the interpeduncular nucleus (IPN), but not to the thalamus.

767 **Movie 5. GAD65/67 label in a zebrafish larva**

768 z-stack of a *s1011Et:GAL4, UAS:GCaMP3* transgenic fish, after immuno-labelling
769 with an antibody to GAD65/67 (magenta). The stack goes from dorsal to ventral.
770 GAD65/67 label is visible in neuropils of the habenula; puncta can be seen between
771 cells in the lateral regions of the habenula in more ventral planes. GAD65/67 labeled
772 cells are visible in the deep focal planes, but these do not express GCaMP3. The
773 location of GAD65/67 expressing cells correlates with the thalamus. S1011Et drives
774 GAL4 expression in the habenula, medial pallium and anterior-lateral pallium. This is a
775 dorsal view, with anterior to the left.

776 **Movie 6. Z-stack of 6 day old *gad1b:RFP, elav13:GCaMP6f* fish**

777 GABAergic neurons (magenta) are visible in the thalamus, below the habenula.
778 Anterior is to the left. The stack goes from dorsal to ventral.

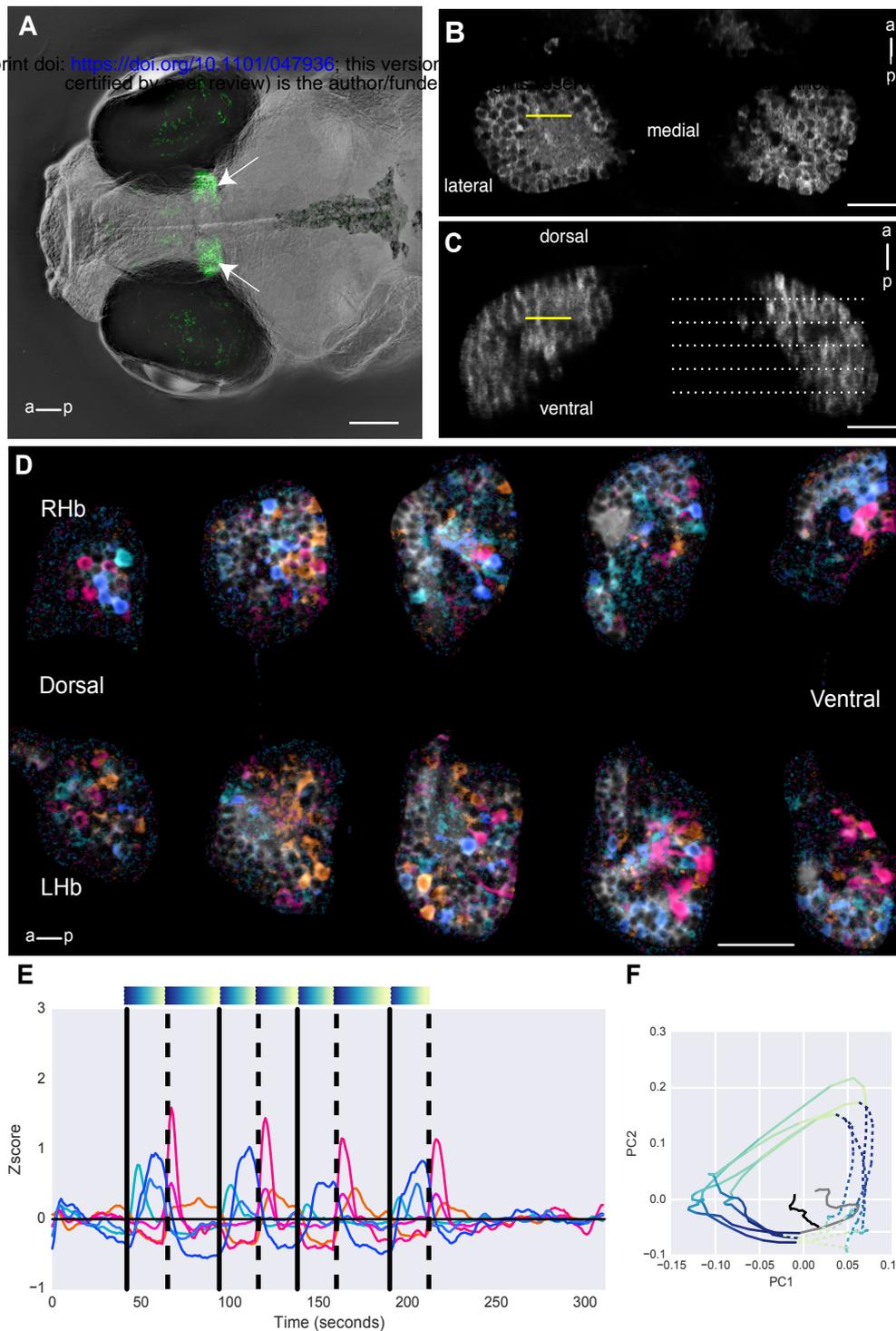


Figure 1. The habenula responds to change in irradiance. (A) Dorsal view of a live 7 day-old fish, with GCaMP3 expression in the habenula (arrows) under the control of the *s1011tEt* GAL4 driver. (B) A single two-photon slice through the habenula of the fish in panel A. (C) A yz reconstruction at the point indicated by the yellow line in panel B. The dotted lines indicate imaging planes separated by 10 μm. The yellow line indicates the plane imaged in B. (D) Spatial distribution of major responses in the habenula of one fish. 5 planes are shown here. The colors are coded according to the temporal pattern of response, as indicated in (E). Images were collected at a rate of 1 stack/second, and four pulses of light were delivered for 20 seconds each, with variable inter-stimulus interval. (E) Centers of *k*-means clusters corresponding to colors of pixels in (D). (F) Trajectory of the habenula response in (D-E) is shown in two-dimensional state space, using the first two principal components (PC1 and PC2). The first two principal components explain 79.7% of the total variance. Traces are color-coded according to the wedges in panel E to represent direction in which change in irradiance drives the neural state. In panels E and F, the bold lines correspond to light onset while the dashed lines indicate offset.

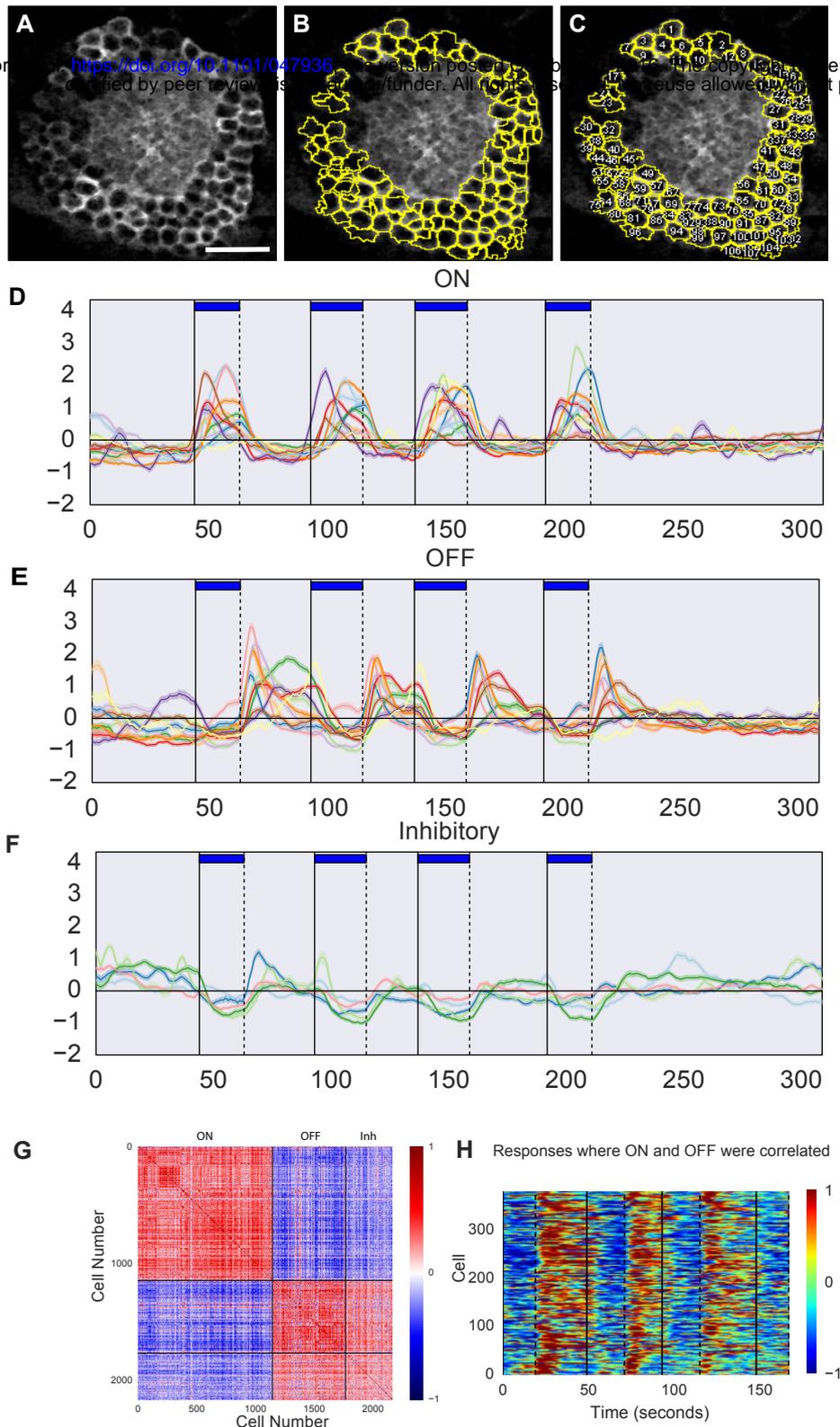


Figure 2. Habenula response to irradiance change is reproducible. (A-C) Semi-automated segmentation of habenula neurons. (D-F) K-means cluster analysis of segmented habenula neuron responses to pulses of blue light in 6 fish. Cells with excitation to light (ON, top) or darkness (OFF, middle), or inhibition to light (Inhibitory, bottom) were seen. Traces show cluster centroids, with shaded regions indicating standard error of the mean. (G) Correlation between activity of cells belonging to ON, OFF and Inhibitory (Inh) clusters shown in panels D-F. In general, the ON and OFF responding cells were uncorrelated (correlation coefficient < 0). (H) Activity traces of cells in ON and OFF clusters that showed high correlation with the other category (381 of 1767 cells). The traces showed that this correlation may be due to OFF cells showing slow decay in fluorescence following light ON. Manual inspection of the traces also did not reveal any that responded reliably to both light ON and OFF. Scale bar = 25 μ m.

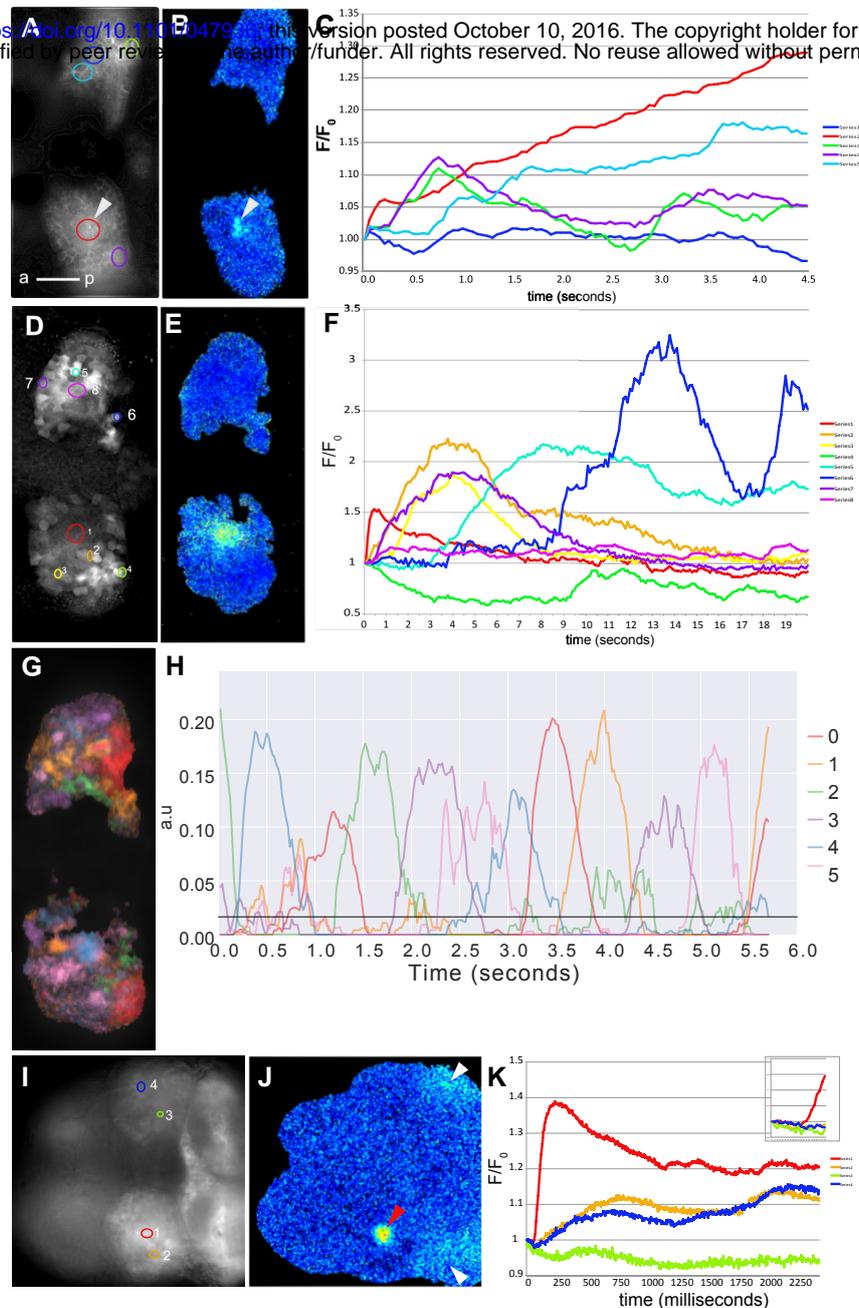


Figure 3. High-speed imaging identifies the dorsal left neuropil as a site of prominent response to irradiance change. (A) Dorsal view of the habenula in a 5 day old *Tg(s1011t:GAL4, UAS:GCaMP3)* fish. (B) Relative change in fluorescence (F/F_0) 250 msec after the start of imaging. The arrowhead indicates the dorsal left neuropil. (C) F/F_0 in five different regions of the habenula. (D-H) Light-evoked calcium change in the habenula of a 5 day old *Tg(s1011t:GAL4, UAS: R-GECO)* fish. (D) Average projection of one 20 second time-lapse, showing habenula neurons. (E) F/F_0 200 msec after light onset. (F) Traces from 8 different regions, indicated in panel D. (G) Averaged response from 4 recordings of the same fish, imaged at 50 hz, analyzed using non-negative matrix factorization. Arrowhead indicates the dorsal left neuropil. Pixels are colored according to the traces (NMF components) in (H). (I-K) 200 Hz widefield imaging of an (*elav13:GCaMP6f*) 5 day old fish at the level of the dorsal habenula. (I) Average projection of the initial 2.475 seconds of the timelapse. Relative fluorescence change in the colored regions are shown in panel I. (J) Relative change in GCaMP6f fluorescence 100 milliseconds after the onset of light. Increase is seen in the left habenula (red arrowhead) and in the thalamus (white arrowheads). (K) Region 1, which is the dorsal left neuropil, shows a sharp increase in fluorescence. Region 3 shows gradual decrease. The inset shows a zoom of the first 100 milliseconds. No rise is detected in the right habenula at this time. Scale bar = 25 μ m.

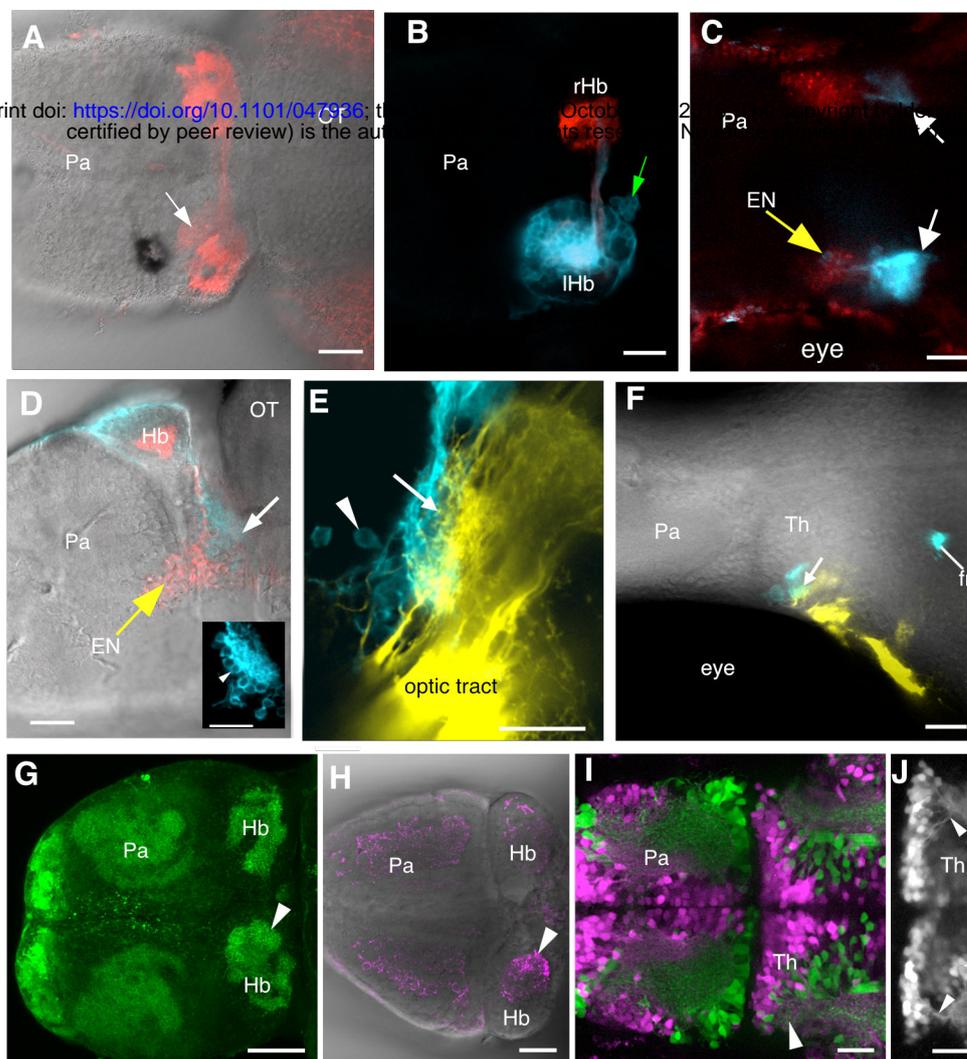


Figure 4. The thalamus projection to the habenula. (A) Dorsal view of the forebrain of a *Et(SqKR11)* larva, in which habenula afferents from the entopeduncular nucleus are fluorescently labeled. The dorsal left neuropil (arrow) is weakly labeled. (B) Dorsal view of the habenula of a *Et(SqKR11)* larva, following DiD injection into the dorsal neuropil of the left habenula. The parapineal (arrow) has been retrogradely labeled. Habenula afferents from the entopeduncular nucleus are labeled by the transgene, and shown in red here. (C) 80 μm deeper in the same fish, showing bilateral label in the thalamus (white arrows). A labeled cell in the entopeduncular nucleus is also indicated (yellow arrow). (D) Lateral view of another larva, in which the dorsal left neuropil had been injected with DiD. The retrogradely labeled thalamic neuropil is indicated (white arrow). The entopeduncular nucleus is indicated by the yellow arrow. The inset shows a higher magnification labeled thalamic neuropil. Cell bodies are labeled (white arrowhead). (E) A close up view of the neuropil retrogradely labeled by DiD (cyan), in a fish where retinal ganglion cells had been labeled with Dil (yellow). RGC terminals intermingle with fibers from DiD-labeled cells innervating the neuropil (arrow). The arrowhead indicates a DiD thalamic neuron labeled retrogradely. (F) Dorsal view of another fish with Dil injection (yellow) into the right retina, and DiD (cyan) into the left habenula. The thalamic neuropil is indicated (arrowhead). (G) Dorsal view of a 6-day-old fish, labeled with an anti-vGlut1/2, which marks glutamatergic pre-synapses. There is strong label in the dorsal left neuropil (arrowhead). (H) Dorsal view showing label with an anti-GAD65/67 antibody. Labeled puncta are visible in the dorsal left neuropil (arrowhead). No labeled cell bodies were detected. (I) A *Tg(gad1b:RFP, vGlut2:GAL4, UAS:eGFP)* fish, with GABAergic cells indicated in magenta, and glutamatergic cells shown in green. Both cell types can be detected in the thalamus. The arrowhead indicates the region of the thalamus that is labeled in panel D. (J) RFP expression in the thalamus of a *Tg(gad1b:RFP)* fish. Arrowheads indicate neurites extending to the thalamic neuropil. All panels except (G) are single optical sections. EN: entopeduncular nucleus; Pa: pallium; OT: optic tectum; Hb: habenula; Th: thalamus; fr: fasciculus retroflexus. Scale bar = 25 μm.

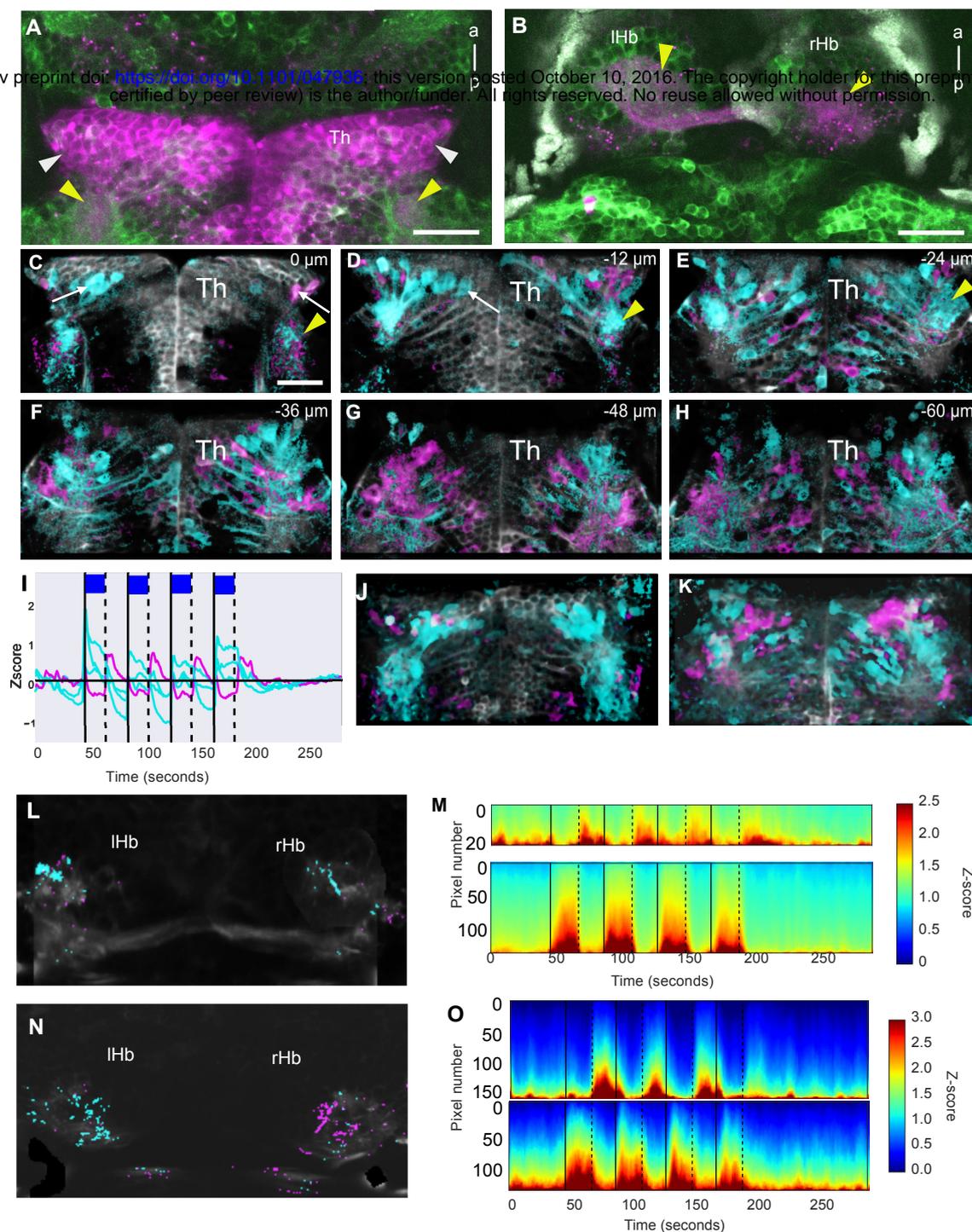


Figure 5. The thalamus has non-overlapping responses to increase and decrease irradiance. Dorsal view of the thalamus (A) and habenula (B) of a fish expressing mCherry (magenta) under the control of the *GAL4s1020t* driver. Cell bodies are labeled in the thalamus (A, white arrowheads). Labeled neurites are visible in the thalamic neuropil (A, yellow arrowheads) and in the habenula neuropils (B, arrowheads). The puncta appear to be secreted fragments of the labeled cells. GCaMP6f (green) is broadly expressed in this fish. (C-H) Six different focal planes, from dorsal to ventral, of a fish expressing GCaMP6f in thalamic neurons. The colors of the pixels represent clusters obtained from *k-means* as shown in (I). Responses are seen in the thalamic neuropil (arrowhead) and in neurons (arrows). (I) Cluster centroids obtained from running *k-means* on data in panels (C-H). (J-K) Color coded activity obtained by registering and performing *k-means* on 4 fish, in the dorsal (J) and ventral (K) thalamus. Colored pixels are a maximum projection and superimposed on an image obtained by averaging the 4 fish. Pixels showing an excitation to light are colored cyan and those showing an excitation to darkness are in magenta. (L-O) Thalamic afferents in neuropils in the dorsal (L) and ventral (N) habenula. Pixels in cyan show a calcium increase during light ON and traces are plotted as heatmaps in the bottom panel of (M) and (O). Pixels in magenta show an increase during light OFF and their heatmaps are shown in the top panel of (M) and (O). In panels I, M and O, blue light was delivered for 20 seconds, with an inter-stimulus interval of 20 seconds. Light onset is indicated by the solid line, while light offset is indicated by the dashed line. a: anterior; p: posterior; Th: thalamus; rHb: right habenula; IHb: left habenula. Anterior is to the top in all cases. Scale bar = 25 μ m.

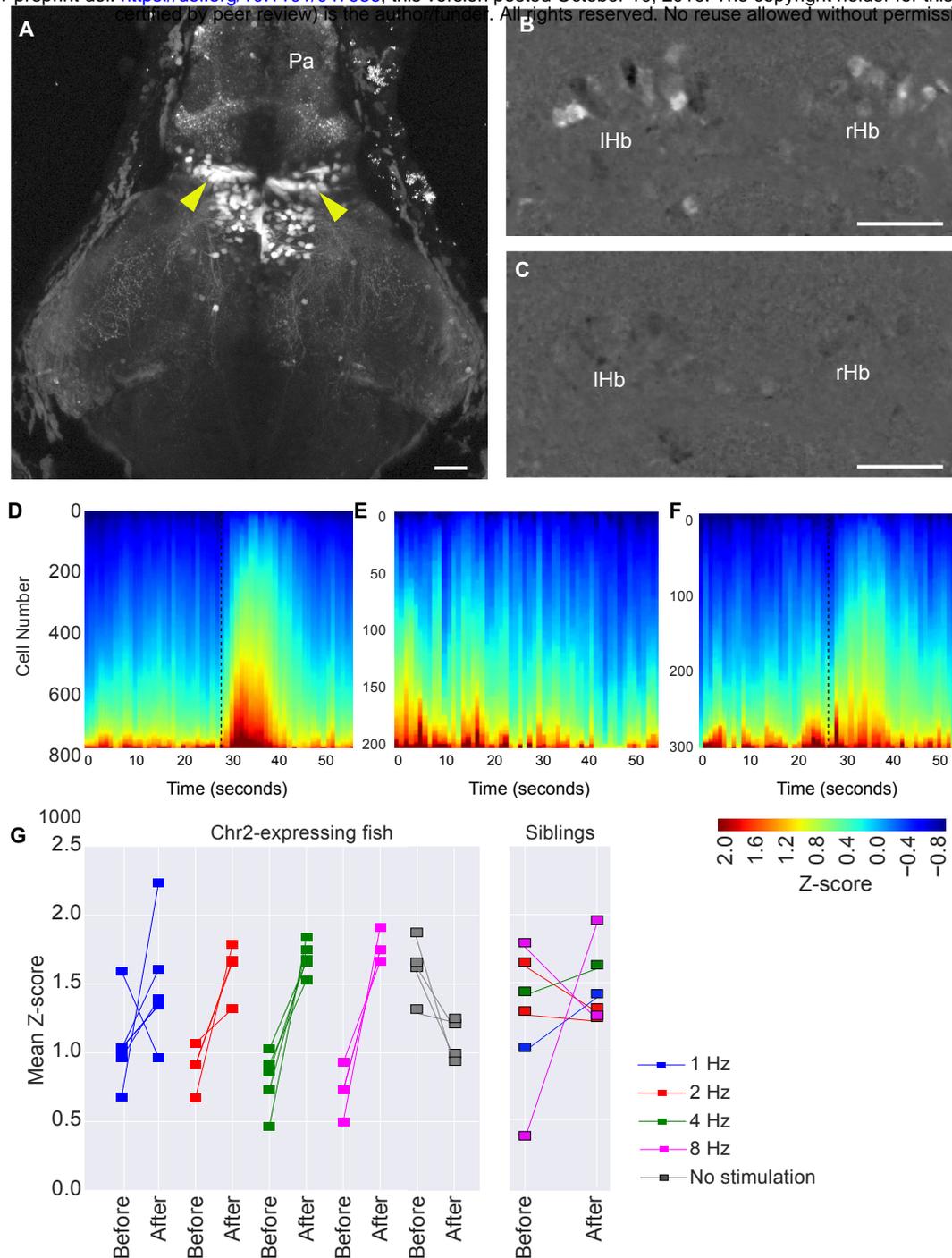


Figure 6. Optogenetic stimulation of the thalamus triggers habenula activity. (A) Expression of ChR2-eYFP in the thalamus (arrowheads) of a 5 day old *Tg(s1020t:GAL4, UAS:ChR2-eYFP, elav13:GCaMP6f)* fish. (B, C) Activity in the habenula of a Chr2-expressing fish, with (B) and without (C) blue LED stimulation of the thalamus. The images show the maximum projections of F/F_0 images for a 25-second period after blue LED illumination, following subtraction of maximum projections of the period before illumination (i.e difference in activity before and after stimulation). (D-F) Heatmaps showing temporal activity from cells segmented in fish with (D, E) and without (F) Chr2. In D ($n = 5$ fish) and F ($n = 2$ fish), blue light pulse was given at the time indicated by the black dashed line. No blue light stimulation was given in E ($n = 4$ fish). Z-scores were calculated by subtracting each time traces by the total mean and dividing by the standard deviation. (G) Mean amplitude of z-scores before and after optogenetic stimulation. Each square stands for a stimulus trial. Amplitude difference before and after stimulation, mean \pm 95% CI: 1 Hz: 0.43 ± 0.56 , 2 Hz: 0.72 ± 0.35 , 4 Hz: 0.89 ± 0.28 and 8 Hz : 1.05 ± 0.18 . Scale bar = 25 μ m.

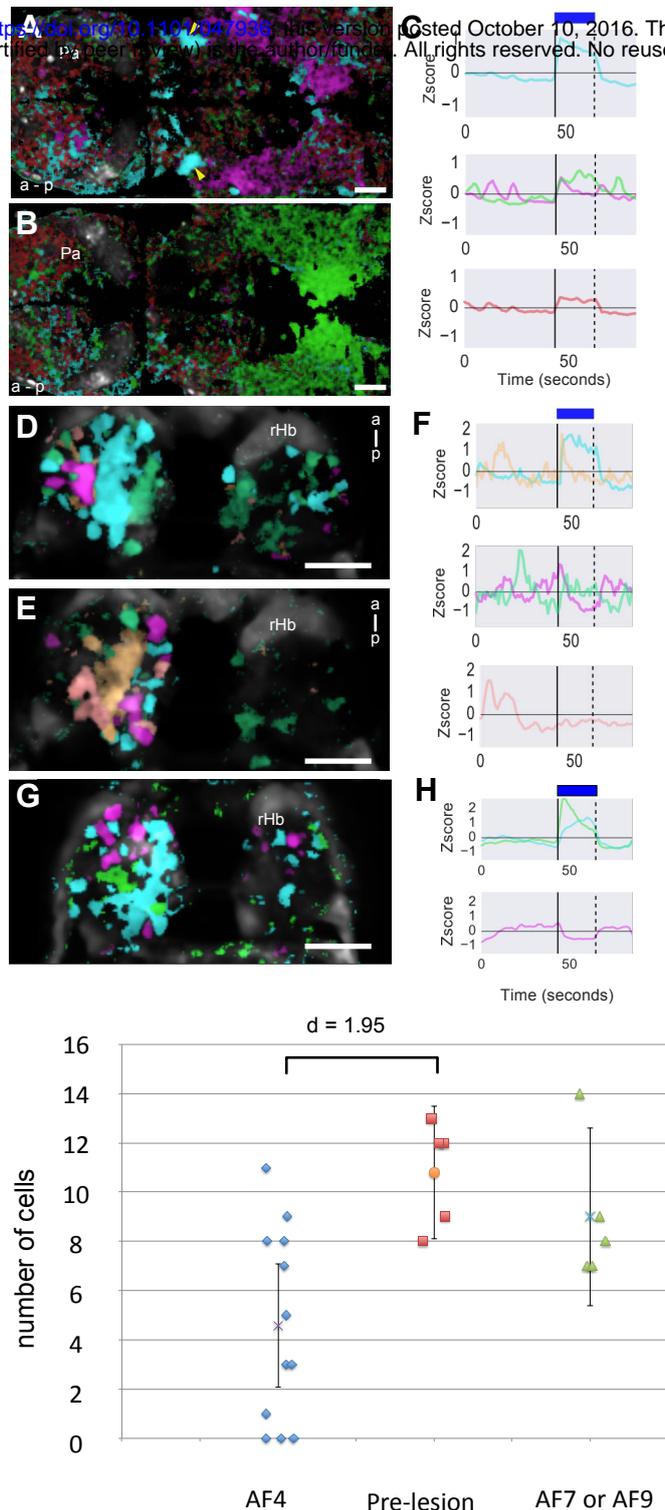
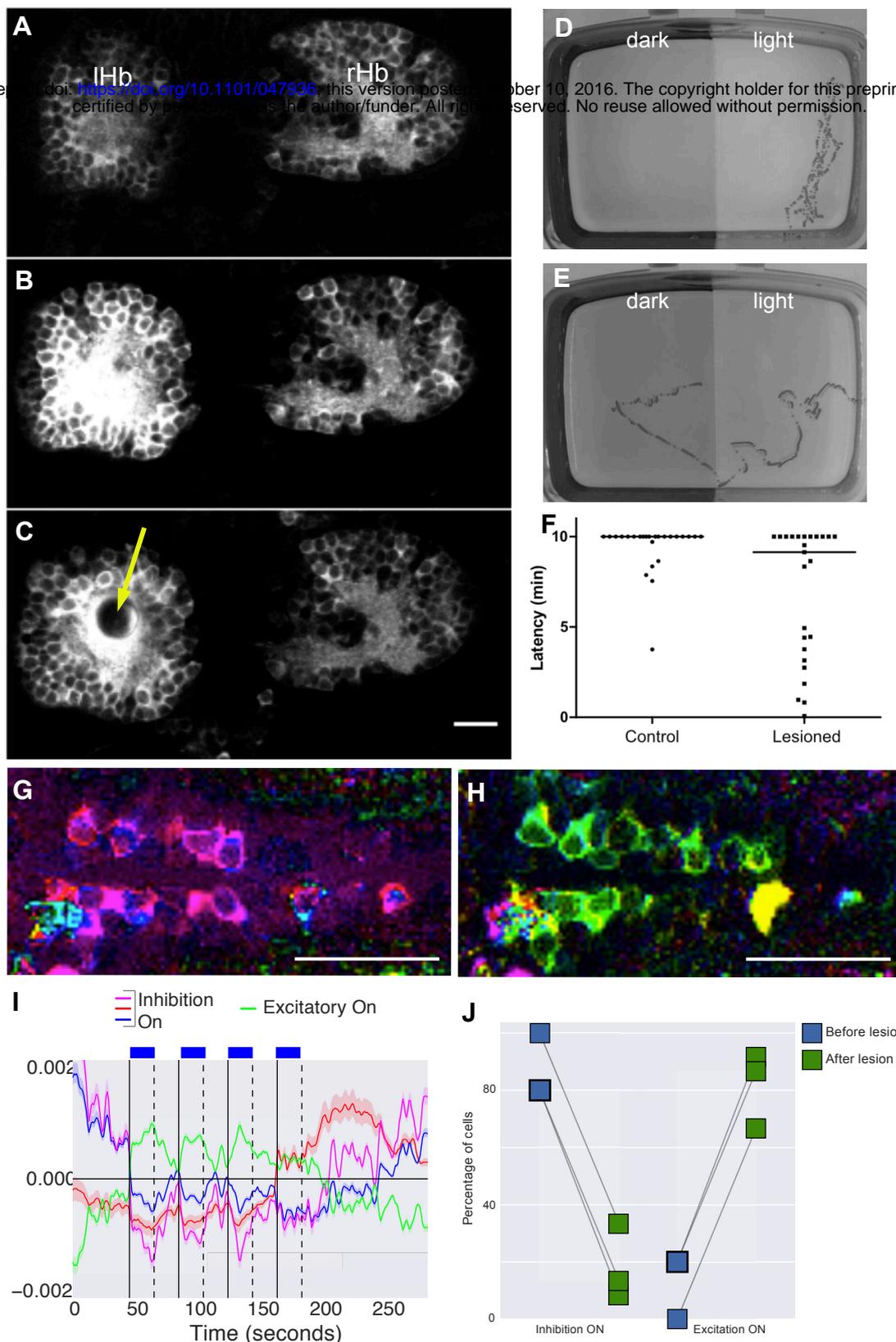


Figure 7. The effect of AF4 lesion on habenula response to irradiance change. (A, B) Dorsal view of an 8-day old Tg(elavl3:GCaMP6f) fish, before (A) and after (B) lesion of AF4 (arrowheads in panel A). Pixels are colored according to their activity, as indicated by traces in (C). The prominent AF4 sustained response to blue light (cyan pixels) is reduced after lesion, although some response is still detected in the brain (brown pixels). (D, E) Habenula activity before (D) and after (E) lesion of AF4. Pixels are colored according to the traces in (F). There is a reduction in the sustained response to light, but some activity that was not stimulus-locked was still detected. (G) The habenula after lesion of AF9, with pixels colored according to the traces in panel (H). (I) Number of cells in one plane of the dorsal left habenula that are excited by blue light, following lesion of AF4 (n = 12), or AF7 (n = 2) or AF9 (n = 3), or before lesion (n = 5). Error bars represent 95% CI. a: anterior; p: posterior; Pa: pallium; rHb: right habenula. Images are all single optical sections. Scale bar = 25 μ m.



Supplemental Figure 1. Dorsal left habenula lesion affects light-dependent behavior and raphe activity.

(A-C) A sequence of frames taken during lesioning of the dorsal left neuropil in a *elavl3:GCaMP6f* fish. The laser caused an increase in intracellular calcium in the left habenula, and eventually led to the formation of a bubble at the lesion site (arrow). (D, E) A minimal projection of a time-lapse of control (D) and lesioned (E) fish, imaged at 15 Hz using infra-red illumination. The lesioned fish crossed into the dark side, whereas the control did not. (F) Latency to cross into the dark side ($n = 25$ fish for each group). The bar indicates median. As the assay was run for 10 minutes, a value of 10 indicates a failure to cross. (G-J) Activity in dorsal raphe in an *elavl3:GCaMP6f* fish, before (G) and after (H) lesioning of the dorsal left neuropil. Pixels are color-coded according to the traces in panel (I). Instead of inhibition (magenta), cells show excitation (green) to increase in irradiance. (J) The effect of lesioning the left dorsal habenula neuropil on raphe response in three different fish. Scale bar = 25 μm .