Section: Neurosystems

Identification of GABAergic neurons innervating the zebrafish lateral habenula

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Running title: GABAergic inputs to the zebrafish lateral habenula

Pages: 22

Figures: 4

Tables: 0

Equations: 0

Total number of words: 6333

Number of words in abstract: 197

Abstract

Habenula neurons are constantly active. The level of activity affects mood and behaviour, with increased activity in the lateral habenula reflecting exposure to punishment and a switch to passive coping and depression. Here, we identify GABAergic neurons that could reduce activity in the lateral habenula of larval zebrafish. GAD65/67 immunohistochemistry and imaging of *gad1b:DsRed* transgenic fish suggest the presence of GABAergic terminals in the neuropil and between cell bodies in the lateral habenula. Retrograde tracing with the lipophilic dye DiD suggests that the former derives from the thalamus, while the latter originates from a group of cells in the posterior hypothalamus that are located between the posterior tuberal nucleus and hypothalamic lobes. Two-photon calcium imaging indicates that blue light causes excitation of thalamic GABAergic neurons and terminals in the neuropil, while a subpopulation of lateral habenula neurons show reduced intracellular calcium levels. Whole-cell electrophysiological recording indicates that blue-light reduces membrane potential of lateral habenula neurons. These observations suggest that GABAergic input from the thalamus may mediate inhibition in the zebrafish lateral habenula. Mechanisms governing release of GABA from the neurons in the posterior hypothalamus, which are likely to be in the tuberomammillary nucleus, remain to be defined.

Introduction

The habenula is a regulator of mood and behaviour in vertebrates (Lecourtier & Kelly, 2007; Namboodiri *et al.*, 2016). It consists of two major subdivisions, the medial and lateral habenula, and receives input from several regions of the forebrain (Mok & Mogenson, 1974; Herkenham & Nauta, 1977; Turner *et al.*, 2016). The lateral habenula has become a focus of substantial interest recently, as activity here controls the release of broadly acting neuromodulators such as dopamine and serotonin (Wang & Aghajanian, 1977; Hikosaka, 2010; Proulx *et al.*, 2014).

44 In addition to transient activity that is evoked by sensory stimuli (Dreosti *et al.*, 2014; Krishnan
45 *et al.*, 2014; Cheng *et al.*, 2017; Zhang *et al.*, 2017) and punishment (Matsumoto & Hikosaka,
46 2009), the habenula displays persistent or spontaneous activity (Gao *et al.*, 1996; Jetti *et al.*,
47 2014; Sakhi *et al.*, 2014; Baño-Otálora & Piggins, 2017). This activity is dependent on synaptic
48 input (Kim & Chang, 2005), and changes with time of day (Zhao & Rusak, 2005; Sakhi *et al.*,
49 2014) or as a result of a recent experience. Persistent elevated activity in the lateral habenula
50 is implicated in depression (Li *et al.*, 2011; Yang *et al.*, 2018; Andalman *et al.*, 2019).
51
52 Given these effects, there is a growing interest in understanding how activity in the lateral
53 habenula is controlled. In many brain regions, excitation is provided by afferent neurons, with
54 inhibition coming from local GABAergic interneurons. The habenula largely lacks GABAergic
55 neurons (Smith *et al.*, 1987; Pandey *et al.*, 2018; Zhang *et al.*, 2018), but receives GABAergic
56 input (Gottesfeld *et al.*, 1979). Although GABAergic input is normally inhibitory, a subset of
57 habenula neurons show extended firing following transient hyperpolarization (Chang & Kim,
58 2004). In the lateral habenula of mice, afferent neurons from the entopeduncular nucleus and
59 the VTA co-release glutamate and GABA (Root *et al.*, 2014; Shabel *et al.*, 2014). A small
60 population of GABAergic neurons in the lateral preoptic area (LPO) of rodents projects to the
61 lateral habenula (Barker *et al.*, 2017). Another input source to the habenula is the antero-dorsal
62 thalamus (Cheng *et al.*, 2017; Zhang *et al.*, 2017; Fernandez *et al.*, 2018), a region that is rich
63 in GABAergic neurons (Mueller, 2012). In the mouse, GABAergic neurons from the ventral
64 lateral geniculate nucleus and intergeniculate leaflet project to the lateral habenula, and enable
65 light-dependent inhibition (Huang *et al.*, 2019). Here we examine the sources of GABAergic
66 input to the lateral habenula of zebrafish, an experimental system with an easily accessible
67 habenula due to eversion of the brain during development (Mueller *et al.*, 2011; Folgueira *et*
68 *al.*, 2012). Our data suggests that there are two sources - the thalamus and the posterior

69 hypothalamus. This data on connectivity is expected to shed light on the mechanisms by which
70 brain states are controlled.

71 Materials and Methods

72 Zebrafish lines

73 Transgenic lines used include *SqKR11Et (Lee et al., 2010; Teh et al., 2010)*, *Et(-*
74 *0.6hsp70l:Gal4-VP16)s1020t* (Scott & Baier, 2009), *Tg(UAS:Kaede)s1999t* (Scott *et al.*,
75 2007), *Tg(elavl3:GCaMP6f)a12200* (Wolf *et al.*, 2017), *TgBAC(gad1b:DsRed)nns26* (Satou
76 *et al.*, 2013), *Tg(vGlut2:GFP)nns43 (Satou et al., 2012)*, *Tg(dao:GAL4VP16)rw0148a* (Amo
77 *et al.*, 2014), *Tg(UAS:GCaMP6s)sq205* and *Gt(hspGFFDMC76A)* (Muto *et al.*, 2017) . The
78 GAL4 enhancer trap line is referred to as *s1020t* in the text for brevity.
79 All experiments were carried out under guidelines approved by the IACUC of Biopolis
80 (number 191420).

81 Neural tracing

82 A saturated solution of DiD (ThermoFisher Scientific) was made by dissolving a small crystal
83 in 50µL of ethanol. Larval fish were anesthetized with tricaine, mounted in 2% low-melting
84 agarose and placed under a compound microscope (Zeiss Examiner). A small amount of dye
85 was pressure injected into either the left or right habenula by visualizing the fish under a water-
86 immersion 40x objective. The fish was rested for 30 minutes to allow the dye to diffuse and
87 then imaged using a Zeiss LSM800 confocal microscope, under a 40x water-immersion
88 objective. Fish with labelled cells outside the habenula were not used.

Antibody label

Larvae were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight and then rinsed several times with PBS. After peeling the skin off the brains, samples were incubated in 1%BSA and then overnight at 4 degrees in primary antibody (anti-GAD65/67, Abcam ab11070, 1:500 (Cheng *et al.*, 2017)). The following day the brains were washed several times with PBS and then incubated in secondary antibody (Alexa 488 goat anti-rabbit, 1:1000) overnight at 4˚C. After multiple washes with PBS, brains were mounted in low-melting agarose and imaged on a laser scanning confocal microscope (Zeiss LSM800) using a 40x water dipping objective. A total of 7 fish were imaged.

Electrophysiology

Whole-cell patch clamp and loose-patch recordings were performed from lateral habenula neurons in 5-10 days post-fertilization larvae using procedures previously described in Lupton et al. (2017), except that fish in the current study were not pinned onto a Sylgard dish. Fish were anesthetized by mivacurium before being mounted in a 2% low-melting agarose in a glass-bottom dish that was then immersed with external saline (composition in mM: 134 NaCl, 2.9 KCl, 1.2 MgCl2, 10 Hepes, 10 Glucose, 2.1 CaCl2, pH 7.8). In loose-patch recordings, the pipette was loaded with standard Ringer's solution to record neural signals from extracellular space while in whole-cell patch recordings, the pipette was loaded with a potassium gluconate–based internal solution (composition in mM: 115 K gluconate, 15 KCl, 2 MgCl2,10 Hepes, 10 EGTA, 4 MgATP, pH 7.2) to record neural signals intracellularly. The pipettes were pulled with thick-walled borosilicate capillaries (1.5 mm OD; 0.86 mm ID; Warner Instruments) using a Flaming Brown P-1000 pipette puller (Sutter Instruments) to give a tip diameter of 1–1.5 µm with an initial resistance of 10–20 MΩ for loose-patch recording. Neural signals were acquired using Multiclamp 700B amplifier, Digidata 1440A digitizer, and pCLAMP software v.10 (all

113 from Molecular Devices). In whole-cell patch recordings, the resistance reached gigaohm seal
114 before breaking into the cells. The data were low-pass filtered at 2 kHz using a Bessel filter
115 and sampled at 20 kHz at a gain of 1. Spike events were detected offline using Clampfit v.10.7
116 (downloaded from Molecular Devices). Graphs were plotted using Microsoft Excel for whole-
117 cell patch data and in Clampfit for loose-patch data.

118 Calcium imaging

119 Larval fish were imaged as described previously (Cheng *et al.*, 2017). Imaging was carried out
120 using a Nikon A1RMP two-photon microscope with a 25x water-dipping objective (NA=1.1),
121 with the Ti-Sapphire tuned to 930 nm and galvano scanning. 5 mm blue LEDs (460-490 nm),
122 controlled by the Nikon Elements software, were used to provide 20 second pulses of light. For
123 imaging the thalamus, multiple planes were captured sequentially.

124 Image analysis

125 Time-series registration, ROI extraction and rastermap calculation were performed using
126 Suite2p (Pachitariu *et al.*, 2017). Data was normalized into z-scores using Matlab.

127 Results

128 The zebrafish habenula is innervated by GABAergic neurons

129 GAD65/67 immunofluorescence indicates that GABAergic terminals are present in the
130 neuropil of medial (also called dorsal, based on adult morphology (Amo *et al.*, 2010)) and
131 lateral (or ventral) habenula of larval zebrafish (Figure 1A, B; arrowheads). In addition,
132 GABAergic neurites were visible between cell soma in the ventro-lateral habenula. The
133 GAD65/67 label in the habenula was distinct from the uniform label seen in cell bodies (see
134 white arrow in Figure 1A), and was seen in the absence of habenula neuron label. As an

135 independent means of visualizing the cell body and neurites of GABAergic neurons, we imaged
136 *gad1b:DsRed, vGlut2:GFP* double transgenic fish, which express DsRed and GFP in
137 GABAergic and glutamatergic neurons respectively. DsRed could be detected in neurites in
138 the lateral habenula, surrounding GFP-expressing neurons (Figure 1C). No DsRed labelled cell
139 was detected in the habenula, in contrast to other brain regions that contained fully labelled cell
140 bodies. This observation, in conjunction with the GAD65/67 antibody labelling, indicates that
141 the zebrafish habenula receives GABAergic input.

142 Neuronal tracing identifies sources of GABAergic afferents targeting

143 To identify the source of the GABAergic inputs, we examined transgenic lines in which
144 habenula afferents are labelled. Two major sources of input to the zebrafish habenula are the
145 entopeduncular nucleus (Turner *et al.*, 2016) and the thalamus (Cheng *et al.*, 2017). The
146 entopeduncular nucleus is located in the ventral telencephalon, in the vicinity of the lateral
147 forebrain bundle (Turner *et al.*, 2016). Neurons in this location, which are labelled in the
148 *Et(sqKR11)* line (Lee *et al.*, 2010) (Supplementary Movie 1), were seen to innervate the
149 neuropil of lateral habenula, but no neurites could be detected in between cell soma in lateral
150 habenula (Figure 1D). In contrast, neurites that are labelled by the *s1020tEt* driver
151 (Supplementary Movie 2), were visible between cells of the lateral habenula (Figure 1E-G).
152 These neurites co-expressed DsRed under the control of the *gad1b* promoter (Figure 1H),
153 indicating that they are GABAergic. GABAergic cell bodies were seen in the thalamus in
154 neurons expressing GCaMP6s under the control of the *s1020tEt* driver, but not in the
155 entopeduncular nucleus, which had few 1020tEt positive cells (Supplementary Movie 2). Thus,
156 GABAergic afferents to the lateral habenula may derive from the thalamus.
157

158 To confirm the location of the GABAergic afferents, the lipophilic tracer DiD was injected into
159 the lateral habenula of *Tg(gad1b:DsRed)* fish (Fig 2A; n = 17 fish; see Movie 3 for an example).
160 In all cases, terminals between soma were visible in the contralateral lateral habenula,
161 suggesting that there may be bilateral or contralateral origin of these afferents. Retrogradely
162 labelled neurons were visible in the ipsilateral thalamus in only 12 of these fish (Fig 2B, D-G),
163 and out of 42 labelled thalamic neurons, 16 were GABAergic, as judged by *gad1b:DsRed*
164 expression. The fact that 5 fish did not contain any labelled neurons in the thalamus, despite
165 the presence of terminals in the lateral habenula (Figure 2L; see also Movie 20190422)
166 indicates that there is an additional or different source of the soma-targeted terminals.
167 Retrogradely labelled neurons in the entopeduncular nucleus were DsRed-negative (Fig 2C),
168 indicating that this is not the source.
169
170 It has been reported that the posterior hypothalamus provides input to the habenula (Turner *et*
171 *al.*, 2016). Indeed, injection into the lateral habenula retrogradely labelled scattered cells in the
172 posterior hypothalamus of all fish where this area was imaged (Figure. 2H-K; n = 7 fish
173 imaged). Label was bilateral in all cases, and 20 out of 46 neurons were positive for DsRed,
174 indicating that this nucleus sends GABAergic projections to the lateral habenula. The labelled
175 neurons were located lateral to the posterior tuberal nucleus, and medial to the inferior lobe of
176 the hypothalamus (IHL), as judged by tracing experiments in the *Tg(76A:GAL4,*
177 *UAS:GCaMP6s)* line (Muto *et al.*, 2017) (Figure 2L-N). Neurons in this region expressed
178 GCaMP6s under the control of the *s1020t* GAL4 driver as well as DsRed under the control of
179 the *gad1b* promoter (Figure 2O-Q). Together, these observations suggest that GABAergic
180 terminals seen between cells in the lateral habenula of the s1020t fish derive from the posterior
181 hypothalamus, and imply that GABAergic terminals in the neuropils derive from the thalamus.

182 ## Blue light triggers activity in thalamic GABAergic neurons and afferents
183 targeting the lateral habenula neuropil

184 It has been reported previously that cells in the anterior thalamus of zebrafish are reproducibly
185 activated by pulses of blue light (Cheng *et al.*, 2017; Zhang *et al.*, 2017). To test whether the
186 responding thalamic cells include GABAergic neurons, two photon calcium imaging was
187 performed on larvae expressing GCaMP6s under the control of the s1020t GAL4 driver. An
188 increase in GCaMP6s fluorescence was seen in an average of 46 ± 23 thalamic neurons (mean
189 ± standard deviation; n = 6 fish). Of these, 20 ± 13 cells (mean ± standard deviation;
190 approximately 43 ± 18%) were GABAergic (Figure 3A-C). To determine whether terminals in
191 the lateral habenula are also affected, imaging was also carried out in this region (n = 3 fish).
192 In response to blue light, a rise in the fluorescence of this reporter was seen in a subset of
193 terminals within the neuropil of the lateral habenula (Figure 3D-F). No response to blue light
194 was seen in terminals between soma, although activity there was correlated to one another
195 (Figure 3G-I'). These observations are consistent with the notion that blue light can influence
196 the lateral habenula via GABAergic afferents from the thalamus that target the neuropils, but
197 not the cell bodies.

198 ## Blue light transiently reduces intracellular calcium in a subset of lateral habenula
199 neurons

200 If thalamic neurons provide GABAergic input to the neuropil of lateral habenula, then
201 stimulation of these neurons may inhibit the lateral habenula. Previous two photon calcium
202 imaging has shown that blue light causes both increase and decrease in ongoing activity
203 throughout the habenula (Cheng *et al.*, 2017; Zhang *et al.*, 2017). In these recordings, however,
204 it was not clear if inhibition also occurs in the lateral habenula. To determine this, we imaged
205 fish expressing GCaMP6s under the control of the *dao* promoter (n = 6 fish). A minority of

206 cells (30/154) showed a loss of activity during exposure to light (Figure 4A-D). We also
207 performed electrophysiology on intact animals. Both increase and decrease in membrane
208 potential (Figure 4E, F) was detected in lateral habenula neurons by whole-cell recording.
209 When loose-patch recording was employed, the onset of blue light was seen to either trigger or
210 dampen neural spikes (Figure 4G, H). Hence, in addition to excitation, blue light-dependent
211 reduction in the activity of lateral habenula cells is supported by both calcium imaging and
212 electrophysiological recordings.

213 Discussion

214 We have examined the source of neurons that can influence neural activity in the lateral
215 habenula of larval zebrafish through the release of GABA. GAD65/67 labelling and imaging
216 of a transgenic line which expresses DsRed under the control of the *gad1b* promoter indicate
217 that GABAergic terminals can be found in the habenula neuropils and also in between cell
218 soma. This suggests that GABAergic neurons target both dendrites and the soma of lateral
219 habenula neurons. Targeting onto the soma was not seen in the medial habenula of larval
220 zebrafish, but only in the lateral habenula. Thus, while GABAergic neurons may influence the
221 effect of excitatory input throughout the habenula by inhibition of dendrites, the lateral
222 habenula could experience stronger inhibition due to signalling onto the soma. Tracing data
223 indicates that GABAergic innervation targeting the neuropil and soma derive from different
224 populations.
225
226 A recent study in mice demonstrated that GABAergic neurons from the lateral geniculate
227 nucleus innervate and inhibit the lateral habenula in mice (Huang *et al.*, 2019). Consistent with
228 these findings, we see that retrograde labelling of lateral habenula neurons in the zebrafish
229 reveals *gad1b*-positive afferents from the thalamus. Further, we show that GABAergic

230 thalamic neurons as well as terminals in the lateral habenula are excited by light, while a
231 number of lateral habenula cells are inhibited. Electrical recordings of the lateral habenula
232 indicate that the onset of blue light led to a reduction in membrane potential and in the number
233 of spikes. The link between illumination levels and mood has been well-studied in animals and
234 humans (Viola *et al.*, 2008; Vandewalle *et al.*, 2010; Bedrosian *et al.*, 2011; LeGates *et al.*,
235 2014). Given that increased activity in the lateral habenula has negative effects (Lecca *et al.*,
236 2014; Proulx *et al.*, 2014; Lawson *et al.*, 2017; Howe & Kenny, 2018), inhibition of these
237 neurons by thalamic GABAergic neurons may contribute to the positive effects of light (Huang
238 *et al.*, 2019). However, it should be noted that only a subset of neurons were inhibited by light,
239 indicating that the effect of blue light on inhibition of the lateral habenula, at least in the
240 conditions used here, are relatively modest.
241
242 Retrograde tracing experiments also revealed *gad1b*-positive inputs from a cluster of cells
243 lateral to the posterior tuberal nucleus and medial to the inferior lobe of the hypothalamus.
244 Projections from the posterior tuberculum (PT) to the habenula have been previously reported
245 (Hendricks & Jesuthasan, 2007; Turner *et al.*, 2016). However, neurons in the posterior
246 tuberculum have been characterized as largely non-GABAergic (Filippi *et al.*, 2014; Heap *et*
247 *al.*, 2017). Similar scattered afferents have been identified by retrograde label in the habenula
248 of adult zebrafish (Turner *et al.*, 2016), where these were suggested to be located in the
249 posterior hypothalamus, and in the trout (Yañez & Anadón, 1996), where the cells were named
250 the "tuberculohabenular nucleus". We propose that these cells are located in the
251 tuberomammillary nucleus, which is located in the posterior hypothalamus (Chen *et al.*, 2017).
252 This would be consistent with the existence of histaminergic fibers in the lateral habenula
253 (Kaslin & Panula, 2001), the fact that neurons in the tuberomammillary nucleus in the posterior
254 hypothalamus are the sole source histamine in vertebrates, and that histaminergic neurons co-

release GABA (Sundvik & Panula, 2012; Yu *et al.*, 2015). Future experiments to define the function of these cells should yield fresh insight into the mechanisms by which activity in the lateral habenula is inhibited.

Acknowledgements

This research was supported by a Lee Kong Chian School of Medicine, Nanyang Technological University Singapore Start-Up Grant, and the Singapore Ministry of Education under its Academic Research Fund Tier 2 (MOE2017-T2-058) and Tier 1 (MOE2016-T1-001-152) awards. We thank Vatsala Thirumalai and members of her lab for instruction on techniques in zebrafish electrophysiology.

Conflict of Interest Statement

The authors declare no conflict of interest.

Author contributions

MR performed antibody label, retrograde tracing and microscopy. RKC performed electrophysiology and functional imaging. SJ imaged transgenic lines, analyzed data and wrote the manuscript.

Data Accessibility

All confocal stacks used here are available on Figshare.

272 # Figure captions

273 **Figure 1. Detection of GABAergic terminals in the habenula.** (A) A 6 day old fish, labelled
274 with an antibody to GAD65/67. Terminals are visible in the lateral habenula (yellow
275 arrowheads). The red arrows indicate GABAergic neurons in the pallium. (B) A 2 week old
276 fish, showing GAD65/67-labelled puncta in the neuropil of the medial habenula (white arrow),
277 and in between cells in the lateral habenula (yellow arrowhead). No habenula cell bodies are
278 labelled. This sample is slightly tilted. (C) A 7 day old *Tg(vGlut2:GFP), Tg(gad1b:DsRed)*
279 double transgenic fish. DsRed (magenta) is visible in terminals in the lateral habenula
280 (arrowheads), while habenula cell bodies express GFP (green). (D) An 8 day old fish *SqKR11Et*
281 fish, with afferents from the entopeduncular nucleus labelled in red. Terminals are restricted to
282 neuropil of the lateral habenula (white arrow), and do not overlap with GAD65/67 label
283 between cells (yellow arrowhead). (E) Afferents from thalamic neurons, labelled with Kaede
284 under the control of the *s1020tEt* driver. Terminals are visible in the neuropil (arrow) and also
285 between cells (arrowhead). (F-H) The left habenula of a 6 day old fish, with terminals labelled
286 by GCaMP6s, which is expressed under the control of the *s1020tEt* driver (G), and DsRed
287 under the control of the *gad1b* promoter (H). The white arrowhead in F indicates
288 autofluorescing pterinosomes in xanthophores (Le Guyader & Jesuthasan, 2002). Scale bar =
289 25 µm for A-E, 10 µm for F-H . All images are dorsal view, with anterior to the left.
290

291 **Figure 2. Retrograde label of lateral habenula afferents.** (A-C) An example of DiD
292 injection (cyan) into the left lateral habenula (A, red arrowhead) of a 7 day old
293 *Tg(elavl3:GCaMP6f), Tg(gad1b:DsRed)* fish. (B) Retrogradely labelled cell bodies
294 (arrowheads) in the thalamic region. (C) A deeper focal plane. Entopeduncular nucleus (EN)
295 cell bodies are also labelled by DiD but are *gad1b*-negative. (D-G) The boxed area in panel B
296 shown at higher magnification. The arrowheads indicate neurons in the thalamus expressing

297 GCaMP6f (E) and DsRed (F), which have been retrogradely labelled by DiD (H). (H-K) DiD
298 injection (cyan) into the right lateral habenula of another double transgenic 7 day old fish (H,
299 red arrowhead). Lateral habenula neurons express GCaMP6s under the *dao* promoter. (I)
300 Bilateral label of cell bodies in the posterior hypothalamus. (J, K) The boxed region in panel I
301 at higher magnification, showing retrogradely labelled neurons that are *gad1b* positive (yellow
302 arrowhead) or negative (green arrowhead). (L-N) DiD injection into the left lateral habenula
303 of a 7 day old *Tg(76A:GAL4, UAS:GCaMP6s, gad1b:DsRed)* fish. (L) The injection site (red
304 arrowhead). (M) Retrogradely labelled cells (yellow arrowheads), at the level of the inferior
305 lobe of the hypothalamus. (N) GCaMP6s expression in the inferior lobe of the hypothalamus.
306 (O-Q) Expression of GCaMP6s and DsRed in a neuron (yellow arrowhead) in the posterior
307 hypothalamus of 10 day old *s1020t:GAL4, UAS:GCaMP6s, gad1b:DsRed* fish. All panels are
308 dorsal views, with anterior to the left. Pa: pallium, OT: optic tectum, fr: fasciculus retroflexus,
309 rHb: right habenula, PT: posterior tuberculum, IPN: interpeduncular nucleus.
310
311 **Figure 3. Effect of blue light on thalamic GABAergic neurons**. (A-C) The thalamic response
312 of a 6 day old fish to 3 pulses of blue light. (A) Confocal image showing thalamic neurons,
313 some of which express DsRed under the control of the gad1b promoter (magenta). (B) Change
314 in GCaMP6f fluorescence of thalamic neurons, correlated to change in the AF4 neuropil (red
315 arrow), which receives retinal input. Cells indicated by arrowheads have a high correlation with
316 activity in this neuropil and express DsRed, as shown in panel A. (C) Rastermap of cells and
317 neuropils extracted from the time-series. Two major responses are phasic and tonic response
318 to light. (D - F) Calcium imaging of terminals in the neuropil of the ventral habenula of a 5 day
319 old fish exposed to 1 pulse of blue light. (D) Average projection of the time series, showing
320 neuropils (yellow arrowheads). (E) Correlation map of response, with red pixels showing
321 terminals with a highly correlated calcium increase in response to a pulse of blue light. (F)

322 Rastermap showing fluorescence change with time. (G - I) Response in terminals between cell
323 bodies. (G) Average of the time-series, showing the location of terminals (yellow arrowheads).
324 (H) Map of response, correlated to terminals in the thalamic neuropil AF4 (white arrowhead).
325 (H') Rastermap of 5 ROIs that are highly correlated with blue light pulses. (I) Map of response
326 correlated to terminals in the lateral habenula (white arrowhead). (I') Rastermap of 6 ROIs in
327 the lateral habenula that are highly correlated to one another. These have no relationship to the
328 light pulses. All panels are dorsal views, with anterior to the top. Th, thalamus. The wedge in
329 panel B indicates the lookup table used for correlation maps.
330
331 **Figure 4. Calcium imaging and electrophysiological recordings in the lateral habenula.**
332 (A-D Response of neurons in the lateral habenula of a 5 day old fish exposed to four pulses of
333 blue light. (A) Average of a time-series, showing distribution of cells labelled in a *dao:GAL4,*
334 *UAS:GCaMP6s* fish. (B) Correlation map of response to 4 pulses of light, with red showing a
335 highly correlated cell, and blue showing an anti-correlated cell. (C) z-scores of the two cells
336 indicated by the blue and red arrowheads in panel B. (D) Rastermap of the response of all cells.
337 Blue bars the presence of blue light. (E, F) Whole-cell recordings showing membrane
338 depolarization (E) or hyperpolarization (F) by blue light. The blue light was 13 seconds in
339 duration in both cases and the range of voltage change (peak-to-trough) was on average 2-3
340 mV in both cases. Solid horizontal lines represent the means of the membrane potentials before,
341 during and after the blue light in accordance with the trace colors. Each trace in panel E is a
342 separate cell. (G, H) Loose-patch recordings. (G) There are spikes of two different heights,
343 implying signals from two cells. Both show an increase in spikes in the presence of blue light.
344 (H) A recording in another fish, showing a reduction in spiking during blue light delivery. The
345 ticks in the upper trace in (G) and (H) were taken from spike detection from the main trace, as
346 indicated by the blue squares. Scale bar = 25 μm.

347

Supplementary Material

349 **Supplementary Movie 1. Z-stack of a 5 day old SqKR11Et, elavl3:GCaMP6f fish.** The
350 yellow arrowheads indicate the lateral forebrain bundle in the ventral telencephalon. The
351 white arrowhead indicates cells in the entopeduncular nucleus, which project to neuropils in
352 the habenula.

353 **Supplementary Movie 2. Z-stack of a 6 day old Tg(s1020tGAL4, UAS:GCaMP6s,**
354 **gad1b:DsRed) fish.** The yellow arrowheads indicate the lateral forebrain bundle in the ventral
355 telencephalon. The white arrowhead indicates the entopeduncular nucleus, which contains no
356 DsRed expressing cells. Cells in the thalamus are strongly labelled by the s1020t driver.

357 **Supplementary Movie 3. Retrograde label of cells in the posterior hypothalamus.** Z-stack
358 of a Tg(dao:GAL4, UAS:GCaMP6s, gad1b:DsRed) fish, after DiD label of the left lateral
359 habenula. Labelled cells are visible in the posterior hypothalamus. Habenula projections to the
360 raphe are also visible. There are no projections to the IPN. Note that GCaMP6s is expressed in
361 the posterior hypothalamus.

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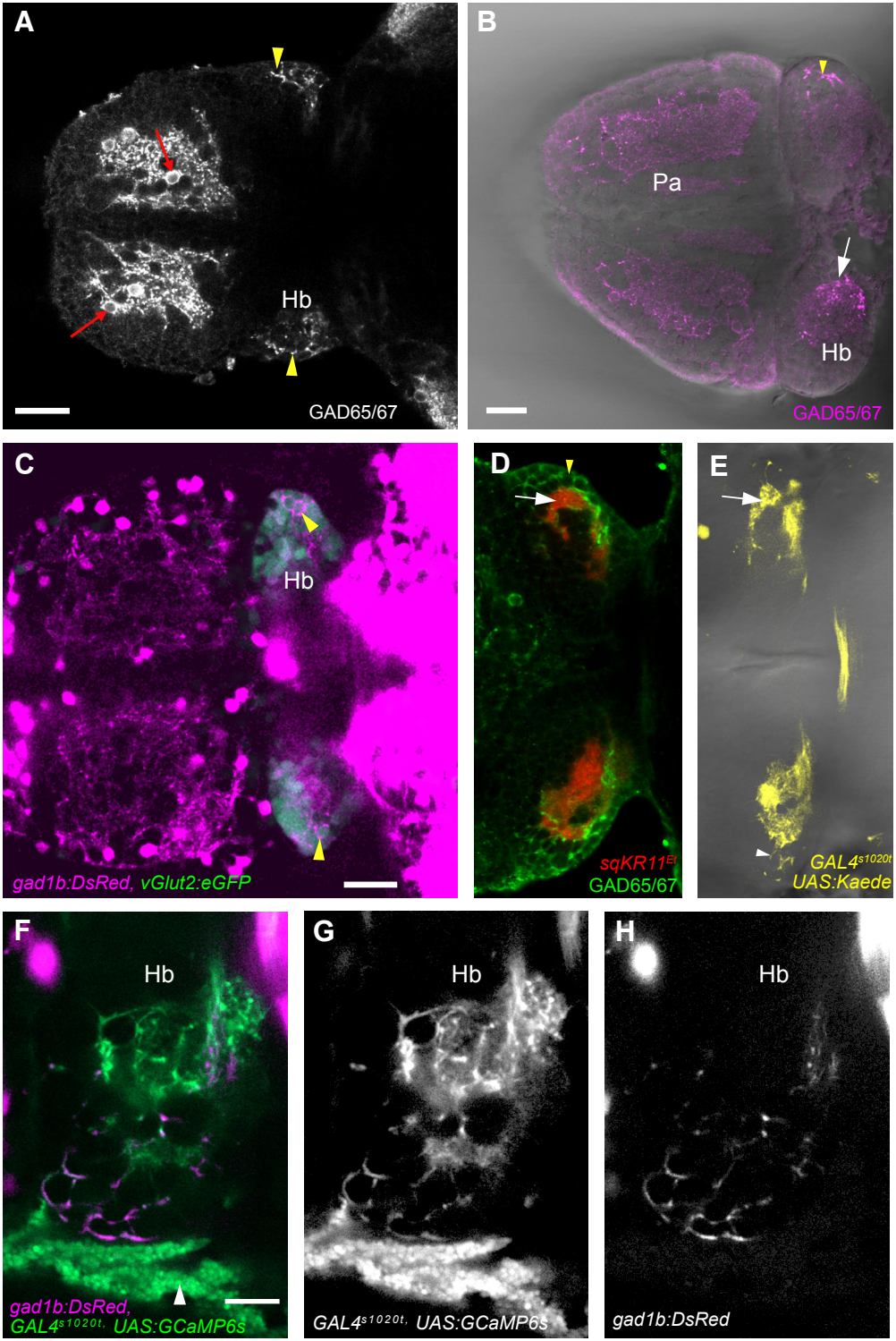
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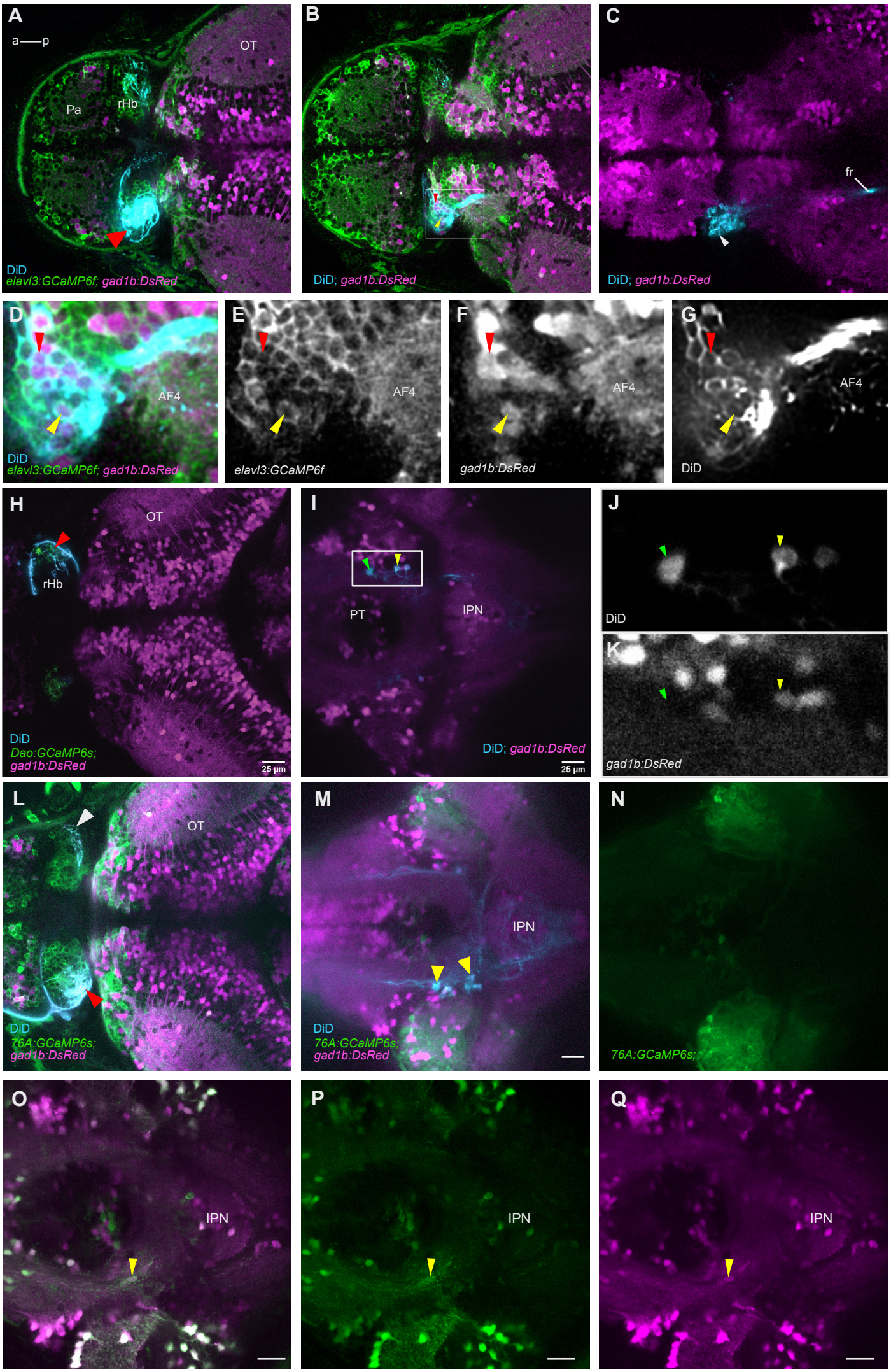
Figure 1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D
AF4
DiD
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Figure 2

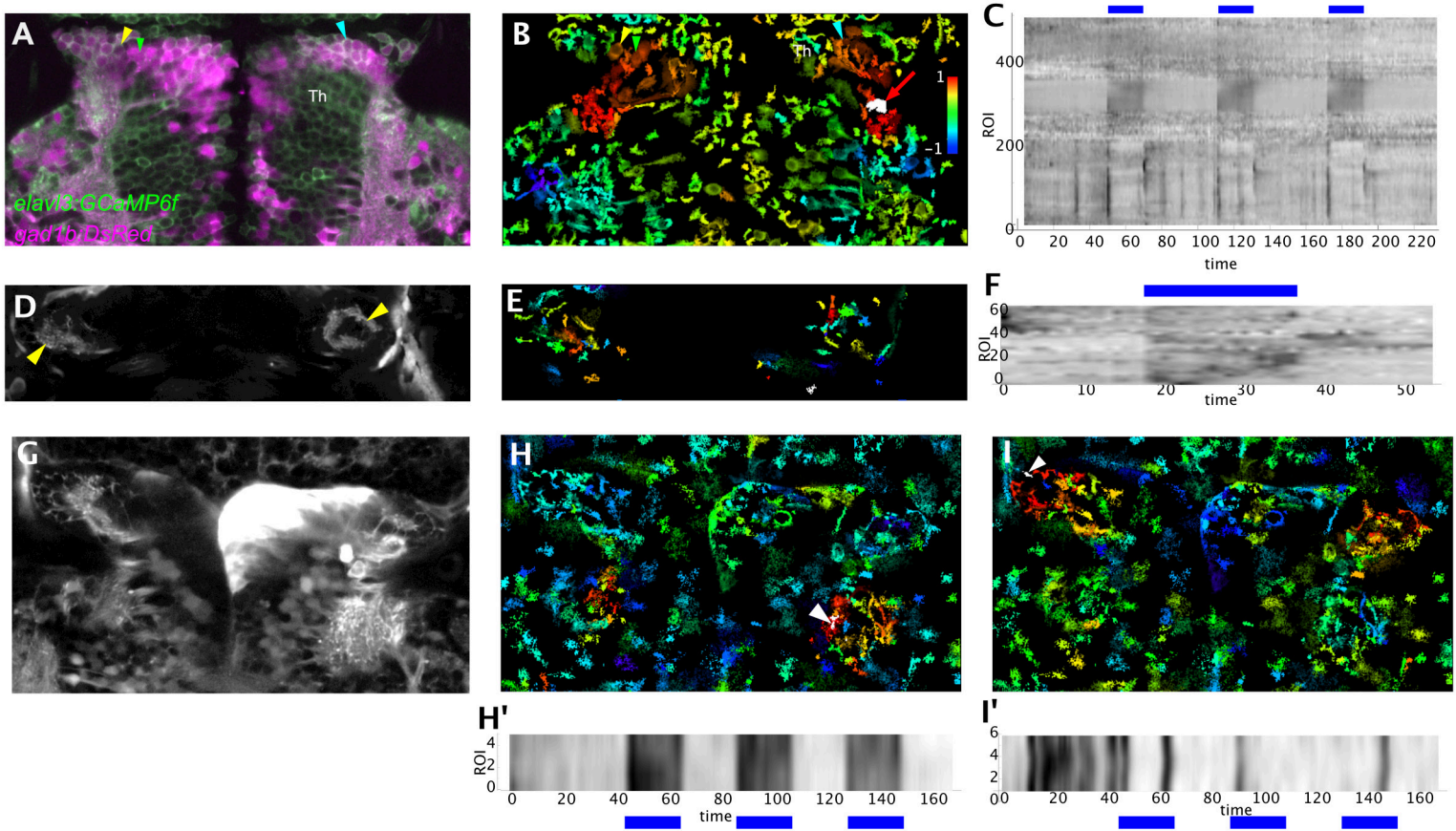
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Figure 3

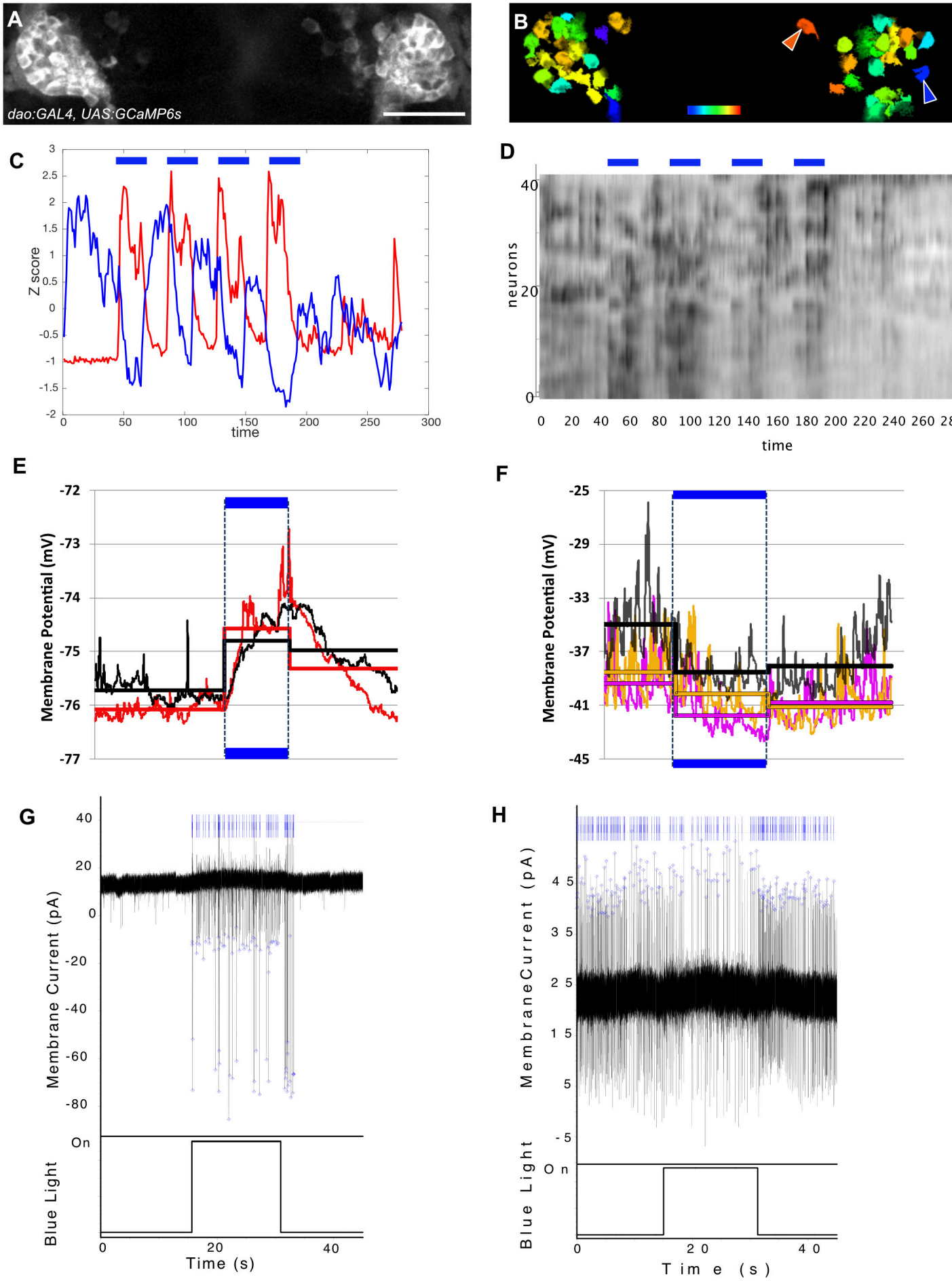
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Figure 4