Section: Neurosystems

Identification of GABAergic neurons innervating the zebrafish lateral habenula

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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 influence 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 lateral habenula. Retrograde tracing with the lipophilic dye DiD indicates that this GABAergic innervation derives from the thalamus and a nucleus lateral to the posterior tuberculum, the putative M2 nucleus. Two-photon calcium imaging indicates that blue light causes excitation of thalamic GABAergic neurons and concomitant inhibition of a subpopulation neurons in the lateral habenula. Whole-cell recording confirmed that blue-light induced hyperpolarization of lateral habenula neurons. No response to blue light was detected in the putative M2 neurons. These observations suggest that GABAergic input from the thalamus mediates inhibition of the zebrafish lateral habenula, possibly contributing to the beneficial effects of blue light. Stimuli acting via the putative preglomerular complex remain to be defined.

Introduction

The habenula is an evolutionarily conserved 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). In addition to transient activity that is evoked by sensory stimuli (Dreosti *et al.*, 2014; Krishnan *et al.*, 2014; Cheng *et al.*, 2017; Zhang *et al.*, 2017) and

punishment (Matsumoto & Hikosaka, 2009), the habenula displays persistent or spontaneous activity (Gao *et al.*, 1996; Jetti *et al.*, 2014; Sakhi *et al.*, 2014; Baño-Otálora & Piggins, 2017). This activity is dependent on synaptic input (Kim & Chang, 2005), and changes with time of day (Zhao & Rusak, 2005; Sakhi *et al.*, 2014) or as a result of a recent experience. Persistent elevated activity in the lateral habenula is implicated in depression (Li *et al.*, 2011; Yang *et al.*, 2018; Andalman *et al.*, 2019).

Given these effects, there is a growing interest in understanding how activity in the lateral habenula is controlled. In many brain regions, excitation is provided by afferent neurons, with inhibition coming from local GABAergic interneurons. The habenula largely lacks GABAergic neurons (Smith *et al.*, 1987; Pandey *et al.*, 2018; Zhang *et al.*, 2018), but receives GABAergic input (Gottesfeld *et al.*, 1979). Although GABAergic input is normally inhibitory, a subset of habenula neurons show extended firing following transient hyperpolarization (Chang & Kim, 2004). In the lateral habenula of mice, afferent neurons from the entopeduncular nucleus and the VTA co-release glutamate and GABA (Root *et al.*, 2014; Shabel *et al.*, 2014). A small population of GABAergic neurons in the lateral preoptic area (LPO) of rodents projects to the lateral habenula (Barker *et al.*, 2017). Another input source to the habenula is the antero-dorsal thalamus (Cheng *et al.*, 2017; Zhang *et al.*, 2017; Fernandez *et al.*, 2018), a region that is rich in GABAergic neurons (Mueller, 2012). In the mouse, GABAergic neurons from the ventral lateral geniculate nucleus and intergeniculate leaflet project to the lateral habenula, and enable light-dependent inhibition (Huang *et al.*, 2019). Here we examine the sources of GABAergic input to the lateral habenula of zebrafish, an experimental system with an easily accessible habenula due to eversion of the brain during development (Mueller *et al.*, 2011; Folgueira *et al.*, 2012). This data on connectivity is expected to shed light on the mechanisms by which brain states are controlled.

Materials and Methods

Zebrafish lines

Transgenic lines used include *SqKR11Et (Lee et al., 2010; Teh et al., 2010)*, *Et(-0.6hsp70l:Gal4-VP16)s1020t* (Scott & Baier, 2009), *Tg(UAS:Kaede)* (Scott *et al.*, 2007), *Tg(elavl3:GCaMP6f^{a12200}) (Wolf et al., 2017)*, *Tg(gad1b:DsRed)* (Satou *et al.*, 2013), *Tg(vGlut2:GFP) (Satou et al., 2012), Tg(dao:GAL4VP16)* (Amo *et al.*, 2014), *Tg(UAS:GCaMP6s^{sq205})*. The GAL4 enhancer trap line is referred to as *s1020tEt* in the text for brevity.

All experiments were carried out under guidelines approved by the IACUC of Biopolis (number 191420).

Neural tracing

A saturated solution of DiD (ThermoFisher Scientific) was made by dissolving a small crystal in 50µL of ethanol. Larval fish were anesthetized with tricaine, mounted in 2% low-melting agarose and placed under a compound microscope (Zeiss Examiner). A small amount of dye was pressure injected into either the left or right habenula by visualizing the fish under a water-immersion 40x objective. The fish was rested for 30 minutes to allow the dye to diffuse and then imaged using a Zeiss LSM800 confocal microscope, under a 40x water-immersion objective.

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 (dpf) 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 from Molecular Devices). In whole-cell patch recordings, the resistance reached gigaohm seal before breaking into the cells. The data were low-pass filtered at 2 kHz using a Bessel filter and sampled at 20 kHz at a gain of 1. Spike events were detected

offline using Clampfit v.10.7 (downloaded from Molecular Devices). Graphs were plotted using Microsoft Excel for whole-cell patch data and in Clampfit for loose-patch data.

Calcium imaging

Larval fish were imaged as described previously (Cheng *et al.*, 2017). Imaging was carried out using a Nikon A1RMP two-photon microscope with a 25x water-dipping objective (NA=1.1), with the Ti-Sapphire tuned to 930 nm. 5 mm blue LEDs (460-490 nm), controlled by the Nikon Elements software, were used to provide 10 or 20 second pulses of light.

Image analysis

Time-series of neuronal cell bodies in the thalamus were processed using Suite2p (Pachitariu *et al.*, 2017). Time series of terminals in the lateral habenula was analyzed using the Thunder platform (Freeman *et al.*, 2014). For the latter, raw images were first registered to correct for XY drift using the Stackreg plugin on FIJI (Thévenaz *et al.*, 1998; Schindelin *et al.*, 2012). K-means cluster analysis was carried out as described previously (Cheng *et al.*, 2017). Data was normalized into z-scores.

Results

The zebrafish habenula is innervated by GABAergic neurons

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

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

Neuronal tracing identifies source of GABAergic afferents

To identify the source of the GABAergic inputs, we examined transgenic lines in which habenula afferents are labelled. Two major sources of input to the zebrafish habenula are the entopeduncular nucleus (Turner *et al.*, 2016) and the thalamus (Cheng *et al.*, 2017). Entopeduncular nucleus neurons, which are labelled in the Et(sqKR11) line (Lee *et al.*, 2010), were seen to innervate the neuropil of lateral habenula, but no neurites could be detected in between cell soma in lateral habenula (Figure 1D). In contrast, neurites from thalamic neurons, which are labelled by the s1020tEt driver, were clearly visible between cells of the lateral habenula (Figure 1E).

To confirm the location of the GABAergic afferents, the lipophilic tracer DiD was injected into the lateral habenula of *Tg(gad1b:DsRed)* fish (Fig 2A). Retrogradely labelled neurons, which were positive for DsRed, were visible in the thalamus (Fig 2B; n = 8 fish imaged). Retrogradely labelled neurons in the entopeduncular nucleus were DsRed-negative (Fig 2C). Injection into either left or right lateral habenula also retrogradely labelled cells lateral to the posterior tuberculum (Figure. 2D, E; n = 9 fish imaged), which is likely to be the migrated M2 nucleus of the preglomerular complex (Heap *et al.*, 2017). Label was bilateral, and a small number of

the neurons were positive for DsRed, indicating that this nucleus sends GABAergic projections to the lateral habenula. The posterior tuberculum itself was also retrogradely labelled, but appeared gad1b-negative.

Blue light triggers activity in thalamic neurons and afferents to vHb

It has been shown that the anterior thalamus of zebrafish is activated by pulses of blue light, and that this can increase activity in the medial habenula (Cheng *et al.*, 2017; Zhang *et al.*, 2017). To test whether GABAergic thalamic neurons are activated by light, two photon calcium imaging was performed on transgenic larvae with broad expression of GCaMP6f and DsRed only in GABAergic neurons. Following the onset of blue light, DsRed-positive as well as DsRed-negative neurons in the thalamus exhibited an increase in fluorescence levels (Figure 3A-C). To determine whether terminals in the lateral habenula are also affected, calcium imaging was carried out on larvae expressing GCaMP6s under the control of the s1020t GAL4 driver. In response to blue light, a clear rise in the fluorescence of this reporter was seen (Figure 3D; n =3 fish), suggesting that thalamic neurons innervating to lateral habenula neurons are activated by light.

Blue light transiently reduces intracellular calcium in a subset of ventral habenula neurons

If thalamic neurons provide GABAergic input to the soma of lateral habenula neurons, then stimulation of these neurons should inhibit the lateral habenula. Two photon calcium imaging indicates that blue light causes both increase and decrease in ongoing activity in lateral habenula neurons (Figure 4 A,B; n = 4 fish). Cells where calcium was reduced during the period of blue light delivery showed an increase at light OFF, suggesting depolarization following hyperpolarization. To confirm that there is inhibition, rather than just a loss of excitation,

electrophysiology was performed on intact animals. Both hyperpolarization (Figure 4C) and depolarization (Figure 4D) was detected in lateral habenula neurons by whole-cell recording. When loose-patch recording was employed, the onset of blue light was seen to either trigger or dampen neural spikes (Figure 4 E,F). Hence, in addition to excitation, blue light-dependent inhibition in lateral habenula cells is supported by both calcium imaging and electrophysiological recordings.

The preglomerular complex, which is adjacent to the posterior tuberculum, is thought to receive input from various sensory systems including the eye and lateral line (Vernier & Wullimann, 2009). We examined the response of these neurons to blue light. No response was seen, indicating that blue light mediated inhibition of the lateral habenula in zebrafish is unlikely to be mediated by this nucleus.

Discussion

We have examined the source of neurons that can influence neural activity in the lateral habenula of larval zebrafish through the release of GABA. GAD65/67 labelling and imaging of a transgenic line which expresses DsRed under the control of the *gad1b* promoter indicate that GABAergic terminals can be found in the habenula neuropils and also in between cell soma. This suggests that GABAergic neurons target both dendrites and the soma of lateral habenula neurons. Targeting onto the soma, which would have a stronger effect on the activity of the postsynaptic neuron, was not seen in the medial habenula.

A recent study in mice demonstrated that GABAergic neurons from the lateral geniculate nucleus innervate and inhibit the lateral habenula (Huang *et al.*, 2019). Consistent with these findings, we see that retrograde labelling of lateral habenula neurons in the zebrafish reveals

gad1b-positive afferents from the thalamus. Further, we show that GABAergic thalamic neurons as well as thalamic terminals in the lateral habenula are excited by light, while a number of lateral habenula cells are inhibited. Electrical recordings of the lateral habenula indicates that the onset of blue light produced hyperpolarization and a reduction in the number of spikes. The link between illumination levels and mood has been well-studied in animals and humans (Viola *et al.*, 2008; Vandewalle *et al.*, 2010; Bedrosian *et al.*, 2011; LeGates *et al.*, 2014). Given that increased activity in the lateral habenula has negative effects (Lecca *et al.*, 2014; Proulx *et al.*, 2014; Lawson *et al.*, 2017; Howe & Kenny, 2018), inhibition of these neurons by thalamic GABAergic neurons may contribute to the positive effects of light (Huang *et al.*, 2019). Additionally, since habenula neurons show prolonged excitation following hyperpolarization (Chang & Kim, 2004; Cheng *et al.*, 2017), offset of light may be aversive.

Retrograde tracing experiments also revealed *gad1b*-positive inputs from a nucleus lateral to the posterior tuberculum. Projections from the posterior tuberculum (PT) to the habenula have been previously reported (Hendricks & Jesuthasan, 2007; Turner *et al.*, 2016). However, neurons in the posterior tuberculum have been characterized as largely non-GABAergic (Filippi *et al.*, 2014; Heap *et al.*, 2017). The nucleus adjacent to the posterior tuberculum is likely to be the M2 migrated nuclei of the posterior tuberculum, which is a part of the preglomerular complex that receives sensory information from various sensory systems, including the lateral line, gustatory, and auditory system. Future experiments will determine what stimulus inhibits the lateral habenula via this nucleus.

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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.

Figure captions

Figure 1. Detection of GABAergic terminals in the habenula. (A) A 6 day old fish, labelled with an antibody to GAD65/67. Terminals are visible in the lateral habenula (yellow arrowheads). The red arrows indicate GABAergic neurons in the pallium. (B) A 2 week old fish, showing GAD65/67-labelled puncta in the neuropil of the medial habenula (white arrow), and in between cells in the lateral habenula (yellow arrowhead). No habenula cell bodies are labelled. This sample is slightly tilted. (C) A 7 day old *Tg(vGlut2:GFP), Tg(gad1b:DsRed)* double transgenic fish. DsRed (magenta) is visible in terminals in the lateral habenula

(arrowheads), while habenula cell bodies express GFP (green). (D) An 8 day old fish *SqKR11Et* fish, with afferents from the entopeduncular nucleus labelled in red. Terminals are restricted to neuropil of the lateral habenula (white arrow), and do not overlap with GAD65/67 label between cells (yellow arrowhead). (E) Afferents from thalamic neurons, labelled with Kaede under the control of the *s1020tEt* driver. Terminals are visible in the neuropil (arrow) and also between cells (arrowhead). Scale bar = 25 μm. All images are dorsal view, with anterior to the left.

Figure 2. Retrograde label of lateral habenula afferents. (A-C) An example of DiD injection (cyan) into the left lateral habenula (A, red arrowhead) of a 7 day old *Tg(elavl3:GCaMP6f), Tg(gad1b:DsRed)* fish. (B) Retrogradely labelled cell bodies (arrowheads) in the thalamic region. (B', B'') The boxed area at higher magnification. One retrogradely labelled cell is *gad1b*-negative (green arrowhead), while the other is positive (yellow arrowhead). (C) A deeper focal plane. Entopeduncular nucleus (EN) cell bodies are also labelled by DiD but are *gad1b*-negative. (D-F) DiD injection (cyan) into the right lateral habenula of another double transgenic 7 day old fish (D, red arrowhead). Lateral habenula neurons express GCaMP6s under the *dao* promoter while GABAergic neurons express DsRed under the *gad1b* promoter. (E) Bilateral label of cell bodies adjacent to the posterior tuberculum (M2). (E', E'') The boxed region in E at higher magnification, showing retrogradely labelled neurons that are *gad1b* positive (yellow arrowhead) or negative (green arrowhead). All panels are dorsal views, with anterior to the left. Pa: pallium, OT: optic tectum, fr: fasciculus retroflexus, rHb: right habenula, PT: posterior tuberculum, IPN: interpeduncular nucleus.

Figure 3. Effect of blue light on thalamic GABAergic neurons. (A-C) The thalamic response of a 6 day old fish to 3 pulses of blue light. (A) Confocal image showing thalamic neurons,

some of which express DsRed under the control of the gad1b promoter (magenta). (B) Change in GCaMP6f fluorescence of thalamic neurons, correlated to change in the AF4 neuropil (circled), which receives retinal input. Cells indicated by arrowheads have a high correlation with activity in this neuropil (C) and express DsRed, as shown in panel A. (D, E) Calcium imaging of thalamic terminals in the ventral habenula. (D) Blue pixels depict thalamic terminals with a calcium increase in response to blue light. (E) Traces showing average change in fluorescence over time. All panels are dorsal views, with anterior to the top. Th, thalamus.

Figure 4. Calcium imaging and electrophysiological recordings in the lateral habenula. (A, B) Change in GCaMP6f fluorescence in lateral habenula neurons of a 7 day old fish exposed to four pulses of blue light. Colours represent the k-means clusters shown in the panel B. Traces represent average changes in fluorescence over time. Both increase (pink traces) and decrease (green traces, white arrows) in fluorescence in response to stimulus can be seen. Cells with a decrease show an increase at light OFF. Solid lines indicate light ON, dotted lines indicate light OFF. Blue bars the duration of the stimulus. a, anterior; p, posterior. (C, D) Whole-cell recordings showing membrane depolarization (C) or hyperpolarization (D) by blue light. The blue light was 13 seconds in duration in both cases and the range of voltage change was 2-3 mV in both cases. Each trace is a separate cell. (E, F) Loose-patch recordings. (E) There are spikes of two different heights, implying signals from two cells. Both show an increase in spikes in the presence of blue light. (F) A recording in another fish, showing a reduction in spiking during blue light delivery. The ticks in the upper trace in (E) and (F) were taken from spike detection from the main trace, as indicated by the blue squares.

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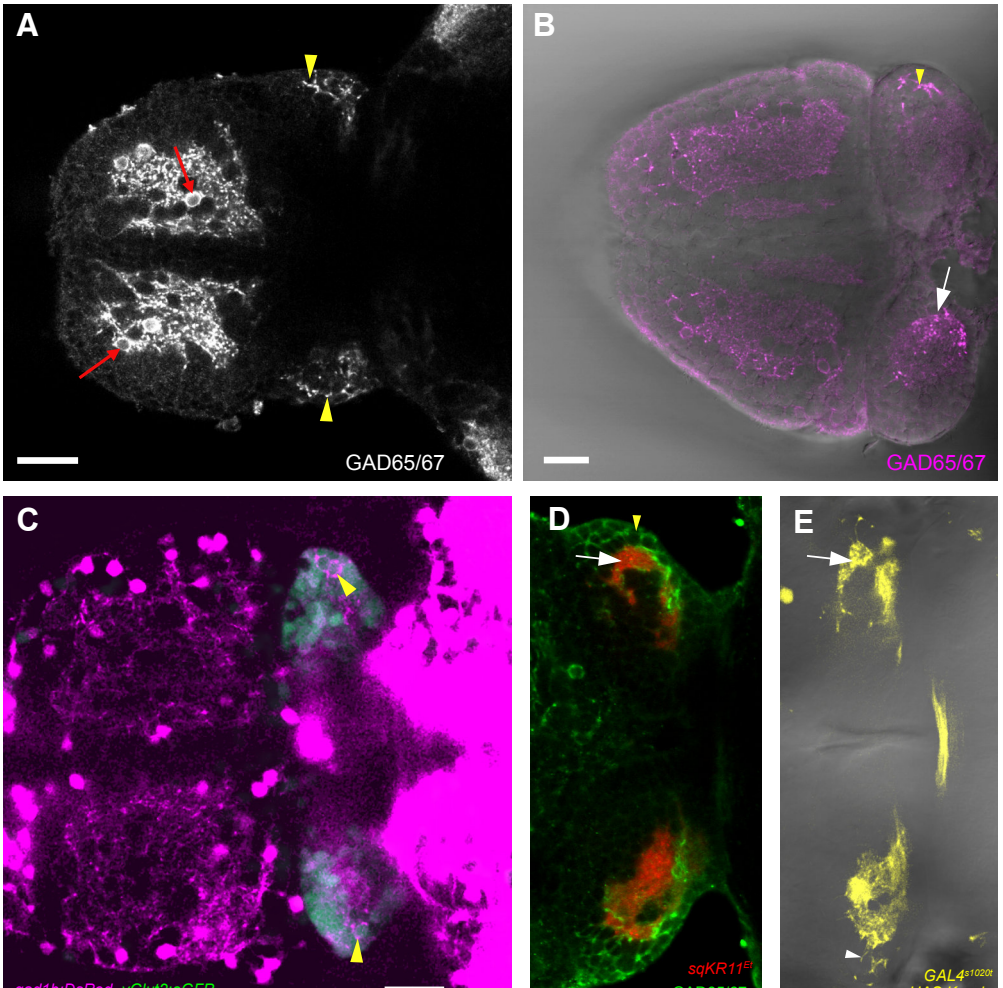
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GAD65/67
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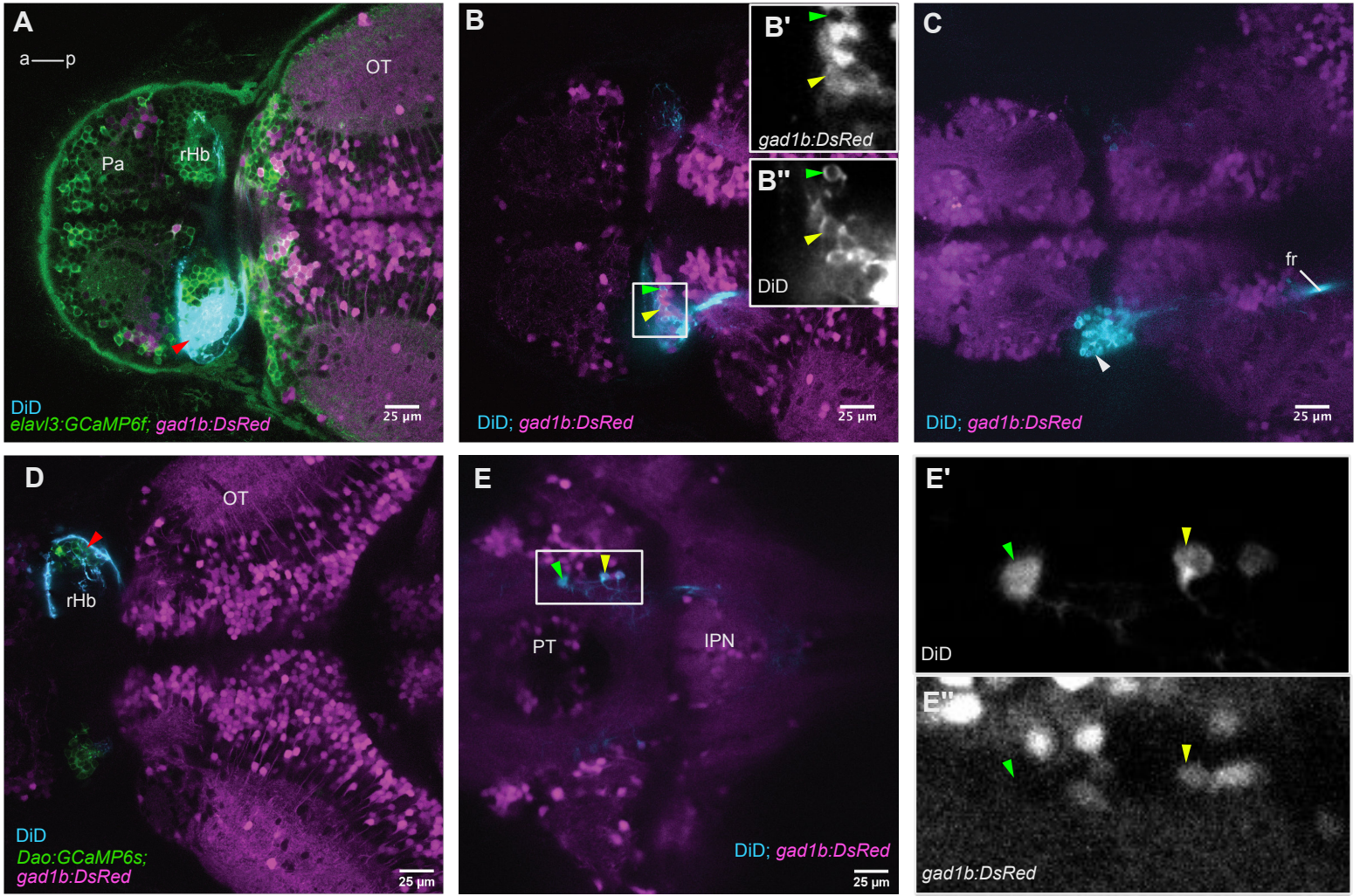
Figure 1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B''
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25 µm
C
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DiD; gad1b:DsRed
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D
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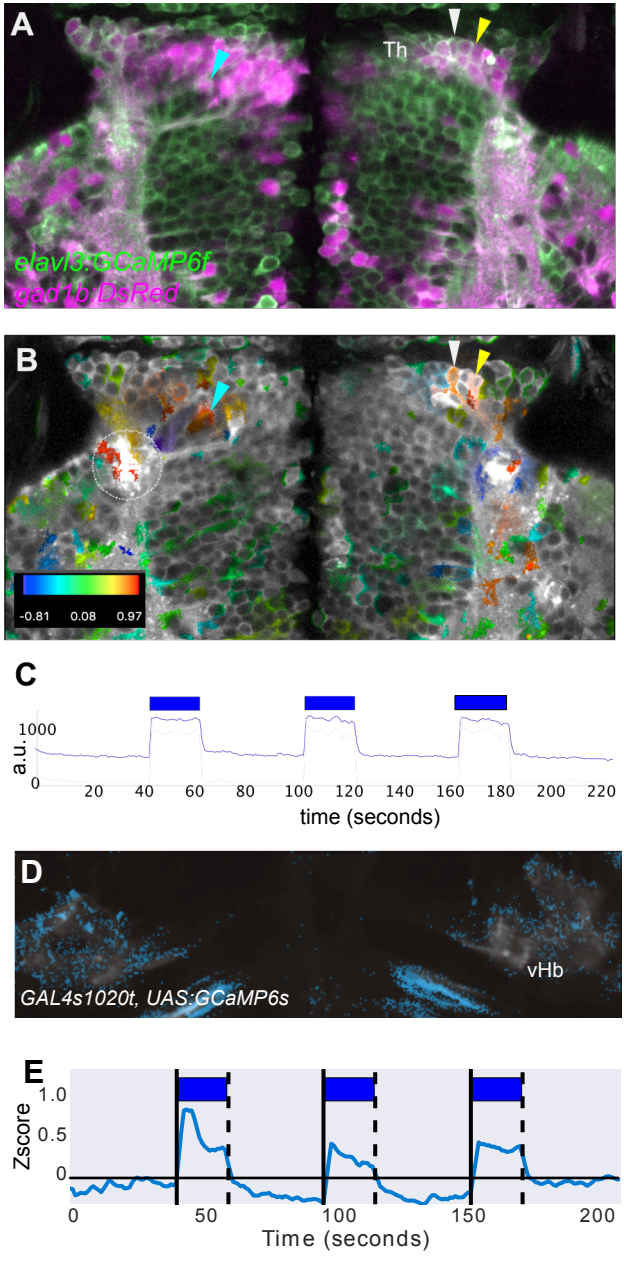
Figure 2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120
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160
180
200
220
time (seconds)
D
vHb
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Figure 3

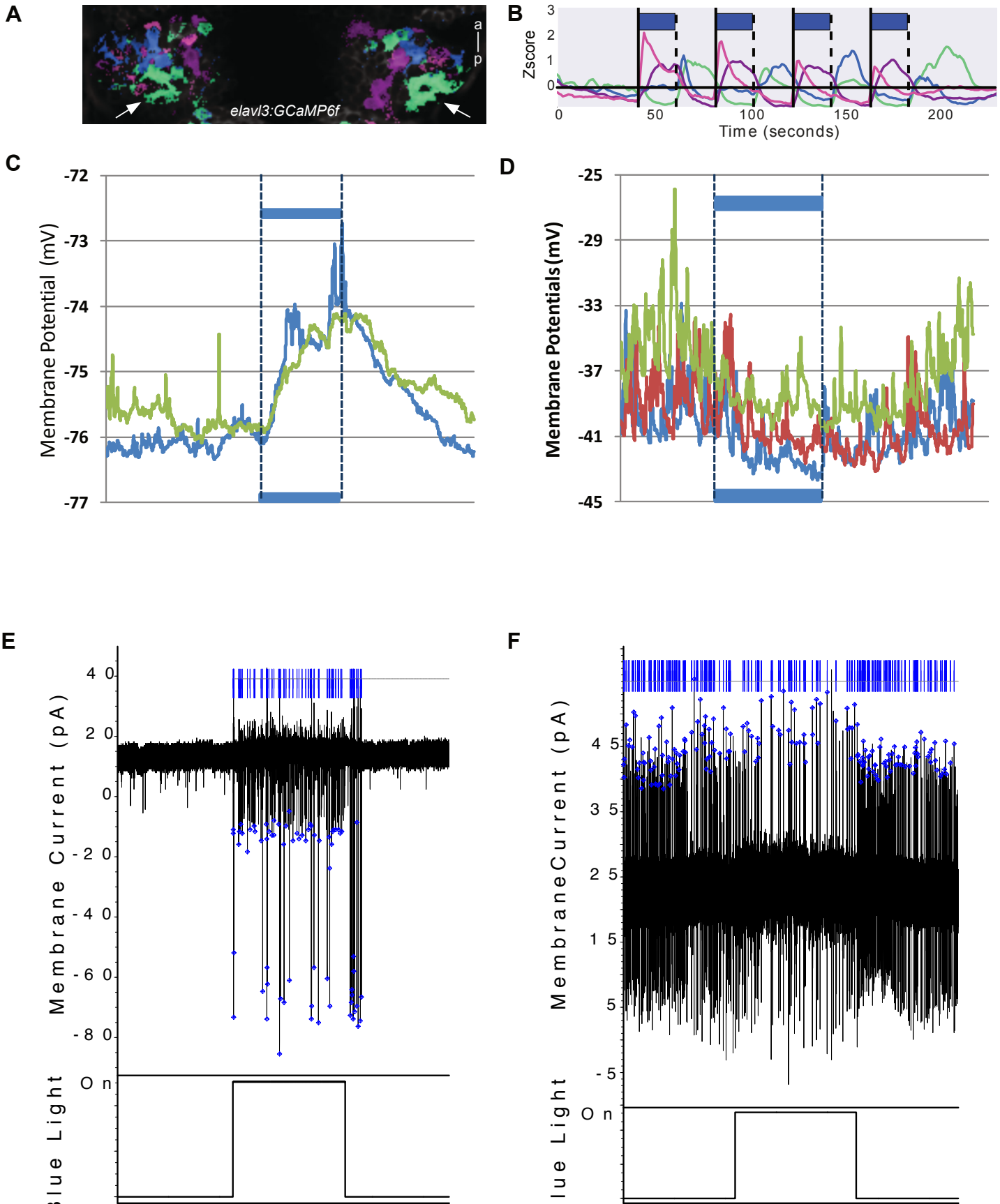

Figure 4